THE EFFECTIVENESS OF OZONATION FOR VIRAL INACTIVATION

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THE EFFECTIVENESS OF OZONATION FOR VIRAL INACTIVATION

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ABSTRACT

Ozone was found to be an effective disinfectant for 15 selected enteric viruses. The mean $\log_{10}$ inactivation observed in 30 seconds, by 0.1 mg/l ozone, was 3.0. Comparisons of the literature data to the results of this investigation were limited, due to the widely different conditions employed.

A two stage inactivation was observed for all the viruses studied. However, viral inactivation was very rapid and the rate of the inactivation reaction could not be determined.

The effectiveness of ozone for viral inactivation was found to be reduced when ozone demanding substances were present in the virus preparation. Several routine aspects of virus pool preparation were found to influence the extent of inactivation observed. Chloroform treatment, phenol red, freezing, centrifugation and dilution of the virus preparation affected the virus inactivation substantially. Resuspension of poliovirus in sewage effluents or nutrient broth decreased the extent of inactivation compared to virus resuspended in ozone demand free water.

The relative resistance of selected enteric viruses could not be unequivocally determined due to differences in virus preparations. The extent of virus inactivation was found to be correlated with the total organic carbon content (TOC) of the preparation. However, only some of the differences in the extent of inactivations could be explained by differences

in TOC. The factors influencing virus inactivation during ozonation were found to be complex and simple, general measurements such as TOC gave only a partial picture.

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INTRODUCTION

INTRODUCTION

The ready contamination of water with physical, chemical and especially biological pollutants frequently makes water a source of illness and hazard, instead of a source of health. Public health officials are now most concerned about virogenic disease, particularly with the advent of water reuse for residential, agricultural and industrial purposes. More than 100 different viruses may be transmitted by water, although the true extent to which such transmission occurs is not known.

Drinking water disinfection practices, begun in the early 1900's, have led to a rapid decline in the incidence of water-borne diseases. Chlorine is the most widely used water disinfectant in the world today. However, in spite of all the successes attributed to chlorine, there are problems encountered in its use in certain disinfection applications. Potential carcinogens, in the form of haloforms, have been detected in the Mississippi River. These compounds have also been found in New Orleans' drinking water and in the blood plasma of local residents (Dowty et al., 1975). A further study showed a statistically significant relationship between cancer mortality rates in Louisiana residents and use of drinking water obtained from the Mississippi River (Page et al., 1976).

Recently, a wild strain of poliovirus 1 was isolated from an Ottawa sewage plant effluent (Sattar and Westwood, 1976). This was judged as a cause for concern, since antibody levels in the general Canadian population are decreasing (McLeod et al., 1975).

Thus the production of toxic end-products by chlorination of waters in the presence of organic matter, and the incomplete virus removal by existing treatment facilities, have renewed interest in the ozonation process. However, although ozonation techniques are very effective, as yet there is little rational and scientific basis for their practical application.

Numerous reports have shown the enteric viruses to be more resistant than bacteria to chlorination and other treatments. Also, the bactericidal properties of ozone are well known, whereas the virucidal properties of ozone have been neglected. For these reasons, it was decided to study the inactivation of viruses by ozone. Human enteric viruses -- strains of poliovirus, coxsackievirus, echovirus, adenovirus and reovirus were chosen, since they represent the majority of viruses of concern in water and wastewater transmission. The objective of this

study was to determine the relative ozone resistance of 15 different viruses and to investigate some of the factors influencing virus inactivation, in the hope of adding to the slowly emerging rationale behind ozonation techniques.

LITERATURE REVIEW

LITERATURE REVIEW

A. PROPERTIES OF OZONE

a) Introduction

The first recorded detection of ozone is that of a "sweet smell" due to the operation of electrical machinery, which was described by Van Marum in 1785. The name ozone was applied by Schönbein (1840), who devoted his life to a study of this gas. However, Schönbein made only semi-quantitative observations on the chemistry of ozone and the first precise information of its chemical properties is derived from the definitive work of Brodie (1872).

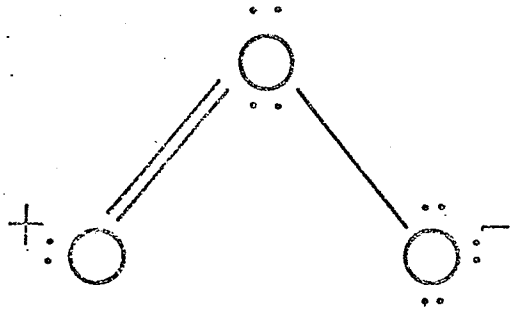
Ozone is a triatomic allotrope of oxygen. Its exact structure remains controversial; however, four resonance structures are generally considered to be the most important, as shown in Figure 1 (Rosenthal, 1974).

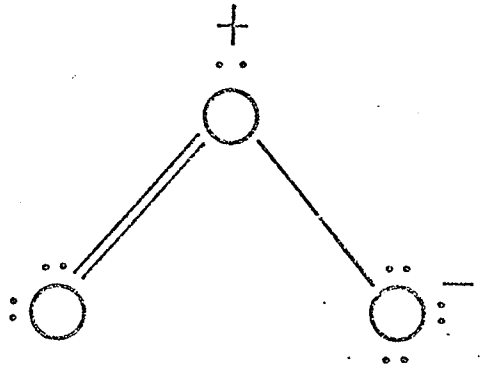
The high oxidation potential of ozone, and its instability and low solubility, are the major considerations in disinfection applications and studies.

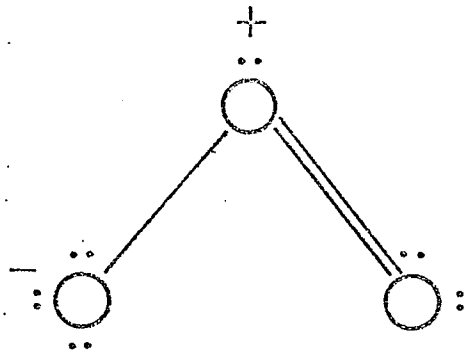
b) Solubility of Ozone

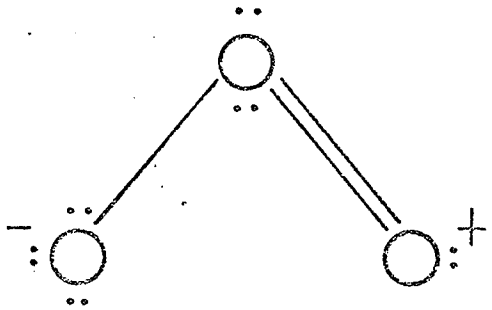
The object of most ozone uses is to transfer the greatest amount of ozone from the gas to the liquid phase. This depends on several factors. Solubility

Figure 1. Resonance hybrid structure of ozone.









increases with decreasing temperature and pH (Stumm, 1956), and thus low temperatures and pH must be employed to optimize ozone concentrations in aqueous solutions. Also, ozone obeys Henry's Law, which states that the solubility of a gas is directly proportional to the partial pressure of the gas above the solution (Diaper, 1975). Since ozone is normally produced in low concentrations, its partial pressure is correspondingly low.

c) Ozone Decomposition

One of the difficulties in working with ozone is its instability in aqueous solution. Decomposition increases rapidly with increasing pH and temperature (Kilpatrick et al., 1956).

The mechanism and kinetics of ozone decomposition in water are uncertain. Efforts to find the rate of decomposition in pure aqueous solution have been hampered because the ability to remove all impurities and reproducibility has been poor (Hewes and Davison, 1971). The decomposition of ozone is much slower than the oxidation of organic substances present in solution.

d) Oxidizing Capacity of Ozone

Houzeau (1872) was the first to report the formation of a compound between ozone and an organic material.

Unfortunately, very few detailed studies have been made into the chemical pathways involved in the ozonation of organic or inorganic substances in water. However, on the basis of its resonance hybrid structure, ozone may react as an electrophile, a nucleophile, or as a 1,3-dipole (Bailey, 1975).

There is no reason for ozone itself to behave as a radical since it is non-paramagnetic; however, a free radical mechanism of decomposition has been proposed (Alder and Hill, 1950) for ozone in aqueous solution:

$$O_3 + H_2O \longrightarrow HO_3^+ + OH^-$$

$$HO_3^+ + OH^- \longrightarrow 2HO_2^+$$

$$O_3 + HO_2^+ \longrightarrow HO^- + 2O_2$$

$$HO^- + HO_2^+ \longrightarrow H_2O + O_2$$

All intermediates are very reactive and short-lived (McCarthy and Smith, 1974). Apparently the free radicals are the principal reacting species, and they react preferentially with inorganic and soluble organic materials before encountering dispersed particles such as viruses (Hewes and Davison, 1971; Hoigné and Bader, 1975).

B. OZONE GENERATION

Because of its inherent instability, ozone must be produced on site. Ozone is generated when an oxygen molecule is sufficiently excited to dissociate into atomic oxygen; further collisions with oxygen molecules cause formation of the ozone molecule. The reaction $3O_2 = 2O_3$, - 69 kc, is endothermic (Diaper, 1972).

Excitation may occur in one of three ways. Ultraviolet radiation and electrolysis both give low ozone yields (Lenard, 1894). Silent electric discharge has been used in the laboratory for many years (Von Siemens, 1857) and is the basis for all commercial ozonators (Murray, 1969). Such generators produce a reproducible source of ozone which can be varied over a fairly wide range. The ozone produced however, is actually a dilution of ozone in a relatively large volume of air or oxygen. Pure ozone has been obtained by fractional distillation of ozone-oxygen mixtures.

The basic configuration of the ozonator is an alternating current established between two parallel electrodes separated by an air gap. To ensure uniformity of the current flow, a dielectric material is placed in intimate contact with one or both electrodes.

When oxygen or low pressure clean dry air flows through the air gap, stray electrons within the gap are excited to an energy level high enough to split some of the oxygen molecules (McCarthy and Smith, 1974). Air must be dried and cooled when used as a feed gas, due to the accumulation of corrosive nitric acid and nitrogen oxides.

The process is inefficient, as only approximately 10% of the energy applied is used to make ozone and the remainder is lost as light, sound and heat (Rosen, 1972). Because the mechanism of production requires collisions, the laws of probability dictate that only a portion of oxygen exposed in the gas flow will be converted to ozone. The maximum yield is 6% by weight of ozone. To obtain the maximum amount of ozone, many parameters must be optimized. These include: temperature, pressure, voltage applied, frequency of the voltage and capacitance of the air gap and dielectric (Klein, et al., 1975).

C. MEASUREMENT OF OZONE

a) Introduction

Quantitative determinations of ozone in aqueous solution have been troublesome. There are several characteristics of ozone in water which cause error in measurement. These are: loss of ozone from solution to the atmosphere above, decomposition of ozone in water (Alder and Hill, 1950) and the reaction of ozone with constituents in water, including large organic compounds, inorganic compounds and most metals (Hewes and Davison, 1971).

The three basic approaches to the measurement of ozone in aqueous solution are: direct, colorimetric and titrimetric. While there have been no comparative studies on measurements obtained with all the methods available, it is widely accepted that quantitative measurements may vary depending on the method used. For example, some methods are specific for ozone, some are not. The latter methods measure anything in the system which reacts in the chemical conversion on which the method is based. This fact makes it difficult to compare studies on virus inactivation at a particular ozone concentration, when different measurement methods have been used.

b) Direct Measurement Methods

The direct methods generate absolute measurements of ozone concentration and are used to standardize and compare all other methods. The direct absorbance procedure (Hann and Manley, 1952) is based on the fact that ozone in water exhibits a maximum absorption at 253.7 nm. Prior determination of the extinction coefficient of ozone is necessary. Although simple in principle, this method is applicable only to very pure solutions free from turbidity, bacteria or other absorbing material. Another absolute measure is gas-phase titration with nitric oxide (Saltzman and Gilbert, 1959). This method is based on the reaction $O_3 + NO \longrightarrow NO_2 + O_2$. It is specific for ozone and free from interferences by organic oxidants and reducing gases.

c) Colorimetric Measurement Methods

The principle of colorimetric methods is the reaction of ozone and a chemical, to produce a colored product, which is usually measured photometrically. There are many methods in this category, but the two most widely used are the orthotolidine-manganese sulfate (OTM) method and the leuco crystal violet (LCV) method. The OTM test (Standard Methods for the Examination of Water and Wastewater, 1975) involves the oxidation by

ozone of manganese to the manganic ion and the subsequent reaction with the acid orthotolidine reagent, to produce color which is measured photometrically. The limitation of the method is that it is only semi-quantitative and is affected by nitrite and oxidized iron. The LCV test (Kinman, 1975) has some advantages in that it is reliable and precise. However, it also requires a large sample volume and because the basis is spectrophotometry, it cannot be used with solutions that absorb light energy.

d) Titrimetric Measurement Methods

The titrimetric methods involve the oxidation of potassium iodide to iodine in the presence of excess iodide. This is the present standard method (Standard Methods, 1975) of analysis for ozone in water. The method is quantitative, capable of precision and subject to the fewest interferences.

There are several ways to determine the end point of the reaction. Use of a starch indicator is the traditional method for end point determination. However, there are some difficulties associated with it and a large error when the ozone concentration is less than about 1 mg/l. A better way to measure iodine formed by the reaction of ozone and KI is the amperometric titration method (Foulk and Bawden, 1926)

using phenylarsene oxide (PAO) as the titrant. The basic reaction is:

$$O_3 + 2KI + H_2O \longrightarrow I_2 + 2KOH + O_2$$

$$\underset{(PAO)}{C_6H_5AsO} + I_2 + 2H_2O \longrightarrow C_6H_5AsO(OH)_2 + 2HI$$

The stoichiometry of the reaction (molar ratio between iodine released and ozone absorbed) has been a controversial point. Ratios between 0.65 and 1.53 have been obtained using iodide solutions of varying pH levels. Several researchers (Ingols et al., 1959; Dietz et al., 1973) have reported an effect of pH on the stoichiometry of the reaction. A neutral phosphate-buffered reagent gives a stoichiometry of $O_3 = I_2$, and this was shown to be the most accurate solution by comparison with independent absolute methods (Boelter et al., 1950; Saltzman and Gilbert, 1959).

The amperometric titration has some problems associated with it. Certain ions in solution, for example copper and aluminum, may poison the electrodes. Also, some losses may occur due to the violent agitation required to make the system function well, and large sample volumes are required for the technique (Kinman, 1975). The iodometric technique is not specific for ozone, as anything in solution that has the ability to oxidize iodide will be determined as ozone (Sproul, 1975).

D. GERMICIDAL CHARACTERISTICS OF OZONE

a) Introduction

Ozone has been used since the beginning of the century to disinfect water supplies. Today, more than 500 municipalities in 50 countries use ozone as a disinfectant (Rosenthal, 1974). In Canada there are 20 plants in operation. Drinking water supplied to Quebec City is ozonated at the rate of 60 million gallons per day (mgd). A further installation, completed in 1976, treats 250 mgd to the city of Montreal (Nadeau and Pigeon, 1975).

The germicidal properties of ozone were studied in 1886 by De Meritans. The bactericidal nature of ozonation is well known (Hann, 1956) but comparatively little information is available on virus inactivation by ozone.

In 1943, Kessel et al., prompted by reports of a resistant strain of poliovirus, (Trask and Paul, 1941) compared chlorine, hypochlorite and ozone as virucidal agents. Two strains of poliovirus (MV and Le) were compared, after treatment and neutralization, by intracerebral injection into monkeys (Macaca mulatta). Identical dilutions of the same strain and pool of poliomyelitis virus were exposed to chlorine in residual amounts between 0.5 and 1.0 ppm and to ozone in residual amounts between 0.05 and 0.45 ppm, under the same

controlled experimental conditions. The virus was inactivated in 2 minutes by ozone compared to an interval between 90 and 180 minutes for inactivation by chlorine.

The rapid inactivation of viruses by ozone and the low concentrations of ozone required, in comparison with other disinfectants, has been further documented. For poliovirus, Katzenelson et al., (1974) found a 99% inactivation by ozone in less than 10 seconds, compared with several minutes required for chlorine (Scarpino et al., 1972) and iodine (Berg et al., 1964) of the same concentrations.

b) Two Stage Inactivation

Katzenelson et al. (1974) found a two stage inactivation curve for poliovirus suspended in phosphate buffer. Stage one, of less than 10 seconds duration, resulted in 99% inactivation and stage two continued for several minutes up to complete inactivation. They postulated that the second plateau stage was due to viral aggregation since it could be eliminated by ultrasonication.

It has never been verified that disaggregation of microorganism flocs is responsible for the synergistic effect of sonozonation (Dahi, 1976). Viral aggregation is well documented in the literature (Sharp, 1965).

However, electron microscopy in individual studies is required for verification (Galasso and Sharp, 1962).

Burleson et al. (1975) presented data complementary to that of Katzenelson and coworkers and described a synergistic effect of ultrasonication and ozonation on virus inactivation. Vesicular stomatitis virus, encephalomyocarditis virus and GD VII virus, suspended in phosphate buffered saline, were completely inactivated in 15 seconds after treatment with ozone.

Neither of these investigators was able to measure the rate of inactivation in stage one. Although dose response may have occurred in the first 8-15 seconds, it was impossible to sample in that period with the apparatus used. Two stage inactivation curves were also obtained for coliphage T2 and Escherichia coli (E. coli.) (Katzenelson et al., 1974).

c) "All or None" Phenomenon

Several groups have found an "all or none" die-away phenomenon associated with ozone. Generally, a level of ozone was found to be the threshold value below which inactivation was not satisfactory and above which more than 99.9% inactivation was achieved.

Majumdar et al. (1973;1974) found a sudden drop in the survival rate for poliovirus in triple distilled water, when the initial ozone concentration reached 1.0 mg/l. They also attempted to quantitate the ozonation process as it applied to poliovirus inactivation and formulated two rate equations to explain the inactivation kinetics above and below the threshold value of 1.0 mg/l. The equations, relating ozone concentration, contact time and virus survival, were deemed valid in both a batch and continuous flow situation. However, Farooq and Chian (1976) concluded that the kinetic rate equations proposed by Majumdar and coworkers were applicable only in a limited range of ozone concentrations and contact times and that for a valid comparison, substantially more experimental data was required.

Various ozone threshold values have been reported for bacteria and viruses. Coin et al. (1964) found that 0.7 mg/l of ozone was a threshold level for poliovirus inactivation. Fetner and Ingols (1956) found a critical value of 0.4-0.5 mg/l for E. coli inactivation. An "all or none" effect of ozonation has also been observed for f2 bacteriophage in a pilot plant study (Longley et al., 1974).

Katzenelson et al. (1974) did not find a threshold level of ozone for poliovirus inactivation. However, they did find inconsistencies in the amount of inactivation obtained at ozone concentrations less than or equal to 0.15 mg/l. In some experiments no inactivation was seen with these concentrations and in other experiments rapid inactivation was obtained with 0.1 and 0.05 mg/l.

Broadwater et al. (1973) suggests that a demand for ozone is exerted by some trace contaminants in the system, which causes decomposition of the ozone before it can come in contact with the organisms and react with them. Thus the "all or none" phenomenon often observed in ozone disinfection work may be caused by ozone demanding substances in the system.

Proponents of the free radical theory of ozone disinfection offer another explanation for the threshold effect. It has been suggested that the "all or none" event may not be a toxilogical phenomenon of microbial response to ozone molecules, but rather may be a stage where a proper free radical activity has not been established (Dahi, 1976; Hoigné and Bader, 1975).

A recent report (Nebel et al., 1975) has questioned the validity of the "all or none" concept of ozone disinfection by demonstrating that the destruction of microorganisms and the oxidation of pollutants parallel one another.

E. FACTORS INFLUENCING VIRUS INACTIVATION BY OZONE

a) Introduction

The major observation in comparing virus inactivation observed in different studies, was the large disparity in the amount of inactivation obtained by the same ozone concentrations. Sproul (1975), in comparing literature data from three different sources, noted unusually large differences in the amount of poliovirus inactivation obtained at a constant concentration x time (Cxt) product of 0.3 mg/min/l. The survivals varied from 0.075 to 40 per cent, about two and one-half orders of magnitude. Reasons for these unusually large variations are unknown, but may be due to differences in the experimental conditions. Some of the differences that Sproul noticed between these studies were: temperature, pH, strain of poliovirus 1 and the cell system used for the assay.

Although the effect of these factors on virus inactivation has not been extensively studied, some speculations may be drawn from the various literature data.

b) Ozone Measurement Method

The ozone measurement method may affect the inactivation obtained since different methods are based on different chemical reactions, are subject to

different interferences and some are more accurate than others, particularly at low ozone concentrations (Kinman, 1975). The other aspect is that the active disinfecting chemical present in ozonated water is unknown, as there are no analytical methods available to determine the true disinfectant. The principal reacting species may be free radicals (Peleg, 1976) in which case the amount of inactivation may not solely depend on the amount of ozone present, but rather on the optimization of free radical formation.

c) Temperature

Temperature may also influence the extent of virus inactivation. Most of the studies in the literature have been done at temperatures between 5°C and 25°C. In this temperature range enteric viruses are stable, however changes in temperature will affect the decomposition rate of ozone to some degree (Hewes and Davison, 1971).

However, Kinman (1975) reported that ozone is more independent of temperature effects than iodine or chlorine, and noted little difference in the rate of destruction of coliform organisms by ozone between 0°C and 30°C. This finding is supported by another study in which temperature changes between 5°C and 25°C had no significant effect on the inactivation of enteroviruses by ozone (Evison, 1973).

However, Ingram and Barnes (1954) found that temperature has an appreciable influence on the bactericidal ability of ozone. Similarly, Yakovleva and Il'nitskii (1967) observed a temperature effect for adenovirus inactivation by ozone. They found less inactivation at 20-21^{o}C than at 13-15^{o}C. However, it was uncertain whether the initial ozone concentrations used were the same at both temperatures. The influence of temperature on virus inactivation by ozone remains controversial.

d) Effect of pH

There have been few investigations on the effect of pH on virus inactivation by ozone. Evison (1973) found that pH changes from 6 to 8 had no significant influence on the disinfecting powers of ozone. Most of the studies in the literature have not mentioned the pH at which virus inactivations were carried out. Although it is well known that pH affects the decomposition of ozone, (Alder and Hill, 1950) the influence of ozone decomposition on microorganism inactivation is not established.

e) Virus Aggregation

There has been no direct evidence that aggregated virus is more resistant to ozone than suspensions of single particles. However, this type of resistance has been shown for poliovirus and reovirus inactivation by bromine (Sharp et al., 1975; Floyd et al., 1976). Thus it is possible that differences in the degree of viral aggregation could account for disparities in the extent of inactivation observed in various studies.

Viral aggregation is a complicated phenomenon that may be induced at various stages during inactivation experiments. The virus suspending fluid, storage temperature of the virus and the composition of the ozone neutralizer and virus diluent may promote either aggregation or deaggregation. Interpretation of the results of disinfection experiments with viruses in water, requires knowledge of the state of particle aggregation at the time they were exposed to the disinfectant and at the time of assay (Floyd and Sharp, 1977).

A dilution of poliovirus or reovirus in distilled water induces aggregation of these viruses, whereas dilution in Dulbecco's phosphate buffered saline or 0.14M NaCl reverses the poliovirus aggregation, but not the aggregation of reovirus. Also acid buffers are reported to induce poliovirus aggregation, which is

reversed when the pH is returned to 7.0 (Floyd and Sharp, 1977; Young and Sharp, 1977). Aggregation effects are also dependent on virus particle concentration, since at low concentrations collisions are less likely to occur. Consequently, the ionic strength, pH and concentration of virus in the suspending fluid, neutralizer and diluent may influence the amount of inactivation recorded. Also, this effect may differ depending on the virus strain.

The storage temperature of virus preparations may also influence the extent of inactivation. Katzenelson et al. (1974) found increased resistance of poliovirus stored at -15°C compared with virus stored at -70°C. They explained this phenomenon as due to clumping of the virus at -15°C which conferred resistance to the virus upon ozonation. Other physical changes of the virus cannot be excluded as possibilities for this resistance, but this shows the importance of storing virus at the same temperature if changes in virus sensitivity are to be avoided.

F. RELATIVE RESISTANCE OF ENTERIC VIRUSES TO OZONATION

a) Introduction

The importance of water as a vehicle for transmission of pathogenic viruses is now generally acknowledged. Although any human virus excreted in the

feces may appear in drinking water, most concern is assigned to those that grow in or near the intestinal tract and are discharged in large numbers. This group includes the enteroviruses (poliovirus, coxsackievirus and echovirus), adenoviruses, reoviruses and infectious hepatitis virus. Diseases caused by these viruses are shown in Table I. Methods for identification and quantitative measurement of human viruses in water samples are inadequate and require involved and expensive techniques (Michele et al., 1974).

Since laboratory tests have generally shown that enteric viruses are more resistant to water disinfectants than organisms of the coliform group, the validity of the coliform test as an indicator of potential pathogenic microbial water pollution has been increasingly questioned (Shah and McCamish, 1972; Ludovici et al., 1975). This challenge has led to many studies of the relative resistance of enteric viruses to various disinfectants, in an effort to find the most resistant virus. Presumably the virus would be used as an index of microbial pollution; its absence signifying the absence of other potential pathogens.

The relative resistance of bacteria, bacteriophage and enteric viruses to chlorine, iodine and bromine has been extensively studied (Kabler et al., 1961; Kott et al., 1974; Ludovici et al., 1975). However,

TABLE I *

Waterborne Enteric Viruses and Diseases

Group	Subgroup	Number of Types or Subtypes	Diseases caused by Virus
Enterovirus	Poliovirus	3	muscular paralysis aseptic meningitis febrile episode
	Echovirus	34	aseptic meningitis muscular paralysis respiratory diseases diarrhea pericarditis and myocarditis hepatitis
	Coxsackievirus A	24	aseptic meningitis respiratory disease infantile diarrhea hepatitis pericarditis and myocarditis
	Coxsackievirus B	6	aseptic meningitis muscular paralysis pericarditis, endocarditis, myocarditis respiratory disease hepatitis or rash spontaneous abortion
Reovirus		3	Role in human disease not well known
Adenovirus		31	respiratory diseases acute conjunctivitis acute appendicitis
Hepatitis		2	infectious hepatitis serum hepatitis

* Tabulated from: Taylor, 1974.

there has been only one major study which compares the resistance of enteric viruses to ozonation (Snyder and Chang, 1975).

b) Problems Encountered in Relative Resistance Studies

Since it has been found that aggregates of poliovirus and reovirus are substantially more resistant to bromine than suspensions of single particles, (Floyd and Sharp, 1977) ideally any comparison of the relative resistance of two different viruses to a disinfectant should be made first on suspensions of single particles. However the aggregated state is difficult to avoid in the laboratory, so there have been no attempts to minimize or monitor the degree of aggregation of different viruses tested in these studies. The question of whether viruses occurring naturally in water are aggregated or present mainly as single particles, may also be raised.

There is also no reason to believe that different enteric viruses destroy tissue culture cells to the same extent or in the same way, and thus it cannot be assumed that different virus preparations are identical even if prepared by a standard method.

Several studies have attempted to overcome this variability by mixing the viruses to be compared. Viruses were treated in the same reaction flask, thereby eliminating any inherent differences due to virus preparations and replicate systems. Cramer *et al.*

(1976) compared the inactivation of poliovirus 3 (Leon) and f2 bacteriophage, by chlorine and iodine in a mixed system and found that f2 phage in each case was at least as, or more resistant to, chlorine and iodine than poliovirus 3. Kott et al. (1974),using a similar system for chlorination experiments, found bacteriophages f2 and MS2 to be at least as, or more resistant than poliovirus 1 (LSc,2ab).

All of the ozonation and most of the halogenation studies have been done by separately inactivating the viruses and then comparing either the $\log_{10}$ reduction of different viruses in a specific time, or the time required for 99.99% inactivation.

c) Relative Ozone Resistance of Bacteria and Viruses

Some discrepancy has resulted in comparing the relative resistance of bacteria and viruses to ozonation. The general observation in the literature is that viruses are more resistant to chemical agents than bacteria (Ludovici et al., 1975). Current literature states that when chlorine (in the HOCl form) is used as the disinfecting agent, bacterial kill occurs first and with greater efficiency than does viral inactivation (Scarpino et al., 1972).

Several pilot plant studies (Pavoni and Tittlebaum, 1974; Longley et al., 1974) employing ozone, have found that viral inactivation occurred more completely and at a faster rate than bacterial kill. The authors concluded that the mechanisms of virus inactivation and bacterial kill may be different for ozone as opposed to other disinfectants. However, comparison of these studies to those with chlorine may not be valid as the former studies were carried out in dynamic large scale reactors in which many more variables come into effect.

Katzenelson et al. (1974), using a batch reactor found that E. coli was more sensitive to ozonation than bacteriophage T2 and poliovirus 1 (Brunhilde).

d) Relative Halogen Resistance of Enteric Viruses

A review of the literature on the relative chlorine resistance of enteric viruses revealed large differences in virus sensitivities. A recent investigation (Liu et al., 1971) indicated that the time required to inactivate 20 different viruses with 0.5 mg/l of free chlorine ranged from 2.7 minutes to 60 minutes.

The relative resistance pattern of the enteric viruses differs for chlorine and iodine, and in different chlorination studies. The most resistant viruses to chlorination found in several studies were:

poliovirus 1 and coxsackievirus A2 (Clarke et al., 1968), poliovirus 1 (strain (MK500) (Kelley and Sanderson, 1958), f2 bacteriophage (Shah and McCamish, 1972) and poliovirus 1 and coxsackievirus A9 (Scarpino et al., 1975). The most resistant viruses to iodination found were: coxsackievirus A2 and B1 (Chang, 1958) and coxsackievirus A9 (Berg et al., 1964).

Olivieri et al. (1975) have suggested that the differences in virus resistance to chlorine and iodine may be due to differences in the disinfectant's mode of action on viruses. However, reasons for the discrepancies in chlorine resistance patterns in various studies are inapparent, except that the viruses were studied under widely different conditions.

c) Relative Ozone Resistance of Enteric Viruses

A review of the ozone literature showed a total of 11 different viruses have been studied by different investigators. These studies are shown in Table II. Their chief characteristic is their disagreement. Because they were carried out using varying procedures and under widely different conditions, a direct comparison of the resistance of the viruses to ozone cannot be made. None of the studies evaluated the effect of soluble organic compounds present in the virus suspensions.

TABLE II

Literature Studies on the Inactivation of Viruses by Ozone

Author and Year	Virus Studied	Condition	Ozone Measurement Method
Kessel et al., (1943)	Polio (MV)	-	OTM
Coin et al., (1964)	Polio 1 (Mahoney)	distilled water	Iodometric (starch indicator)
Yakovleva and Il'nitskii (1967)	Adeno 7a	autoclaved tap water	Iodometric
Carazzone and Vanini (1969)	Bacterio-phage T1	distilled water	Iodometric
Majumdar et al., (1973,1974)	Polio	triple distilled water primary effluent secondary effluent	Iodometric
Katzenelson et al., (1974)	Polio 1 (Brunhilde)	ozone demand free water	Iodometric spectroscopic
Pavoni and Tittlebaum (1974)	Bacterio-phage f2	secondary effluent	-
Longley et al., (1974)	Bacterio-phage f2	secondary effluent	Iodometric
Snyder and Chang (1975)	Polio 1, 2, 3 Coxsackie B3, B5 Echo 12, 29 Adeno 7a	ozone demand free water filtered river water	OTM
Katzenelson and Biederman (1976)	Polio 1 (Brunhilde)	filtered raw sewage	Iodometric spectroscopic

The major study on relative ozone resistance was done by Snyder and Chang (1975) on eight enteric viruses. The inactivations were carried out in ozone demand free water at 2°C, with a constant ozone dose of 1g/hour. The only variable was contact time. The relative resistances found, in comparison with the pattern found for chlorine by Liu et al. in 1971, are shown in Table III. The data indicates that there was a virus strain difference in resistance to both ozone and chlorine and that the pattern was different for the two disinfectants.

Of particular interest is the wide difference in response to ozone by echovirus 12 and 29. Echovirus 12 required a contact time approximately 10 times longer than echovirus 29 for the same degree of inactivation. Since they belong to the same family and show an antigenic cross relationship (Melnick and Wenner, 1969), a basic question is raised as to the action of ozone in virus inactivation. Snyder and Chang proposed that echovirus 12 be used as an indicator virus for future studies of virus-ozone interaction under various conditions.

TABLE III

Relative Resistance of Eight Human Enteric Viruses to Chlorine and Ozone*

Chlorine		Ozone	
Virus	Minutes[a]	Virus	Minutes[a]
Adeno 7a	12.5	Echo 29	2.5
Echo 12	14.5	Adeno 7a	7.2
Polio 1	16.2	Polio 1	7.8
Coxsackie B3	16.2	Polio 3	8.4
Polio 3	16.7	Coxsackie B3	9.8
Echo 29	20.0	Coxsackie B5	10.4
Coxsackie B5	39.5	Polio 2	10.9
Polio 2	40.0	Echo 12	12.2

a Minutes required for 99.99% inactivation

* tabulated from: Liu et al., 1971 and Snyder and Chang, 1975.

G. INACTIVATION BY OZONE OF VIRUSES SUSPENDED IN SEWAGE EFFLUENTS

Different kinds of water to be disinfected (drinking water, surface water, renovated water, or sewage effluents) at all times contain a certain amount of organic matter which reacts with ozone creating an ozone demand. A number of articles (Coin et al., 1964; Majumdar et al., 1973; 1974) indeed point out that the ozone concentrations required to inactivate viruses in water possessing ozone demand are considerably higher than the concentrations needed under optimal conditions.

Katzenelson and Biederman (1976) investigated the effect of secondary effluent on the inactivation of poliovirus by ozone. Sewage effluent containing 10^8 pfu/ml purified poliovirus was injected into buffer with a known ozone concentration. The final concentration of sewage effluent was either 5% or 10%.

Complete disappearance of the ozone was observed on addition of the virus-effluent mixture. This was accompanied by a sharp reduction (90-99.9%) in virus titer. Inactivation took place in the first 10 seconds, followed by an insignificant change. The degree of inactivation correlated with the ozone concentration up to 1.3 mg/l, above which there was only a slight increase in inactivation. Less inactivation was observed with 10% effluent than with 5% effluent at the same ozone concentration.

Snyder and Chang (1975) investigated the relative resistance of eight human enteric viruses to ozone and found that virus inactivation in filtered river water required approximately twice the contact time as that of ozone demand free water, to achieve the same inactivation. The pattern of resistance found in river water was the same as that in ozone demand free water.

Most of these investigations were comprised of laboratory scale experiments which do not represent field conditions. The majority of the studies used a relatively small volume of water and a high concentration of seed virus.

MATERIALS AND METHODS

MATERIALS AND METHODS

A. GENERATION OF OZONE

Ozone was generated by the silent electric discharge process in a Welsbach Laboratory Ozonator, Model T-408[1], using medical grade oxygen (dew point -85°F.) as a feed gas. The oxygen was passed through a bed of silica gel before entering the ozonator. Ozone produced was channeled out of the ozonator through Tygon tubing connected to a fritted glass gas sparger. The yield was determined by gas flow rate, gas pressure, input voltage and electrode cooling.

B. PREPARATION OF OZONE SOLUTIONS

Ozone demand free water (ODFW) was used for making ozone solutions. Double glass distilled water was saturated with ozone to remove ozone demanding organic materials. The residual ozone was heat dissipated by autoclaving at 121°C for 30 minutes.

Ozone solutions for inactivation experiments were prepared by bubbling ozone into ODFW to slightly greater than the required concentration. Excess ozone was removed by vigorously stirring the ozone solution on a magnetic stirring assembly.

1 Welsbach Ozone Corp., Philadelphia, Pennsylvania.

C. MEASUREMENT OF OZONE CONCENTRATIONS

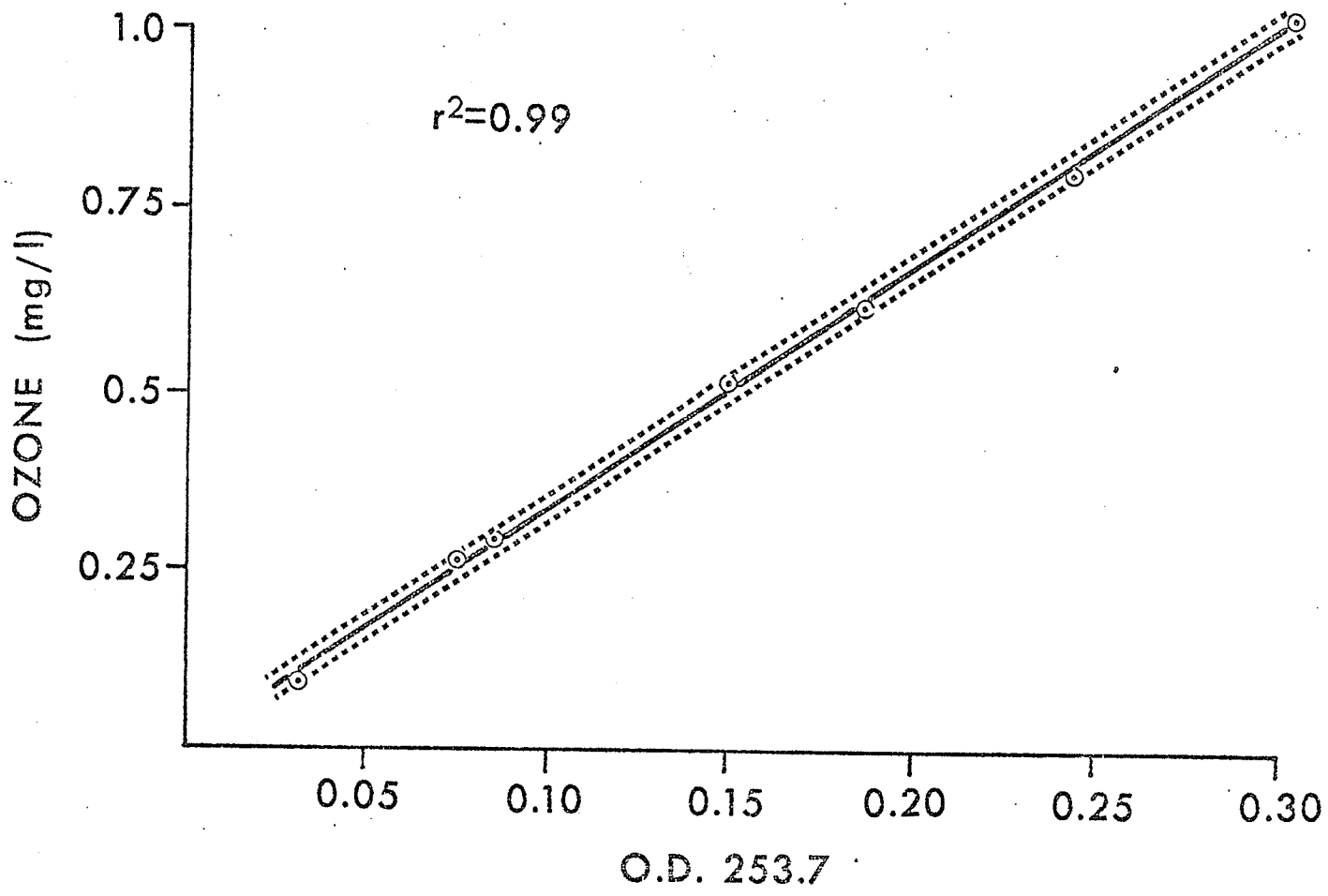
Solutions were measured for ozone concentration immediately prior to their use for experimental work. The absorbance of solutions at 253.7 nm was read in a Unicam SP500 spectrophotometer.[2] A 40 mm path length was used for all ozone concentrations. ODFW was used as a blank.

A calibration curve was prepared, which related ozone concentrations to absorbance at 253.7 nm (Figure 2). The ozone concentrations were determined by the iodometric method (Standard Methods, 1975), employing a Wallace and Tiernan Amperometric Titrator and reagents.[3] See Appendix A for the method.

2 Unicam Instruments Ltd., Cambridge, England.

3 Penwalt Corp., Wallace and Tiernan Division, Scarborough, Ontario.

Figure 2. Correlation between absorbance at 253.7 nm and ozone concentration. A 40 mm light path quartz cuvette was used. 95% confidence limits are shown.


1.0
0.75
0.5
0.25
OZONE (mg/l)
r^2=0.99
0.05
0.10
0.15
0.20
0.25
0.30
O.D. 253.7

D. CELL CULTURES

BGM cells were obtained from Dr. G. Berg, U. S. Environmental Protection Agency, Cincinnati, Ohio. HeLa cells were obtained from Dr. W. Wold, Institute for Molecular Virology, Saint Louis University School of Medicine, Saint Louis, Missouri.

Both cell lines were maintained in Blake bottles. Complete cell monolayers, 3-4 days old, were treated with 0.25% trypsin and 0.1% versene (Appendix B) in Hanks' balanced salt solution (HBSS, Appendix C) for approximately 15 minutes at 37°C, to detach and disperse the cells. The trypsin solution was neutralized with 5 ml of Eagle's minimal essential medium (MEM) containing 8% calf serum (Appendix D). Cell culture bottles were reseeded with approximately 2×10^7 cells and 90 ml of Eagle's MEM with 8% calf serum was added to each bottle. Phenol red indicator was deleted from the growth media and trypsin solutions.

E. VIRUS PRODUCTION

Table IV lists the low passage virus strains used.

a) Polioviruses, Coxsackieviruses, and Echoviruses

Falcon flasks[4] (75 cm^2) containing a complete monolayer of BGM cells (approximately 1.5×10^6 cells) were used to produce virus pools. Cells were rinsed twice with

4 Falcon Plastics, Los Angeles, California.

TABLE IV

Virus Strains

Source	Virus	Type	Strain	Previous Passage	Passage in this Laboratory (Cell Line)
1	Polio	1	gauze pad (GP) 103 (non vaccine)	Low passage	5 (BGM)
1	Polio	1	GP100 (vaccine)	Low passage	4 (BGM)
5	Polio	2	raw sludge (RS) 7778	2 (MK)	3 (BGM)
1	Polio	3	GP30	Low passage	4 (BGM)
1	Coxsackie	B3	septic tank (ST) 335	Low passage	4 (BGM)
5	Coxsackie	B4	GP1000	2 (MK)	4 (BGM)
1	Coxsackie	B5	RS 24	Low passage	4 (BGM)
5	Echo	11	clinical isolate (C)-2491	2 (RhMK)	3 (BGM)
4	Echo	12	Travis (ATCC VR-42)	2 (RhMK)	3 (BGM)
4	Echo	29	JV-10 (ATCC VR-227)	2 (RhMK)	3 (BGM)
3	Reo	1	Lang (ATCC VR-230)	1 (RhMK)	1 (RhMK) 5 (BGM)
6	Reo	3	CAN230	-	1 (RhMK) 5 (BGM)
3	Adeno	4	RI-67 (ATCC VR-4)	3 (KB) 5 (FL)	3 (HeLa)
5	Adeno	7	C-7312		3 (HeLa)
2	Adeno	8	C-45	3 (MK)	3 (HeLa)

1 Dr. S. A. Sattar, Department of Microbiology, University of Ottawa, Ottawa, Ontario.
2 Dr. R. S. Faulkner, Department of Microbiology, Victoria General Hospital, Halifax, Nova Scotia.
3 Center for Disease Control, Ottawa, Ontario.
4 Dr. O. Liu, Northeastern Water Hygiene Laboratory, Public Health Service, U. S. Department of Health, Education and Welfare, Narragansett, Rhode Island.
5 W. Stackiw, Manitoba Provincial Virology Laboratory.
6 Dr. J. Levy, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Maryland.

phosphate buffered saline (PBS, Appendix E) and infected with a virus suspension to give an input multiplicity of approximately 5, when the titer of the seed virus was known. The virus inoculum was made up to 3 ml with PBS and the virus was allowed to adsorb for 60 minutes at 37°C; flasks were rocked gently every 10-15 minutes. Eagle's MEM (2% calf serum, without phenol red) was added to make the volume up to 25 ml.

Usually within 20-48 hours, maximal cytopathic effect (CPE, approximately 90% of the cells off the flask) was observed and the virus was harvested. Virus fluids were centrifuged at 500g for 15 minutes to remove cell debris. Aliquots of this virus preparation were stored at 4°C and referred to as stock virus preparations.

Further purification consisted of centrifugation at 12,000g for 30 minutes and ultracentrifugation in a Beckman Ultracentrifuge Model L2-65B, using a type 42.1 fixed angle rotor. Twenty-two ml of virus suspension was aseptically transferred into each tube and the tubes were centrifuged at 100,000g for one hour. The supernatant was discarded and the pellet was washed twice in ODFW and then resuspended in 22 ml of ODFW. An hour at 4°C was allowed to soften the pellet and aid resuspension, which was accomplished by thorough mixing on a Vortex mixer. The virus suspensions were aliquoted into ozone demand free tubes and stored at 4°C. These virus suspensions were referred to as reference virus pools.

b) Reoviruses

Reovirus pools were prepared in the same manner as enterovirus pools, with the following differences. Virus pools were harvested before maximal CPE occurred, (usually 3-4 days) by three alternate cycles of freezing at -20°C and thawing at 37°C.

The reoviruses required one passage in primary rhesus monkey kidney cells[5] before a detectable CPE was observed on BGM cells.

c) Adenoviruses

Adenovirus pools were prepared in the same manner as enterovirus pools, with the following exceptions. Falcon flasks (75 cm^2) were seeded with approximately $4x10^6$ HeLa cells and allowed to form a complete monolayer for 24-48 hours. The virus inoculum was allowed to adsorb for 3 hours with gentle rocking of the flasks every 20 minutes.

The maintenance medium was changed after 3 days and the virus was harvested when maximal CPE was observed (3-7 days). Harvesting was accomplished by three cycles of alternate freezing at -20°C and thawing at room temperature.

5 Obtained from W. Stackiw,
Manitoba Provincial Virology Laboratory.

F. PLAQUE ASSAY PROCEDURE

a) Polioviruses, Coxsackieviruses, and Echoviruses

Plastic tissue culture dishes[6] (52 x 13 mm) were used for all the plaque assay experiments. The dishes were seeded with approximately $2x10^6$ BGM cells in 5 ml of an enriched growth medium (Appendix F). The cells were allowed to form a complete monolayer for 4-6 days in a 37°C humidified incubator in an atmosphere of 5% CO_2 in air.

The growth medium was then removed and the cell monolayers inoculated with the appropriate virus dilution (0.5 ml/plate) in a prepared diluent (Appendix G). The infected plates were incubated at 37°C for 1 hour and 5 ml of an agar overlay with 2% fetal calf serum (Appendix H) was carefully added to each plate. The plates were placed in a humidified CO_2 incubator at 37°C.

In 38-42 hours the plates were overlaid with 5 ml of a neutral red agar solution (Appendix I). They were then placed in the dark at room temperature and plaques were counted 12-24 hours later.

6 Lux Scientific Co., Newberry Park, California.

b) Reoviruses and Echovirus 11

The plaque assay procedure was the same as for the enteroviruses, with the following differences. BGM cell monolayers in petri dishes were used for virus inoculation 3-4 days after seeding the plates. Plates were overlaid with an agar overlay containing 8% fetal calf serum (Appendix H) and fed after 4 days with another 3 ml of the overlay. Neutral red agar was added 7 days after virus inoculation for reoviruses. For echovirus 11, neutral red was added 5 days post infection, as the greatest number of plaques were obtained at this time.

c) Adenoviruses

The plaque technique was the same as for the enteroviruses, with the following differences. Tissue culture dishes were seeded with approximately $1x10^6$ HeLa cells and allowed to form a complete monolayer for 1-2 days in a CO2 incubator at 37°C. After virus inoculation, plates were incubated for 90 minutes at 37°C in a CO_2 incubator and were rocked every 20 minutes. Five ml of an enriched agar overlay (Appendix D) was added to each plate. After 5 days incubation, the plates were fed with 5 ml of the same overlay and after 10 days they were fed with another 3 ml of the same overlay. Neutral red was added 15 days after virus inoculation.

G. BACTERIOPHAGE

f2 bacteriophage stock was obtained from Dr. N. Zinder, Rockefeller University, 66th Street and York Avenue, New York.

a) Preparation of Bacteriophage Stock

Bacteriophage stock was produced by the agar layer method (Swanstrom and Adams, 1951), using E. coli $K37^+$ as a host bacterium. Host bacteria were grown overnight on 1.5% agar tryptone slants and resuspended in broth before use.

Bacteriophage dilution (0.1 ml) was mixed with host bacterium (0.1 ml); this was added to 2.5 ml of melted tryptone agar (0.7%) which was mixed and poured over a 25 ml layer of 1.5% tryptone agar (Appendix K). Plates were incubated overnight at 37°C. Tryptone broth was added to those plates showing semi-confluent lysis. This was incubated at room temperature for 4 hours. The broth was removed and centrifuged at 12,000g for 30 minutes. The supernatant, containing approximately 10^{11} pfu/ml , was stored at 4° C.

b) Plaque Assay for Bacteriophage

One half ml of serial tenfold dilutions was added to a mixture of 2.5 ml melted tryptone agar (0.7%) and 0.1 ml host bacterium. See Appendix K for constituents of the diluent. This was quickly poured over a

25 ml layer of tryptone agar in a petri dish and allowed to harden. The assay plates were incubated for 24 hours at 37°C and plaques subsequently counted and expressed in terms of plaque forming units/ml (pfu/ml).

H. SEWAGE SAMPLES

Primary and secondary effluents were obtained from the City of Winnipeg, North End Treatment Plant which had the operating characteristics outlined in Appendix L.

a) Total Organic Carbon Analysis

Total carbon and inorganic carbon were estimated by the combustion-infrared method described in Standard Methods (1975). Total organic carbon was determined as the difference of total carbon and inorganic carbon. Samples were reproducible with a precision of less than ± 2.0 mg/l.

I. EXPERIMENTAL DESIGN

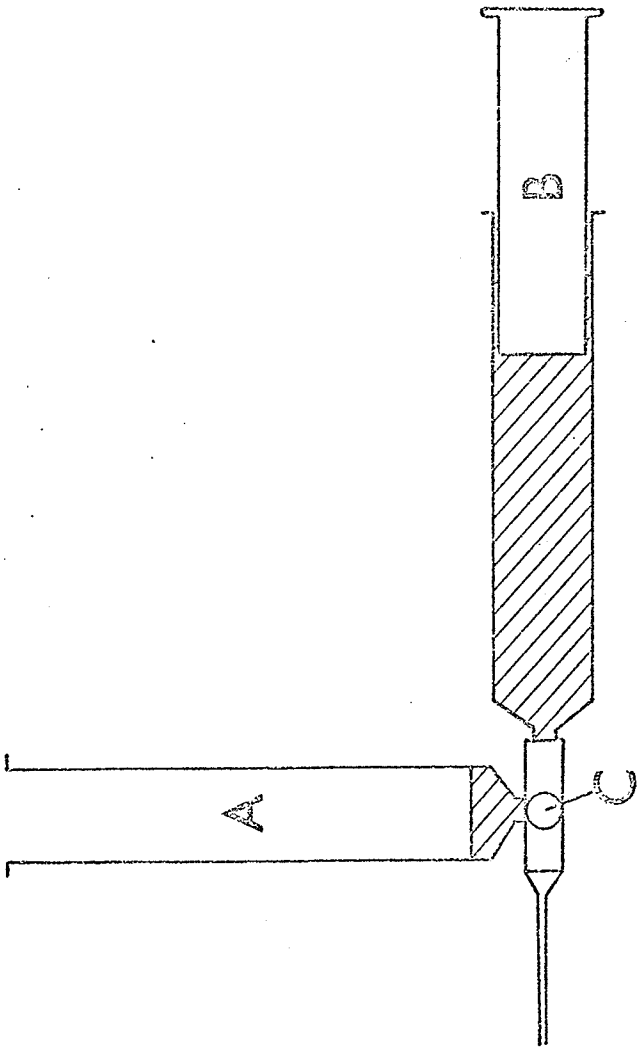
A standard system was devised to optimize the virus-ozone mixing process, which combined maximum accuracy with minimum losses. The most difficult step to overcome in working with ozone, which is a labile gas, was the preparation of and measurement of ozone solutions. To

overcome the problems of mass transfer, ozone was prepared and used as an aqueous solution in ozone demand free distilled water.

To interact the virus preparation with the ozone solution, an apparatus was constructed which consisted of two 10 ml glass syringes connected by a 3-way stopcock (Figure 3). The apparatus was made ozone-demand free by drawing a saturated ozone solution into syringe B and passing it into syringe A. The ozone solution was flushed out through the needle and each syringe was reciprocally washed with six changes of ozone demand free water.

Nine ml of ozonated ODFW was drawn into syringe B and one ml of virus suspension was pipetted into syringe A. The stopcock was then adjusted to allow mixing of the two solutions and the first 2 ml was discarded. One ml time samples were released into tubes containing one ml of neutralizer (Appendix G) and immediately mixed with a Vortex mixer. Neutralized samples were then serially diluted and assayed in duplicate, as outlined in the plaque technique. Composition of the virus diluent is given in Appendix G. Controls were done in the same manner, using ODFW in syringe B. All experiments were carried out at the temperature of the laminar flow safety cabinet ($26^{o}C \pm 2^{o}C$).

Figure 3. Ozone-virus reaction apparatus. Two 10 ml syringes are interconnected by a 3-way stopcock (C). Syringe A contains the virus suspension. Syringe B contains the ozonated ozone demand free water.


B
A
C

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENTAL PROCEDURES AND RESULTS

A. INTRODUCTION

The objective of this investigation was to determine the effectiveness of ozonation, to reduce viral infectivity. Initial experiments to investigate the kinetics of inactivation, showed that viral inactivation was very rapid and development of measurement techniques to monitor the reaction, were outside the scope of this investigation. With the aid of Bryn Easterbrook, University of Manitoba glassblower, an aspirator-static mixer device was constructed which allowed samples to be taken 200 m seconds after initiation of the reaction. However, this was still not a sufficiently short time to determine the time course of inactivation. It was therefore decided that, for all practical purposes, the inactivation reaction *per se* could be considered instantaneous.

A second objective was to determine the relative resistance, if any, of selected enteric viruses to ozonation, using fixed initial ozone concentrations. A system where ozone was added continuously to the reaction vessel was not used, since the rate limiting step would then be determined by mass transfer of ozone into solution (Nebel *et al.*, 1975).

Initially, inconsistencies in the extent of virus inactivations were observed. Therefore it was decided to investigate some of the factors influencing virus inactivation in the ozonation process, using poliovirus 1 (GP103) as a model. Poliovirus has frequently shown resistance to water disinfectants (Kelly and Sanderson, 1958; Clarke et al., 1968; Scarpino et al., 1975).

The first part of this study describes a standardization of the procedures used for comparing virus inactivations.

B. VIRUS INACTIVATIONS BY OZONE

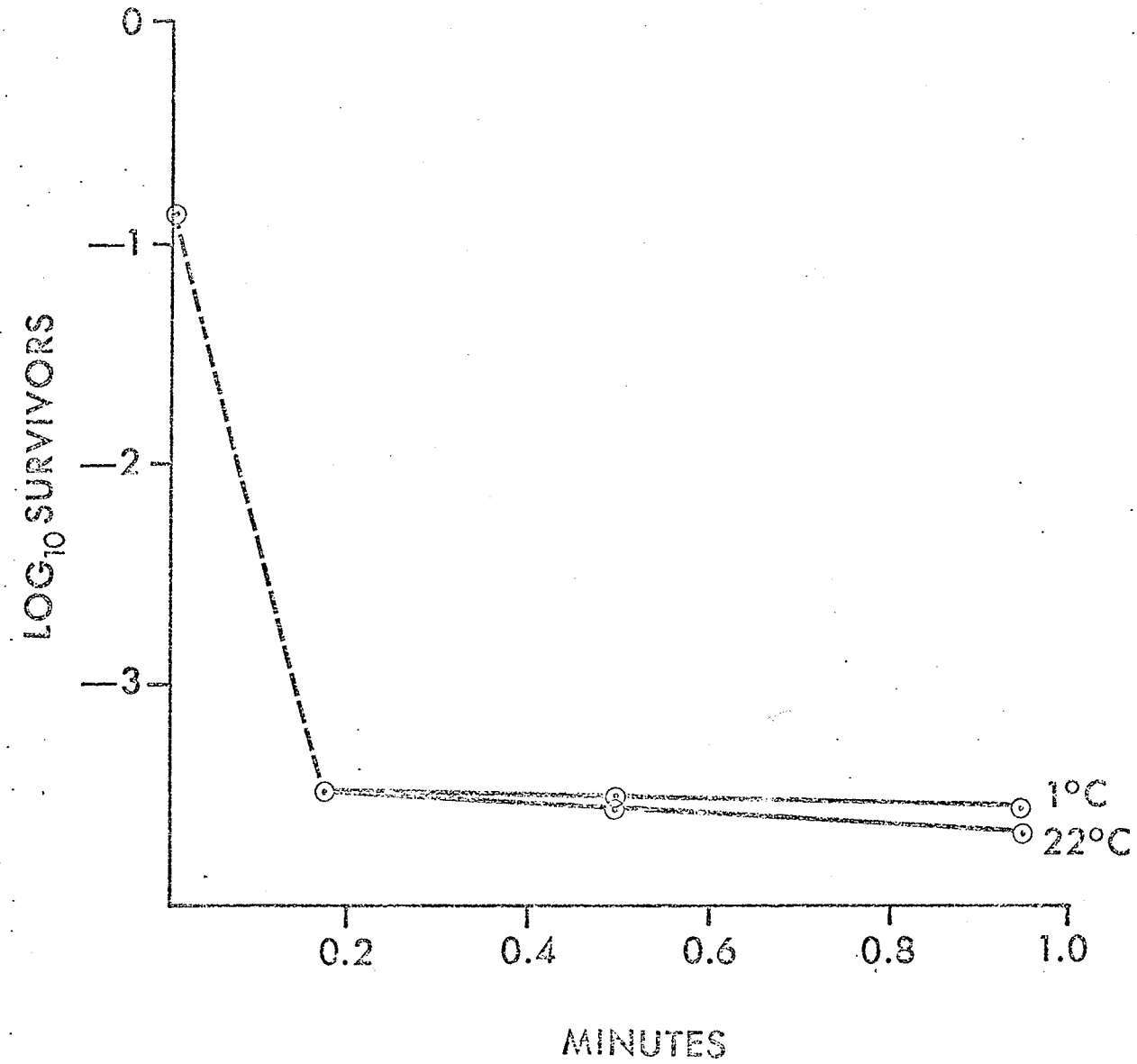
a) Two Stage Inactivation

Fifty-five experiments were performed, encompassing more than 500 separate virus inactivations. The single most important observation was the nature of the inactivation, as shown in Figure 4. Without exception, the pattern of virus inactivation, after ozone treatment, was a rapid loss of infectivity. This was sometimes followed by a gradually diminishing inactivation on prolonged exposure to ozone. Time samples were taken at 30 and 60 second intervals, for convenience, as preliminary experiments showed that the initial inactivation occurred in less than 200 m seconds.

When the reference virus preparations were inactivated, there was no further inactivation after 30 seconds. However, when stock virus preparations were inactivated, a gradually diminishing inactivation was observed between 30 and 60 seconds.

An experiment was performed, in which ozonated ODFW was added twice to the reaction vessel. Five ml of a poliovirus 1 suspension was inactivated by five ml of ozone

Figure 4. Typical Inactivation Curve of Poliovirus 1 (GP103) at 1°C and 22°C. Ozone dose was 0.1 mg/l.


0
1
2
3
LOG_{10} SURVIVORS
1°C
22°C
0.2
0.4
0.6
0.8
1.0
MINUTES

(0.1 mg/l) in the reaction vessel described in Materials and Methods. The first two ml were discarded. Three one ml timed samples were released up to two minutes. Another five ml of ozone (0.1 mg/l) was then added to the reaction vessel, and was immediately mixed. A one ml sample was taken and assayed by the plaque technique.

Figure 5 shows that the infective virus particles present at two minutes were rapidly inactivated by the second addition of ozone. The inactivation obtained was similar to that observed in the first stage of all inactivation curves.

b) Response of Poliovirus 1 (GP103) to Ozone Dose

The inactivation of poliovirus 1 (GP103) was exponentially related to ozone dose. Figure 6 depicts a typical response to ozone dose, of the reference poliovirus 1 preparation. Log_{10} inactivation is defined as the negative logarithm of the surviving fraction.

Log_{10} inactivation = $- Log_{10} (N/No)$ where

N = titer of surviving particles (pfu/ml)

No = initial titer of virus suspension (pfu/ml)

Since $- Log_{10} (N/No) = Log_{10} (No/N) = Log_{10} No - Log_{10} N$, the working relationship for determining the log_{10} inactivation is to subtract the log_{10} of the surviving pfu/ml from the log_{10} of the initial pfu/ml ($Log_{10} No - Log_{10} N$).

Figure 5 Inactivation Curve of Poliovirus 1 (GP103) by 0.1 mg/l Ozone added twice: at time 0 and 2.0 minutes after initiation of the reaction.

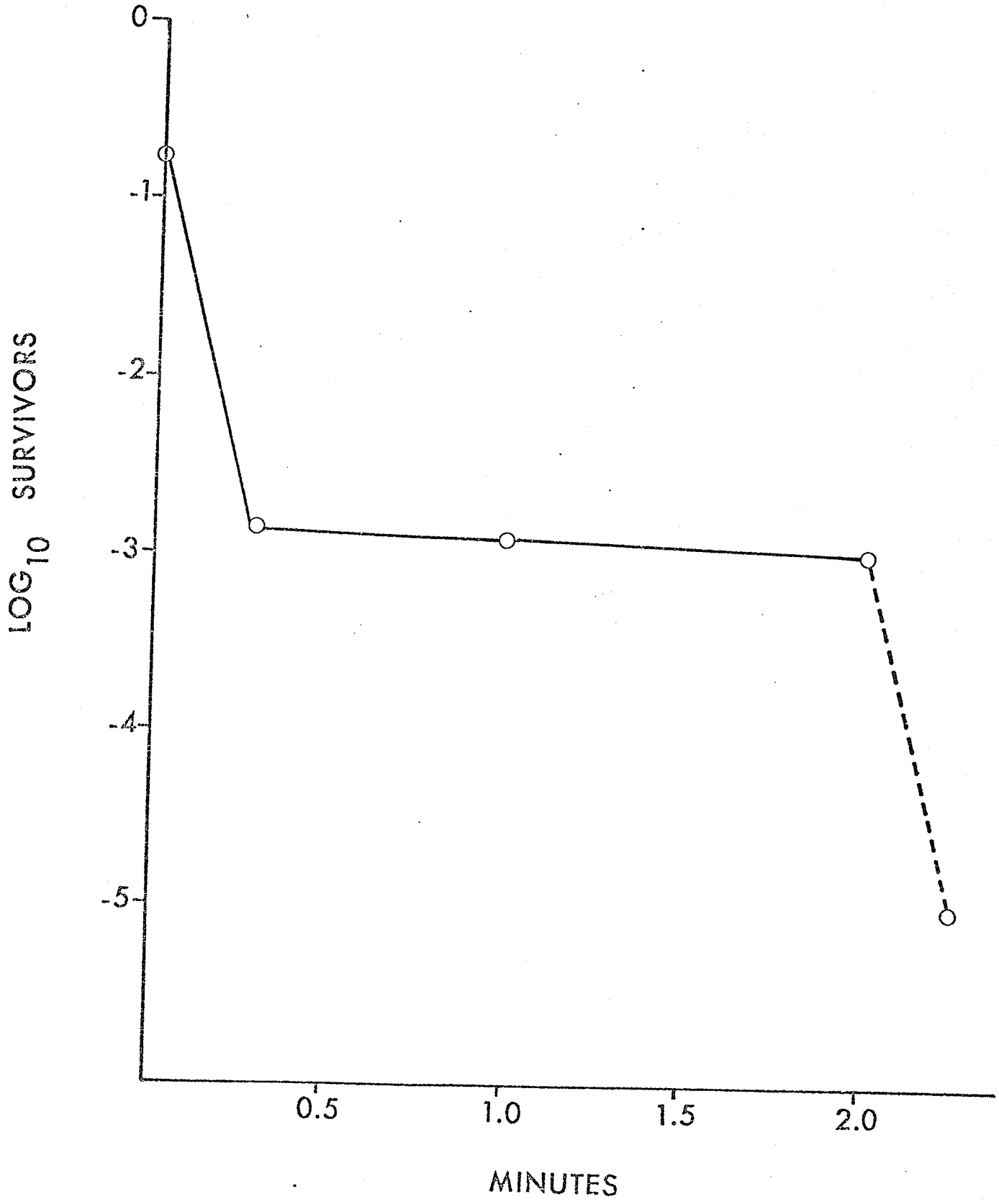
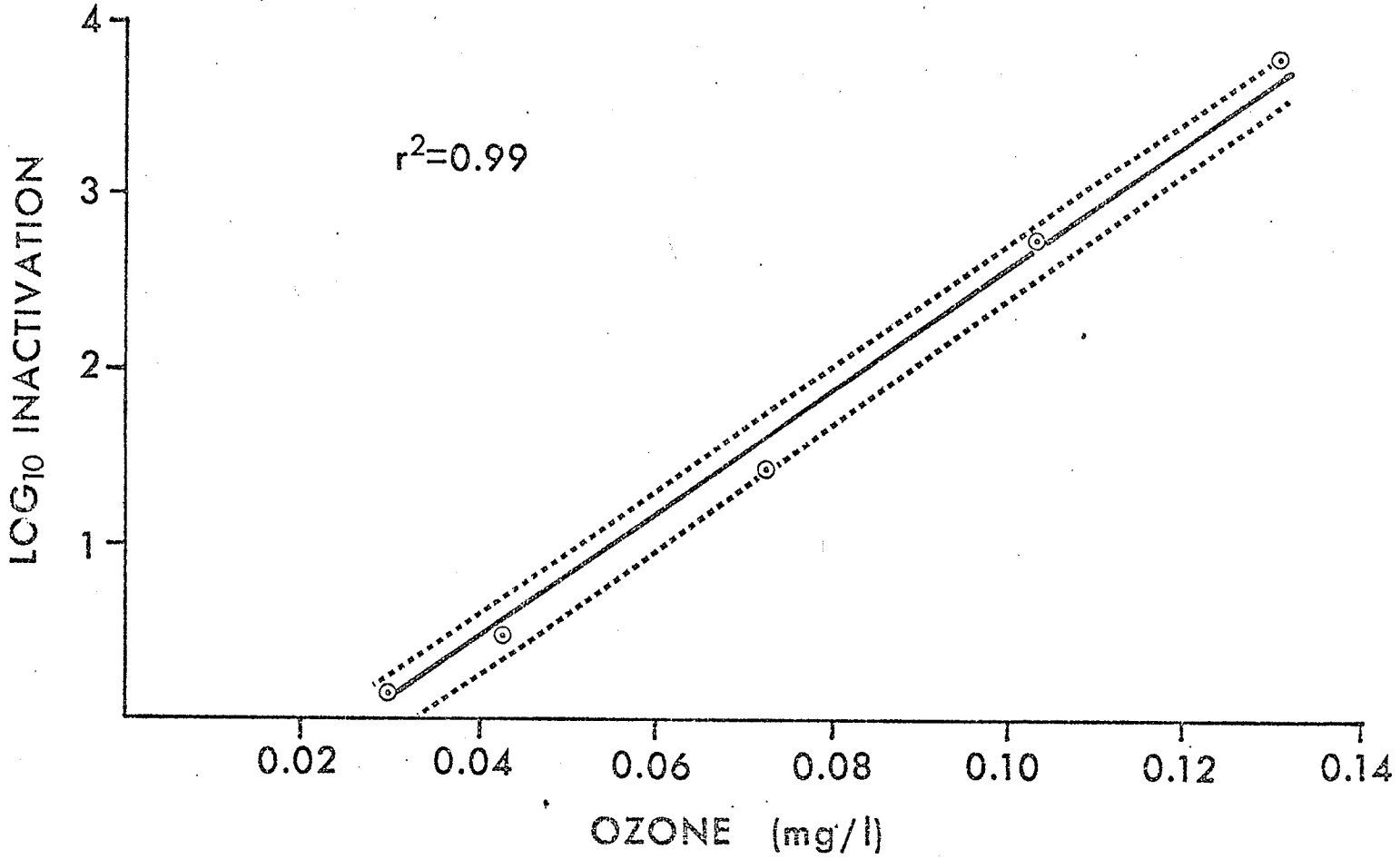

0
-1
-2
-3
-4
-5
LOG10 SURVIVORS
0.5
1.0
1.5
2.0
MINUTES

Figure 6 Correlation between Poliovirus 1 (GP103) Inactivation and Initial Ozone Dose, showing 95% confidence limits. Each point represents the mean of at least four separate inactivations observed in 30 seconds, with 2-4 replicates in each.


4
3
2
1
LCG10 INACTIVATION
r2=0.99
0.02
0.04
0.06
0.08
0.10
0.12
0.14
OZONE (mg/l)

This presentation provides a simple visual interpretation of the extent of inactivation and avoids the cumbersome designation of percent inactivation, where it is difficult to discern the important 10-fold difference between, for example, 99.90% and 99.99% inactivation. The term $\log_{10}$ inactivation is similar to the familiar pH term and mathematically can be treated with the same ease. For example, assume a situation where 1×10^{5} pfu/ml of virus is inactivated by addition of ozone, to a final 2×10^{2} pfu/ml. Then:

$$N/No = 2 \times 10^{2}/1 \times 10^{5} = 2 \times 10^{-3}$$

$$\text{Log}_{10} \text{ inactivation} = - \text{Log}_{10} (N/No) = \text{Log}_{10} (2 \times 10^{-3})$$

$$= - (\text{Log}_{10} 2 + \text{Log}_{10} 10^{-3})$$

$$= - (0.301 - 3) = 2.7$$

Similarly, $\log_{10}$ inactivation = Log_{10} No - Log_{10} N = 5.0 - 2.301 = 2.7.

It is important to note that in Figure 6, a small increase in ozone dose resulted in a very large inactivation. This shows that the extent of viral inactivatiion was exponentially related to the ozone dose. No inactivation occurred until the ozone concentration exceeded 0.03 mg/l.

C. FACTORS INFLUENCING VIRUS INACTIVATION BY OZONE AND THE STANDARDIZATION OF PROCEDURES FOR COMPARING VIRUS INACTIVATIONS

a) Ozone Decomposition

To determine the stability of ozone solutions, the change in concentration of various solutions was measured over a period of 15 minutes (Figure 7). Ozone solutions of different initial concentrations were prepared as outlined in Materials and Methods. The change in ozone concentration was measured at room temperature ($22^{o}C \pm 2^{o}C$) and at the pH of the ODFW (5.6 ± 0.3), which was used for all experiments.

Figure 7 shows that slightly less decomposition was observed in solutions of the lowest ozone concentrations. Because ozone solutions were unstable over a period of time, all ozone solutions were used immediately (within one minute) for virus inactivation experiments.

Although the effect of temperature on ozone decomposition was not determined, it was established that temperatures of 1^{o} and $22^{o}C$ had the same effect on virus inactivation (Figure 4).

Ozone decomposition was affected by the ionic content of the solution in which ozone was prepared. A 10 times concentrated solution of Dulbecco's phosphate buffer saline, without Ca^{2+} or Mg^{2+}, (Appendix E) was used as a source of inorganic ions and was diluted to various concentrations. Figure 8 shows the effect of

Figure 7. Decomposition of Ozone in Ozone Demand Free Water. Each point is the mean of two determinations.

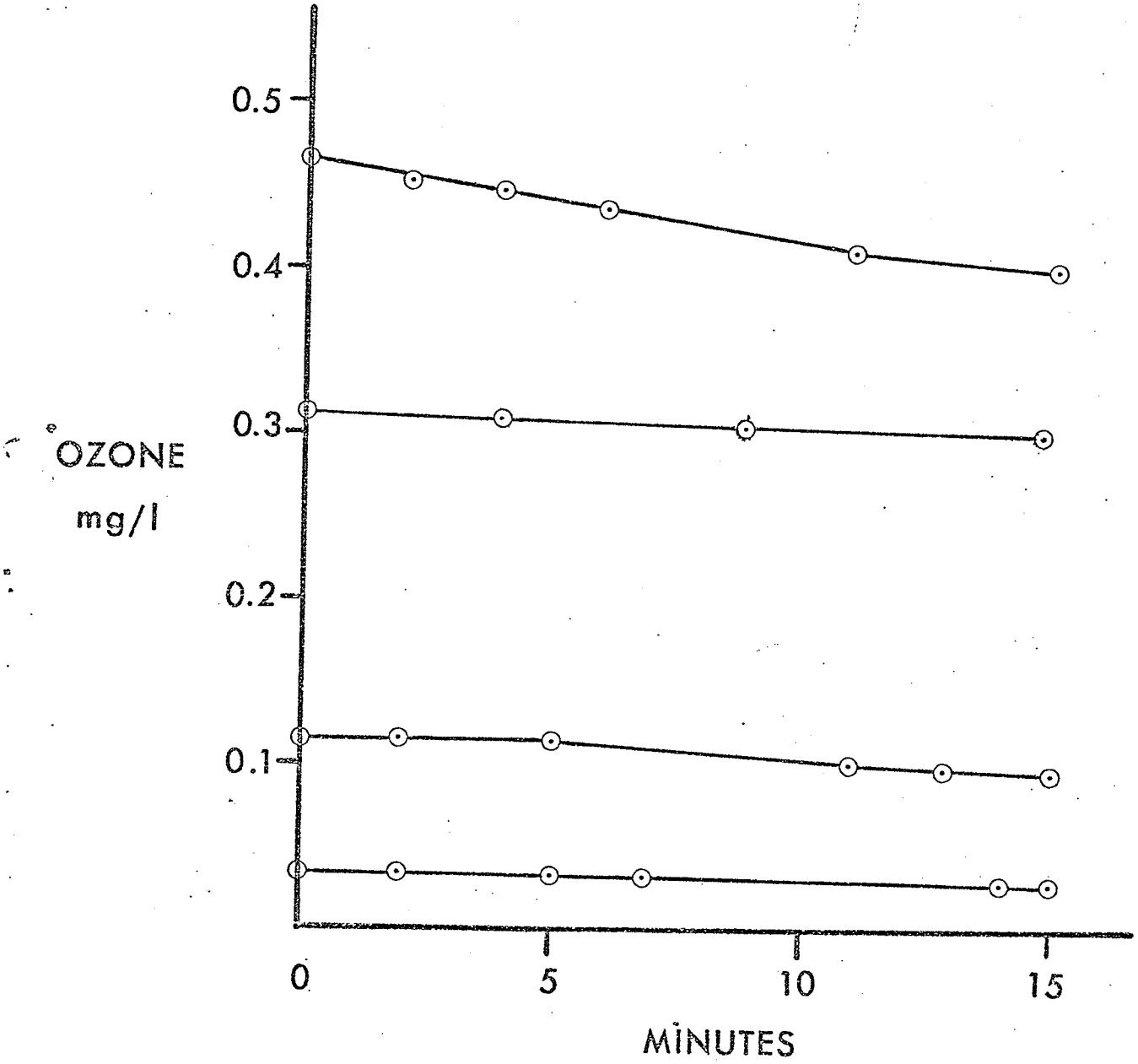
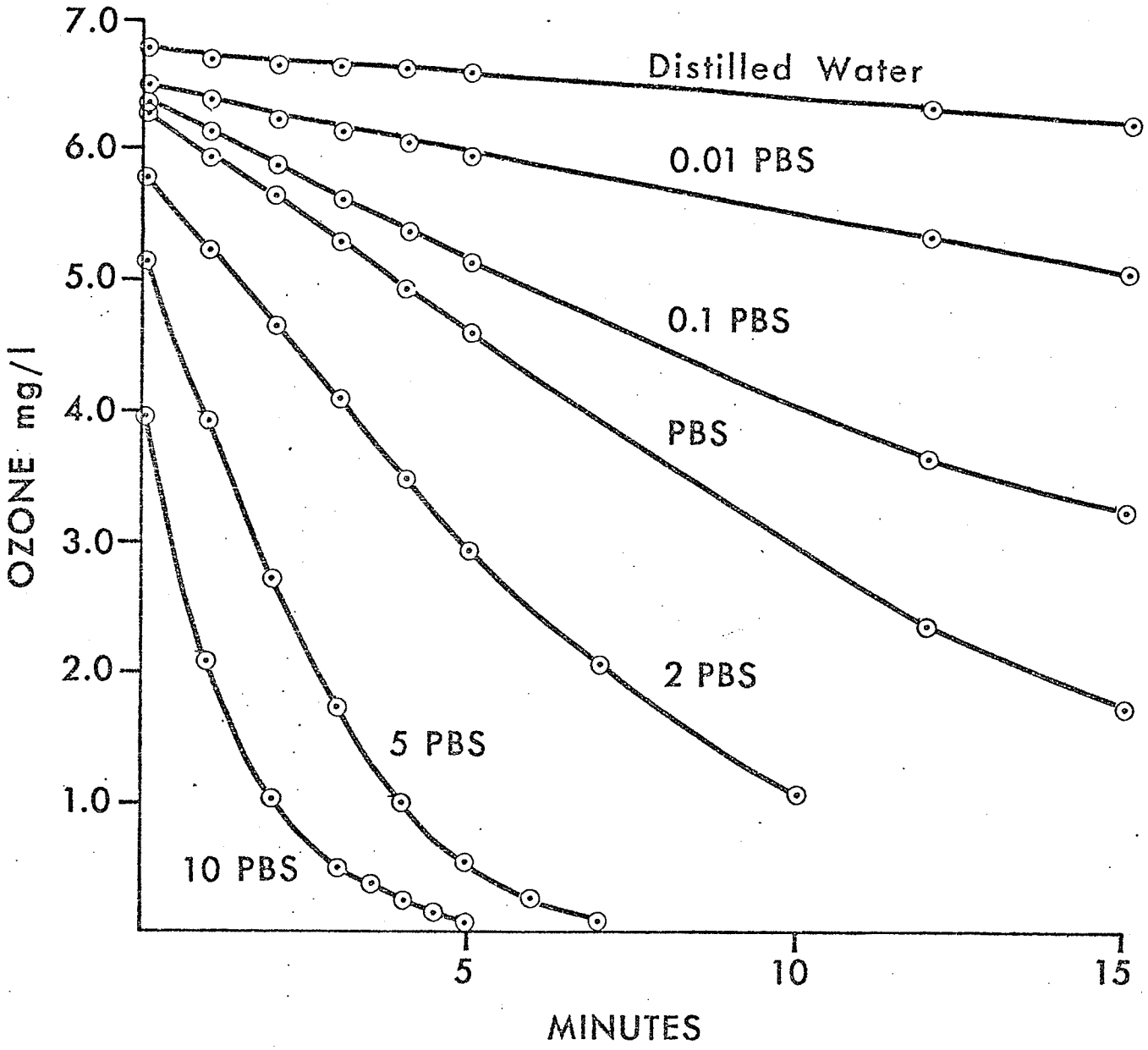

0.5
0.4
0.3
0.2
0.1
OZONE
mg/l
0
5
10
15
MINUTES

Figure 8. Decomposition of Ozone in Dulbecco's Phosphate Buffer Saline, without Ca^{2+} or Mg^{2+} (PBS).


7.0
6.0
5.0
4.0
3.0
2.0
1.0
OZONE mg/l
Distilled Water
0.01 PBS
0.1 PBS
PBS
2 PBS
5 PBS
10 PBS
5
10
15
MINUTES

inorganic ions on ozone stability. The rate of decomposition increased with increased concentrations of inorganic ions.

b) Cell Susceptibility Controls

Cell susceptibility controls (Wallbank, in preparation) were done for each virus, to test the ability of the neutralizer to stop the action of ozone. Cell susceptibility viruses (CSV) were prepared by diluting the reference virus preparations in ODFW, to the extent that another 1:9 dilution of the suspension would yield approximately 30-100 plaques/0.5 ml. The cell susceptibility titer was determined by a plaque assay of the CSV diluted 1:9 in neutralizer. The cell susceptibility control was determined by adding 1 ml of CSV to 4.5 ml neutralizer. Four and one-half ml of ozone was then added. This was immediately mixed with a Vortex mixer and assayed for plaques. The CSV titer results were then compared to the CSV control results. When control results were less than titer results, the Students' t test was used at the 95% confidence level. Results are shown in Table V. No significant difference was observed between the plaque numbers obtained in neutralizer and ozone and the plaque numbers obtained in neutralizer.

TABLE V

Cell Susceptibility Controls*

Virus	Plaque Numbers in Neutralizer	Plaque Numbers in Neutralizer and Ozone (0.4 mg/l)
Polio 1 (GP103)	59,61,70	63,70,71
Polio 1 (GP100)	33,24,25	34,38,31
Polio 2	70,76,87	81,80,95
Polio 3	44,47,38	40,51,45
Coxsackie B3	100,77,74	80,96,75
Coxsackie B4	27,30,30	28,30,29
Coxsackie B5	185,164	185,168
Echo 11	43,34	45,36
Echo 12	34,32	32,36
Echo 29	37,38	37,42
Reo 1	69,87	74,80
Reo 3	44,39	45,35
Adeno 4	87,99	85,90
Adeno 7	80,75	81,73
Adeno 8	26,16	18,28

* the plaque numbers in neutralizer and in neutralizer + ozone were compared by the Students' t test at the 95% level. No significant difference was found for any of the viruses

c) Effects of the Assay System on Determination of Poliovirus 1 Inactivation

To determine the effects of the assay system on determination of poliovirus 1 inactivation, three different cell lines were used for the assay of one inactivation. Table VI shows the $\log_{10}$ inactivation of poliovirus 1 by 0.5 mg/l ozone. Cell lines used for the assay were: BGM, Vero and HeLa. Although a difference in the susceptibility of the cell lines to poliovirus was observed, no difference was observed in the $\log_{10}$ inactivation detected. One cell line (BGM) was used for all virus assays except adenovirus.

d) Plaque Assay Reproducibility

To determine the reproducibility of the plaque assay used to quantitate virus inactivation, five separate assays were done on the reference poliovirus 1 preparation. These values, expressed in $\log_{10}$ and ranging from 6.0 to 6.3, were tested by analysis of variance. The F-value obtained experimentally was 0.25 compared to $F_{0.05}(4,9) = 3.63$. Thus there was no significant difference between the assays of poliovirus 1 (GP103) at $\alpha = 0.05$. This indicates that the plaque assay used to detect poliovirus 1 infectivity was reproducible.

TABLE VI

The Effects of the Assay System on Determination of Poliovirus 1 Inactivation by 0.5 mg/l Ozone

Cell Line used for Plaque Assay	Log_{10} Initial* pfu/ml	Log_{10} Inactivation* in 12 seconds ($\pm$ SD)
BGM	6.5	4.3 ± 0.2
Vero	6.3	4.3 ± 0.3
HeLa	5.8	4.2 ± 0.2

* calculated from three observations

e) Variations in the Inactivations obtained by Different Ozone Concentrations

Ozone solutions were prepared according to a standard procedure, as outlined in Materials and Methods and the reference poliovirus preparation was used throughout the course of all experiments. However, inconsistencies in the $\log_{10}$ inactivations were observed. These may be the result of variation in three areas:

1) variation within a single inactivation - expressed by differences in the replicate counts of a single trial.

2) variation within a single experiment - expressed by differences in the $\log_{10}$ inactivation observed in different trials in the same experiment.

3) variation in the $\log_{10}$ inactivation in trials from different experiments.

The most widely used measure of relative variation (magnitude of the variation is expressed relative to the mean) is percent coefficient of variation, CV, which is defined as:

$$CV = s/\bar{x} \times 100\% \quad \text{where}$$

s = standard deviation of a set of data and

$\bar{x}$ = mean

The percent coefficient of variation for poliovirus 1 inactivation in the three areas, at different ozone concentrations, is shown in Table VII.

TABLE VII

Variation in Poliovirus 1 Inactivations
Obtained with Different Initial Ozone Concentrations

Ozone Concentration (mg/l)	Percent Coefficient of Variation In a single inactivation (replicate plates)	N	In a single experiment (replicate inactivations)	N	In inactivations from different experiments	N
0.03	13.2	4	24.6	4	32.5	4
0.04	6.2	4	11.9	3	10.4	4
0.07	0.7	4	10.1	4	14.4	4
0.10	1.4	4	8.0	4	11.2	4
0.13	0.9	4	4.1	3	3.8	4

N = number of observations

The variation in a single inactivation was the least, ranging from 0.7 to 13.2%. It is important to note that the variation within a single experiment and the variation in different experiments was much larger than the variation within a trial. Also interesting, was the increase in variation in the inactivation as the initial ozone concentration decreased.

f) Influence of Ozone Demanding Substances on the Inactivation of Poliovirus 1

A greater variation, than shown in Table VII, was observed before the preparation of virus suspensions was standardized. Different treatments, used in the preparation of virus suspensions, were investigated for their effect on poliovirus 1 inactivation.

i) Centrifugation and Dilution

Table VIII shows the effect of centrifugation and dilution of a stock poliovirus preparation, on the extent of inactivation observed at 0.1 mg/l ozone. The reference suspension, to which all others are compared, was prepared and purified as outlined in Materials and Methods, and inactivated without prior dilution. The $\log_{10}$ inactivation in 30 seconds was 3.0.

To determine what purification steps were necessary to achieve the greatest inactivation that could be measured accurately, a stock poliovirus suspension was subjected to various centrifugation and dilution treatments. After each treatment, an aliquot

TABLE VIII

The Effects of Centrifugation and Dilution on Poliovirus 1 Inactivation by 0.1 mg/l Ozone

Centrifugation Treatment of Stock* Virus Preparation	Dilution	Total Organic Carbon (mg/l)	Log_{10} Inactivation in 30 seconds
none	-	1,450	0.1
	1/10	ND	0.5
	1/100	ND	2.0
12,000 g for 30 minutes	-	ND	0.4
12,000 g for 30 minutes, 100,000 g for 60 minutes; pellet resuspended in ODFW	-	26.3	3.0+
	1/10	ND	$\geq$ 4.8
12,000 g for 30 minutes, 100,000 g for 60 minutes; pellet resuspended in ODFW, 12,000 g for 30 minutes	-	ND	3.2
12,000 g for 30 minutes, 100,000 g for 60 minutes; pellet resuspended in ODFW, 12,000 g for 30 minutes, 100,000 g for 60 minutes; pellet resuspended in ODFW	-	ND	2.7

* virus was grown in MEM without phenol red and the suspension was centrifuged at 500 g for 15 minutes

ND not determined

\+ inactivation of reference virus preparation

of virus suspension was inactivated by 0.1 mg/l ozone and assayed in duplicate or triplicate.

Very little inactivation was obtained with the stock virus suspension. An analysis of the total organic carbon (TOC) content of the preparation revealed 1,450 mg/l. Although a large pellet was obtained when the stock preparation was centrifuged at 12,000 g for 30 minutes, the inactivation increased only slightly, from $\log_{10} = 0.1$ to $\log_{10} = 0.4$. However, when this preparation was then centrifuged at 100,000 g for 1 hour, the $\log_{10}$ inactivation increased to 3.0, concomitant with a decrease in TOC to 26.3 mg/l.

Further centrifugations were done to determine their effect on inactivation, compared to the reference preparation. No further increase in the inactivation was observed when the preparation was again centrifuged at 12,000 g and then at 100,000 g. Because there is some loss in virus titer at each purification step and no increase in the inactivation was obtained with further centrifugation, it was decided to routinely centrifuge stock virus preparations at 12,000 g for 30 minutes and 100,000 g for 60 minutes.

The effect of dilution of the virus suspensions, on inactivation by 0.1 mg/l ozone, is also shown in Table VIII. All dilutions were made in ODFW and in ozone demand free tubes.

When a 10- or 100-fold dilution of the stock virus suspension was made prior to inactivation, an increase in the $\log_{10}$ reduction was observed. Also, when the reference suspension was diluted 10-fold, an increase in the $\log_{10}$ inactivation from 3.0 to $\geq$ 4.8 was observed. However, it was not possible to dilute reference virus preparations, prior to inactivation, due to the decrease in virus titer. Meaningful inactivation data could not be obtained with virus pools of low titer.

ii) Freezing

Enterovirus suspensions are often stored at -70°C, where their infectivity is stable over a period of several years or more. They may also be stored at 4°C for a shorter period of time.

The $\log_{10}$ inactivation of the reference poliovirus preparation (stored at 4°C) was compared to the inactivation obtained with the same virus preparation stored at -70°C. Results are shown in Table IX.

Less inactivation ($\log_{10}$ = 2.2) was obtained with previously frozen virus than with a suspension stored at 4°C ($\log_{10}$ inactivation = 3.0). This effect had been observed earlier, when standard conditions for virus preparation and storage were not yet formulated. For example, the $\log_{10}$ reduction of coxsackievirus B4

TABLE IX

The Effects of Freezing, Chloroform and Phenol Red on Poliovirus 1 Inactivation by 0.1 mg/l Ozone

Virus Preparation	Treatments of Virus Preparation	Log_{10} Inactivation in 30 Seconds
virus grown in MEM without phenol red; centrifuged at 500 g for 15 minutes	-	0.1 *
	centrifuged at 12,000 g for 30 minutes and 100,000 g for 60 minutes, pellet resuspended in ODFW, stored at 4°C	3.0 +
	centrifuged at 12,000 g for 30 minutes and 100,000 g for 60 minutes, pellet resuspended in ODFW, stored at -70°C	2.2
	chloroform (final concentration = 5%) at 4°C for 60 minutes, centrifuged at 500 g for 15 minutes	0
	chloroform (final concentration = 5%) at 4°C for 60 minutes, centrifuged at 500 g for 15 minutes, 12,000 g for 30 minutes and 100,000 g for 60 minutes, pellet resuspended in ODFW	0.6
virus grown in MEM with phenol red; centrifuged at 500 g for 15 minutes	-	0.1
	centrifuged at 12,000 g for 30 minutes and 100,000 g for 60 minutes, pellet resuspended in ODFW	1.6

* inactivation obtained with stock virus preparation
\+ inactivation obtained with reference virus preparation

stored at $4^{o}C$ was 3.0, compared to 2.0 for previously frozen virus. Similarly a $\log_{10}$ inactivation of 4.0 was observed for coxsackievirus B5 ($4^{o}C$) compared to 2.0 for a previously frozen virus preparation. All virus preparations used to compare virus inactivations were stored at $4^{o}C$.

iii) Phenol red

The effect of phenol red on poliovirus 1 inactivation, by 0.1 mg/l ozone, is shown in Table IX. Two poliovirus preparations were made. Both were grown in MEM containing 2% calf serum, and phenol red in a final concentration of 0.002% was added to the media, for one of the preparations.

The effect of phenol red on inactivation is masked when the inactivations of stock virus preparations are compared. Centrifugation at 12,000 g for 30 minutes and 100,000 g for 60 minutes, with two washes of the pellet, did not eliminate all of the phenol red from the virus preparation. This is seen by the $\log_{10}$ reduction of 1.6, compared to 3.0 obtained with reference virus preparation (without phenol red).

Phenol red was deleted from the growth medium and trypsin solutions, for all cell lines which were used for virus preparation.

iv) Chloroform

Table IX shows the effect of chloroform treatment on poliovirus inactivation by 0.1 mg/l ozone.

An aliquot of stock poliovirus preparation (without phenol red) was treated with chloroform at a final concentration of 5%. This was shaken manually for five minutes and the chloroform allowed to settle out, for 1 hour at $4^{o}C$. The layer of virus preparation was then centrifuged at 500 g for 15 minutes.

The dramatic effect of chloroform treatment on inactivation was seen when the preparation was then centrifuged at 12,000 g for 30 minutes and 100,000 g for 60 minutes. The $\log_{10}$ reduction = 0.6 for the previously chloroformed preparation, compared to 3.0 for the reference virus preparation.

Chloroform was not used in the preparation of virus pools made for comparative purposes.

v) Resuspension Fluid for Virus Pellets

The method of preparation of ODFW, used for the resuspension of virus pellets obtained in ultracentrifugation, was found to effect virus inactivation. The resuspension water used to make the reference poliovirus pool was prepared in graduated media bottles with foil closures. Double glass distilled water was ozonated and autoclaved in the media bottle with foil closures.

When the stock poliovirus preparation was ultracentrifuged and resuspended in ODFW, which had been ozonated and autoclaved in media bottles with screw cap closures, the $\log_{10}$ inactivation was 1.8, compared to 3.0 for the reference suspension. This effect was observed only when the water was autoclaved after ozonation. When the ozone was allowed to dissipate with time in a 37^{o}C incubator, no difference was observed between water prepared in bottles with different closures.

vi) Sewage Effluents and Nutrient Broth

Aliquots of a stock poliovirus 1 preparation were centrifuged at 12,000 g and 100,000 g as outlined in Materials and Methods. After washing the pellets twice in ODFW, they were resuspended in various concentrations of sewage effluents and nutrient broth. Primary and secondary sewage effluents were separately mixed with ODFW to give 100, 50 and 25% effluent solutions. These were used to resuspend the virus pellets. One virus pellet was resuspended in ODFW as a control.

Due to the nature of the reaction apparatus, in which one ml of virus suspension was mixed with 9 ml ozonated ODFW, the final concentrations of effluent used were: 10%, 5%, 2.5% and 0%. After inactivation and neutralization, all samples were filtered through "0.45μ"

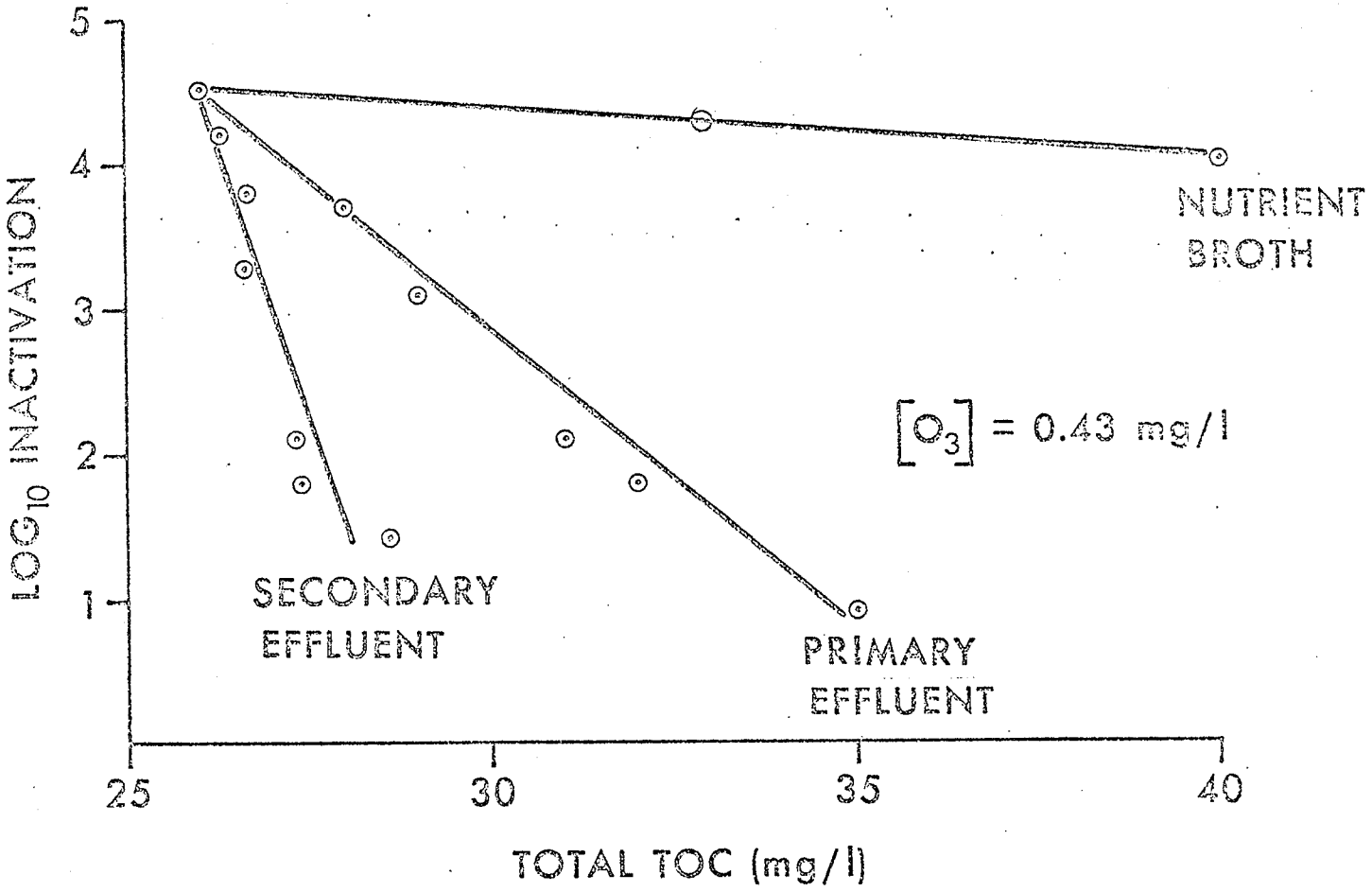
Gellman filters. Controls done to determine the effect of filtering, showed no loss of pfu/ml when "0.45μ" Gellman filters were used.

Controls done to determine the effect of sewage effluents also showed no loss of pfu/ml, provided the samples were filtered and diluted at least 1:1 in neutralizer. However, undiluted effluent samples which were not filtered, yielded microbial contamination and cell toxicity, so that plaques were not detected after virus addition. No indigenous viruses were detected in the primary and secondary effluent samples used.

The effect of primary and secondary effluents on poliovirus inactivation, by 0.43 mg/l ozone, is shown in Figure 9. A concentration of 0.43 mg/l ozone was used, as it gave the greatest inactivation of the reference poliovirus preparation which could be measured accurately. Figure 9 encompasses the results of several experiments using sewage effluents with different TOC analysis.

Greater inactivation was observed when the virus was suspended in secondary effluent, than when suspended in primary effluent. Also, the TOC of secondary effluent is always lower than that of primary effluent (Appendix L). However, the TOC of secondary effluent in this investigation was approximately five times less than the TOC of primary effluent. The $\log_{10}$ inactivation of poliovirus in

Figure 9. The Inactivation of Poliovirus 1 (GP103) suspended in Primary and Secondary Sewage Effluents and Nutrient Broth. The TOC of the virus suspension (0% effluent) was 26 mg/l.


LOG10 INACTIVATION
5
4
3
2
1
NUTRIENT BROTH
[O3] = 0.43 mg/l
SECONDARY EFFLUENT
PRIMARY EFFLUENT
25
30
35
40
TOTAL TOC (mg/l)

secondary effluent was not five times greater than that in primary effluent, as shown in Figure 9. In each case, the contribution to TOC of the virus suspension was 26 mg/l. The TOC shown in Figure 9 is the combined TOC contributed from the virus preparation and suspension medium.

The most striking observation was that the very small contribution of TOC from secondary effluent had a dramatic effect on virus inactivation, more so than the TOC derived from primary effluent or nutrient broth. In general, there was a lesser effect of the TOC from nutrient broth, than the TOC derived from sewage effluents.

D. RELATIVE OZONE RESISTANCE OF SELECTED ENTERIC VIRUSES

a) Introduction

Early in this investigation it became apparent that efforts to determine the relative resistance of a number of viruses to ozonation were fraught with much inconsistency. Extreme care was then taken to prepare all virus suspensions by a standard method as outlined in Materials and Methods, for the reference virus pools. All of the virus pools used for comparisons in this investigation were made on the same day, to avoid differences in the cell system, medium and ODFW used for resuspension. Phenol red was deleted from all laboratory solutions. None of the preparations were treated with chloroform and all viruses were resuspended, after ultracentrifugation, in ODFW prepared in media bottles with foil enclosures. Virus suspensions were stored at 4° C, and used for inactivations without prior dilution.

b) Separate Virus Inactivations

Apparent differences in virus sensitivity to ozone were observed, even when the virus suspensions were prepared under rigidly controlled conditions. Table X shows the $\log_{10}$ inactivation of 15 different viruses by 0.1 mg/l ozone, in 30 seconds. For more than half of the viruses $\geq$ 3.0 logs of inactivation were observed. The virus titer listed in the table represents the pfu/ml of virus present in the syringe, after mixing with ODFW.

TABLE X

The Inactivation of 15 Enteric Viruses by 0.1 mg/l Ozone

Virus	Initial Titer (pfu/ml)	Total Organic Carbon (mg/l)	N*	Log_{10} Inactivation ± SD
POLIO 1 (GP103)	1.4×10^6	26.3	12	3.1 ± 0.5
POLIO 2 (7778)	2.5×10^5	25.8	13	3.2 ± 0.2
POLIO 3 (GP30)	2.0×10^5	25.0	10	3.6 ± 0.1
POLIO 1 (GP100)	9.8×10^4	28.8	8	3.5 ± 0.3
COXSACKIE B3	1.1×10^5	--	6	2.2 ± 0.2
COXSACKIE B4	2.0×10^5	33.8	4	2.7 ± 0.1
COXSACKIE B5	1.5×10^5	33.8	3	1.8 ± 0.1
ADENO 4	2.2×10^4	45.0	6	1.5 ± 0.4
ADENO 7	6.5×10^3	36.3	4	1.7 ± 0.2
ADENO 8	1.4×10^6	45.6	3	1.5 ± 0.1
ECHO 11	1.8×10^4	47.0	8	1.9 ± 0.1
ECHO 12	2.4×10^5	28.8	7	3.7 ± 0.1
ECHO 29	1.1×10^5	25.5	8	3.6 ± 0.3
REO 1	2.4×10^5	28.3	12	3.3 ± 0.3
REO 3	4.0×10^5	27.5	19	3.5 ± 0.3

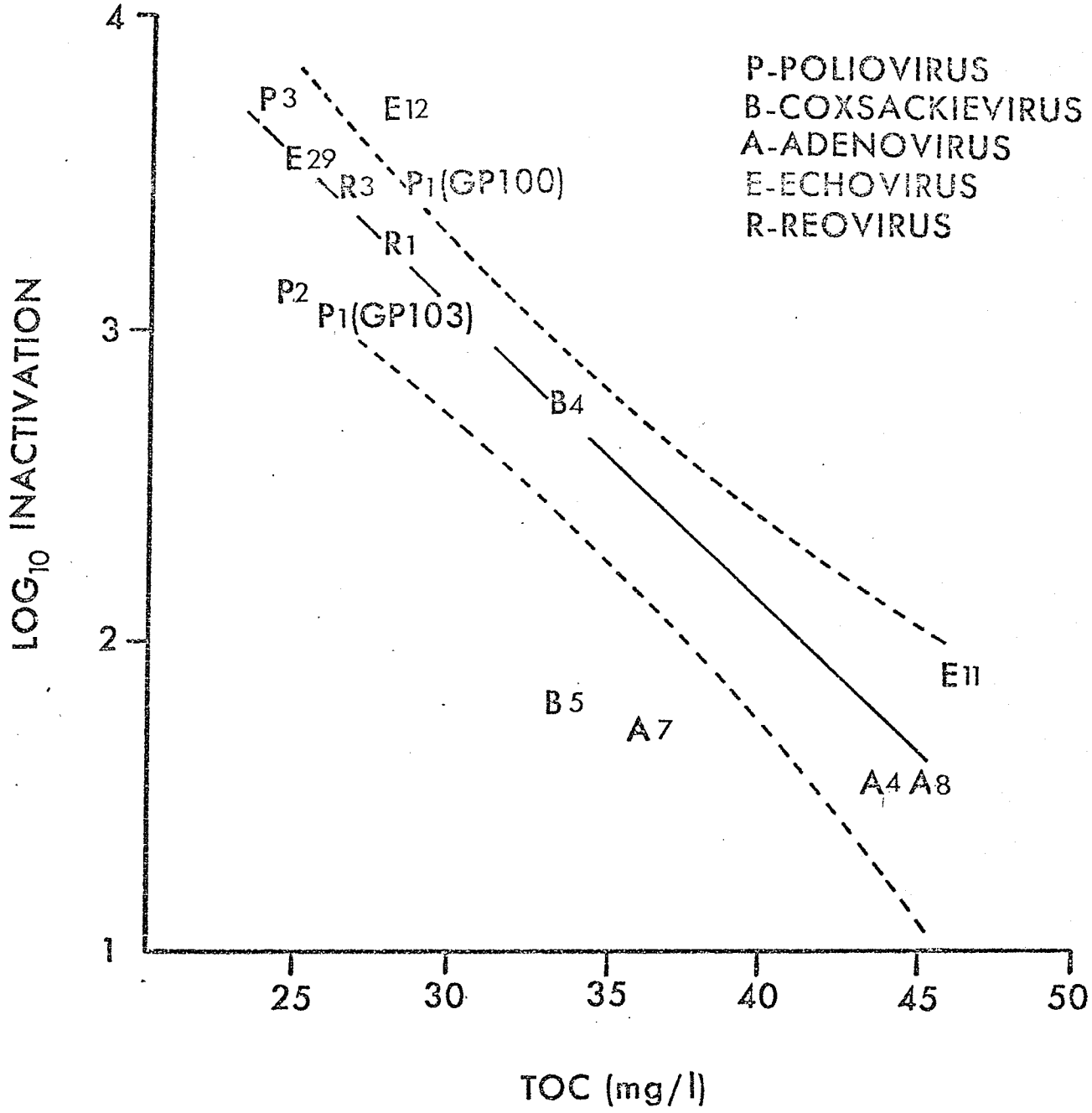
* N - number of observations for virus inactivations

A comparison of the $\log_{10}$ inactivations for 14 of the viruses (excluding coxsackievirus B3 for which there is no measurement of TOC) is highly significant at $\alpha = 0.05$ ($F_{(13,116)} = 64.1$). Thus a statistically significant difference exists between the inactivations of 14 selected enteric viruses by ozone.

The virus suspensions were also compared on the basis of TOC content. As seen in Figure 10, the differences observed in virus inactivations are readily correlated to the different TOC of virus preparations ($r^2 = 0.77$). Even though these suspensions were all prepared and purified in a similar manner, the TOC content ranged from 25 to 47 mg/l. In general, the virus preparations with the lowest TOC (25 to 28.8 mg/l) gave the greatest inactivations (3.1 to 3.7). Virus preparations with higher TOC (33.8 to 47 mg/l) gave the lowest inactivations ($\log_{10}$ = 1.5 to 2.7).

The inactivations were then compared by analysis of variance after adjusting for the differences in TOC. However the $\log_{10}$ inactivations were still significantly different at $\alpha = 0.05$ ($F_{(13, 116)} = 12.77$). Thus the differences in the extent of inactivations, although correlated to TOC, cannot be solely explained by differences in TOC of the preparations.

Figure 10. Correlation between virus inactivation by ozone and total organic carbon (TOC) in the virus suspension. Ozone concentration is 0.1 mg/l. 95% confidence limits are shown. $r = -0.88$, $r^2 = 0.77$.


P-POLIOVIRUS
B-COXSACKIEVIRUS
A-ADENOVIRUS
E-ECHOVIRUS
R-REOVIRUS
P3
E12
E29
R3
P1(GP100)
R1
P2
P1(GP103)
B4
E11
B5
A7
A4 A8
4
3
2
1
25
30
35
40
45
50
LOG10 INACTIVATION
TOC (mg/l)

Several inactivations of viruses which had similar TOC contents were compared. The group of three adenoviruses and three echoviruses could not be compared due to large differences in TOC. For comparisons of small groups of virus inactivations a mean square within = 0.072 and 116 degrees of freedom were used from the analysis of variance of all viruses, so as not to lose power in the comparison. The comparisons made were: echoviruses 12 and 29 ($F(1,14) = 0.54$), coxsackieviruses B4 and B5 ($F(1,6) = 15.87$), reoviruses 1 and 3 ($F(1,30) = 4.86$), polioviruses 1 (GP100), 1 (GP103), 2 and 3 ($F(3, 42) = 8.88$) and polioviruses 1(GP100) and 1 (GP103) ($F(1,19) = 8.16$). At $\alpha = 0.05$, using 116 degrees of freedom in the denominator for analysis of variance, all viruses compared except for echoviruses 12 and 29 were significantly different.

c) Inactivation of Mixed Virus Suspensions

An attempt was made to inactivate different viruses under similar conditions, by ozonating a mixed suspension of viruses and then separating them by applying neutralizing antisera.[7] Poliovirus 1 (GP103), 2 and 3 were mixed in equal volumes in an ozone demand free tube and allowed to sit for several hours. The inactivation was performed using one ml of the mixed suspension and nine ml of ozonated ODFW. After neutralization of the ozone, the sample was divided into three aliquots, each of which was mixed with an equal volume of a mixture of two neutralizing antisera. Controls were done to determine the extent of cross neutralization and none was observed with the antisera used. Results are shown in Table XI.

No difference was observed between the $\log_{10}$ inactivation obtained for poliovirus 1, 2 and 3, inactivated in combined suspension. However, the application of this technique to other viruses was not universally successful, due mainly to the breakthrough of "neutralized" virus. Neutralizing antiserum could not be obtained in sufficient titer to prevent this.

d) Inactivation of Viruses in Combined Suspension with f2 Bacteriophage

Efforts were made to determine relative virus inactivation by mixing each virus with diluted f2 bacteriophage (4.8 mg/l TOC). A natural separation of the two

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TABLE XI

Inactivation of Poliovirus 1, 2 and 3 in Combined Suspension by 0.1 mg/l Ozone*

Virus Determined	Initial Titer (pfu/ml)	Neutralizing Antisera Added	Final Titer (pfu/ml)	Log_{10} Inactivation
Polio 1	8.6×10^5	Polio 2, Polio 3	7.3×10^2	3.07
		Polio 1	0	
Polio 2	3.6×10^5	Polio 1, Polio 3	3.04×10^2	3.01
		Polio 2	0	
Polio 3	2.6×10^5	Polio 1, Polio 2	2.2×10^2	3.07
		Polio 3	0	

* the individual viruses were determined by the use of neutralizing antisera

was possible after ozonation, since enteric viruses and f2 bacteriophage have different host susceptibilities. By mixing each of the viruses with f2 bacteriophage, the TOC of the combined preparation was effectively lowered. It was expected that virus inactivation, by 0.1 mg/l ozone, would therefore increase and the standard phage preparation would be an indicator for virus suspension purity.

Table XII displays the results of this comparison for 14 different viruses. In all but two cases, the bacteriophage was inactivated to a greater extent than the viruses, and again there is some correlation between TOC of the preparation and extent of virus inactivation. However, all of the viruses did not show an increase in inactivation when mixed with f2 bacteriophage. Rather, the seven viruses which had previously shown the least inactivation ($\log_{10}$ = 1.5 to 2.7) when ozonated separately, showed an increase in inactivation when in combination with the phage. The seven viruses which had previously shown the greatest inactivation ($\log_{10}$ = 3.1 to 3.7) when ozonated separately, decreased in inactivation when ozonated in combination with f2 phage.

The inactivation of f2 phage, by 0.1 mg/l ozone, was in all cases lower when in combination with viruses ($\log_{10}$ inactivation = 2.9 to 4.1) than when inactivated alone ($\log_{10}$ inactivation = 4.7).

TABLE XII

Inactivation of Viruses in Combination with f2 Bacteriophage by 0.1 mg/l Ozone

Virus	Total Organic Carbon (mg/l)	$\log_{10}$ Inactivation		
		Virus ± SD	Phage ± SD	Total
Polio 1 (GP103)	15.5	3.0 ± 0.4	3.4 ± 0.4	3.2
Polio 2 (7778)	15.3	2.6 ± 0.2	3.5 ± 0.5	3.1
Polio 3 (GP30)	14.9	3.1 ± 0.2	3.5 ± 0.3	3.3
Polio 1 (GP100)	16.8	2.4 ± 0.2	2.9 ± 0.1	2.7
Coxsackie B3	--	2.6 ± 0.7	4.1 ± 0.5	3.3
Coxsackie B4	19.3	3.9 ± 0.7	3.3 ± 0.4	3.6
Coxsackie B5	19.3	3.3 ± 0.2	3.2 ± 0.4	3.3
Adeno 4	24.9	2.8 ± 0.3	3.0 ± 0.4	2.9
Adeno 7	20.5	1.7 ± 0.3	3.9 ± 1.1	2.8
Adeno 8	25.2	2.6 ± 0.4	3.2 ± 0.7	2.9
Echo 11	25.9	2.3 ± 0.5	3.1 ± 0.1	2.7
Echo 12	16.8	3.2 ± 0.3	3.3 ± 0.5	3.3
Reo 1	16.5	2.5 ± 0.1	3.9 ± 0.5	3.2
Reo 3	16.2	2.0 ± 0.2	3.4 ± 0.4	2.7

DISCUSSION

DISCUSSION

A. INTRODUCTION

The germicidal properties of ozone were studied, to determine the effectiveness of ozonation for inactivation of a selected array of enteric viruses. The influence of several factors on viral inactivation was investigated. A standard procedure for the preparation of virus pools and ozone solutions was then defined, in order to compare virus inactivations uniformly.

B. VIRUS INACTIVATIONS BY OZONE

A two stage inactivation curve was observed for all the viruses studied. Katzenelson et al. (1974) observed a two stage inactivation for poliovirus, T2 bacteriophage and E. coli.

The tailing effect suggests the existence of a minority component of virus particles which are more resistant than the majority component and are not observed until they become a significant fraction of the survivors. Many indeterminate factors might extend the longevity of some of the virus particles being inactivated (Gard, 1960). Katzenelson et al. (1974) postulated that the plateau stage was due to viral aggregation, since it could be eliminated by ultrasonication. Other reasons for apparent virus heterogeneity may be, adsorption of some of the

particles to the walls of the vessel or, encapsulation of some of the virus particles in organic material. Another reason, when the ozone concentration is not maintained, may be the consumption of all the active disinfectant, so that further inactivation is impossible. The results of this investigation suggest the latter possibility, since the plateau effect was obliterated by the addition of more ozone.

The rate of the inactivation reaction could not be determined. Preliminary experiments showed that the reaction occurs in less than 200 m seconds. Other groups (Katzenelson et al., 1974; Burleson et al., 1975) were also unable to determine the dose response which may occur in the first part of the inactivation curve. For all practical purposes, considering that mass transfer of ozone gas into solution requires a considerably greater time than that observed for virus inactivation (McCarthy and Smith, 1974), the inactivation process may be considered instantaneous.

There are several major differences between the inactivation of poliovirus observed in this investigation and the inactivations found in other studies. In this investigation, the extent of virus inactivation was found to increase with increased contact time for the stock poliovirus preparation. However, for the

purified poliovirus, there was no further inactivation after the initial reaction observed at 30 seconds.

Katzenelson et al. (1974) observed an effect of contact time for purified poliovirus. After an initial inactivation of 2 to 3 logs, another 1 to 2 logs of virus was inactivated up to 60 seconds. A possible explanation for the diminishing inactivation described in some two stage inactivation curves, is the appearance of an intermediate oxidation species, triggered by the virus or organic contaminants introduced with the virus preparation (Katzenelson et al., 1974).

In this study, the inactivation of poliovirus was exponentially related to the ozone dose for all concentrations used. The dose response curve shows that no inactivation occurred until the ozone concentration exceeded 0.03 mg/l. This value is similar to the threshold levels of ozone concentration observed by other investigators. Interesting to note is the difference in the values at which the "threshold effect" has occurred in other studies. Katzenelson et al. (1974) observed an effect at 0.2 mg/l, Coin et al. (1964) at 0.7 mg/l and Majumdar et al. (1973;1974) at 1.0 mg/l. Presumably, the threshold effect is due to the finite ozone demand of some trace contaminants of the virus suspension which must be overcome before the inactivation proceeds (Broadwater et al., 1973). Hoigné and Bader (1975)

have indicated that during ozonation, the soluble constituents of the suspension will react with ozone preferentially than with dispersed particles such as viruses. If this effect is truly caused by ozone demanding substances in the virus preparation, then the ozone concentration at which it is observed, may indicate the level of competing material in the preparation.

An increase in poliovirus inactivation was observed with increasing ozone concentrations above 0.03 mg/l. This is in contrast to Katzenelson et al. (1974), who found little increase in inactivation with increasing ozone concentrations above the threshold level. They correlated the lack of dose response above 0.2 mg/l ozone with a levelling off of the redox potentials of ozone solutions, above 0.2 mg/l.

In general, the extent of poliovirus inactivation in this study was greater than that observed in any of the other studies, for the same ozone concentrations.

C. FACTORS INFLUENCING VIRUS INACTIVATION BY OZONE AND STANDARDIZATION OF PROCEDURES FOR COMPARISONS OF VIRUS INACTIVATIONS

Conditions for this investigation were chosen to be mutually favorable to ozone stability and virus stability. Stability of ozone is critically determined by the suspending menstruum and physical conditions. In Freon 12 and at - 78°C, ozone is stable for months (Murray, 1969), but under conditions of practical use, decomposition is affected by temperature, pH and the presence of organic matter and inorganic ions.

Ozone demand-free double glass distilled water (pH 5.6 and 22°C), was found to be the most appropriate suspending fluid for making ozone solutions. Little change in ozone concentrations was observed in 15 minutes, when ozone solutions were prepared under these conditions. All ozone solutions were used within one minute, for virus inactivation, in which time negligible decomposition had occurred.

It is uncertain what major effects on the virus preparation were exerted by suspension in ODFW. Because the enteric viruses are acid stable (Hamparian et al., 1963; Rosen, 1965), it is presumed that the low pH of the ODFW had little effect on virus stability. There was little change in the infectivity of most viruses stored at 4°C, in ODFW. However, echovirus 1 and coxsackievirus A9

rapidly dropped in titer on storage. Floyd and Sharp (1977) have attributed this loss of titer to aggregation in solutions with low ionic content. This may have been a contributing factor, but other mechanisms may also be operating, such as leaching of ions important in the structural integrity of the virus.

Suspension of the viruses in media with a higher ionic content may have stabilized the virus infectivity, however this would have also decreased the stability of ozone. The decomposition of ozone prepared in Dulbecco's phosphate buffered saline (without Ca^{2+} or Mg^{2+}) was shown to be much greater than that observed in ODFW.

Cell susceptibility controls were done for all the viruses studied, to determine the effectiveness of the neutralizer in arresting the activity of ozone. No significant difference was found between the number of plaques observed in the neutralizer and the number observed in neutralizer and ozone, for all the viruses studied. Thus a 1:1 dilution of ozone in MEM containing phenol red was sufficient for immediate neutralization.

No differences were observed between the inactivations of poliovirus detected on different cell lines. Although BGM and Vero cells were found to be more sensitive in detecting poliovirus infectivity than HeLa

cells, the extent of inactivation detected was the same for all three cell lines. Sproul (1975) noted unusually large differences in the extent of virus inactivation observed in different studies. He suggested that one reason for these differences was that different monkey kidney cell lines were used for virus assays. However, this seems unlikely since no difference in the inactivations in this investigation was found when diverse cell lines such as HeLa (human epithelial) and BGM or Vero (monkey kidney) were used for the virus assay.

Although it was shown that the plaque assay for detection of poliovirus infectivity was reproducible, there were variations in the extent of inactivation observed at the same ozone concentrations. This variation was shown to be very small within one inactivation however, was greatly increased in trials within an experiment and in trials from different experiments. The small variation within a trial was probably due to small differences in the virus susceptibility of BGM cells from plate to plate. The greater variation within one experiment and from different experiments may be due to the preparation and manipulation of ozone solutions. Differences caused by changes in cell susceptibility with time, or in time of absorption or overlay composition were not a major source of error, as there was little difference in the percent coefficients of variation from within an experiment and from different experiments.

However, an interesting observation was the increase in variation in the inactivations as the initial ozone concentration decreased. Since decomposition of ozone was slightly less at low ozone concentrations, this can be excluded as a possible source of error. However, possible reasons for this increased variation are: difficulty in accurate measurement of solutions of low ozone concentration and, more importantly, the greater effect that any introduced ozone demanding substances would have on solutions of lower ozone concentration.

In this investigation several routine aspects of virus pool preparation were shown to have an effect on virus inactivation by ozone.

An increase in inactivation was observed when poliovirus 1 was ultracentrifuged and resuspended in ODFW, or diluted in ODFW. This illustrates that as the soluble constituents are removed from the preparation, by ultracentrifugation or dilution, the virus inactivation concomittantly increases. This is in agreement with the concept that soluble constituents of the preparation react with ozone preferentially, than with dispersed particles such as viruses (Hoigné and Bader, 1975). When these constituents are removed, more ozone is available for virus inactivation. Ozone demanding substances were also present in the ultracentrifuged virus preparation, as is

illustrated by the increase in inactivation upon dilution.

An increase in resistance of poliovirus to ozone was observed when the virus preparation had been stored at -70°C compared to 4°C. Katzenelson et al. (1974) found an increased resistance to ozone, of poliovirus 1 stored at -15°C compared to virus stored at -70°C. They explained this phenomenon as due to clumping of the virus at -15°C which conferred resistance to the virus and which could be eliminated by ultrasonication. Virus clumping may also be the reason for the differences in sensitivity observed with different storage temperatures in this investigation. However, other physical changes of the virus preparation cannot be excluded. For example, all of the viruses slightly decreased in titer when stored at -70°C. Thus, it may be speculated that a small fraction of virus particles, presumably inactivated during the freezing process and thus not infective, still reacted with the ozone. This would leave less ozone available for reaction with infective virus particles, thereby decreasing the extent of inactivation observed. Although the reason for this change in sensitivity is unknown, this finding points out the need for a standard storage temperature, if changes in virus sensitivity are to be avoided.

Phenol red was found to have a dramatic effect on poliovirus inactivation, even in the small amount carried over in an ultracentrifuged, washed virus preparation. In most laboratories, phenol red is used as a pH indicator for all tissue culture reagents, such as media, trypsin solutions and balanced salt solutions, since it is non-toxic in concentrations up to 0.05 percent (Paul, 1965). Since the use of phenol red is not mentioned in most literature studies, comparisons of the virus inactivations from different studies are difficult to make.

Chloroform treatment was also found to have a dramatic effect on poliovirus inactivation. Little inactivation was observed for an ultracentrifuged virus preparation that had been previously treated with chloroform. Since the enteric viruses are resistant to chloroform treatment (Feldman and Wang, 1961), it has been used frequently to eliminate contaminating bacteria and promote cell debris dissociation, in the preparation of virus pools. However, the solubility of chloroform in water is 7.42 mg/ml at 25^{o}C and so it would be expected that some chloroform would remain in solution after treatment. Also, the density of chloroform is 1.484 which is very similar to the density of the enteroviruses and so it would be expected that some chloroform would pellet with the virus in ultracentrifugation.

Primary and secondary sewage effluents were both found to have an extensive influence on the inactivation of poliovirus by ozone. The most striking observation was that secondary effluent had a greater effect on virus inactivation than did primary effluent or nutrient broth, even though the TOC content was much lower in secondary effluent. Thus it appears that the quality of TOC has an important effect on virus inactivation, although the constituents contributing to the TOC in any of the preparations are not defined. This is an important concept when one considers that recycled waters may have significantly different organic matter content than fresh waters. It also indicates that the factors influencing virus inactivation during ozonation are complex and simple, general measurements such as TOC, present only a partial picture.

Other groups (Snyder and Chang, 1975; Katzenelson and Biederman, 1976) have also found that waters with an ozone demand have an extensive influence on virus inactivation. However, the results of this investigation are not comparable to others, since different waters (filtered river water, filtered raw domestic sewage) were used and no measurements of TOC were made.

The pattern of virus inactivation observed in sewage effluents was a two stage inactivation similar to that obtained in ODFW. Thus the form of inactivation

does not appear to be influenced by the organic matter present in sewage effluent. Since no lag or shoulder was observed in the inactivation curve, the oxidation of organic matter in sewage and inactivation of viruses probably proceeded at the same time, so far as could be detected with this system.

D. RELATIVE OZONE RESISTANCE OF ENTERIC VIRUSES

The importance of viral resistance is highly documented in the literature and many reports appear which have observed virus strains that are more resistant than others to a particular disinfection treatment.

The relative resistance of selected enteric viruses to ozonation could not be unequivocally determined in this investigation, due to apparent differences in virus preparations. Even though all viruses were prepared by a standard method, differences in the TOC analysis of virus preparations were observed. These were found to be correlated with the extent of inactivation obtained for different viruses. However, only some of the differences in virus inactivations could be explained by differences in TOC. After adjusting for TOC content, the inactivations of selected enteric viruses were still found to be different.

Five different groups of enteric viruses were studied. The inactivation of viruses with similar TOC analysis, within each group, was tested by analysis of variance. It is interesting to note that echovirus 12 and 29 show

no difference in response to ozonation. Previously, Snyder and Chang (1975) had reported a wide difference in response of these two viruses to ozonation. They found that echovirus 12 displayed the greatest resistance of eight viruses studied, and proposed that it be used as an indicator virus in future studies. Also, poliovirus 1 (GP100, vaccine) and poliovirus 1(GP103, non vaccine) were found to be different in response to ozonation. Without further study on ozone virus interactions, the reason for this apparent difference cannot be speculated. However, this difference in inactivation of two strains of poliovirus 1 may explain some of the variation in the extents of inactivation observed in literature studies using different poliovirus 1 strains.

Of the five groups of viruses studied, three (polioviruses, echoviruses and coxsackieviruses) are picornaviruses which contain single stranded RNA and are 20 to 30 nm in size. The reoviruses contain double stranded RNA and are 75 to 80 nm in size, whereas the adenoviruses contain double stranded DNA and are 70 to 90 nm large. However, it was impossible to determine any correlation between the extent of virus inactivation and the type of nucleic acid or size of the virus particle. For example, although the polioviruses, echoviruses and coxackieviruses are homogeneous in size and type of nucleic acid, gross

differences in the extent of virus inactivations were observed. The influence of TOC on virus inactivation makes it impossible to discern true differences in virus sensitivity, or the influences of other factors such as size of the virus particle or type of nucleic acid.

No difference was found between the inactivations observed for poliovirus 1, 2 and 3 when inactivated in mixed suspension and separated by neutralizing antisera. This system avoided differences caused by replicate systems and virus preparations. Because these viruses were found to differ when inactivated separately, it may be speculated that the majority of the observed difference was caused by the TOC of the virus preparations. Similarly, f2 bacteriophage has been reported as less resistant (Clarke et al., 1968) or more resistant (Shah and McCamish, 1972) than poliovirus to chlorination. However, Cramer et al., (1976) found little or no difference in the relative resistance of f2 bacteriophage and poliovirus when a mixed suspension was inactivated by chlorine and iodine. Thus, it may be speculated, that any difference in virus sensitivity observed in this type of system would indicate true differences in response of the viruses to the disinfectant. However, differences in the degree of virus aggregation (Floyd and Sharp, 1977) or the physical association of one of the viruses with some ozone demanding constituent may also cause observed differences in virus sensitivity.

Another attempt was made to determine relative virus inactivation by mixing each virus with diluted f2 bacteriophage. This effectively lowered the total TOC of the combined preparation and it was expected that inactivation would therefore increase and the standard phage preparation would be an indicator of suspension purity. On the basis of the data it is tempting to conclude that there is a difference in the relative resistance of the viruses studied to ozonation, however the picture is more complex than is evident at first glance. The addition of the phage influenced the virus inactivations and appeared to "average" the influence of suspension TOC on virus inactivation. In general those viruses which showed low inactivation when ozonated separately showed an increase in inactivation when ozonated in combination with f2 phage (TOC is decreased) but, the viruses which previously demonstrated high inactivation singly have decreased in inactivation when ozonated in combination with the phage. Thus, no conclusions can be drawn concerning individual virus resistance from this data.

Clearly, the factors influencing virus inactivation during ozonation are complex and simple, general measurements such as TOC give an incomplete picture.

This investigation has set the stage for further studies on the constituents contributing to TOC and their effect on virus inactivation by ozone. Further study is required to determine the effect of virus aggregation on the

ozonation process, as Floyd and Sharp (1977) have suggested that virus aggregates may be the normal state of viruses in nature. Also, very little is known of the effect of ozonation on viruses associated with suspended solids. Paramount for further investigations on the relative ozone resistance of viruses is the elucidation of the mechanism of virus inactivation by ozone.

E. SUMMARY

1) A two stage inactivation curve was observed for all viruses studied.

2) The kinetics of the inactivation reaction could not be determined.

3) Several routine aspects of virus pool preparation were found to have an important influence on virus inactivation.

4) The total organic carbon content of virus preparations was found to be correlated with the extent of inactivation observed.

5) The relative ozone resistance of selected enteric viruses could not be unequivocally determined due to differences in virus preparations.

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APPENDICES

APPENDIX A

Amperometric Measurement of Ozone

(1) Electrolyte tablets (USP sodium chloride) and distilled water were placed in the cell unit and left for at least 24 hours to establish equilibrium of the silver-chloride reference electrode.

(2) 1 ml of buffer solution (pH 7.0) was added to 200 ml of ODFW and was then ozonated.

(3) 1 ml of potassium iodide solution and then 1 ml of pH 4.0 buffer was added to the ozone solution.

(4) The 200 ml sample was placed in the cup on the titrator.

(5) The agitator was turned on and the potentiometer adjusted to make the microammeter pointer read maximum on the scale.

(6) The solution was titrated with phenylarsene oxide solution (PAO, 0.005N).

(7) The endpoint of the reaction was reached when the pointer ceased to move upon further increments of PAO.

(8) The ozone concentration was determined by:

$$O_3 \text{ (mg/l)} = \frac{(A - B) \times N \times F}{V} \text{ where}$$

A = ml of PAO (sample)

B = ml of PAO (blank)

N = normality of PAO = 0.005 N.

F = factor to convert moles to mg/l = 24,000

V = volume of sample = 200 ml

APPENDIX B

Trypsin and Versene Solution in HBSS

CW Trypsin

NaCl	8 gm
KCl	0.4 gm
Na_2HPO_4	0.06 gm
KH_2PO_4	0.06 gm
Glucose	50.0 gm

Double glass distilled water to 1000 ml

Trypsin (Difco, 1:250 or 1:300) 100 gm

This was stirred for 30 minutes at 4°C and centrifuged at 12,000g for 1 hour. The solution was filtered through a series of membrane filters with Dacron separators starting with a pre-filter, then 1.2 μ, 0.8 μ, 0.6 μ and 0.45 μ filter.

Working Solution

HBSS	100 ml
Versene (Baker Chemical Co.)	1 gm

Double glass distilled water to 1000 ml

This was autoclaved at 121°C for 25 minutes and the following were added:

Sodium bicarbonate (7.5%)	4.7 ml
1 N NaOH	2.0 ml
CW Trypsin	25 ml

APPENDIX C

Hanks' Balanced Salt Solution (HBSS)

Solution A - (10X)

NaCl	80 gm
KCl	4 gm
$MgSO_4 7H_2O$	2 gm
Na_2HPO_4	0.5 gm
Glucose	10.0 gm
KH_2PO_4	0.6 gm

Double glass distilled water to 1 liter.

Solution B - (10X)

$CaCl_2$	1.4 gm

Double glass distilled water to 1 liter.

Working solution

Solution A	100 ml
Solution B	100 ml

Double glass distilled water to 1 liter.

The solution was autoclaved at 121^{o}C for 20 minutes and stored at 4^{o}C.

APPENDIX D

Growth Medium for Cell Cultures

MEM concentrate

MEM Auto-Pow without phenol red (Flow Laboratories)	86.48	gm
Double glass distilled water	2000	ml

Autoclave at 121°C for 30 minutes.

1 X MEM

Double glass distilled water (sterile)	7.7	liters
MEM concentrate	2.0	liters
Calf serum (Flow Laboratories, heat inactivated at 57°C for 30 minutes)	800	ml
L-glutamine (29.3 mg/ml, (Sigma Chemical Co.)	96	ml
Sodium bicarbonate (7.5%)	140	ml

1 X MEM solution was dispensed into 500 ml bottles and 5 ml of the following antibiotic solution was added:

Concentrated Antibiotic Solution (PSF)

Penicillin G-Potassium salt (Sigma Chemical Co.)	10,000	units/ml
Streptomycin sulfate (Sigma Chemical Co.)	10,000	ug/ml
Amphotericin B (fungizone) (Squibb)	250	ug/ml

APPENDIX E

Dulbecco's Phosphate Buffer Saline without Ca^{2+} or Mg^{2+} (PBS) pH 7.5

10X Solution

NaCl	80 gm
KCl	2 gm
Na_2HPO_4 (anhydrous) dibasic	11.5 gm
KH_2PO_4 monobasic	2 gm

Double glass distilled water to 1 liter.

Working Solution:

10X PBS	100 ml

Double glass distilled water to 1 liter.

The solution was dispensed into 100 ml bottles, autoclaved at 121°C for 20 minutes and stored at room temperature.

APPENDIX F

R3 Growth Medium for Cell Monolayer Cultures in Petri Plates

Medium 199 (22 mg/ml, Gibco)	820 ml
MEM amino acids (50X, Gibco)	164 ml
MEM vitamins (100X, Gibco)	82 ml
MEM non-essential amino acids (100X, Gibco)	82 ml
L-glutamine (29.3 mg/ml, Sigma Chemical Co.)	82 ml
Sodium pyruvate (22 mg/ml, Gibco)	100 ml
Tryptose phosphate broth (Difco)	1,000 ml
Fetal calf serum (heat inactivated at 57°C for 30 minutes, Flow Laboratories)	800 ml
Double glass distilled water (sterile)	7,500 ml

The medium was dispensed into 500 ml bottles and to each bottle 5 ml of the PSF concentrate was added. The medium was stored at -20°C and thawed just before use. The pH was adjusted to 7.3 by the addition of 5.5 ml sodium bicarbonate (7.5%) and 3 ml 1 N NaOH.

APPENDIX G

Ozone Neutralizer

MEM Auto-Pow with phenol red (Flow Laboratories)	70 gm
Double glass distilled water	4150 ml

The neutralizer was autoclaved at 121 °C for 30 minutes and dispensed into 100 ml bottles. To each bottle was added:

Concentrated PSF solution	1 ml
Eagle's buffer at pH 7.3	1 ml
Sodium bicarbonate (7.5%)	1 ml

Virus Diluent

Neutralizer	1000 ml
Ozone demand free water	1000 ml

The diluent was dispensed into 500 ml bottles and stored at 4°C.

APPENDIX H

Nutrient Agar Overlay with 2% Fetal Calf Serum

MEM Auto-Pow with phenol red (19 mg/ml, (Flow Laboratories)	2110 ml
Fetal calf serum (heat inactivated at 57°C for 30 minutes, Flow Laboratories)	92 ml
L-glutamine (29.3 mg/ml, Sigma Chemical Co.)	46 ml
Concentrated PSF solution	46 ml

The overlay was dispensed into bottles containing 57 ml each and stored at -20°C.

Immediately before use the overlay was thawed and heated to 47°C. To each bottle was added:

Sodium bicarbonate (7.5%)	2.5 ml
Eagle's buffer at pH 7.3	1 ml
$MgCl_2$ (2.5 M)	1 ml

The overlay was added to an equal volume of sterile 1.8% Difco Bacto-agar which was cooled to 47°C.

Nutrient Agar Overlay with 8% Fetal Calf Serum

The overlay was made in the same manner as above with the following differences in constituents:

MEM Auto-Pow with phenol red (19 mg/ml, (Flow Laboratories)	1900 ml
Fetal Calf Serum (heat inactivated at 57°C for 30 minutes, Flow Laboratories)	360 ml

APPENDIX I

Neutral Red Agar

5X Solution

Neutral Red dye (Matheson, Coleman and Bell)	1 gm
Double glass distilled water to	1000 ml

This was filtered through 33 cm Grade 230 Reeve Angel filter paper and stored at room temperature.

Working Agar Solution

5X Neutral Red solution	400 ml
Double glass distilled water	1400 ml
10X PBS (Dulbecco's)	200 ml
Bacto agar (Difco)	18 gm

Components were brought to a boil in a boiling water bath and dispensed into 200 ml bottles, which were then stored at $4^{\circ}C$. Just prior to use the agar solution was melted and cooled to $47^{\circ}C$.

APPENDIX J

Nutrient Agar Overlay for Adenoviruses

MEM Auto-Pow with phenol red (19 mg/ml, Flow Laboratories)	1,900 ml
Fetal calf serum (heat inactivated at 57°C for 30 minutes, Flow Laboratories)	360 ml
L-glutamine (29.3 mg/ml, Sigma Chemical Co.)	46 ml
Concentrated PSF solution	46 ml

The overlay was dispensed into bottles containing 57 ml each and stored at -20°C.

Immediately before use the overlay was thawed and heated to 47°C. To each bottle the following was added:

Sodium bicarbonate (7.5%)	2.8 ml
Eagle's buffer at pH 7.3	1.3 ml
$MgCl_2$ (2.5M)	1.3 ml
MEM non-essential amino acids (100X, Gibco)	1.3 ml
MEM vitamins (100X, Gibco)	2.0 ml
Arginine (2.1%, Sigma Chemical Co.)	0.4 ml
Proteose Peptone (88 mg/ml, Difco)	5.0 ml
Total Volume	71.0 ml

The overlay was added to an equal volume of sterile 1.8% Difco Bacto-agar which was cooled to 47°C.

APPENDIX K

f2 Phage Cultivation Media

Tryptone Broth

Bacto tryptone	10 gm
Yeast extract	1.0 gm
Glucose	1.0 gm
NaCl	8.0 gm
$CaCl_2$	0.33 gm
Double glass distilled water to	1000 ml

This was autoclaved at 121°C for 15 minutes and NaOH was added to obtain a pH of 7.0.

Bacto agar (Difco) was added to the broth to give a 1.5% agar solution and 25 ml was dispensed into petri plates

Overlay

Bacto agar (Difco) was added to the tryptone broth to give a 0.7% agar solution which was melted before use and cooled to 47°C.

Diluent

NaCl	8.0 gm
$CaCl_2$	0.33 gm
Double glass distilled water to	1000 ml

This was autoclaved at 121°C for 20 minutes and stored at 4°C.

Operating Characteristics - City of Winnipeg North End Sewage Treatment Plant *

Monthly Averages - 1975

	J	F	M	A	M	J	J	A	S	O	N	D
FLOW (MGAL/DAY)	45.6	45.1	49.2	71.9	64.3	61.9	60.4	54.2	52.2	50.8	44.8	43.0
pH	7.3	7.3	7.4	7.4	7.4	7.4	7.4	7.5	7.4	7.4	7.3	7.3
TEMPERATURE (^{o}C)	13.6	13.5	12.5	13.5	14.5	16.6	21.7	21.4	19.8	18.8	15.8	14.7
TOC (MG/L)												
RAW	205	202	171	170	155	152	137	141	166	159	162	178
PRIMARY	136	132	113	112	90	86	86	93	108	95	97	114
SECONDARY	26	33	35	40	30	28	31	37	45	29	27	29
BOD (MG OXYGEN/L)												
RAW	320	300	280	210	240	260	216	230	270	270	280	300
FINAL	35	43	43	44	38	38	36	47	40	31	29	46
AMMONIA-NITROGEN (MG/L)												
RAW	26.2	26.0	23.6	17.2	25.1	16.7	13.4	18.3	22.2	25.7	22.7	22.1
FINAL	24.6	24.3	22.5	16.2	24.5	17.3	11.2	14.4	20.5	24.6	20.8	20.4
TOTAL KJELDAHL-NITROGEN (MG/L)												
RAW	38.2	34.8	31.3	25.3	36.8	26.6	22.4	25.9	30.8	32.3	31.0	33.3
FINAL	27.7	26.0	23.3	18.7	27.3	19.5	13.2	17.3	23.3	24.9	21.9	23.6
NITRATE-NITROGEN (MG/L)												
RAW	0.05	0.14	0.24	0.44	0.87	1.62	0.58	0.52	0.73	0.51	1.28	1.13
FINAL	0.01	0.02	0.07	0.31	0.39	1.16	3.03	2.94	2.19	2.32	0.97	0.15

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