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C.W. Kay, C. Barnes, and A.M. Wallbank
Department of Medical Microbiology and Infectious Diseases
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Department of Medical Microbiology and Infectious Diseases
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for

THE MICROBIOLOGY LABORATORIES
APPLIED RESEARCH DIVISION

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M.S. Mahdy, Consultant B.J. Dutka, Head

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ABSTRACT

The effectiveness of ozonation to destroy animal virus infectivity was studied. The misalignment of comparisons of literature data was explained on the basis of varying amounts of contaminating soluble constituents in the virus preparations.

Ozone was determined to be an effective virucide in "clean" water. An initial dose of 0.1 mg/l of ozone provided 99.999% inactivation of poliovirus practically instantaneously. In dynamic systems virus inactivation would be mass transfer limited.

Total organic carbon content of the suspending medium was related to virus inactivation. Varying protective effects were conferred by the soluble constituents of the suspending medium and the degree of protection was dependent on the chemical nature of the soluble components. Virus inactivation by ozone seems to be more closely allied to competing ozone demanding constituents in the suspending menstruum than to inherent properties of the virion.

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INTRODUCTION

The establishment of community water supplies and the concurrent inception of water disinfection practices have led to a rapid decline in the incidence of water-borne diseases. Transmission of disease by the water route has been minimized by successfully controlling the prevalent etiological agent - the bacteria. However, complete removal of disease producing agents from the water supply is not yet possible.

Public health officials are now most concerned about virogenic disease - particularly with the advent of water reuse for residential, recreational, agricultural, and industrial purposes. Virus removal accomplished by most treatment processes is generally incomplete (3). Many viruses pathogenic to man are shed in feces, are present in raw sewage effluents, and are discharged into streams or other surface waters (4) where they may persist for extended periods. Welke, Friedrich, and Mai (70) were able to recover echoviruses 560 days after seeding into river water.

More than 100 viruses, many causing overt disease symptoms, have been isolated from human feces. Those most likely to be important in water transmission include the polioviruses, coxsackie viruses A, coxsackie viruses B, echoviruses, reoviruses, adenoviruses, hepatitis A virus, and probably the agent(s) of infectious non-bacterial gastero-enteritis (38). In addition numerous viruses of non-human origin are present in water bodies. It has been suggested that some viruses when infecting unnatural hosts may produce cancers (35, 67). Considering

that 57% of 14 million head of cattle in Canada are maintained on the prairies and are producing ten times the quantity of feces produced by man this may represent a special hazard (38).

Numerous reports have shown the viruses to be more resistant than bacteria to chlorination and other treatments (4), thus precluding the use of the coliform index as an indicator of disinfection in standard treatment processes. Viruses, also, are difficult to detect, requiring sophisticated concentration techniques. Consideration of the inadequacy of existing water and sewage treatment processes and concern generated by the discovery of the carcinogenic properties or organochloride complexes have led to a search for more effective and more innocuous water treatment processes.

Ozone has been proposed as an alternative to chlorine as a disinfectant for drinking water and sewage effluents. A number of rather thorough studies have been made of the germicidal properties of ozone (8, 9, 11, 13, 22, 25, 30, 31, 39, 40, 42, 59), but their chief characteristic is their disagreement. A review of the literature reveals that studies of the inactivation of viruses by ozone were carried out using varying procedures and under different conditions (Table 1). None of the studies evaluated the effect of soluble organics present in the virus suspensions. Comparisons of the literature data must, therefore, be viewed conservatively.

TABLE 1

Summary of studies on virus inactivation by ozone

Author and year	Virus studied	Condition	Ozone measurement method
Kessel et al, 1943	Poliovirus (MV)	--	OTM
Coin et al, 1964	Poliovirus I (Mahoney)	distilled water	Iodometric (starch indicator)
Yakovleva and Ilinitskii, 1967	Adenovirus 7a	autoclaved tap water	Iodometric
Carazzone and Vanini, 1969	Bacteriophage T1	distilled water	Iodometric
Majumdar et al, 1973, 1974	Poliovirus	triple distilled water primary effluent secondary effluent	Iodometric
Katzenelson et al, 1974	Poliovirus I (Brunhilda)	ozone demand free distilled water	Iodometric spectroscopic
Pavoni and Tittlebaum, 1974	Bacteriophage f2	secondary effluent	--
Longley et al, 1974	Bacteriophage f2	secondary effluent	Iodometric
Snyder and Chang, 1975	Poliovirus 1, 2, 3 Coxsackie B3, B5 Echovirus 12, 29 Adenovirus 7a	ozone demand free distilled water filtered river water	OTM
Katzenelson and Biederman, 1976	Poliovirus I (Brunhilda)	filtered raw sewage	Iodometric spectroscopic

INACTIVATION OF VIRUSES BY OZONE

REVIEW OF THE LITERATURE

Ozone, a labile allotrope of oxygen, is a powerful oxidizing agent. Since its discovery, by Schönbein in 1840, ozone has been found useful in a number of applications. It 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 (15). 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, under construction, will treat 250 mgd supplied to the city of Montreal (45).

In the United States the primary objective of ozonation has been colour, odour and taste control in some water supplies. Chlorine is the most widely used 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.

Kinman (34) reviewed the advantages and disadvantages of using ozone as a disinfectant. He concluded that it will probably never be widely used in the U.S. for municipal wastewater disinfection so long as cost is a major design consideration.

Recently other factors have come to light which may considerably affect the future of ozonation as a wastewater disinfectant. Potential

carcinogens, in the form of chlorine-organic matter complexes, have been detected in the Mississippi River. These same compounds have also been found in New Orleans' drinking water and in the blood plasma of local residents (16). A further study showed a statistically significant relation between cancer mortality rates in Louisiana residents and use of drinking water obtained from the Mississippi River (47).

Recently a wild strain of poliovirus 1 was isolated from an Ottawa sewage plant effluent (55). This was judged as cause for concern since immunity levels in the general Canadian population are decreasing (37).

The production of toxic end-products by chlorination of waters in the presence of organic matter, and the ineffectiveness of existing treatment facilities to remove viruses, have renewed interest in the ozonation process. As natural water supplies diminish, reclamation and reuse become more important.

Although ozonation techniques are very effective, it is surprising that as yet there is little rational and scientific basis for its practical application. The germicidal properties of ozone were studied in 1886 by De Meritans (52). The bactericidal nature of ozonation is well known (6, 20, 25), but comparatively little information is available on the inactivation of viruses by ozone.

In 1943, Kessel et al (32) prompted by reports of a resistant strain of poliovirus (66), 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 varying from 0.5 to 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.

Poliovirus has been the choice for most inactivation studies because of its resistance and ease of assay. Further reports on the inactivation of poliovirus by ozone were made by Coin (11), Perlman (50), Schaffernoth (56), Gevaudian (22), Majumdar (39, 40), Katzenelson (30,31), McLean (42) and Sproul (61).

Other viruses used to study inactivation by ozone include; influenza (43), adenovirus (71), vesicular stomatitis virus, encephalomyocarditis virus, and GD VII virus (8) and bacteriophages f2 (48) and T1 (9).

Sproul (61) noted unusually large differences, for ozone inactivation data, in comparing literature data from different sources. He stressed the need for validation work for local situations, the need for a standard methodology, and analytical methods capable of determining the active disinfectant during ozonation.

A study of the relative resistance of 20 human enteric viruses to free chlorine (36) revealed a range of 2.7 to 60 minutes required for

inactivation with 0.5 mg/l chlorine.

Snyder (59) compared the relative resistance of 8 human enteric viruses (polio 1, 2 and 3; coxsackie B3 and B5; echo 12 and 29; and adeno 7a) to ozonation. The study was carried out in ozone-demand free water and river water at 2°C, with a constant ozone dose of 1 g/hr. The only variable was contact time. The most important aspect of this study was the discovery of an intratype resistance - among members of echoviruses. Echo 12 required a contact-time 10 times longer than echo 29 for the same degree of inactivation.

Attempts have been made to quantitate the ozonation process as it is applied to virus inactivation. Majumdar (39, 40) worked with the inactivation of poliovirus in triple distilled water, primary effluent, and secondary effluent. He formulated two equations to explain the inactivation kinetics above and below a threshold value of 1.0 mg/l ozone residual. The equations, relating ozone concentration, contact time and virus survival were deemed valid for triple distilled water, primary effluent and secondary effluent in both a batch and continuous flow situation.

Katzenelson (30) found a two stage inactivation curve for poliovirus. 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 stage was due to viral clumping since it could be eliminated by ultrasonication. Another finding from this study was that storage of the virus stock at -15° C

instead of the usual -75° C conferred resistance on the virus. This was explained as due to clumping and poses the question if viruses in natural environments are of the sensitive type or if they include a significant portion of clumps - thus following the resistant pattern of inactivation under practical field conditions of water and wastewater disinfection. Viral aggregation is well documented (21, 58, 72).

Burleson (8) presented data complementary to that of Katzenelson and described a synergistic effect of ultrasonication and ozonation on virus inactivation. Vesicular stomatitis virus, encephalomyocarditis virus, and GD VII virus were completely inactivated after treatment with ozone in 15 seconds when suspended in sterile phosphate-buffered saline. Longer times were required when the viruses were suspended in waters which exerted an ozone demand due to organic material present. This time could be reduced by simultaneous ultrasonication and ozonation.

Katzenelson (31) 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 the 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. However, this leaves an amount of residual infective virus of 10^7 to 10^5 PFU/ml. Inactivation took place in the first 10 seconds followed by an insignificant change. The degree of inactivation correlates with the strength of the ozone concentration up to 1.3 mg/l, above which there was only a slight increase in inactivation capacity.

Inactivation was less with 10% effluent than with 5% effluent at the same ozone concentration. Continuous bubbling into effluent resulted in a 90 second lag before residual ozone appeared. Following a 30 second delay the virus was 99% inactivated in 1 minute even though no residual was detected. After 2 minutes virus inactivation was 99.999% and the residual was 0.6 mg/l. This compares favourably with the data of Coin (11) who found a 0.7 mg/l residual required for satisfactory inactivation and Majumdar's 1.0 mg/l "threshold" concentration (40).

A distinction must be made between disinfection and sterilization. Miller (43) evaluated methods to satisfy the rigid 100% sterilization requirement, at Fort Detrick, for sewage containing highly pathogenic organisms. The study concluded that complete inactivation of viruses, pathogenic bacteria, spores, and endotoxins was dependent on ozone dose and contact time. Thirty minutes were required to produce sterility in sewage with a quantity of ozone between 100 and 200 ppm (measured as the difference in the amount of influent and effluent ozone as applied to the weight of the sewage sample). The period of treatment is related to the contact efficiency between ozone and the agent. Removal of suspended solids did not significantly decrease the time required to sterilize nor the quantity of ozone used, but did significantly influence the disinfection time.

Nebel (46) conducted a pilot study with an 80 gallon per minute commercial packaged activated sludge plant. The effluent was ozonated at a constant dose of 15 mg/l in a contact chamber. An average

residual of 0.05 mg/l was produced, which decayed to zero after leaving the contact chamber. Five minutes were required for 100% destruction of 10^{11} pfu/ml f2 bacteriophage. However, disinfection occurred within 3-8 seconds. (The term disinfection is not used in its strict microbiological sense).

A pilot study was carried out by Sommerville and Remple (60) with a 60 gallon per minute package plant treating drinking water to control taste and odour. A retention time of 5 minutes with an ozone residual of 0.1 mg/l was not sufficient for complete disinfection. (i.e. total reduction of microbiological flora).

McLean and Smith (42) added Sabin poliovirus 1 to sewage effluent which was passed down a 12 foot column at 2-4 l/min (17°C). Ozone was bubbled up at 3 l/min. Samples were collected at 18 inch intervals. Reduction of virus titer by 2-3 log $TCID_{50}$ resulted from contact with 0.7-1.2 ppm ozone for 10 seconds. A contact time of 2.5 minutes at 1.6 ppm or greater residual ozone gave a 99.99% reduction in titer.

Dahi (13) described the inactivation of microorganisms by ozone as a three stage process. The first stage is due to ozone demand, then there is a proper inactivation stage followed by a stage due to the inactivation of composed microbial units. The latter stage could be eliminated by prior or simultaneous ultrasonic treatment, with ozonation. Data was presented to show that the germicidal properties of ozonation result from free radical formation when ozone decomposes.

Hoigné and Bader (28) showed that hydroxyl radicals are the predominant decomposition product of ozone decay, catalyzed by the hydroxide ion in water. Rate constants for reactions of hydroxyl radicals with many substrates are very high and these radicals are consumed preferentially by dissolved species (organic and inorganic) before they encounter dispersed particles such as microorganisms. Direct consumption of ozone is slow and selective compared to the fast, less selective, reaction of solutes with hydroxyl radicals. Thus decomposition of ozone can compete with the direct consumption of ozone molecules.

Peleg (49), reviewing the chemistry of ozone in the treatment of water, presents evidence to show that the decomposition product, in particular hydroxyl radicals, may be more important than ozone as the oxidizing and germicidal agent in water.

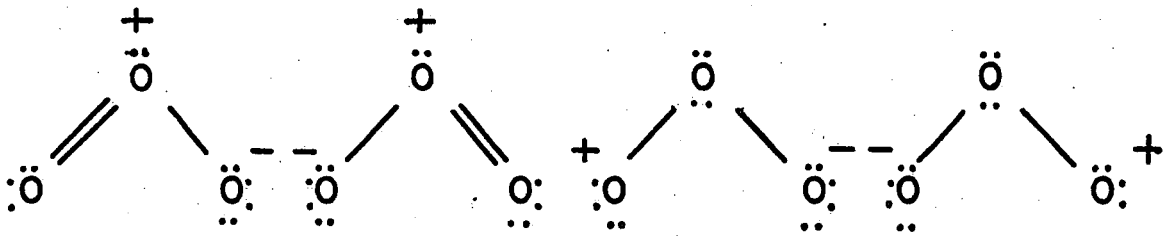
In the above cited studies the data were obtained using varying procedures and under different conditions. Extrapolations and direct comparisons of the resistance of viruses to ozonation must be made with caution.

Properties of Ozone

The first recorded detection of ozone is that of a "sweet smell", in the vicinity of an electric discharge, which was described by Van Marum in 1785 (68). The name ozone was applied by Schönbein (57) in 1840, who devoted his life to a study of the gas. However, Schönbein

made only semi quantitative observations on the chemistry of ozone and the first precise information of its chemical properties are derived from the definitive work of Brodie (7).

Ozone is a triatomic allotrope of oxygen. Its exact structure remains controversial, but it is generally considered to be a resonance hybrid of the following structures:



The solubility of ozone decreases with increasing temperature and pH (64). Ozone obeys Henry's law and, therefore, the solubility is directly proportional to the partial pressure of ozone in the gas phase (14).

Ozone is a labile gas and decomposes rapidly with increasing temperature and pH (33), and in the presence of organic and inorganic impurities (18). The rate of decomposition of ozone is much slower than the oxidation of organic substances present in the solution (2).

Houzeau (29) was the first to report the formation of a compound between ozone and an organic material. Unfortunately very few studies

have been made of the chemical reactions involved in the ozonation of organic and inorganic substances in water. However, on the basis of its structure, ozone may react as an electrophile, a nucleophile, or as a 1,3 - dipole (2). 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 for ozone in aqueous solutions; -

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

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

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

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

All intermediates are very reactive and short lived (1).

In aqueous solutions the germicidal properties of ozone are thought to be due to the free radical decomposition products (13, 28, 49).

MATERIALS AND METHODS

Production of ozone

Ozone was generated by the silent electric discharge process in a model T-408 Welsbach Laboratory Ozonator. (Welsbach Ozone Corp. Philadelphia, Pa.). Dry, medical grade oxygen (dew point -85°F) was used as the feed gas. The ozone produced was delivered, via tygon tubing, to a sintered glass gas sparger. The yield was determined by gas flow rate, gas pressure, input voltage and electrode cooling.

Ozone-demand free water

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.

Preparation of ozone solutions

Ozone solutions for inactivation experiments were prepared immediately before use by introducing ozone into ozone demand free water to slightly greater than the required concentration. Excess ozone was removed by vigorously stirring the ozone solution on a magnetic stirring assembly.

Measurement of ozone concentration in solution

The amount of ozone in solution was determined routinely by absorption at 253.7 nm, previously described by Hann and Manley (26).

A calibration curve was prepared relating absorbance at 253.7 nm to ozone concentration (mg/l) determined by the iodometric method (Fig. 1). A 1 cm path length was used for ozone concentration > 1 mg/l and 4 cm path length for concentrations less than 1 mg/l.

Iodimetric determination of ozone

Excess neutral, buffered, potassium iodide was oxidized by ozone to iodine. The iodine was titrated with standard phenylarsene oxide (0.00564 N) to the amperometric endpoint as described in Standard Methods (63). The end point was determined with a Wallace and Tiernan Amperometric Titrator (Penwalt, Corp., Belleville, N.J.).

In neutral buffered solution the stoichiometry of the reaction is:

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

$$C_6H_5AsO + I_2 + 2\,H_2O \longrightarrow C_6H_5AsO\,(OH)_2 + 2HI$$

This method has been described as the most accurate indirect chemical method available (5, 54).

Cultivation and quantitation of viruses

The viruses used in this study were all low passage viruses and are listed in table 2. With the exception of the adenoviruses, all viruses were cultivated and assayed on BGM cells, which were obtained from Dr. G. Berg, U.S. Environmental Protection Agency, Cincinnati, Ohio.

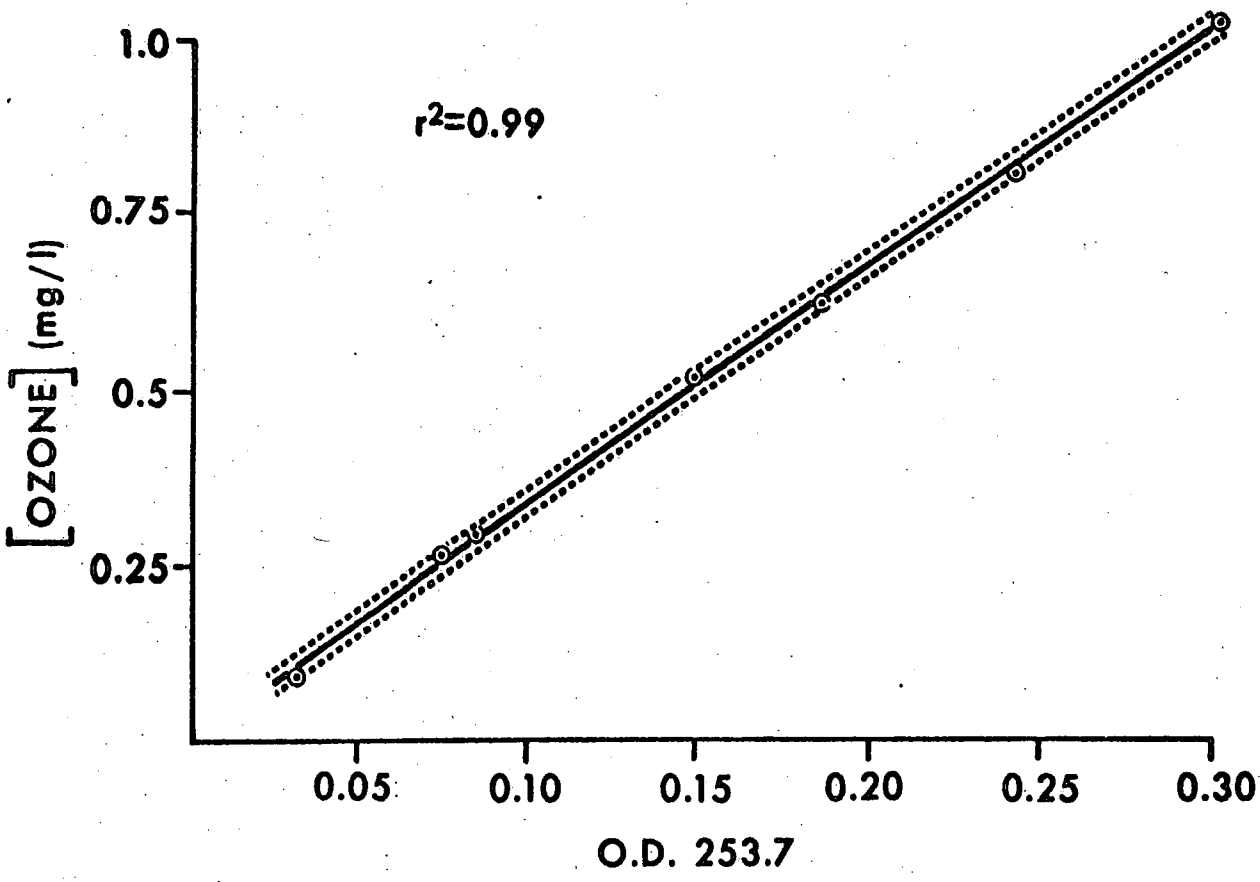


FIG. 1 CORRELATION BETWEEN ABSORBANCE AT 253.7 nm AND OZONE CONCENTRATION. A 40 mm LIGHT PATH QUARTZ CUVETTE WAS USED. 95% CONFIDENCE LIMITS ARE SHOWN.

TABLE 2

Virus strains

Virus	Strain	Isolation	Passage	Source
Polio 1	GP103, non-vaccine	sewage effluent	5 (BGM) previously low passage	Dr. S. A. Sattar, Dept. of Microbiology, U. of Ottawa
Polio 1	GP100, vaccine	sewage effluent	4 (BGM) previously low passage	"
Polio 3	GP30	sewage effluent	4 (BGM) previously low passage	"
Coxsackie B3	335	septic tank	4 (BGM) previously low passage	"
Coxsackie B5	24	raw sludge	4 (BGM) previously low passage	"
Adenovirus 8	45	clinical	3 (HeLa) previously 3 (MK)	Dr. R. S. Faulkner, Dept. of Microbiology, Victoria Gen. Hosp Halifax, Nova Scotia
Adenovirus 4	RI-67	ATCC VR-4	3 (HeLa) previously 3 (KB) 5 (human amnion)	Centre for Disease Control, Otta
Reovirus 1	Lang	ATCC VR-230	1 (RhMK), 5 (BGM) previously 1 (RhMK)	"
Echovirus 12	Travis	ATCC VR-42	3 (BGM) previously 2 (RhMK)	Dr. O. Lui, Northeastern Water Hygiene Laboratory, Public Healt Service, U.S. Dept. of Health, Education and Welfare, Harraganset, Rhode Island
Echovirus 29	JV-10	ATCC VR-227	3 (BGM) previously 2 (RhMK)	
Polio 2	RS 7778	raw sludge	3 (BGM) previously 2 (MK)	Mr. W. Stackiw, Viral Diagnostic Laboratory, Province of Manitoba
Coxsackie B4	GP1000	sewage effluent	4 (BGM) previously 2 (MK)	
Adenovirus 7	C-7312-75	clinical	3 (HeLa)	
Echovirus 11	C-2491	clinical	3 (BGM) previously 2 (RhMK)	

Adenoviruses were grown and assayed on HeLa cells supplied by Dr. W. Wold, Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Missouri.

Both cell lines were maintained in Blake bottles. Complete monolayers, 3-4 days old, were trypsinized for 15 minutes at 37°C to detach and disperse the cells (Appendix VIII). The trypsin solution was then neutralized with 5 ml of Eagle's minimal essential medium (MEM) containing 8% calf serum (Appendix I). Cell culture bottles were reseeded with approximately 2 X 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.

Preparation of stock virus pools

(a) Polioviruses, coxsackieviruses and echoviruses.

Monolayers of BGM cells (approx. 1.5 X 10^6 cells) on Falcon flasks (75 cm^2) were infected with 3 ml of virus suspended in phosphate buffered saline (Appendix VII), at an input multiplicity of approximately 5. Virus suspensions were allowed to adsorb for 60 minutes at 37°C with periodic gentle rocking. Maximum cytopathic affect was usually evident within 20-48 hours. The virus was harvested by centrifugation at 500 g for 15 minutes and the supernatant was further centrifuged at 12,000 g for 30 minutes.

The virus pools were further purified by ultracentrifugation at 100,000 g for 1 hour. The pellet was washed twice in ozone demand free

water and resuspended into 22 ml of ozone demand free water which was further aliquoted into ozone demand free tubes and stored at 4° C.

(b) Reoviruses.

Reovirus pools were prepared in the same manner as the enteroviruses except the pools were harvested before maximal cytopathic effect (usually 3-4 days) by three cycles of alternate freezing and thawing.

The reoviruses required one passage on primary rhesus monkey kidney cells before detectable cytopathic effect was observed on BGM cells.

(c) Adenoviruses.

Pools were prepared as for the enteroviruses except that the Falcon flasks were seeded with HeLa cells (4×10^6 cells). A complete monolayer was formed in 24-48 hours. The virus inoculum was adsorbed for 3 hours with gentle rocking every 20 minutes. The maintenance medium was changed after 3 days. Maximum cytopathic effect was evident in 3-7 days whereupon the virus was harvested by three cycles of alternate freezing and thawing.

Plaque assay

(a) Polioviruses, coxsackieviruses and echoviruses.

Four to six day old monolayers of BGM cells in 5 ml of R3 medium (Appendix II) in plastic tissue culture dishes (52 X 13 mm) were infected with 0.5 ml of diluted virus. The virus was allowed to adsorb for 60 minutes at 37°C and was then overlayed with 5 ml of agar overlay medium

with 2% fetal calf serum (Appendix III). The plates were incubated at 37°C in an atmosphere of humid 5% CO_2. After 38-42 hours the plates were overlaid with 5 ml of neutral red agar (Appendix IX). Plaques were counted 12-24 hours later.

(b) Reoviruses.

The assay procedure was similar to that for the enteroviruses except the BGM monolayer was infected 3-4 days after seeding and the agar overlay medium contained 8% fetal calf serum. The cells were refed with 3 ml of agar overlay 3 days post infection and neutral red agar was added at 7 days. For echovirus 11 neutral red agar was added 5 days after infection.

(c) Adenoviruses.

The assay technique was similar to that above with the exception that 1-2 day old HeLa cells were used. Adsorption was extended to 90 minutes with gentle rocking every 20 minutes. Five ml of an enriched agar overlay (Appendix IV) was added to each plate. After 5 days incubation the plates were refed with 5 ml of the same overlay agar and again with 3 ml after a further 5 days. Neutral red agar was added 15 days after virus infection.

Bacteriophage

f2 bacteriophage stock was obtained from Dr. N. Zinder, Rockefeller University, 66th Street and York Ave., New York. The T series phage were

obtained from the American Type Culture Collection.

Preparation of bacteriophage stock

Bacteriophage stock was produced by the agar layer method of Swanstrom and Adams (65). Escherichia coli $K37^+$ was the host for f2 and E. coli B for the T series phage. 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) and to this was added 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 X). 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 12,000 g for 30 minutes. The supernatant, containing approximately 10^{11} pfu/ml, was stored at 4°C.

In the plaque assay technique 0.5 ml of a serial tenfold dilution, with 0.1 ml of host bacterium, were added to 2.5 ml of melted 0.7% tryptone agar. After mixing this was layered on 25 ml of 1.5% tryptone agar.

Sewage samples

Primary and secondary effluents were obtained from the City of Winnipeg, North End Treatment Plant which had the operating characteristics outlined in Table 6. Bacteriological analysis for fecal coliforms and

fecal streptococci were determined by the membrane filter technique as described in Standard Methods (63) and Pseudomonas aeruginosa was determined by the method described by Dutka and Kwan (17).

Total organic carbon

Total carbon and inorganic carbon were estimated by the combustion-infrared method described in Standard Methods (63). Total organic carbon was determined as the difference of total carbon and inorganic carbon. Samples were reproducible with a precision of less than $\pm$ 2.0 mg/l.

Experimental design

The initial objective of this study was to determine the kinetics of inactivation of viruses suspended in various media. Preliminary experimentation revealed that the inactivation reaction was very fast and measurement techniques were not available to reliably monitor the course of the reaction. An aspirator-static mixer device was constructed which allowed samples to be taken 200 m sec after initiation of the reaction. However this was still not a sufficiently short time interval to determine a time course of reaction. It was therefore decided that, for all practical purposes, the inactivation reaction per se could be considered instantaneous. In a system where ozone was added continuously the rate limiting step would, necessarily, be determined by mass transfer. So our primary objective then became the determination of the relative resistance of viruses to ozonation.

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

To overcome the problems of mass transfer, ozone was prepared and used as an aqueous solution in ozone demand free distilled water. The concentration of ozone in the solution was determined by a direct, quantitative measurement of light absorption at 253.7 nm. To interact the virus preparation with the ozone solution an apparatus was constructed which consisted of two 10 ml syringes connected by a 3-way stopcock (Fig. 2). 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.

Usually, during an inactivation experiment, syringe B contained 9 ml of ozone solution of known concentration and syringe A contained 1 ml of virus preparation. The stopcock was then adjusted to allow mixing of the two solutions and, after discarding the first 2 ml, time samples were taken. The inactivated virus-ozone solution samples were mixed with a neutralizer and diluted for virus assay. The composition of the neutralizer and diluent is given in Appendix X.

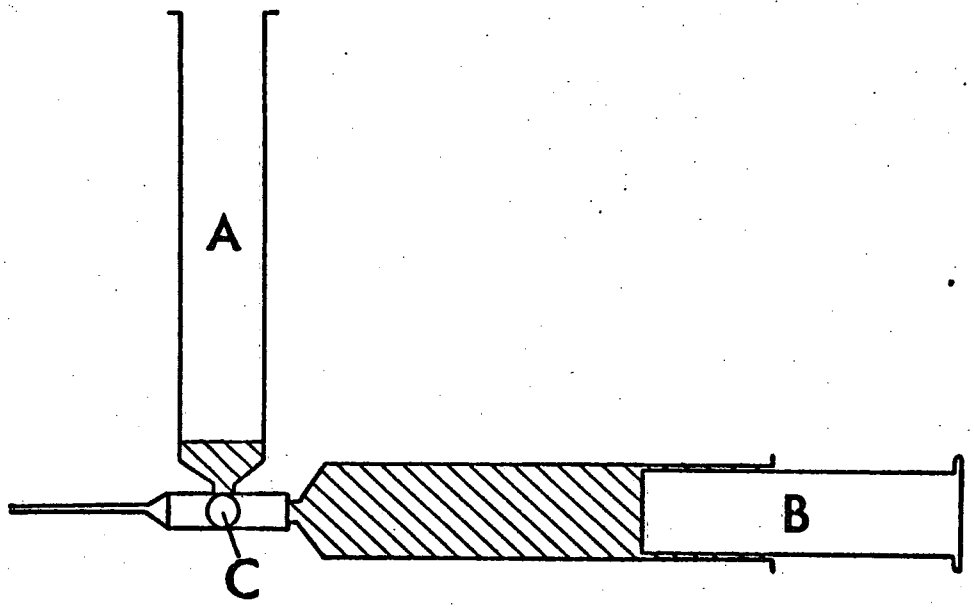


Figure 2. Inactivation Reactor

RESULTS AND DISCUSSION

Stability of Ozone

Ozone is a labile gas which spontaneously decomposes, probably by a free radical mechanism, to oxygen. The stability of ozone is critically determined by the suspending menstruum and physical conditions. In Freon-12 at -78°C (41) ozone is stable for months, but under conditions of practical use, the decomposition is greatly affected by temperature, pH, presence of organic matter and inorganic ions and the suspending phase.

In attempting to evaluate the effect of ozone on virus inactivation a number of compromises have to be reached. The conditions must be determined to be mutually favourable to both ozone stability and virus stability.

Ozone demand-free double glass distilled water pH 5.3 and 22°C, was chosen as the most appropriate suspending fluid for making ozone solutions and Fig. 3 depicts the relative stability of the most commonly used ozone concentrations. In all inactivation experiments the elapsed time between preparation of the ozone solution and addition of virus for inactivation, was less than 1 minute during which time negligible decomposition had occurred. It is uncertain what major effects on the virus preparation were exerted by suspension in water with such low ionic content and low pH. There appeared to be little change in infectivity titre of most viruses which were stored at 4°C in ozone

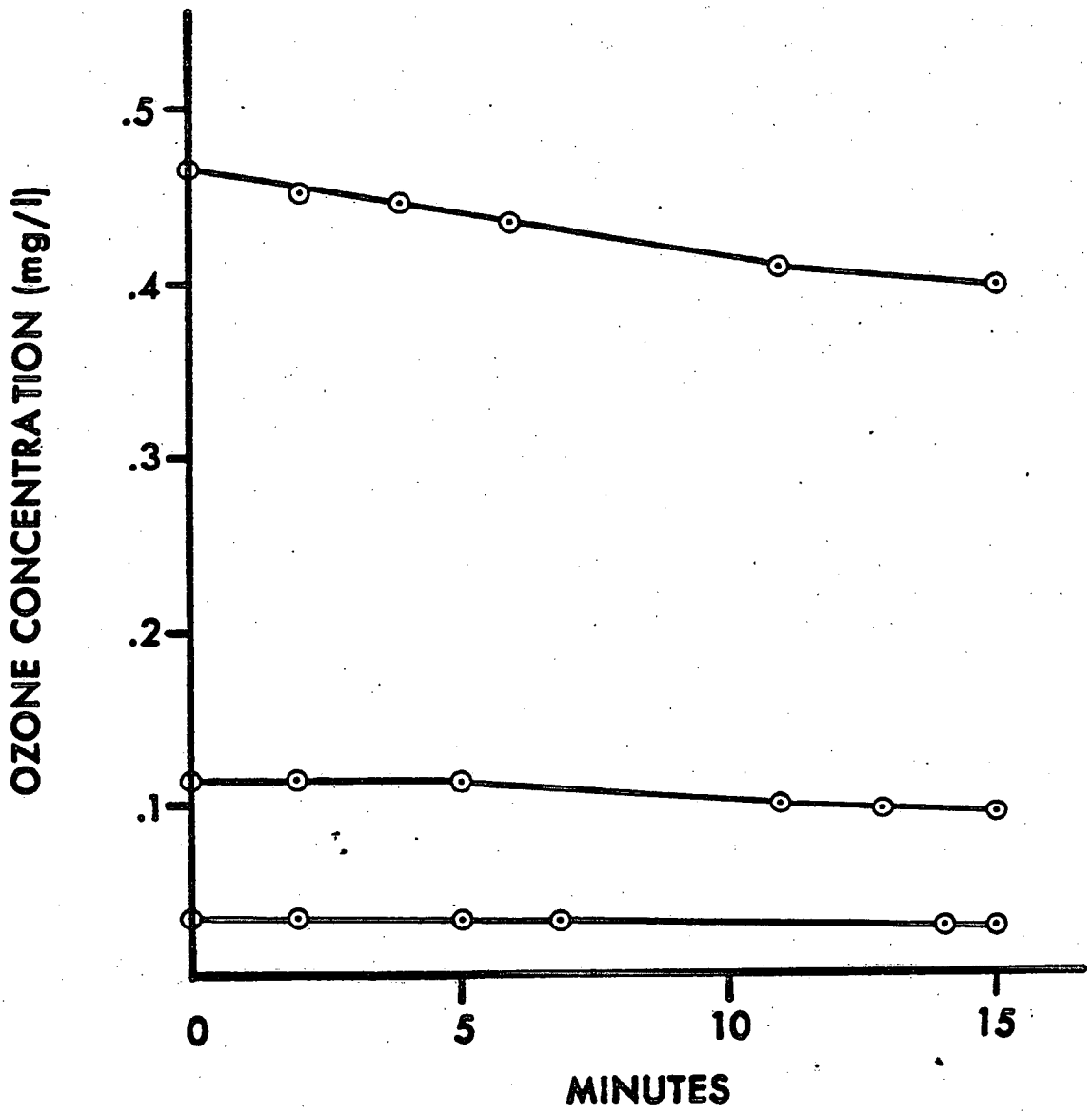


FIG 3. DECOMPOSITION OF OZONE IN OZONE DEMAND-FREE WATER.

demand free water. However, the echoviruses and coxsackie A9 rapidly dropped in titre on storage. Floyd and Sharp (21) have attributed this loss of titre to aggregation, and this indeed may be 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 virus in other media may stabilize the virus infectivity but also leads to increased instability of the ozone. Fig. 4 shows the effect of inorganic ions (supplied as phosphate buffered saline - Appendix VII) on ozone decomposition.

All inactivations were carried out in ozone demand free water, pH 5.3 at room temperature. It was established that temperatures from 1°C to 22°C had the same effect on virus inactivation (Fig. 5). Ozone solutions were unstable at pH greater than 7.5 and the effect of pH on virus inactivation could not be determined.

Inactivation of viruses

Fifty-five experiments were performed encompassing more than 500 separate inactivations. The single most obvious observation was the nature of the inactivation. Without exception, the pattern of virus inactivation, after ozone treatment, was an immediate loss of infectivity followed by a gradually diminishing inactivation on prolonged exposure to ozone. Fig. 5 depicts a typical inactivation curve. The sampling time was selected for convenience. Preliminary experiments showed that the initial inactivation occurred in less than 200 m sec.

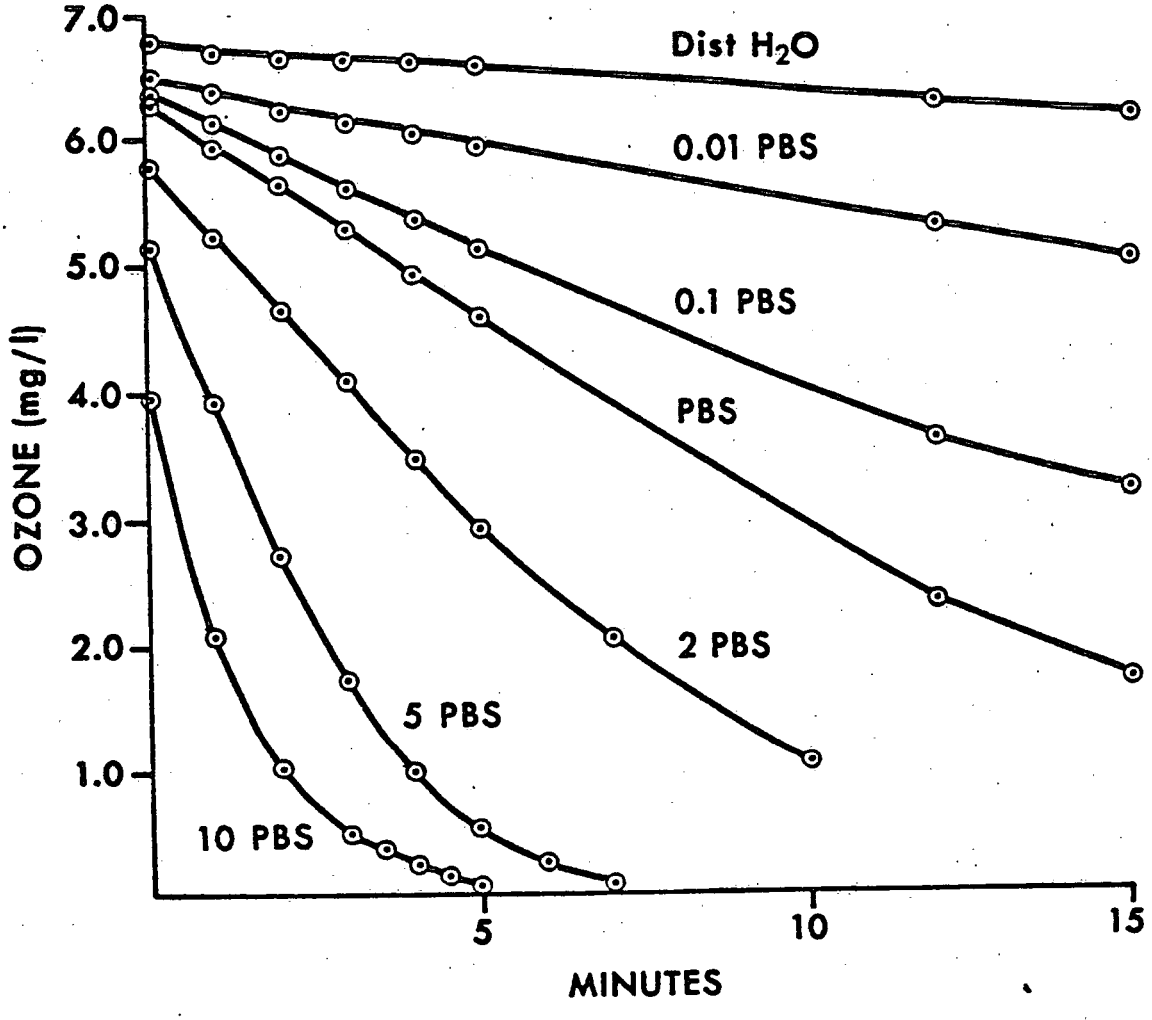


FIG 4. RATE OF DECOMPOSITION OF OZONE IN PHOSPHATE BUFFERED SALINE (PBS)

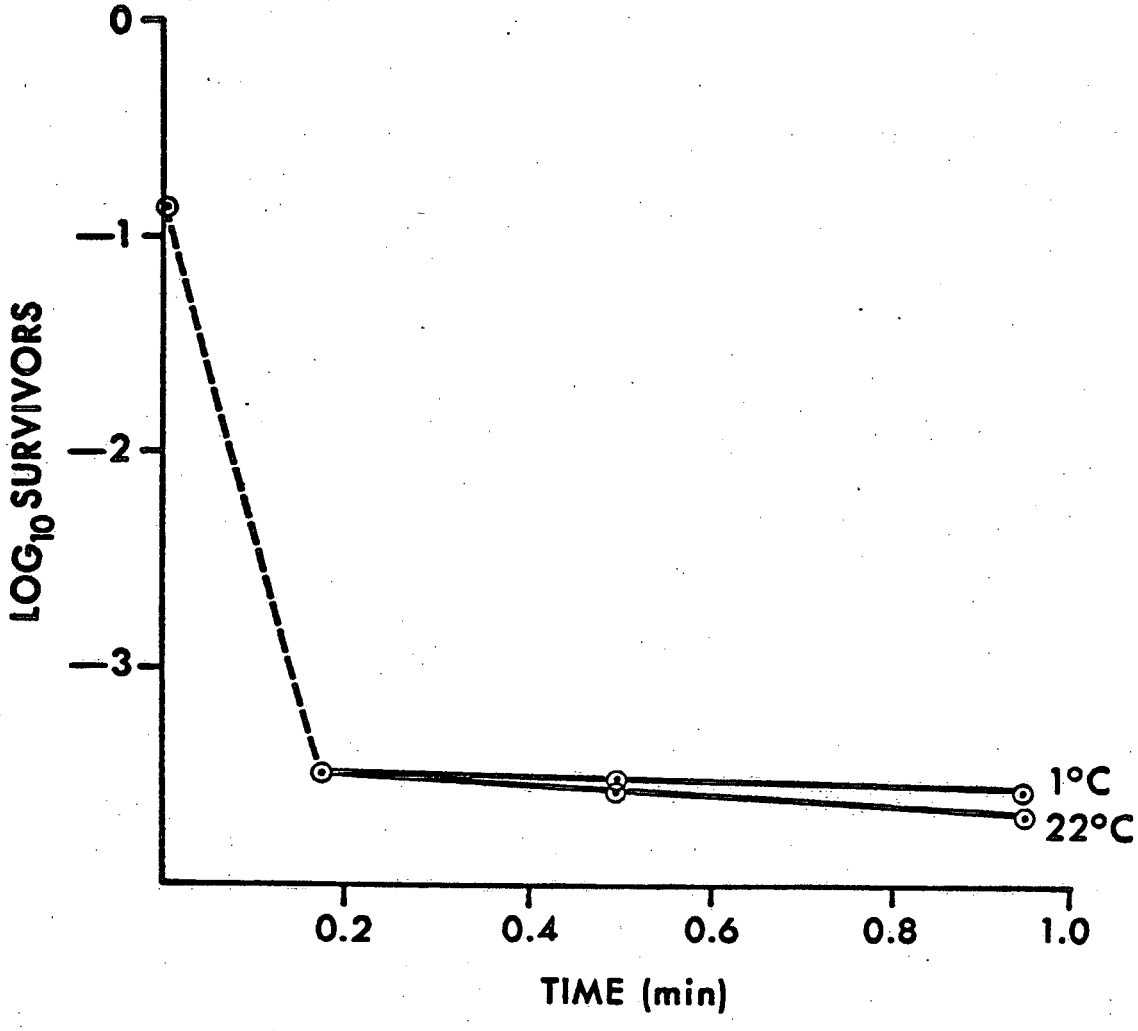


FIG 5. TYPICAL INACTIVATION CURVE. POLIOVIRUS (GP103) AT 1°C AND 22°C. OZONE DOSE 0.1 mg/l.

The extent of viral inactivation was exponentially related to the ozone dose. As shown in Fig. 6, a small increase in ozone dose results in a very large inactivation. Loss of infectivity is measured in terms of $\log_{10}$ inactivation, which is defined as the negative logarithm of the surviving fraction.

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

where N = titre of surviving particles (pfu/ml)

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

Since $- \text{Log}_{10} (N/No) = \text{Log}_{10} (No/N) = \text{Log}_{10} No - \text{Log}_{10} N$

the working relationship for determining the $\log_{10}$ inactivation is to subtract the $\log_{10}$ of the surviving pfu/ml from the log of the initial titre ($\log_{10}No - \log_{10}N$). This presentation provides a simple visual interpretation of the amount of inactivation and avoids the cumbersome designation of per cent 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

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

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

$$= -(\log_{10} 2 + \log_{10} 10^{-3}$$

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

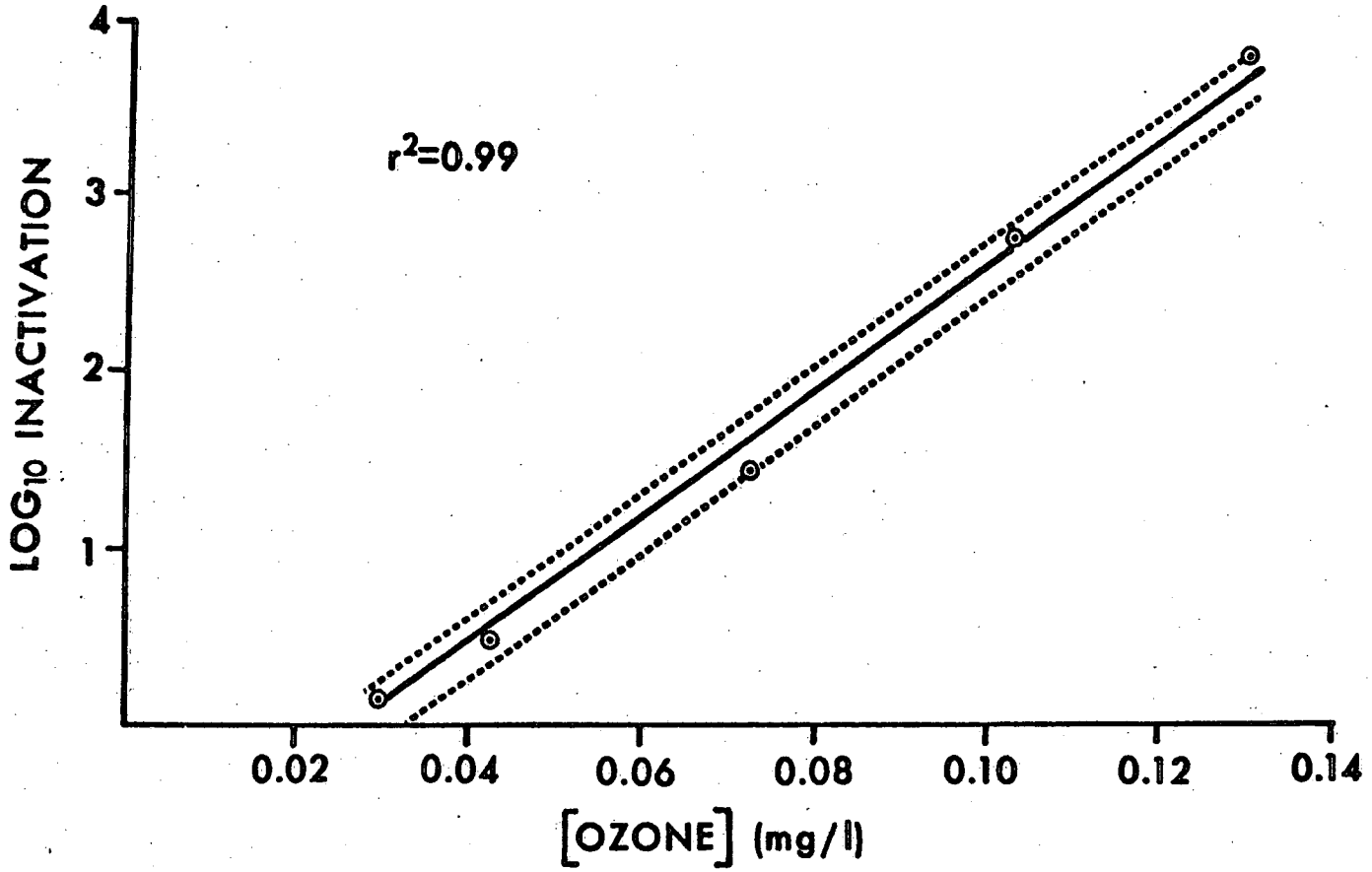


FIG. 6 CORRELATION BETWEEN VIRUS INACTIVATION AND INITIAL OZONE DOSE, SHOWING 95% CONFIDENCE LIMITS.

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

To convert from $\log_{10}$ inactivation to surviving fraction or % inactivation $\left[(1-N/No) \times 100\%\right]$.

$\log_{10}$ inactivation = 2.7 = - log(N/No)

$-2.7 = (0.3 - 3.0) = \log(N/No)$

$10^{0.3} \times 10^{-3} = N/No = 2 \times 10^{-3}$

% inactivation = (1 - N/No) x 100% = (1 - .002) x 100% = 99.8%

It is not necessary to carry out the mathematical manipulations to convert $\log_{10}$ inactivation to % inactivation if it is remembered that

1 $\log_{10}$ inactivation - 90% inactivation

2 $\log_{10}$ inactivation - 99% "

3 $\log_{10}$ inactivation - 99.9% "

4 $\log_{10}$ inactivation - 99.99% "

and so on. The fractional $\log_{10}$ inactivation term can be readily interpolated for example:

2.7 $\log_{10}$ inactivation

2 $\log_{10}$ inactivation - 99%

3 $\log_{10}$ inactivation - 99.9%

antilog 0.7 = 0.84

Therefore 2.7 log inactivation is 99.8%.

It is important to note that in Fig. 6 no virus inactivation occurs until the ozone concentration exceeds approximately 0.03 mg/l. Presumably this is due to the finite ozone demand of the soluble constituents of the virus suspension which must be overcome before inactivation proceeds. Hoigné and Bader (28) have indicated that during ozonation the soluble constituents of the suspension will react preferentially to the reaction of dispersed particles. This point of view is readily displayed in Fig. 7 which clearly indicates that as soluble constituents of the suspension are removed, by dilution or ultracentrifugation, the virus inactivation concomitantly increases. Again, in Fig. 8 is indicated the enhanced dose-response of a purified preparation of poliovirus.

Early in the study it became apparent that efforts to compare the relative resistance of a number of viruses to ozonation were fraught with much inconsistency. Attempts were made to delineate the reason(s) for these inconsistencies. One reason for differences in the relative extent of inactivation of the virus preparations was that the preparations were not similar. The stock virus suspensions were compared, on the basis of total organic carbon (TOC) content. Table 3 indicates the extent of virus inactivation by 0.1 mg/l ozone. Superficially it appears that some viruses are more resistant than others but, as seen in Fig. 9 this difference in "resistance" is most readily correlated to the different TOC of the virus preparations. Even though these suspensions

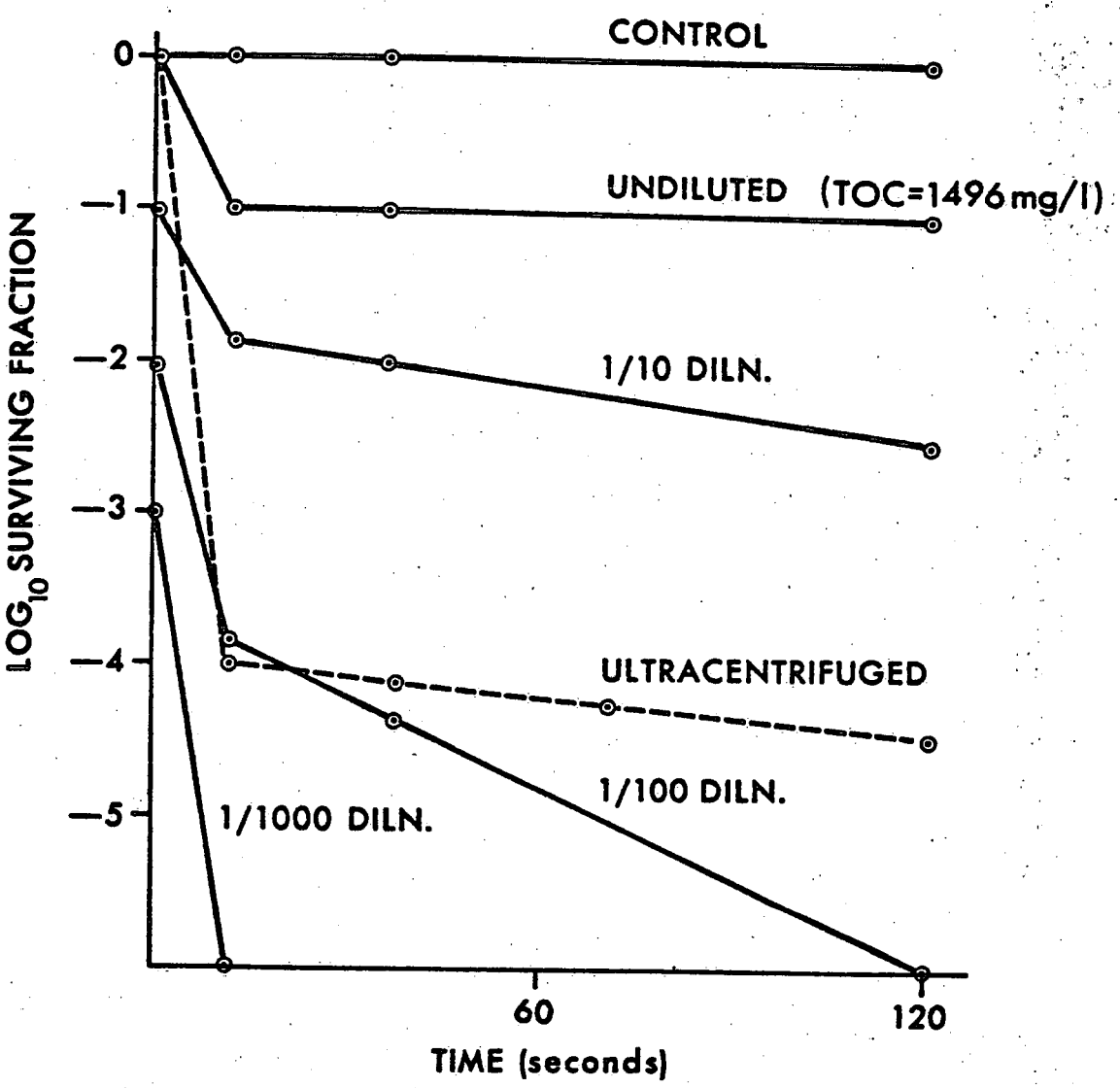


FIG 7. INACTIVATION OF POLIOVIRUS 1 BY 0.5 mg/l OZONE (INITIAL DOSE).

A. ——— EFFECT OF LOG DILUTION OF VIRUS SUSPENSION IMPURITIES

B. – – – – EFFECT OF ULTRACENTRIFUGATION 100,000 × g FOR ONE HOUR. (VIRUS RESUSPENDED IN OZONE DEMAND–FREE WATER).

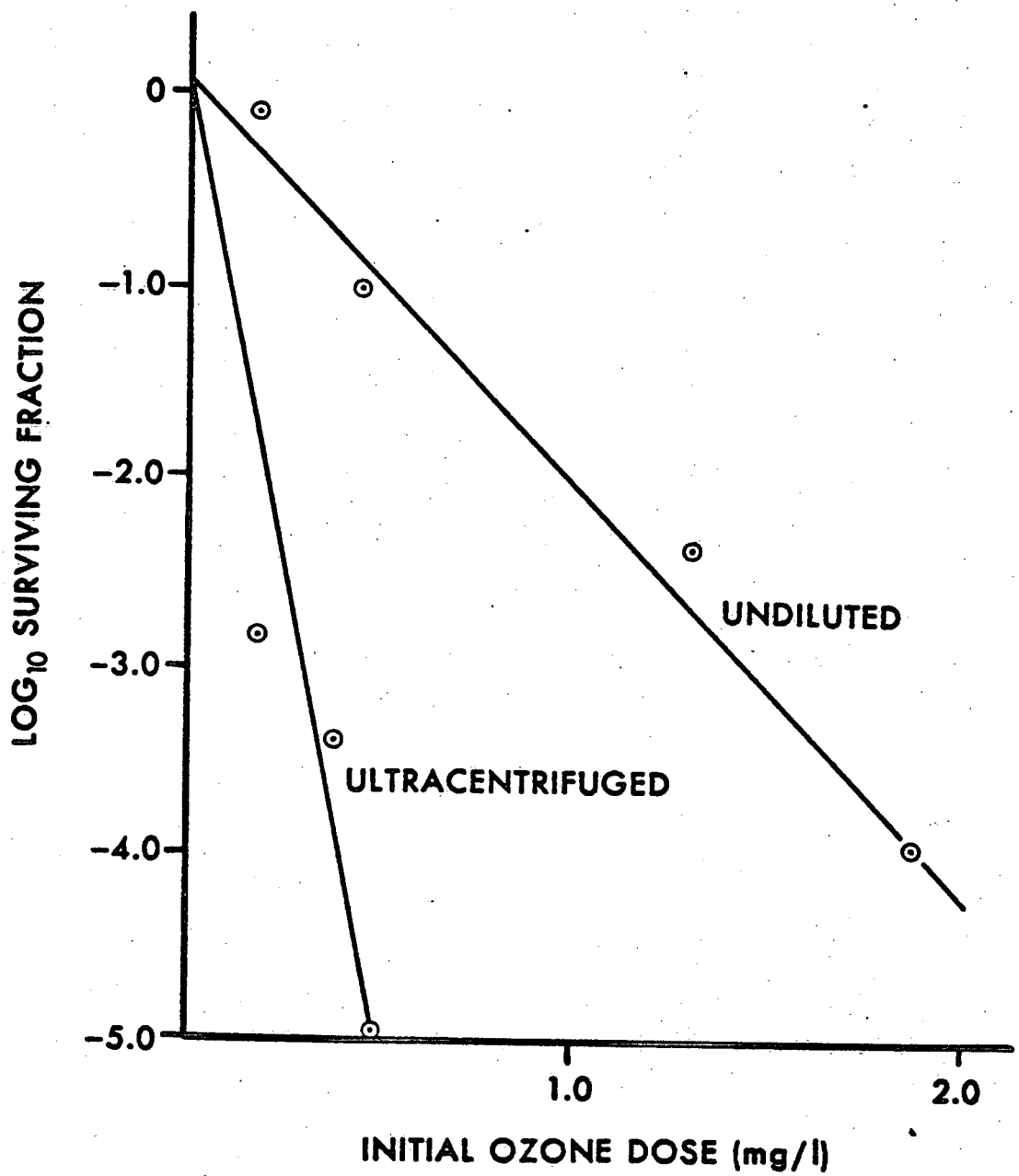


FIG 8. EFFECT OF INITIAL OZONE DOSE ON INACTIVATION OF POLIOVIRUS 1.

TABLE 3

VIRUS	TITRE (pfu/ml)	TOC (mg/l)	N	LOG_{10} INACTIVATION
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	3.7 ± 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	7	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

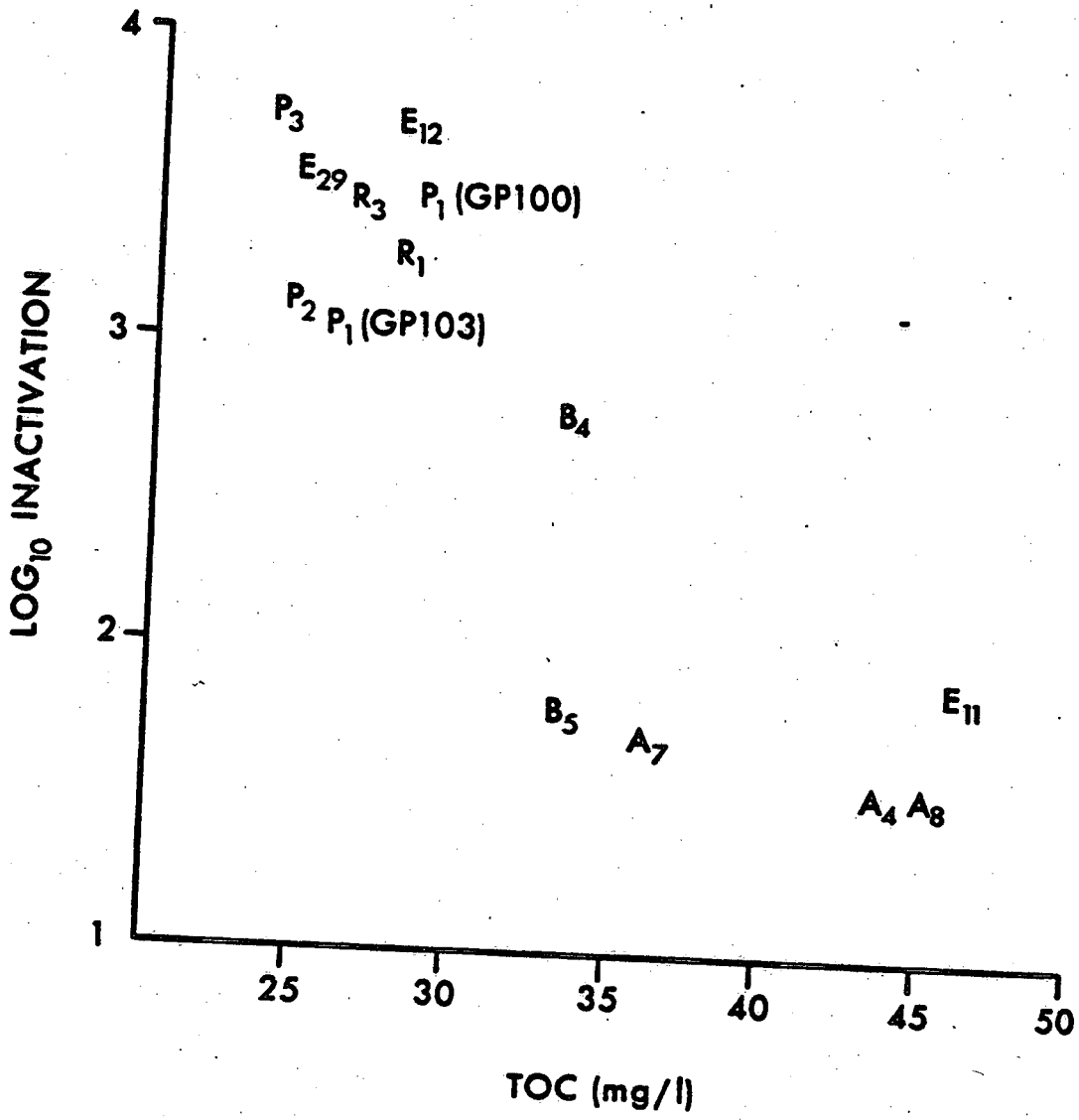


FIG. 9 CORRELATION BETWEEN VIRUS INACTIVATION BY OZONE AND TOTAL ORGANIC CARBON (TOC) IN THE VIRUS SUSPENSION. OZONE CONCENTRATION IS 0.1 mg/l. $r^2 = -.88$, $r^2 = .77$.
SEE TABLE 2 FOR STRAIN DESIGNATION.

were all prepared and ultracentrifuged in a similar manner gross differences are still apparent.

Efforts were made to determine relative inactivation by mixing each virus with diluted f2 bacteriophage (4.8 mg TOC). 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. Table 4 displays the results of this comparison. It is tempting to conclude on the basis of the data in Table 4 that there is a difference in the relative resistance of the viruses studied to ozonation. However, the data presented in Table 4 is the average of a number of inactivations and in the individual cases sometimes the virus and sometimes the phage was inactivated to a greater extent. On the average, however, greater inactivation was observed for the phage than for the viruses, and again there is a correlation between TOC of the preparation and extent of virus inactivation. But the picture is more complex than is evident at first glance. 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 inactivation when ozonated in combination with the phage! The addition of the phage (suspended in nutrient broth) influences the inactivations of the virus and appears to have "averaged" the influence of suspension TOC on virus inactivation.

TABLE 4

VIRUS	TOC(mg/l)	LOG_{10} INACTIVATION VIRUS	LOG_{10} INACTIVATION PHAGE	TOTAL LOG_{10} INACTIVATION
Polio 1 (GP103)	15.5	3.0 ± .4	3.4 ± .4	3.2
Polio 2 7778	15.3	2.6 ± .2	3.5 ± .5	3.1
Polio 3 GP 30	14.9	3.1 ± .2	3.5 ± .3	3.3
Polio 1 GP 100	16.8	2.4 ± .2	2.9 ± .1	2.7
Coxsackie B3	----	2.6 ± .7	4.1 ± .5	3.3
Coxsackie B4	19.3	3.9 ± .7	3.3 ± .4	3.6
Coxsackie B5	19.3	3.3 ± .2	3.2 ± .4	3.3
Adeno 4	24.9	2.8 ± .3	3.0 ± .4	2.9
Adeno 7	20.5	1.7 ± .3	3.9 ±1.1	2.8
Adeno 8	25.2	2.6 ± .4	3.2 ± .7	2.9
Echo 11	25.9	2.3 ± .5	3.1 ± .1	2.7
Echo 12	16.8	3.2 ± .3	3.3 ± .5	2.3
Reo 1	16.5	2.5 ± .1	3.9 ± .5	3.2
Reo 3	16.2	2.0 ± .2	3.4 ± .4	2.6

It is interesting to note at this point that strain GP103 poliovirus 1 and strain GP100 poliovirus 1 are, by application of the Student's T test, different in response to ozonation (at the 95% level of significance). Also, similarly, echovirus 12 and 29 show no difference. Previously Snyder (59) had reported a more than significant difference in the response of these two viruses to ozonation.

Clearly the factors influencing virus inactivation during ozonation are complex and, simple, general measurements give only a partial picture.

An attempt was made to perform inactivations of the different viruses under similar conditions by mixing the virus suspensions and, following ozonation, to determine the extent of inactivation of each virus by applying neutralizing antisera. Table 5 indicates the results of this attempt. No difference is apparent in the inactivation (95% level of significance) of each virus in the presence of the other. No cross-neutralization was evident. The application of this technique to some of the other viruses was not successful due, mainly, to breakthrough of the "neutralized" virus. Neutralizing antisera could not be obtained in sufficient titre to prevent this.

The effect of different kinds of TOC did not become fully apparent until sewage was used as the suspending medium for virus inactivation.

Aliquots of a stock virus suspension were ultracentrifuged and the pellets were resuspended in primary and secondary effluents at varying effluent concentrations (0-10%).

TABLE 5

Inactivation of poliovirus 1, 2 and 3 in combined suspension. 0.1 mg/l ozone. (individual virus was determined by the use of neutralizing antisera)

Virus determined	Initial titre (pfu/ml)	Neutralizing antisera	Final titre (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	3.4×10^2	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 primary and secondary effluents were typical samples obtained from the City of Winnipeg North Main Sewage Treatment Plant. The plant had the operating characteristics shown in Table 6. The bacteriological characteristics of primary and secondary effluent were to be evaluated each time a sample was collected for inactivation studies. However many of the inactivation studies with the effluents did not yield meaningful results, and this data was not obtained. Table 7 gives a typical fecal coliform, fecal streptococci and Pseudomonas aeruginosa count for both primary and secondary effluent.

The TOC of the primary effluent was approximately five times that of the secondary effluent. It would be expected that less inactivation would occur in the primary effluent than in the secondary. However, inspection of Fig. 10 shows that this is not the case. In each suspension the contribution to TOC of the virus suspension was 26 mg/l, and in Fig. 10 the TOC is the combined contribution of that from the virus and that from the suspension medium. The most striking observation is that the very small contribution of TOC from the secondary effluent has a dramatic effect on virus inactivation at the ozone concentration used, more so than that derived from primary effluent or nutrient broth.

Thus it appears that the "quality" of TOC is as important as the quantity of TOC in viral inactivation. This is an important concept when one considers that recycled waters may have significantly different organic matter content than fresh waters.

Fig. 11 shows the effect of ozone dose, at three levels of TOC

Table 6

Operating Characteristics - City of Winnipeg NE Sewage Treatment Plant*

Monthly Average - 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
BOD5 (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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Wastes and Wastewater Disposal
City of Winnipeg

TABLE 7

Bacteriological Quality of Primary and Secondary Effluents

Organism	Primary Effluent (CFU/100 ml)		Secondary Effluent (CFU/100 ml)	
	(1)	(2)	(1)	(2)
Fecal Coliforms	1.8×10^6	2.0×10^6	2.8×10^5	8.0×10^4
Fecal Streptococci	3.4×10^5	4.0×10^5	7.7×10^4	6.0×10^4
Pseudomonas aeruginosa	5.6×10^3	7.3×10^3	2.9×10^3	4.0×10^3

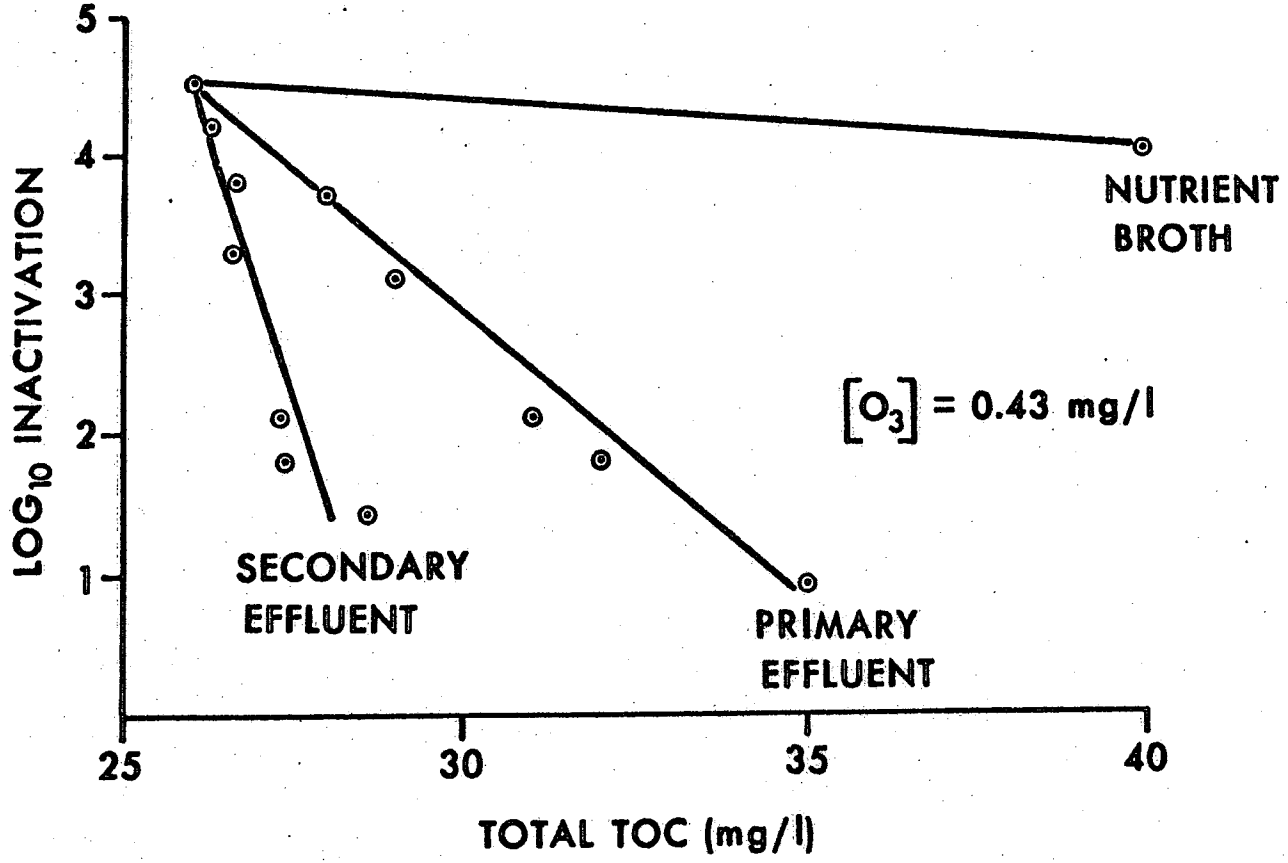

FIG 10. INACTIVATION OF POLIOVIRUS 1 (GP103) SUSPENDED IN PRIMARY AND SECONDARY EFFLUENTS AND NUTRIENT BROTH TOC OF VIRUS SUSPENSION IS 26 mg/l.

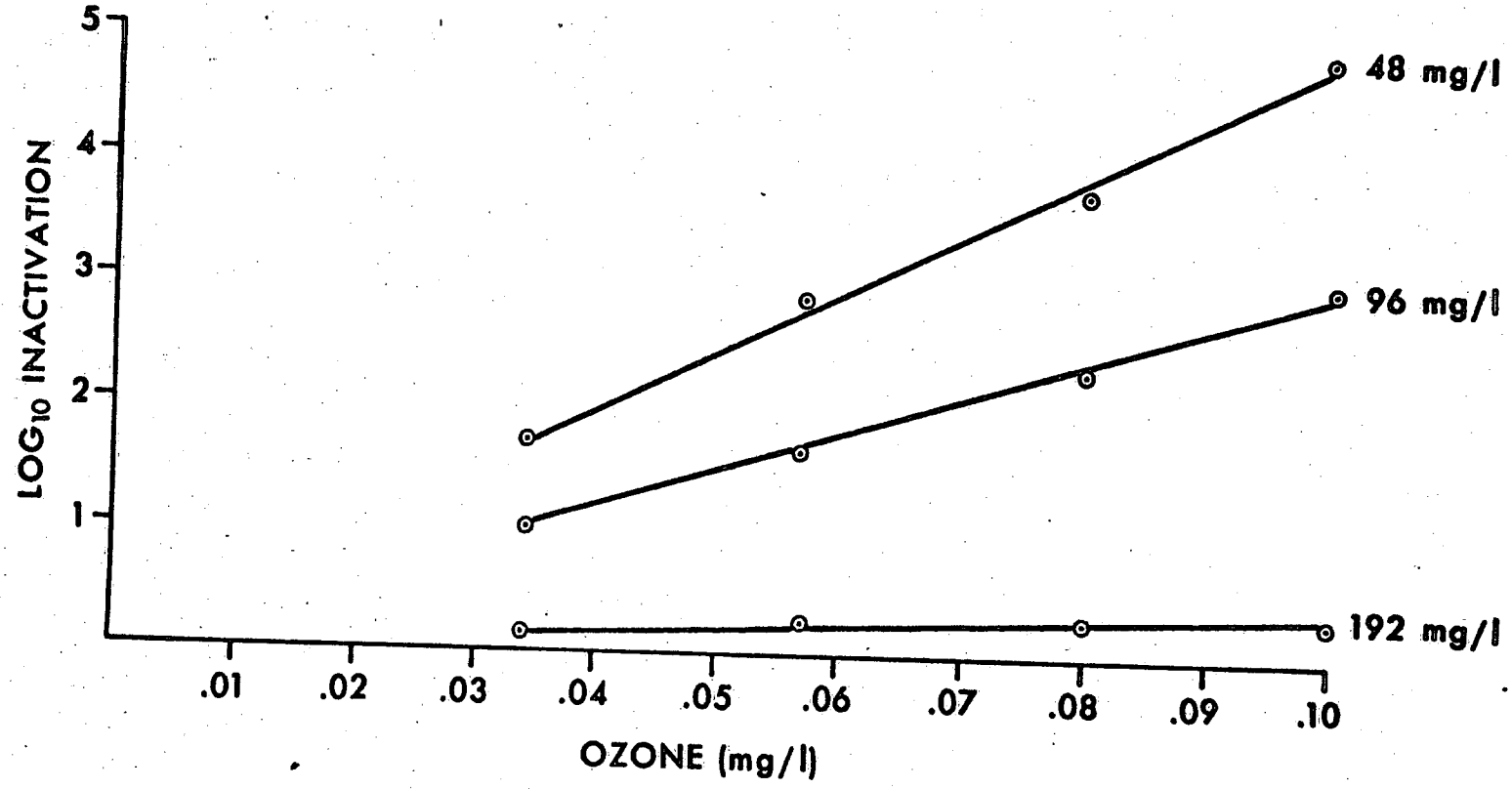

FIG 11. EFFECT OF TOC (SUPPLIED AS NUTRIENT BROTH) ON INACTIVATION OF f2 BACTERIOPHAGE.

(supplied as nutrient broth) on f2 phage inactivation.

The effect of TOC on f2 inactivation at 0.1 mg/l ozone is shown in Fig. 12. Considering the source of TOC it is difficult to equate the resistance to ozonation of f2 phage with that of the virus preparations. Reference to Table 4 shows that the total inactivation in mixed suspensions of phage and virus is correlated well to the total TOC of the mixture but no conclusions can be drawn concerning individual virus resistance from this data. A similar result is obtained when the T series phage are ozonated as tabulated in Table 8.

A standard procedure in the preparation of virus stock pools of entero viruses is to add chloroform to the virus preparation to promote cell debris dissociation and to eliminate contaminating bacteria. Chloroform is not completely removed from the preparation and the small amount of carry over into the virus stock pools has a high ozone demand and a dramatic effect on virus inactivation. Table 9 lists some sources of ozone demand, encountered in virus pool preparations, and their effect on virus inactivation.

The effectiveness of ozonation as a virucidal agent is determined primarily by the chemical and physical state of the virus suspension. It is apparent, from Table 9, that carry over of soluble constituents from the cell fluids to the virus suspension medium influences greatly the extent of inactivation of the virus achieved by ozonation. Indicator dye (phenol red) used in the tissue culture medium and chloroform treatment of the virus suspension both show a particularly

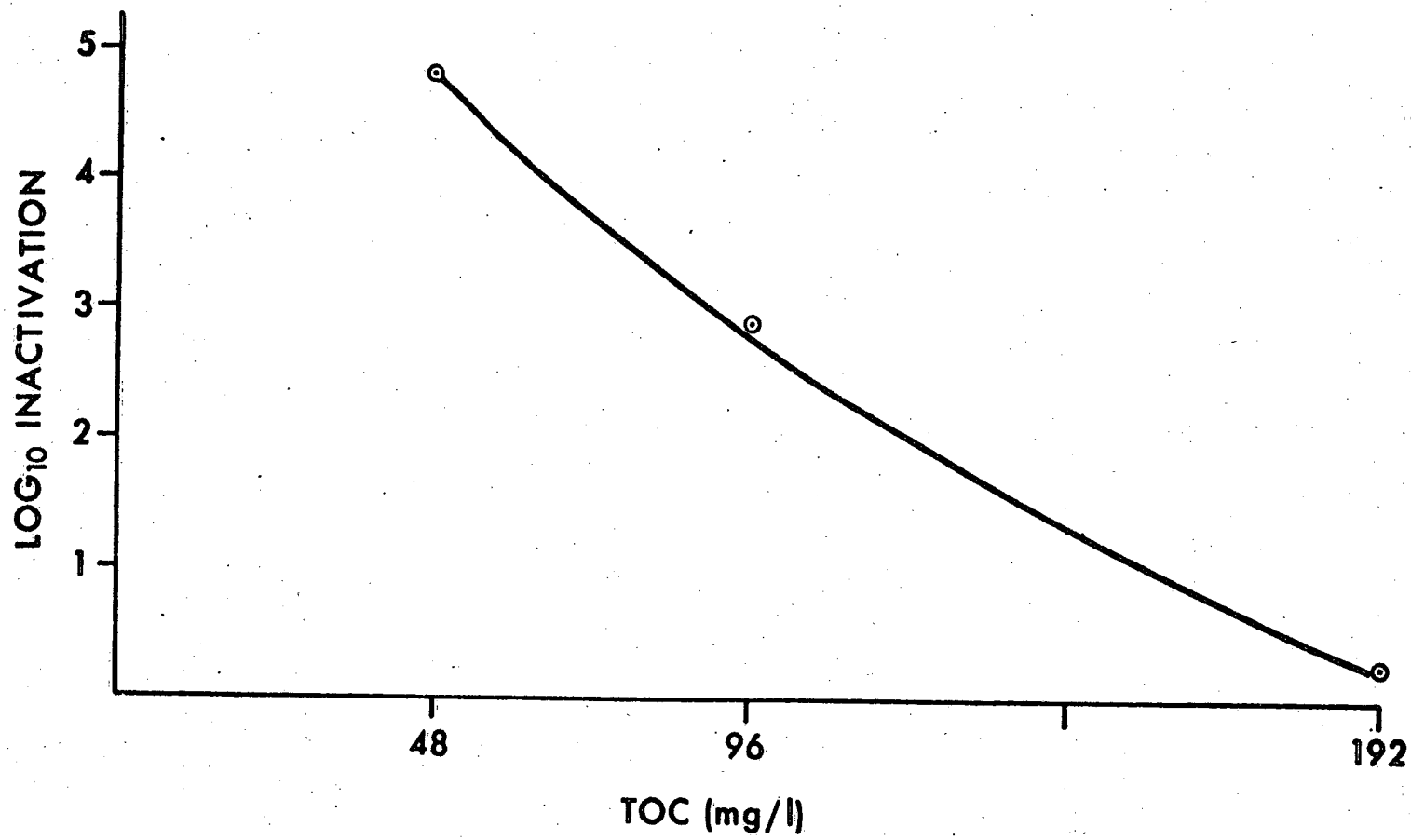

FIG 12. INACTIVATION OF f2 BACTERIOPHAGE — DEPENDENCE ON SUSPENSION TOC. OZONE DOSE IS 0.1 mg/l.

TABLE 8

Inactivation of mixed suspension of f2 and T1, T2 and T3 coliphage by 0.1 mg/l ozone (Coliphage, suspended in nutrient broth was diluted 1/100 and mixed with a similar dilution of f_2)

Mixture	Log_{10} Inactivation	
	coliphage	f_2
f_2 + T1	4.8	3.5
f_2 + T2	4.1	3.7
f_2 + T3	3.5	2.7

TABLE 9

Sources of ozone demand and effect on inactivation of Poliovirus 1* at 0.1 mg/l ozone

Preparation	Log_{10} Inactivation
Stock virus harvested from tissue culture fluids (Phenol red not present in the medium)	0.05
Stock virus diluted 1/10 in ozone demand free water	0.52
Stock virus diluted 1/100 in ozone demand free water	2.44
Stock virus centrifuged 10,000 g for 30 minutes	0.44
Stock virus centrifuged 10,000 g for 30 minutes then 100,000 g for 1 hour, resuspended in ozone demand free water	3.0
Above diluted 1/10	4.8
Preparation No. 5 with further 10,000 g (30 min.) centrifugation	3.2
Preparation No. 5 frozen at -70° C	2.2
Preparation No. 5 previously treated with chloroform (final concentration 5%)	0.6
Preparation No. 5 virus grown in medium containing phenol red	1.58

* All treatments were applied in a single experiment to a common source of stock virus.

dramatic effect. Routine freezing of aliquots of virus suspension also influences the inactivation.

Ultracentrifugation as a single treatment is not sufficient "purification" since further dilution of this preparation greatly increases the amount of inactivation of the virus.

Bioassay of virus may be a source of error in determining the effect of ozonation. It is well known that there is a disparity between the number of particles determined by electron microscopy and plaque forming units (58). The data in Table 10 show that there is no important difference apparent when three cell lines were used for bioassay of ozonated poliovirus.

An attempt was made to standardize the assay procedure by using a common cell line (except for adenoviruses) for virus assays. The effects of physical parameters were not further studied but it was observed that the echoviruses showed a poor response to plaque production on BGM cells which were greater than four days old before infection.

Very little information is available on the mechanism of action of ozone. Direct alternations of DNA and RNA have been suggested because of changes in the absorption spectra (10), reactions with pyrimidine bases (51), mutants formed by Escherichia coli exposed to aqueous ozone solutions, and chromosomal aberrations of mouse ascites, chick embryos (53), and human cells (19). Recently Hamelin and Chung (23) reported a genetic locus involved with DNA repair mechanisms in an ozone resistant E. coli.

The radiometric effects of ozonation suggest that free radicals are involved. The short lived decomposition product of ozone - the

TABLE 10

Effect of assay system on determination of poliovirus inactivation by 0.5 mg/l ozone

Cell line	Log_{10} inactivation
BGM	4.25
Vero	4.26
HeLa	4.18

hydroxyl radical - has been proposed as the germicidal agent in ozonation (13, 28, 49). Hewes and Davis (27) showed that COD removal was proportional to the ozone decomposition rate rather than the concentration of undecomposed ozone.

The importance of viral resistance is highly documented in the literature and many reports appear which report virus strains that are more resistant to a particular disinfection treatment. A good example is the resistance of f2 coliphage which has been reported as less resistant and more resistant than poliovirus to chlorination. Cramer (12) found no difference in the relative resistance of f2 and poliovirus when a mixed suspension was inactivated by chlorine and iodine.

Very little is known of the effect of ozonation on viruses associated with suspended solids. Moore, Sagik and Malina (44) have established that viruses associated with suspended solids are infective.

Further study is also required to determine the effect of virus aggregation in the ozonation process. Floyd and Sharp have suggested that virus aggregates may be the normal state in nature (21).

CONCLUSIONS

Virus inactivation by ozonation is exponentially related to the amount of ozone applied. The extent of inactivation is determined primarily by the amount and kind of soluble constituents in the suspending fluid. Under equivalent conditions the extent of virus inactivation is similar for all viruses and f2 phage.

The relative resistance of virus to ozone could not be unequivocally determined neither could the rate of the inactivation reaction. However for all practical purposes, considering that mass transfer of ozone gas into solution requires a considerably greater time than that observed for virus inactivation, the inactivation process is mass transfer limited in a dynamic system.

Ozone is a potent germicide when not influenced by soluble ozone demanding substances, but becomes dramatically less effective in the presence of certain ozone demanding substances.

The measurement of TOC is not sufficient alone to determine the effect of ozone on virus inactivation.

More work is required to determine the effect of ozonation under actual operating conditions. Laboratory observations can serve only to determine the direction of practical pursuit.

It is misleading to talk in terms of "residual" ozone since it is not possible to determine, with conventional methods, whether a residual exists. Present methods determine only total oxidant, with unknown germicidal properties. It is unlikely that ozone, being so reactive persists for any appreciable time in an organically contaminated environment. In any case it is certainly better to index microbial inactivation to initial ozone dose, which can be accurately measured.

On the basis of presently available information it is difficult to predict a recommended use dose of ozone. It appears that each

situation will require individual evaluation.

The present work sets the stage for a more intensive study into the effectiveness of ozonation as a viral disinfectant. Further investigation is required to elucidate the mechanism of viral inactivation and the effect of virus adsorption to suspended solids. There is little doubt that ozone, in aqueous solution, is a potent virucide but its effectiveness is severely curtailed by soluble organic contaminants.

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APPENDIX I

Growth Medium for Cell Cultures

MEM concentrate

MEM Auto-Pow without phenol red (Flow Laboratories)	86.48	gm.
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-(Δ)	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/mg.
Streptomycin sulfate (Sigma Chemcial Co.)	10,000	ug./ml.
Amphotericin B (Squibb)	250	ug./ml.

APPENDIX II

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 (△, 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 $\underset{\sim}{N}$ NaOH.

APPENDIX III

Nutrient Agar Overlay with 2% Fetal Calf Serum

MEM Auto-Pow with phenol red (19 mg./ml., Flow Laboratories)	2110	ml.
Fetal calf serum (Δ, 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 (Δ, Flow Laboratories)	360	ml.

APPENDIX IV

Nutrient Agar Overlay for Adenoviruses

MEM Auto-Pow with phenol red (19 mg./ml., Flow Laboratories)	1,900 ml.
Fetal calf serum (Δ, 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 V

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 500 ml. bottles. To each bottle was added:

Concentrated PSF solution	5 ml.
Eagle's buffer at pH 7.3	5 ml.

Virus Diluent

Neutralizer (prepared above)	1000 ml.
Ozone demand free water	1000 ml.

The diluent was dispensed into 100 ml. bottles and stored at 4°C.

APPENDIX VI

Hanks' Balanced Salt Solution (HBSS)

Solution A - (10X)

NaCl	80	gm.
KCl	4	gm.
$Mg \cdot SO_4 \cdot 7H_2O$	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 solutions were autoclaved at 121°C. for 20 minutes and stored at 4°C.

APPENDIX VII

Dulbecco's Phosphate Buffer Saline

Without Ca^{2+} or Mg^{2+} (PBS) pH 7.5

10X Solution

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

Double glass distilled water to 1000 ml.

Working Solution:

10X PBS	100	ml.

Double glass distilled water to 1000 ml.

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

APPENDIX VIII

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 12,000 g for 1 hour. The solution was filtered through a series of membrane filters with Dacron separators starting with a pre-filter, then a 1.2 u, 0.8 u, 0.6 u and 0.45 u filter.

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 $\underline{\sim}$ NaOH	2.0 ml.
CW Trypsin	25 ml.

APPENDIX IX

Neutral Red Agar

10X Solution

Neutral Red dye and NR redundant (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

10X 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 temperature in a boiling water bath and dispensed into 200 ml. bottles, which were then stored at 4°C. Just prior to use the agar solution was melted and cooled to 47°C.

APPENDIX X

f2 phage cultivation media

Tryptone broth	g/l
Bacto tryptone	10.0
Yeast extract	1.0
Glucose	1.0
NaCl	8.0
$CaCl_2$	0.33

pH to 7.0 with NaOH

Autoclave 121°C

For plates add 1.5% Bacto agar and dispense 25 ml into plates.

Overlay - above broth with 0.7% agar

Diluent	g/l
NaCl	8.0
$CaCl_2$	0.33

Sterilize by autoclaving.

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