RICE UNIVERSITY

Inactivation of Solids-Associated Virus by Hypochlorous Acid

by

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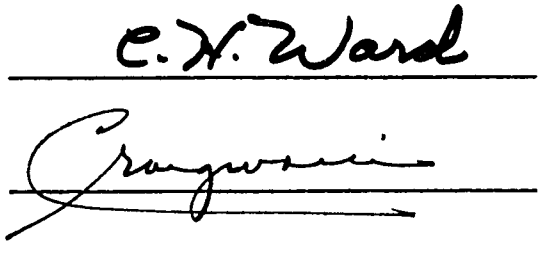
Signatures of Thesis Directors:



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DEDICATION AND ACKNOWLEDGMENTS

This work is dedicated to C. Wallis and C.H. Ward; without their encouragement, counsel and financial assistance, it could not have been completed.

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M. Kessick and J. Hightower were especially helpful in interpreting adsorption and inactivation data. The hours I have spent in discussion and experimentation with classmates and friends both at Rice University and Baylor College of Medicine have been among the most valuable and memorable of my life.

Special thanks go to Carla; she has supported my efforts and has made life interesting and exciting even when pregnant ideas and discoveries were few and far between.

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1. Literature Survey: Chlorination of Virus

1.1 Susceptibility of Viruses to Chlorination

Evaluation of chlorine and chlorine-containing compounds as viral disinfectants began in the 1940's. Kessel et al. (1943) showed that for complete inactivation of poliovirus it was necessary for the virus to be in contact with total residual chlorine levels of 0.5 to 1.0 mg/l for at least 3 hours. Trask, Melnick and Wenner (1945) performed inactivation tests using poliovirus obtained from stools of paralytic patients, from passaged monkey cord suspensions, and from Theiler's mouse poliovirus. It was found that (1) 10-minute contact periods of exposure to 0.5 mg/l residual chlorine did not totally inactivate the monkey-adapted virus, (2) 10% diluted suspensions of stools from poliomyelitic patients protected viruses from inactivation, (3) inactivation of mouse virus was dependent upon the molecular form of the chlorine applied, and (4) extraneous organic matter interfered with the disinfection process.

Kelly and Sanderson (1958) utilized three poliovirus strains and two coxsackievirus B strains to show that complete inactivation of these viruses was not achieved by the usual conditions for bacterial disinfection of water supplies. Viral inactivation by chlorine was shown to depend upon the type of virus, pH level, free chlorine concentration, contact time, and temperature. In water at pH 7 and 25°C, a minimum free chlorine residual of 0.3 mg/l for 30 minutes was required; at higher pH levels or lower temperatures, more chlorine was needed.

Lothrop and Sproul (1969) demonstrated that present chlorination practices requiring a total residual of 1 mg/l chlorine are inadequate

for a high level of virus inactivation. A 99.99% destruction of type 1 poliovirus demanded 40 mg/l combined chlorine residual and a contact time of 30 minutes in settled wastewater. Significantly, free chlorine residuals were extremely virucidal at levels of only 0.2 to 0.4 mg/l for 30 minutes.

Since the work of Clarke et al. (1956) and Weidenkopf (1958), hypochlorous acid has been the form of free chlorine found to be the most effective against viruses. Hypochlorous acid is not charged, and it was felt that an uncharged molecule may have the best chance of penetrating the ionized capsid. Hypochlorite ion has recently been shown by Scarpino et al. (1972) to be an effective virucide, although the high pH level, pH 10, used in these experiments probably precludes its usefulness in practical applications.

It is evident that viruses can be inactivated by chlorine compounds, but this involves much more than just exposure of the infectious particle to disinfectant molecules: controlling factors are the type of virus, type and concentration of chlorine, hydrogen ion concentration, temperature, time of contact and presence of interfering substances.

1.2 Mode of Action of Chlorine

The enteric viruses and the RNA-containing bacteriophages consist of a tightly packaged interior strand of messenger RNA which is surrounded by a proteinaceous capsid of icosahedral geometric design.

Ingols (1963) assumed that the mode of action of chlorine on viruses consisted of an attack on many sites in the virus proteins. Evidence for this hypothesis was that chlorine is capable of disrupting peptide

linkages, substituting onto the benzene ring of tyrosine, deaminating free ammonia radicals, and changing viral antigenic characteristics.

Olivieri et al. (1973) reported on inactivation of phage f2 by chlorine, iodine and bromine. Phage f2 appeared to be inactivated by chlorine attack on the nucleic acid. Chlorine inactivated free RNA at a rate similar to that of the whole virus, and the protein was still capable of functioning in adsorption after the virus was inactivated. These workers found that f2 was more susceptible to hypochlorous acid than to hypochlorite ion and theorized that the hypochlorite ion was hindered in its passage through the net-negatively charged capsid by electrostatic repulsive forces.

1.3 Inactivation Kinetics

Beginning with Chick (1908), there has been much interest in describing the inactivation process kinetically. Although this early work was with bacteria and bacterial spores, Chick's postulate that with an excess of disinfectant the inactivation follows a first-order course with respect to remaining viable organisms is the basis for most interpretations of viral disinfection data.

Results of disinfection tests have traditionally been presented in the form of survival curves. Survival curves display either the logarithm of the remaining viable viruses or the percentage of remaining viable viruses against elapsed contact time. First-order behavior, also known as exponential law behavior, is evidenced when the logarithm of surviving virus plots as a straight line of downward slope against increasing elapsed time. Fair et al. (1948) plotted the logarithm of the

concentration of free chlorine required to inactivate a given percentage of starting virus, say 99% inactivation, versus the logarithm of contact time. The resulting straight line of negative slope was represented by an empirical equation. This equation is $C^n t = K$, in which "C" is the chlorine concentration, "n" is an exponent called the coefficient of dilution, "t" is the time necessary to achieve some required inactivation, and "K" is a constant.

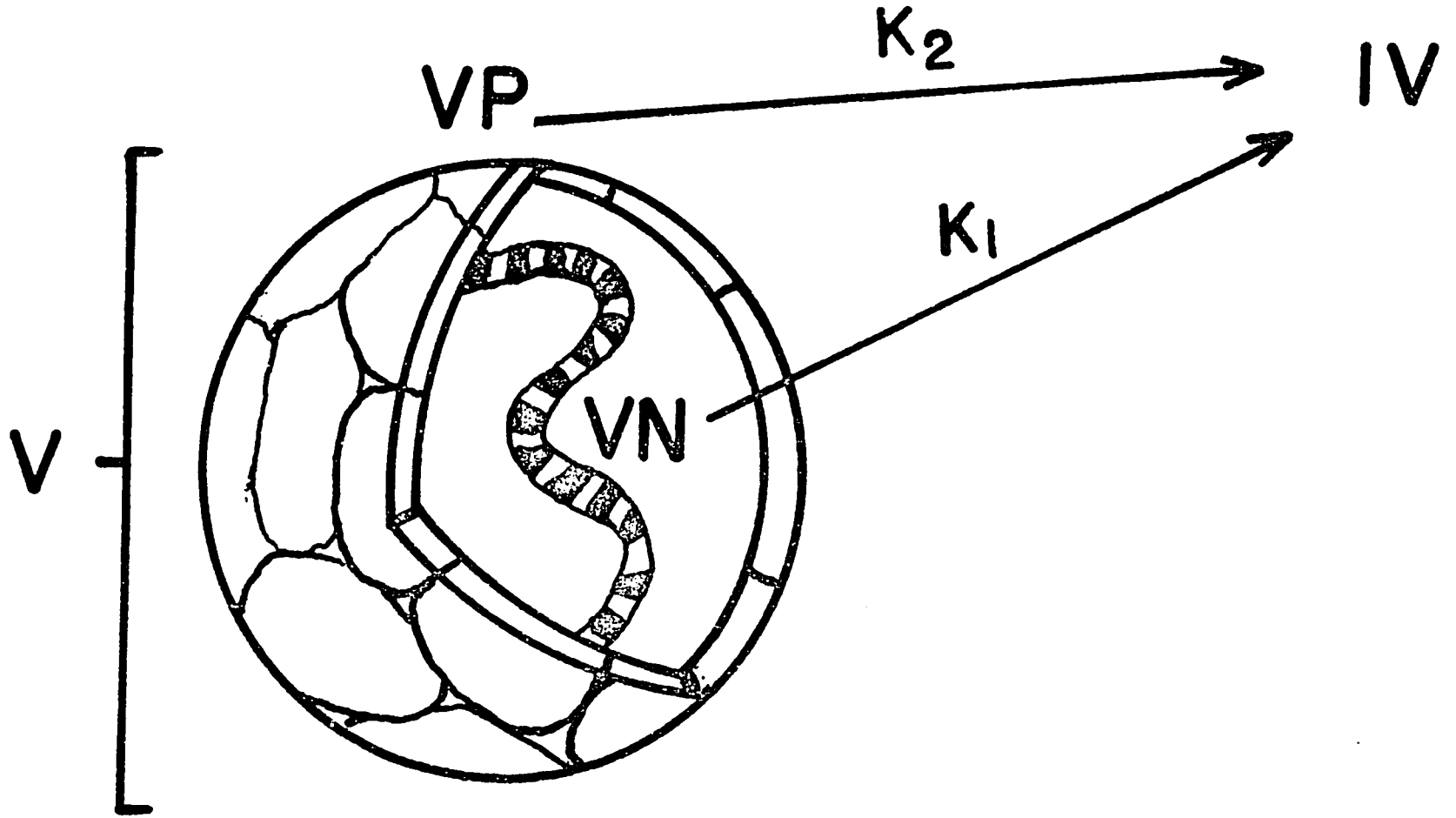
Salk (1956) reported that inactivation of poliovirus by formaldehyde could be described by first-order kinetics. Gard (1957) contended with Salk's findings by stating that according to his experiments with formaldehyde the rate of inactivation does not remain constant but decreases continually in the course of the process. He postulated that "tanning" occurs in which early exposure to the disinfectant alters the capsid proteins, making it increasingly difficult for subsequently appearing disinfectant molecules to approach the viral nucleic acid. Weidenkopf (1958) reported behavior similar to that of Gard for type 1 poliovirus exposed to free available chlorine at pH 6 and pH 7 at 0°C. Inactivation rates were greatest at the beginning of the exposure period; survival curves displayed two slopes with an initial steep grade followed by a more shallow slope. Weidenkopf incorrectly concluded that the inactivation rate was independent of virus concentration because he found the same percentage survivals occurring at the same times for a given concentration of chlorine regardless of the starting concentration of virus. Consideration of the survival curves presented in his paper indicates that a plot of the time required to attain 99% inactivation as

a function of starting virus concentration plots as a horizontal line which is indicative of a first-order reaction. Hiatt (1964) considered some of the factors which cause observed inactivation rates to deviate from the exponential law behavior and stated that these phenomena are most likely due to errors in dosimetry (decreasing concentration of disinfectant throughout the contact time) or heterogeneity of the viral population.

It is a reasonable assumption that a virus may be rendered non-infectious either by chlorine attack on the nucleic acid or by chlorine attack on the proteinaceous capsid. Based on the report of Olivieri, it may be assumed that the inactivation rate for the nucleic acid is the faster of the two rates. The model for a reaction system such as this is analogous to that presented by Hightower (1975) for the chemical reaction of A and B by two pathways, each first order in A and in B, to product C with reaction rate constants K_1 and K_2. Virus inactivation by two pathways is shown in Figure 1-1. Although it is not the purpose of this research to pursue the applicability of such a model for chlorination, it is worthy of comment inasmuch as it has not appeared in the literature and raising the question of its possible usefulness may prompt future experimentation to test the model.

Majumdar, Ceckler and Sproul (1972) have set a good example for future chlorination studies by their publication of inactivation data for ozonation of poliovirus in the form of definite rate equations. Rate equations express the dependency of inactivation upon the concentration of virus, concentration of disinfectant, and contact time in the power

Figure 1-1. Model for virus inactivation by two pathways. Virion (V), viral nucleic acid (VN), viral protein capsid (VP), inactivated virus (IV).


VP
K2
IV
K1
VN
V

law form. This form of presentation allows the disinfection data to serve as the basis for selection of the type and size of disinfectant reactor and the conditions under which the reactor is to be operated for best performance.

1.4 Effects of Aggregates

Waterborne viruses may exist as singly suspended particles or they may exist as a member of an aggregate of viral particles. Evidence for aggregation has appeared from electron microscopy and from immunological studies. For example, Wallis and Melnick (1967) showed that the cause of the non-neutralizable persistent fraction of echovirus type 4 (Pesacek) was virus aggregation. Virus in the non-neutralizable aggregate was found to constitute 30% of the infective units of unfiltered Pesacek.

Berg, Clark, Berman and Chang (1967) developed a mathematical model for virus disinfection which took into account aggregation as the cause of aberrations in survival curves of viruses exposed to disinfectants. These aberrations normally appear as a bimodal rate of inactivation; that is, the slope of the percent survival curve versus time is much steeper for short exposure times than for longer exposure times. Some have proposed that monomers are inactivated first; whereas, the aggregated viruses are more resistant to inactivation. Poduska and Hershey (1972) presented a model for virus inactivation by chlorine in which the factor of aggregate size was included. The model was based on the principles of first-order, irreversible chemical reaction kinetics and it was assumed that viruses in clumps were inactivated at a slower

rate than single viruses. It was not possible to determine the clump size distribution; therefore, it was assumed that only two sizes existed. Experimentation was not performed by the modelers, rather published and unpublished data were analyzed for fit to the aggregate model. The model validity is questionable since no information is available on whether aggregates were actually present in the tests shown or if the bimodal survival curves could have resulted from errors of dosimetry.

1.5 Effects of Solids

To the possibility that a virus may exist as a freely suspended monomer or as a member of an aggregate of viral particles must be added a third possibility: the virus may be adsorbed to the surface of or be enmeshed within particulate matter.

In the keynote address at the recent Water Resources Symposium, Gerald Berg (1974) stated: "To be sure, hypochlorous acid is an excellent disinfectant for waters that do not contain solids, ammonia, or organics. But we still need to know the extent to which solids affect disinfection . . . " Culp (1974) has reviewed those factors influencing virus inactivation by chlorination and has stated that viral disinfection is improved in waters of turbidity not greater than one Jackson Turbidity Unit. Culp theorized: "At higher turbidities, viruses, because of their small size, may become enmeshed in a protective coating of turbidity contributing matter."

To this date, few studies have been reported concerning the role of suspended solids on disinfection. Lee and Waller (1970) looked at the effect of solids on sewage disinfection, but the indicator organism

here was a coliform. Lund (1973) briefly noted that the viral inactivation rate constant may be dependent upon the oxidation potential and, that the presence of reducing compounds in the form of suspended solids may reduce the oxidation potential. Lund notes that the measurements of such potentials would be both inconvenient and difficult. Boardman and Sproul (1975) reported that bacteriophage T7 adsorbed to bentonite and then exposed to chlorine is not any more resistant to inactivation than if the virus was freely suspended. In their words: "It has been found with the T7 virus in triple distilled water at a pH of 7.0 with free chlorine residuals as low as 0.07 mg/l that the inactivation was 100% in 10 minutes. When the T7 virus was adsorbed to 50 mg/l of kaolinite clay under salt conditions which did not lead to aggregation of the clay particles, complete inactivation of the virus was also noted at 0.07 mg/l of free chlorine residual with 10 minutes of contact time. Adsorption of this virus to kaolinite does not result in a physical orientation of the virus such that it cannot be attacked by the free chlorine." The last sentence of this quotation from Boardman and Sproul (1975) is not justifiable. Bacteriophages are extremely susceptible to chlorination (Poduska and Hershey, 1972): 4- or 5-logs of these phages are lost within 4 minutes with exposure to less than 1 mg/l HOCl. It is not surprising then that complete inactivation was found after 10 minutes. The taking of samples initially and then after 10 minutes does not allow any conclusions regarding the inactivation rate to be drawn. It is possible that the inactivation rate for the solids-associated virus was less than that for the freely suspended virus but that all virus in both cases were inactivated

after 10 minutes.

What types and concentrations of solids are present in sewage? Camp and Meserve (1974) state that in municipal sewage of medium strength, the total solids content is about 800 mg/l: 40% of which is suspended and 60% is colloidal and dissolved. About 70% of the suspended solids are organic, and the rest are mineral. Faust and Hunter (1971) have found that activated sludge plant effluents contain 2-4 mg/l of colloidal organics.

Several important questions must be answered with regard to disinfection of solids-associated viruses. Do mineral solids protect attached viruses? Do organic solids protect attached viruses? Can the inactivation of solids-associated virus be expressed in the form of rate equations applicable to process design?

2. Literature Survey: Interaction of Virus with Particulate Matter

2.1 The Virus in Suspension

Because of their size and their hydrophilic proteinaceous capsids, the enteric viruses and the bacteriophages behave as biocolloids. Ionizable carboxylic and amino groups on the capsid surface are influenced by the presence of charged solutes such as hydrogen ions, hydroxyl ions, and soluble salts. Throughout the normal pH range in natural waters, viruses, as well as the common suspended clay particulates, exhibit a net negative surface charge. Both chemical and physical theories have been advanced to explain the aggregation of such like-charged particles; Stumm and Morgan (1970) have discussed in detail the electrical double-layer theories of colloidal stability which hypothesize that multivalent cations compress the double layers of neighboring particles sufficiently to allow van der Waal's forces to hold the interacting particles together.

2.2 Adsorption to and Elution from Particulates

Shepard and Woodend (1951) suspended bacteriophage T2 with glass powder, Celite AFA and aluminum oxide in various concentrations of sodium chloride at neutral pH and found that adsorption of the phage was markedly dependent upon the salt molarity. Adsorption was retarded under basic pH conditions. The addition of meat infusion broth or bovine serum albumin to the medium limited adsorption; it was assumed that the organic material blocked adsorption sites since infection of the host cell was not inhibited by broth or albumin.

Working with a tryptophan-dependent bacteriophage, Fildes and Kay (1963) showed that adsorption to kaolin depended upon the molarity

of sodium chloride. In the range from 0.02 M to 0.08 M, adsorption of the phage displayed an A-shaped curve with the maximum adsorption occurring at 0.05 M.

Dunn and Hitchborn (1965) found that adsorption of plant viruses to bentonite at pH 7.4 was influenced by the concentration of magnesium ions. Seventeen plant viruses were tested for adsorption to bentonite at four magnesium sulfate concentrations ranging from 10^{-4} M to 5×10^{-3} M. These plant viruses could be classified into three groups according to their adsorption characteristics. Tobacco rattle virus, for example, avidly adsorbed even at the lowest magnesium concentration. Tobacco mosaic virus and wild cucumber mosaic virus only adsorbed at high magnesium concentrations. The third class, which included turnip yellow mosaic virus, did not adsorb at any magnesium concentration. These investigators suggested that the magnesium-dependent adsorption results from the linkage of virus protein to bentonite through the mediation of magnesium ions. Differences then between viruses in their affinity for bentonite may be due to differences in the amounts of magnesium bound to the surfaces of the different viruses.

Cookson (1966) utilized agitated suspensions of bacteriophage T4 and activated carbon to study the kinetics and mechanisms of virus adsorption. Optimal conditions for adsorption of this phage were pH 7 and an ionic strength of 0.08. The adsorption data were well fitted by the Langmuir isotherm. It was concluded that the adsorption mechanism was of a physical nature, resulting from electrostatic forces between the virus and the carbon surface. Most likely the charged carboxyl

groups and charged amino groups are involved. Esterification of carboxyl groups on the carbon reduced adsorption, indicating that these particular surface groups played a role in the attachment phenomenon.

Carlson et al. (1968) attached bacteriophage T2 to three different clays: for sodium chloride concentrations of at least 0.02 M, more than 90% of the phages adsorbed to kaolinite, montmorillonite, and illite. The presence of egg albumin, bovine albumin and sewage organics inhibited the attachment of phages by competition for adsorption to the clay surface; and, importantly, such organics displaced viruses that had previously attached to the clay.

Roper and Marshall (1974) adsorbed and desorbed an E. coli strain-13 lysogenic phage to montmorillonite by adjustment of salinity. Examination under the electron microscope showed that the phage adsorbed either in a face-to-face or tail-to-face orientation. Based on the mechanism set forth by Marshall et al. (1971) for adsorption of bacteria to surfaces, Roper and Marshall (1974) proposed that, in like manner, attraction of a negatively charged phage to a negatively charged surface occurs when the electrolyte concentration is high enough to compress the electrical double layers at the interacting surfaces to a point where the van der Waal's attractive forces become effective. Lowering of the electrolyte concentration results in restoration of the diffuse electrical double layers and the repulsion forces separate the interacting surfaces.

Bitton and Mitchell (1974) investigated the adsorption of bacteriophage T7 to magnetite and found that addition of 250 ppm of $CaCl_2$

to the virus-magnetite suspension increased adsorption of the phage from 34 to 95%. These experimenters theorized that the divalent cations bridge the virus to the iron oxide, thereby forming a magnetite-cation-virus complex.

Gerba (1973) and Gerba and Schaiberger (1975) have implicated virus adsorption to particulate matter as a possible explanation for the loss of viral titer in seawater. These investigators indicated that attachment of the viruses to particulates or to other viruses results in aggregates which assay as only one virus. Although titers are decreased, longevity is increased, presumably because the virus-virus or virus-particulate association affords protection from free proteolytic enzymes or direct microbial degradation.

Schaub (1972) and Moore et al. (1974) have shown that viruses associated with solids retain their capacity to infect. Most recently, Moore, Sagik and Malina (1975) studied the association of viruses with raw wastewater suspended solids, final effluent solids, bentonite clay, and kaolinite clay. Significantly, poliovirus was found to be infective in the adsorbed state.

3. Materials and Methods

3.1 Virus and Virus Assay

Bacteriophage MS-2 was obtained from the American Type Culture Collection and was catalogued as ATCC 15597-B. Chaudhuri and Engelbrecht (1969) have compiled the general characteristics of MS-2. Pertinent to its function as a physical enterovirus model are these properties: diameter, 25 mμ; molecular weight, 3.7×10^6 gm; isoelectric point, pH 3.9; and nucleic acid, single-stranded RNA. Chaudhuri and Engelbrecht (1969) and Chaudhuri (1974) employed MS-2 in filtration, adsorption, and chemical coagulation tests. Kott et al. (1974) used MS-2 in a study of the validity of coliphages as pollution indicators.

Davis and Sinsheimer (1963) described the conditions and procedures for replication and assay of this phage. Host cell was E. coli ATCC 15597. Growth broth contained per liter: 30 gm trypticase soy broth, 3 gm NaCl, and 2 ml 1 M $CaCl_2$. Top agar for plating contained the same ingredients as growth broth plus 15 gm Bacto-agar. Bottom TSA agar was purchased in plates from Micro-Media, Houston, Texas.

Plating procedure was that of Adams (1959) and Rovozzo and Burke (1973) with the modification of Gerba (1973) of 3 ml top agar, 0.5 ml sample, and 1.0 ml log-phase host cell suspension.

3.2 Preparation of Virus Stock

Plates confluent with plaques were overlaid with 5 ml of trypticase soy broth. After 3 hours at room temperature, the plates were gently swirled several times and the broth decanted. The suspension of free bacterial cells and bacteriophages was spun down at 3,000 rpm with a

10.5-cm rotor radius for 15 minutes to sediment the bacterial cells. The supernatant fluid was passed through a washed 0.45-μ Millipore HA membrane. 0.4-ml aliquots of the filtrate were stored in ampules at -70°C.

3.3 Purified Water

Water containing 1.5 mg/l or less of dissolved solids and no detectable organics was obtained by passing tapwater through the Carborundum Aquella water purification system and was used in place of distilled water.

3.4 Cellulose Nitrate Membranes

Cellulose nitrate membranes, Millipore HA, 25 mm in diameter with a porosity of 0.45 μ availed as a model adsorption surface. Exposed surface area is 4.0 cm^2. Membranes were pretreated to remove residual detergent and preconditioned for adsorption by passing 10 ml of adsorption enhancing solution through the membranes.

3.5 Preparation of Bentonite Suspension

Bentonite (catalog number B-235) was obtained from Fisher Scientific Company, Fair Lawn, New Jersey. Five grams of the powdered clay was added to 2,000 ml of purified water. After 2 hours of stirring at 200 rpm in a 2-liter graduated cylinder, the suspension was allowed to settle for 24 hours.

One liter of supernatant suspension was divided into 100-ml aliquots. Each aliquot was placed in a number 8422 centrifuge tube and spun down for 15 minutes at 805 g. The supernatant fluid was discarded,

the pellet was resuspended in purified water, and the centrifugation and resuspension were repeated three times.

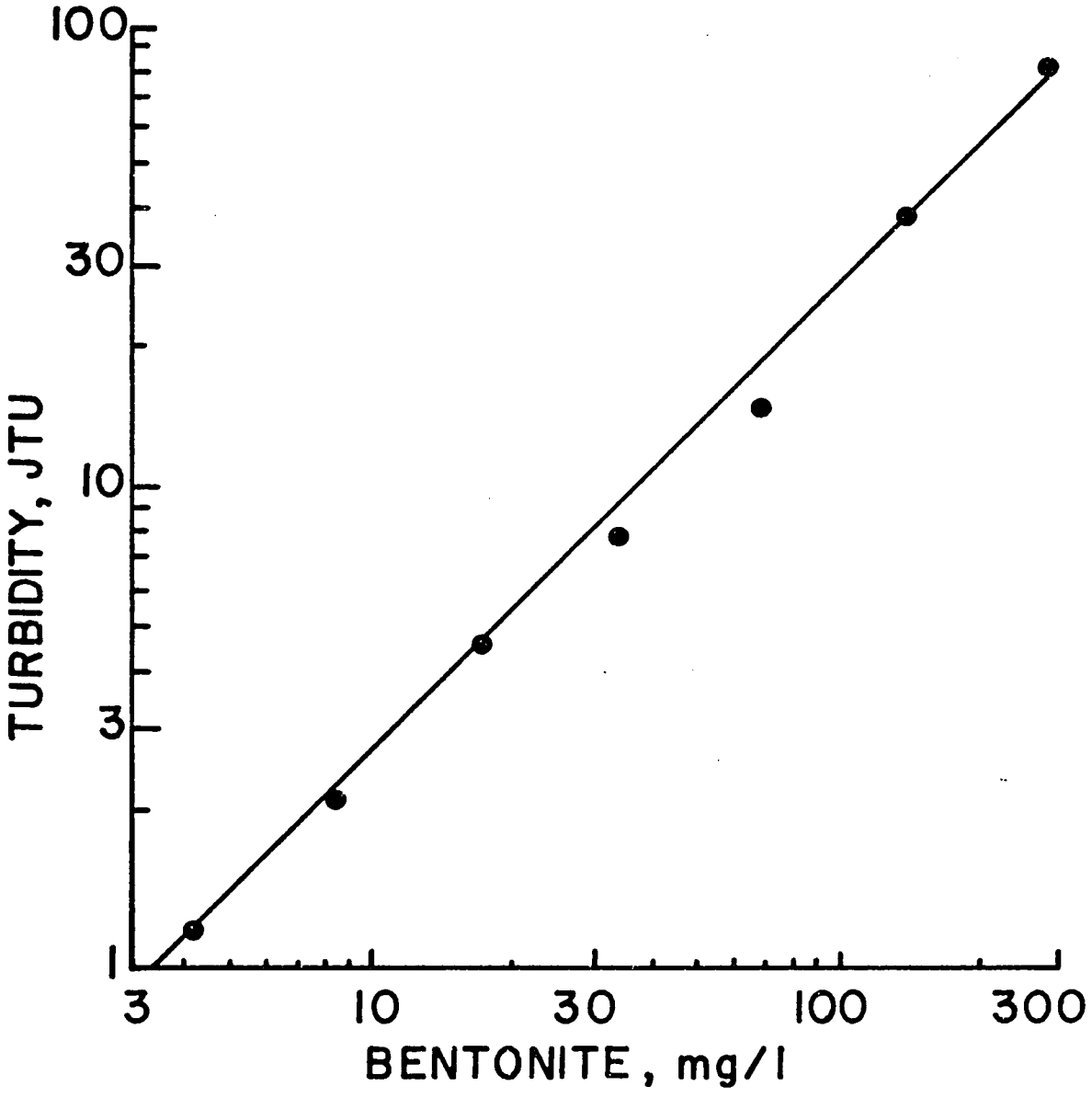
After final resuspension, 100-ml volumes were diluted with 100 ml of purified water and passed through a washed 90-mm Millipore HA membrane of 0.45-μ porosity. The filtrate was discarded and the membrane was removed and scraped for recovery of particulates greater than 0.45 μ in diameter. Particulates from each membrane were resuspended in 100 ml of purified water.

Each filtered 100-ml aliquot was then placed in a number 8422 centrifuge tube and spun down for 20 minutes at 2100 g, resuspended, pooled with the other aliquots, and stored at 4°C.

Suspended (non-filterable) solids were determined by using Whatman GF/C membranes. These membranes were first washed, dried, and weighed; then they were mounted in a suction apparatus for filtration of 25-ml volumes of the stock suspension. Following filtration the filter and associated solids were dried to a constant weight at 103°C. Difference in filter weight before and after filtration and volume filtered were used to calculate the concentration of suspended solids. An average of three filtrations was taken as suspended solids concentration in mg/l.

A standard curve relating suspended solids to Jackson Turbidity Units was prepared by making appropriate dilutions of the stock suspension in purified water and determining the corresponding JTU value on a Hach Model 2100A turbidimeter. This standard curve is shown in Figure 3-1.

Figure 3-1. Relationship of turbidity to suspended solids concentration for bentonite.


100
30
10
3
1
TURBIDITY, JTU
3
10
30
100
300
BENTONITE, mg/l

A 12-ml sample of a 30 JTU suspension was presented to Ms. Mayra Bisnow, Fine Particle Lab technician, of the Coulter Electronics, Inc., Hialeah, Florida. The sample was run in Isoton electrolyte on a Coulter Counter Model TA II using a bi-aperture technique. The sample was shaken well prior to analysis. Analysis yielded counts per milliliter and the cumulative and differential weight-percent versus equivalent spherical diameter. These data are given in Table 3-1. Diameter versus weight-percent distribution and particle counts are illustrated in Figures 3-2 and 3-3. From the particle counts it can be calculated that 94% of the particles are of 2 μm or less in equivalent spherical diameter. Particles of this size are classified as coarse-clay according to the scheme of Jackson (1956).

A 1-ml sample of a 30 JTU suspension was prepared for electron microscopy and photographed by Dr. Pierre Payment (visiting fellow at Baylor College of Medicine from the Université de Montréal), using an RCA Model EMU-3. Various sizes and irregular morphologies of the particles can be seen in Figure 3-4, which is at 11,000X magnification. Ciaccio (1971) has commented on the difficulties encountered in seeing surface details because of the opaqueness of the particles.

3.6 Chlorine

Hypochlorous acid, HOCl, was procured by dissolving calcium hypochlorite (Olin HTH) in purified water with subsequent addition of 1 N HCl to reduce pH to 5.90 to 6.25. Stock solutions were prepared at a concentration of about 1000 mg/l HOCl and dispensed in 25 or 100-μl volumes.

Table 3-1. Analysis of Bentonite Particulates at 30 JTU

Diameter, microns	Weight-Percent		Counts/ml
	Cumulative	Differential	
0.5	100.0	0.4	3.3×10^7
0.63	99.6	2.6	1.0×10^8
0.79	97.0	3.4	7.0×10^7
1.00	93.6	1.7	1.7×10^7
1.26	91.8	2.3	1.2×10^7
1.59	89.5	4.4	1.1×10^7
2.00	85.1	7.9	1.0×10^7
2.52	77.2	13.7	8.7×10^6
3.17	63.5	16.7	5.3×10^6
4.00	46.9	16.2	2.6×10^6
5.04	30.7	13.6	1.1×10^6
6.35	17.0	7.0	2.8×10^5
8.00	10.0	3.2	6.4×10^4
10.10	6.8	2.0	2.0×10^4
12.70	4.8	2.0	1.0×10^4
16.00	2.8	2.0	5.0×10^3
20.20	0.8	0.8	1.0×10^3

Figure 3-2. Weight-percent distribution for 30 JTU bentonite.

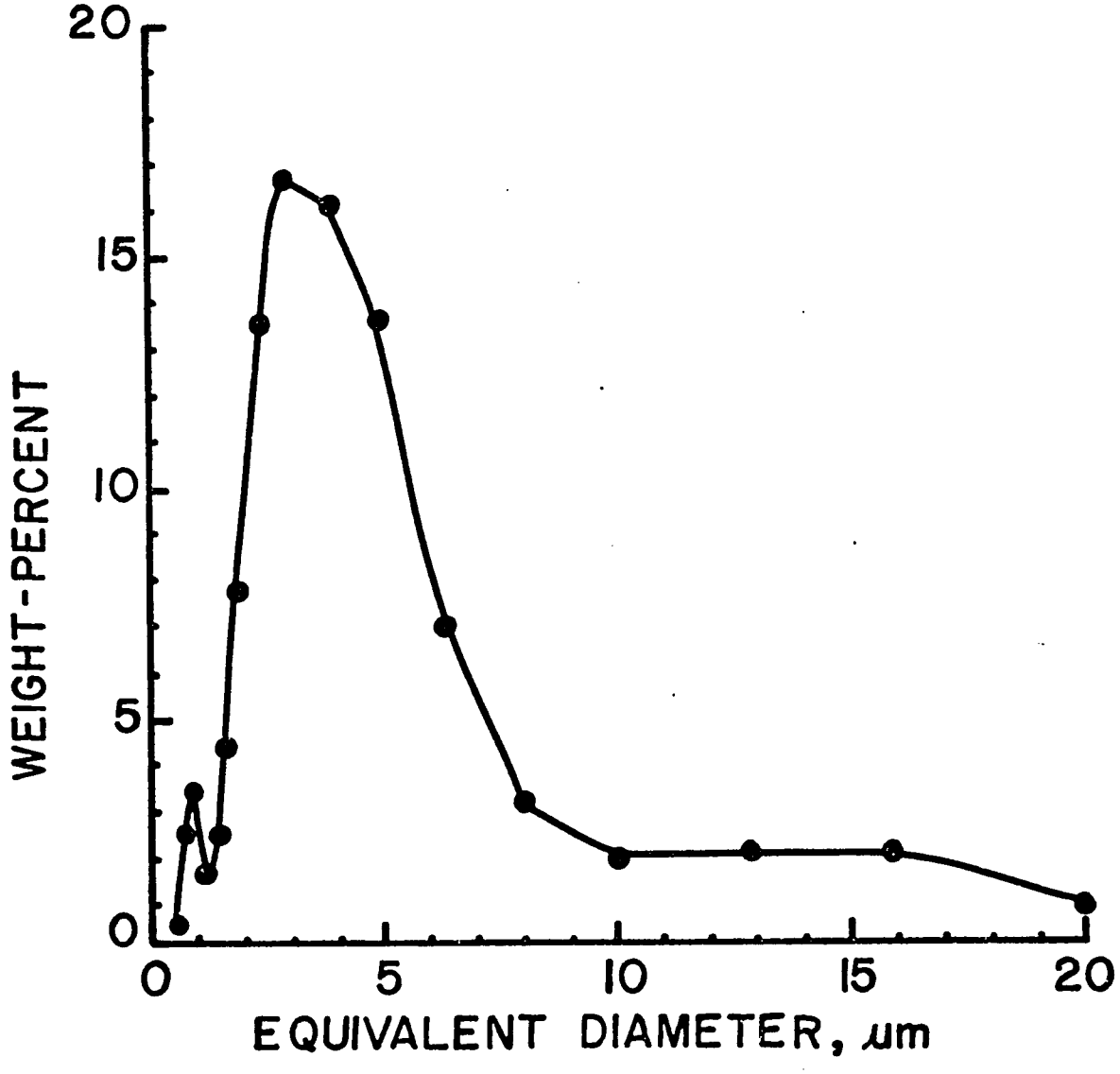

20
15
10
5
0
WEIGHT-PERCENT
0
5
10
15
20
EQUIVALENT DIAMETER, μm

Figure 3-3. Particle density distribution for 30 JTU bentonite.

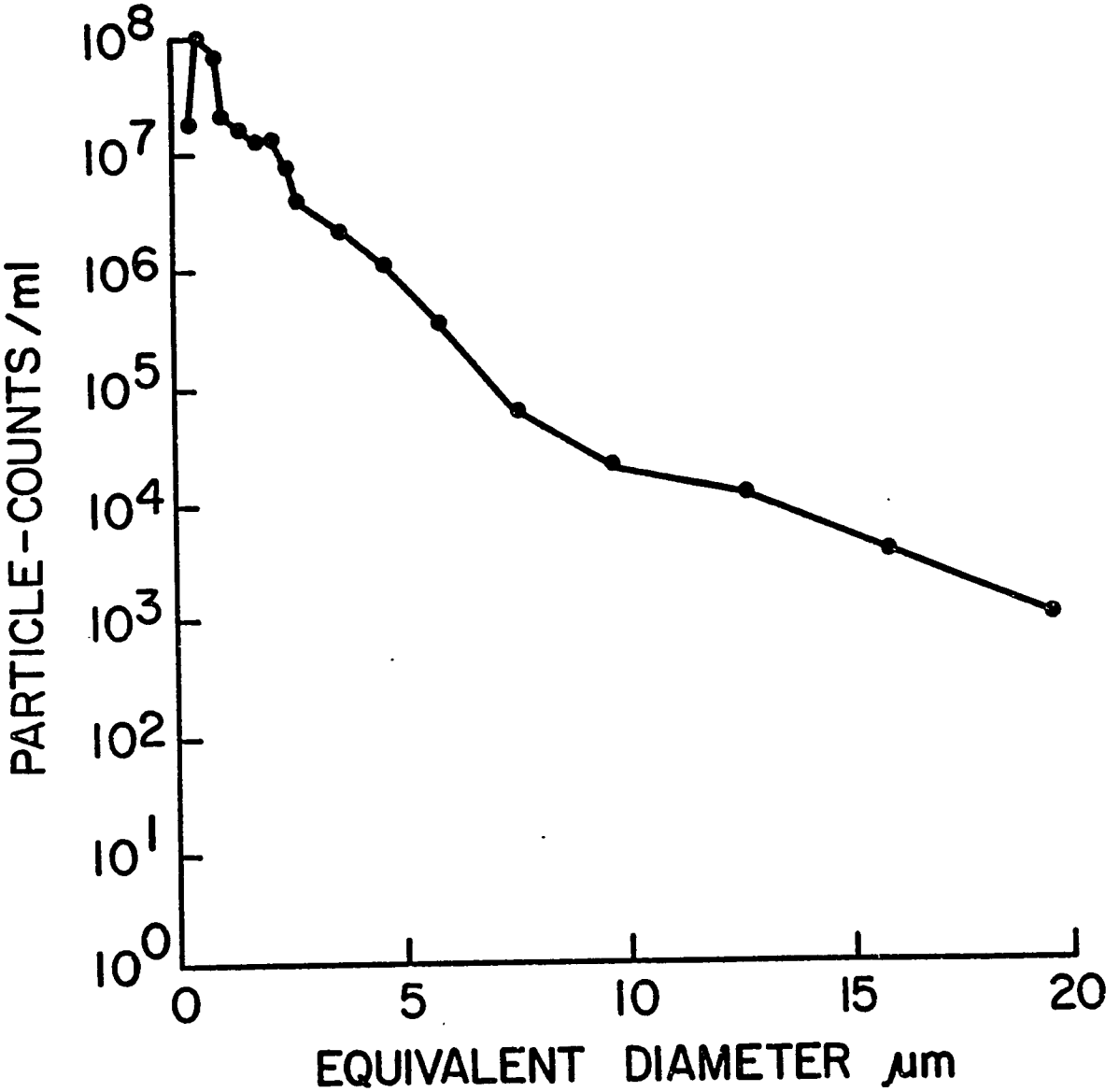

PARTICLE-COUNTS/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
5
10
15
20
EQUIVALENT DIAMETER μm

Figure 3-4. Electron micrograph of bentonite particulates.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3.7 Preparation of Combined Chlorine

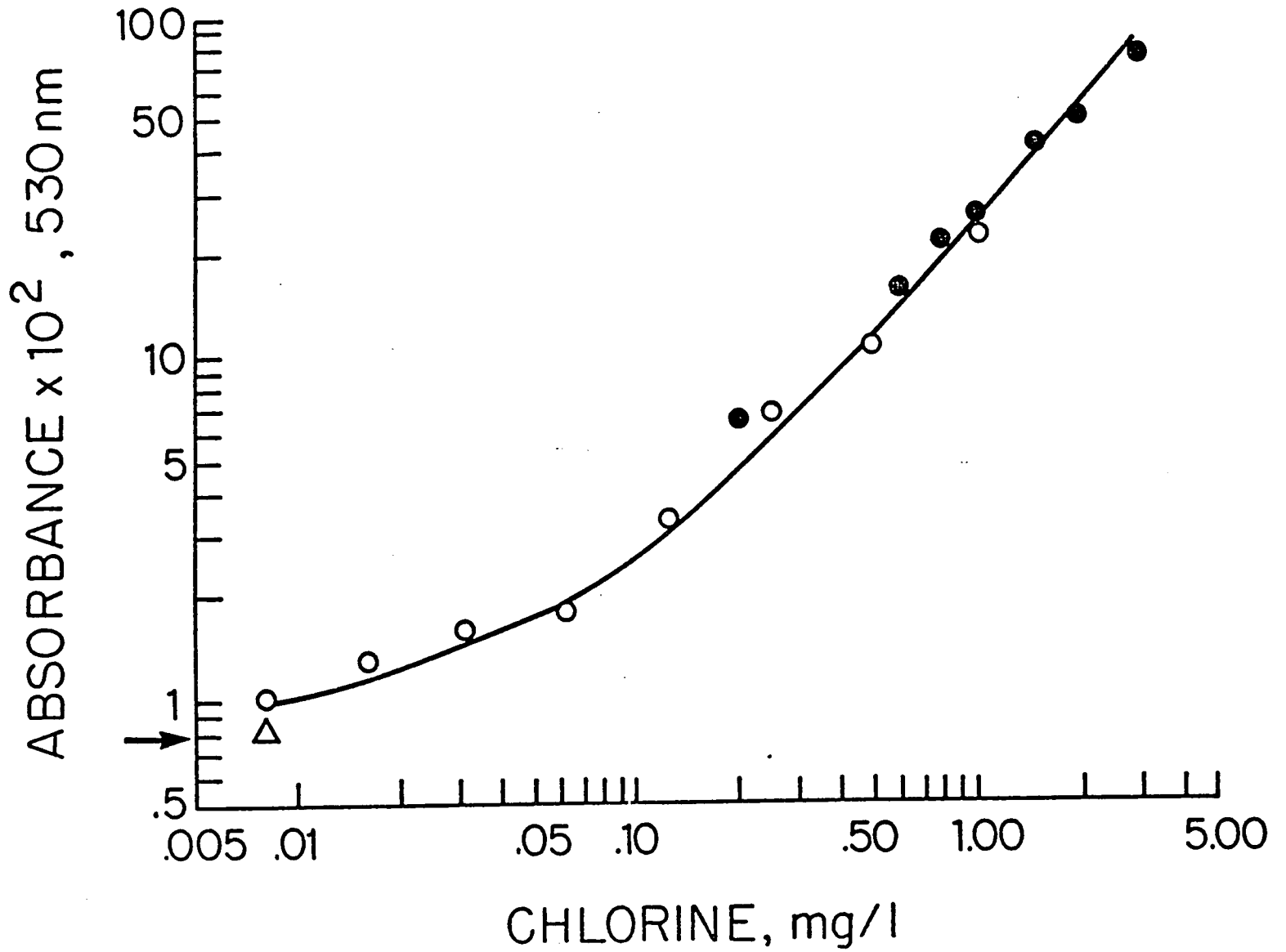
An ammonium chloride stock solution was prepared in purified water to a final concentration of approximately 1000 mg/l ammonia; 100-ml aliquots of this stock solution were added to reaction flasks. To each flask was added granular calcium hypochlorite to a desired final concentration; addition of 1 N hydrochloric acid was necessary to maintain pH 6. The flasks were stirred magnetically at room temperature for 24 hours. Speciation of combined chlorine forms was determined by the Lamotte-Palin DPD method.

3.8 Detection of Chlorine

Chlorine was detected by the Lamotte-Palin DPD method and by the ortho-tolidine molar absorptivity method. Colorimetric determination consisted of placing 2.5-ml reagent-reacted samples in 1-cm cuvettes of the Varian 635 spectrophotometer. Absorption wavelength was 435 nm for OT and 530 nm for Lamotte-Palin DPD.

The absorbance-concentration curve shown in Figure 3-5 was made with Lamotte standards and with a potassium permanganate standard prepared according to the 13th edition of Standard Methods (1971). Because the standards supplied by the Lamotte Chemical Products Company did not include any of less than 0.2 mg/l chlorine, the potassium permanganate standard was necessary to extend detection to the lower concentration values. Then too, the close agreement of the standards from two sources substantiates the reliability of the chlorine concentrations reported.

Figure 3-5. Absorbance versus chlorine concentration standard curve: Lamotte standards (●); potassium permanganate (O); reaction control (Δ).


ABSORBANCE x 10², 530nm
100
50
10
5
1
.5
.005
.01
.05
.10
.50
1.00
5.00
CHLORINE, mg/l

The DPD reagents are supplied in the form of pills; each pill is to be reacted with 10 ml of sample. It was found that one-half pill reacted with 5 ml of sample yielded identical absorbance values as the recommended procedure; therefore, since this alteration reduced experimental costs, it was followed in all detection steps. The 530 nm absorbance of a control reaction sample, 5 ml purified water plus one-half DPD pill, was 0.008 ± 0.001. Since colloidal particles produced during dissolution of the pill contributed to this absorbance, and since these particles settled with time, the control reaction sample was not placed in the reference cell of the dual beam Varian 635 spectrophotometer. Reproducible results were obtained by filling the reference cell and test cell with purified water and zeroing the absorbance display; then, DPD-developed test samples were placed into the test cell (within one minute after the start of the chromogenic reaction) and their absorbance was recorded. This procedure avoided the interference of settling particles in the reference cell. For those experiments involving solids-associated virus, the 530 nm absorbance of a 5-ml sample (without the reagent pill added) was subtracted from the absorbance value of a color-developed test sample of equivalent solids concentration.

Speciation of combined chlorine was determined by using DPD-1 reagent for free chlorine, DPD-2 reagent for mono-chloramine, DPD-3 reagent for di-chloramine, and DPD-4 reagent for total residual chlorine.

3.9 Chlorine Demand of Experimental Equipment and Membranes

Tubing, syringes, microsyringe holders, valves, membranes and other ancillary equipment were soaked overnight in 50 mg/l hypochlorous acid. The next day all equipment was mounted and rinsed with distilled water until no chlorine was detectable in the effluent. Representative treated membranes were then challenged with 5 ml of 1 mg/l hypochlorous acid and the filtrate was found to contain the same amount of chlorine, which indicated satisfaction of the membrane chlorine demand. These trial membranes were discarded.

3.10 Physiological State of Host Cell

Adams (1959) has emphasized the requirement of using log-phase-growth cells as hosts for phage by stating: "It is usually necessary to work with cultures of the host organism which are actively growing at an exponential rate, since in such cultures the percentage of nonviable organisms is small. Presence in the culture of any considerable percentage of dead bacteria will lead to difficulties, since virus particles which become adsorbed to such bacteria do not reproduce themselves or form plaques on agar plates."

Davis and Sinsheimer (1963) infected an exponentially growing 37°C culture of MS-2 host cells and found that within 5 minutes 90% or more of the input phage was adsorbed. Replicated phage first appeared after 25 minutes. Burst sizes were of the order of 10^4 phage per bacterium.

Host cell, ATCC 15597, for this work was obtained from the American Type Culture Collection. In order to determine the time

necessary to achieve exponential growth, a growth curve was developed for this bacterium suspended in TSB at 37°C and shaking at 180 rpm. A suspension of cells was prepared by serially diluting a plate colony in purified water. One-tenth ml of an approximate 10^8 cells per ml suspension was added to 100 ml of TSB. One-ml samples were taken at various intervals and plated on Levine EMB. Growth of this plate-colony seed is shown in Figure 3-6. Exponential growth occurs between 3 and 6 hours after inoculation. Stationary phase begins after 7 hours with maximum concentration of cells about $10^{9.3}$ CFU/ml.

A more convenient means of obtaining exponential-growth cells was achieved by adding 1 ml of a 12-hour stationary cell culture to 100 ml of TSB. From Figure 3-6 it is evident that this seed attains log growth in about 2.5 hours and is exponentially reproducing until 5 hours elapsed time. In all phage assays, this procedure was followed with host cells taken after 3 hours of growth.

3.11 Preparation of Organic-Solids Suspension

Humic acid (catalog number H1,675-2) was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. A 200 mg/l solution was prepared by adding 0.200 gr to 100 ml purified water followed by dropwise addition of 10 N NaOH to alkaline pH. A standard curve was prepared by diluting this stock in purified water and recording the corresponding absorbance at 254 nm. This curve is shown in Figure 3-7.

Activated sludge colloidal solids were obtained by collection of 2 gallons of activated sludge from the Southwest Sewage Treatment Plant, Houston, Texas. Two liters of this material was blended in a

Figure 3-6. Host cell growth curves: plate-colony seed (●); stationary-phase-suspension seed (O).

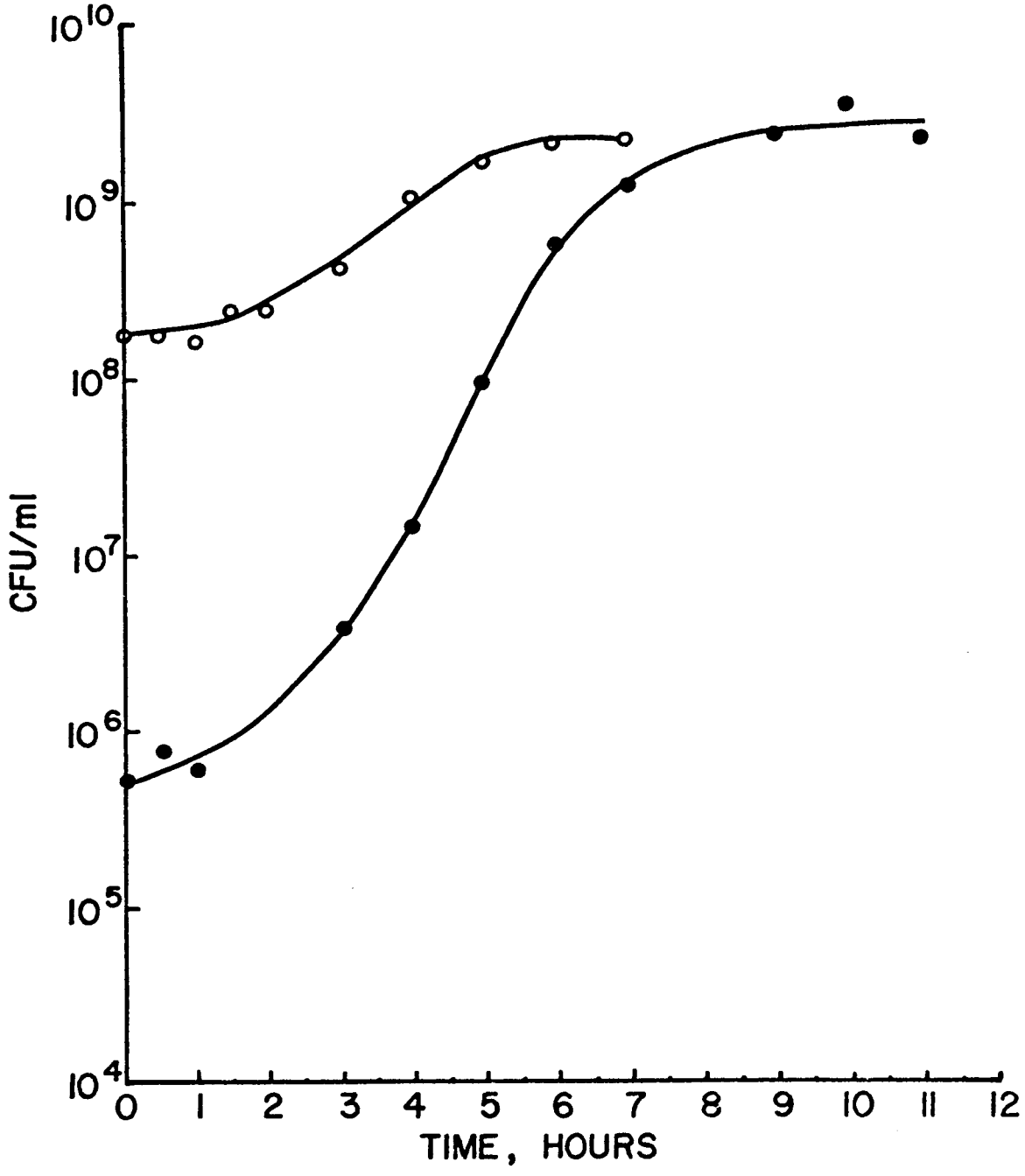
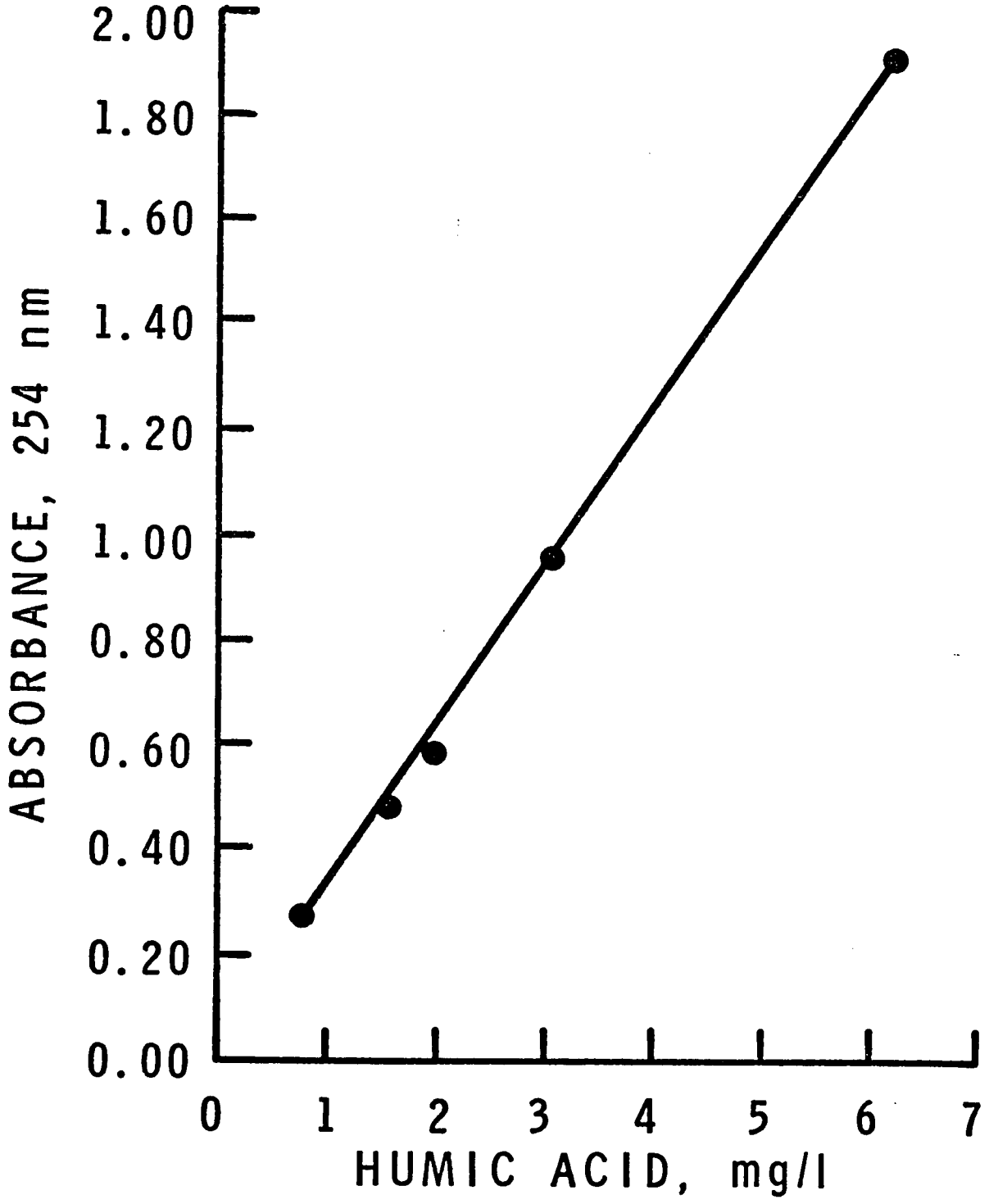

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
CFU/ml
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME, HOURS

Figure 3-7. Absorbance versus humic acid concentration standard curve.


2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
ABSORBANCE, 254 nm
0
1
2
3
4
5
6
7
HUMIC ACID, mg/l

Waring commercial blender at high speed for 5 minutes. The blended suspension was allowed to settle overnight at 4°C in a graduated 2-liter cylinder. The top 1 liter was withdrawn and autoclaved. Suspended solids concentration of the sterile material was determined to be 72 mg/l. JTU versus mg/l standard curve is shown in Figure 3-8.

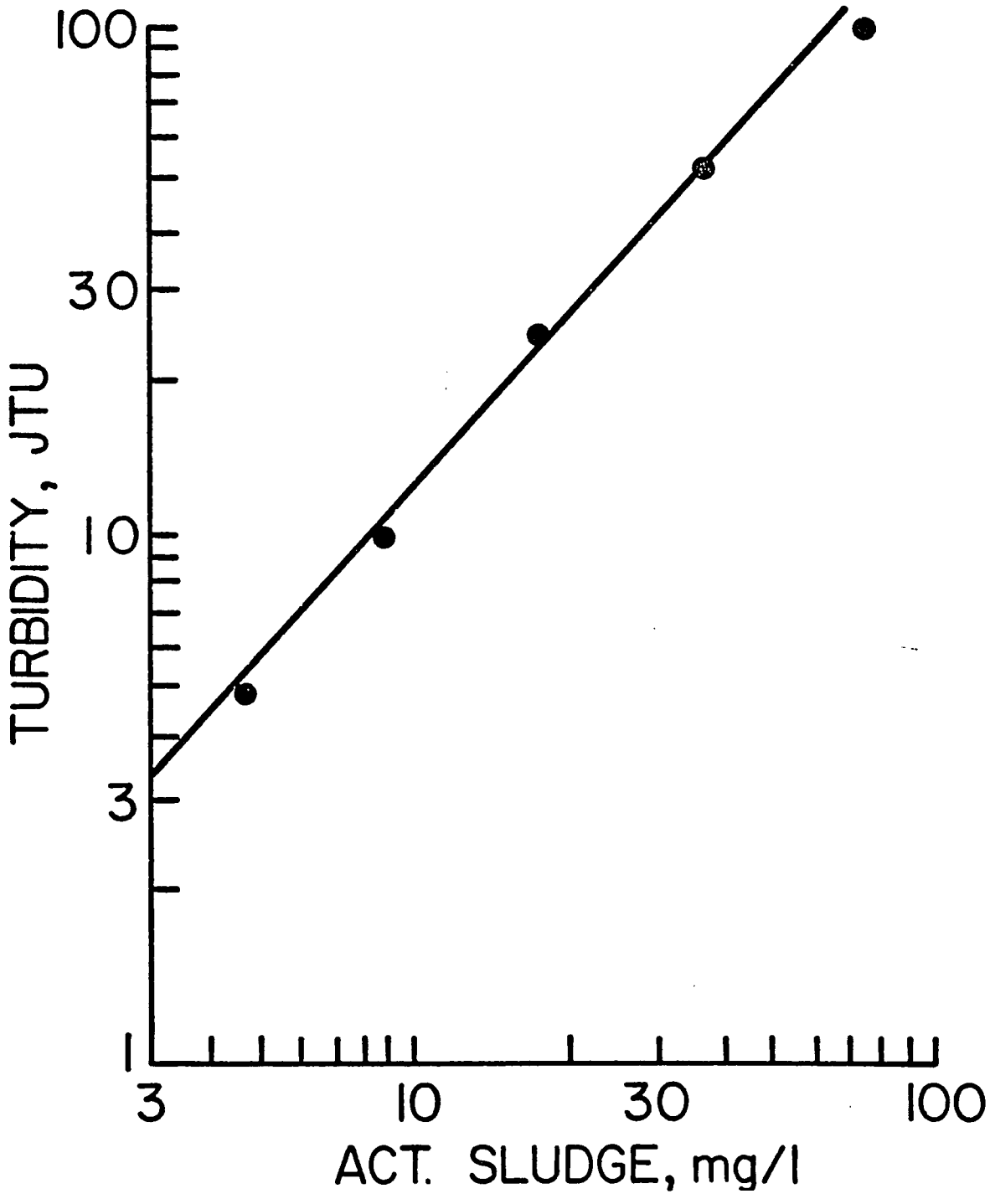
3.12 Clarified Sewage

On January 7, 1976, 1 gallon of clarified effluent was collected at the Southwest Treatment Plant. This material was centrifuged at 2000 g for 10 minutes in a Servall RC-3 to remove most of the particulates. The supernatant was then filtered through a 0.45-μ HA membrane to obtain soluble effluent constituents. Absorbance of this filtrate at 254 nm was 0.133. According to Dobbs et al. (1972), this is equivalent to 7 mg/l total organic carbon (TOC). By the method of Nessler adapted by the Hach Chemical Company for a direct-reading colorimeter, DR-A 2725, ammonia was found to be present at a concentration of 10.3 mg/l.

3.13 Removal of Ammonia from Clarified Sewage by Clinoptilolite

Clinoptilolite was obtained from the Baroid Division of the National Lead Company, Houston, Texas. An ion exchange column, 1.5 cm in diameter and 10 cm long, was loaded with washed 4-mesh clinoptilolite. This zeolite was charged with 10 bed-volumes of 0.3 M $CaCl_2$ and then rinsed with 5 bed-volumes of purified water. A 75-ml sample of 0.45-μ filtered sewage effluent from the Southwest Treatment Plant was adjusted to pH 6 and then recycled 10 times through the column at dropwise flow rate. Required column operating parameters were ob-

Figure 3-8. Relationship of turbidity to suspended solids concentration for activated sludge.


100
30
10
3
1
TURBIDITY, JTU
3
10
30
100
ACT. SLUDGE, mg/l

tained from the work of Koon and Kaufman (1975). Removal of organics was checked by comparing absorbance at 254 nm before and after passage through the column. Removal of ammonia was checked by comparing ammonia concentration before and after passage by the Nessler method.

4. Experimental Procedures

4.1 Determination of Virus Dispersion

Two 0.22-μ, Millipore GS, 25-mm membranes were mounted in stainless steel holders. Each membrane was then washed with 5 ml of tris(hydroxymethyl)amino-methane (Tris) buffer, then 5 ml of 10% fetal calf serum in Tris buffer was passed through each membrane. These treated membranes were then washed three times with 10 ml of Tris buffer to remove non-adsorbed organics.

Stock phage at a titer of 2.2×10^{11} PFU/ml was diluted 1×10^{7} fold in a final volume of 200 ml of Tris buffer. This suspended phage was then sampled and plated prior to challenging the first membrane with 30 ml of suspension. Filtrate from the first membrane was collected, sampled and plated. The collected filtrate was then passed through the second membrane and the filtrate was collected, sampled and plated.

4.2 Virus pH Sensitivity Tests

To a dilution tube containing 1.9 ml of pH-adjusted 0.05 M glycerine were added and mixed 100 μl of stock phage to yield a final titer of 5×10^{4} PFU/ml. At elapsed times of 5, 30 and 60 minutes, 0.3 ml was withdrawn from the tube and diluted 10, 100, 1000, and 10,000 times in pH 7.6 Tris buffer and plated. At each time period the data were normalized to the titer of the pH 7.0 control tube.

4.3 Adsorption of Virus to Cellulose Nitrate Membranes

Ten-ml samples of appropriately diluted virus suspended in various salt and hydrogen ion concentrations made in purified water were loaded

into 20-ml syringes. By hand pressure, fluid was passed at acceptable flow rates through the cellulose membrane mounted in a Millipore stainless steel 25-mm filter holder. About 3 ml of the same medium without virus was then passed through the membrane until the 10-ml mark on the collection beaker was reached. This procedure was necessary because of the residual volume beneath the membrane mounting in the holder. Samples were taken for viral assay before and after passage through the membrane.

4.4 Pressure and Flow Rate Relationship for Cellulose Nitrate Membrane

Flow rate data were obtained for various constant pressures. Purified water was placed in a 1-liter reservoir equipped with pressure gauge and relief valve. Fluid was led to the membrane holder with quick-connect and BD valve fittings. A stopwatch and graduated cylinder were used to obtain flow rate data at any required constant pressure. In a similar manner, data were taken to find the maximum flow rate at which no breakthrough occurs during virus adsorption.

4.5 Effect of Oxidation of Membranes on Virus Adsorption

Membranes were mounted in Millipore stainless steel holders. A 30-ml syringe was placed onto the holder inlet, held upright, and loaded with chlorine at known concentrations. Free hypochlorous acid accounted for 70% of the total chlorine present. Five-ml volumes were passed by syringe plunger through the membrane intermittently during the working day; 15-20 ml was allowed to remain above the membrane overnight. All membranes were soaked in pH 5.5-6.0 chlorine solutions made in purified water at room temperature for 15 to 22 hours. These treated membranes were then challenged with diluted stock virus in 0.05 M $MgCl_2$

at neutral pH.

4.6 Elution of Virus from Cellulose Nitrate Membranes

Viruses adsorbed to membranes were treated with 5 ml of eluent. Eluent was passed by syringe through the membrane and collected in a 10-ml beaker. Five ml of pH buffer, if necessary, was immediately filtered through the same membrane and this effluent was collected in the same beaker with the eluate (if a basic eluent had been used), attaining a final volume of 10 ml and affecting neutralization.

Time of exposure to organic eluents was increased by collecting the eluate and passing it through the membrane several times.

4.7 Adsorption of Virus to Clay Particulates

Clay suspensions at a desired Jackson Turbidity Unit (JTU) and neutral pH were prepared in either purified water or 0.05 M $MgCl_2$ solutions made in purified water. Viruses were diluted from stock ampules in sterile purified water, and 1 ml of the appropriate dilution was added to 49 ml of the clay suspension which was in a number 146401 reaction flask shaking at 150 rpm. Temperatures were within the range of 22-24°C.

Fifty minutes were allowed for equilibration of adsorbed and non-adsorbed viruses. At the end of this time, 25 ml of the clay-virus suspension were poured into a 40-ml centrifuge tube and spun down at 5800 g for 15 minutes in a Servall RC-2B centrifuge equipped with an HB-4 head. As a control, 1 ml of the diluted stock virus was also added to 49 ml of $MgCl_2$ without clay, shaken at 150 rpm for 50 minutes, and centrifuged. Supernatants were sampled and discarded. The pellet was resuspended

in 25 ml of $MgCl_2$ and centrifuged as before. After this second supernatant was sampled and plated, it was discarded. The pellet was either stored or resuspended for immediate testing.

In order to follow the rate of virus adsorption to particulate matter, two methods have been tried: centrifugation with sampling of the supernatant, and filtration with sampling of the filtrate. Colloidal solids can be taken down within 5 minutes at 12,000 g; yet, viruses continue to adsorb to the colloidal particulates as they migrate to the tube base. Oza and Chaudhuri (1975) filtered a virus and granular charcoal suspension through a number 42 Whatman, 25-mm, paper disc which had been soaked in 3% beef extract for 4 hours and then mounted in a glass filter-apparatus.

In this study Millex HA, 25-mm membranes were pretreated by mounting on a 5-ml Stylex syringe and passing through, in succession, 5 ml Tris buffer, 5 ml 10% fetal calf serum in Tris, and 5 ml Tris buffer. At any desired time, 5 ml of the shaking suspension could be withdrawn and filtered through such a membrane. Filter porosity is 0.45 μ, and the smallest particulates are 0.50 μ; therefore, all clay particulates and any attached virus are intercepted by the membrane. A 5-ml sample of the control suspension is also filtered to account for any adsorption to the treated membrane.

Temperature dependence was determined by agitating the virus-clay suspension on a rotary table placed within a Napco incubator. The control and clay suspensions were allowed to equilibrate at 37°C before addition of 1 ml of diluted stock virus to 49-ml test volumes. Similarly, suspensions were equilibrated at 5°C in the 5°C cold-laboratory. Adsorption as a func-

tion of time was followed by withdrawing 5-ml volumes at 10, 20, 30, 50 and 75 minutes elapsed time and immediately filtering through a serum-treated membrane.

4.8 Elution of Virus from Clay Particulates

4.8.1 Evaluation of Candidate Eluents

A virus-bentonite suspension was prepared according to section 4.7. A 2-ml volume of the suspension was added to 2 ml of eluent contained in a large dilution tube. This tube was immediately vortex mixed for 1 minute and then passed through a Millipore GS 25-mm membrane. The filtrate was added to either an equal volume of pH neutralizer or to Tris buffer.

4.8.2 Time as a Factor

Virus-bentonite suspensions were prepared according to section 4.7. Four-ml volumes of the virus-bentonite suspension were added to 4-ml volumes of the eluent which were contained within Costar 30-ml plastic tissue culture bottles. The elution suspensions were then shaken at 100 rpm on a New Brunswick Model G2 rotary table. At various elapsed times a bottle was taken off the table, a 4-ml sample was withdrawn, and the eluate suspension was filtered through a Millipore HA 25-mm membrane which had been treated with 5 ml of 10% fetal calf serum in Tris to inhibit virus adsorption to reactive sites. The filtrates were diluted in 2% TSB-Tris. An equal volume of agitated control-eluent was processed in like manner to indicate any loss of titer due to passage through the membrane.

4.8.3 Immobilization Followed by Elution

Virus was first adsorbed to bentonite, then a 5-ml volume of the resuspended solids with attached viruses was passed through a 25-mm HA membrane pretreated with fetal calf serum as described in section 4.7. The filtrate was sampled to detect any viruses which were released from the solids during the immobilization step.

A 5-ml volume of eluent was passed through the membrane at a flux of about 3 ml/minute/cm^2. The eluate was collected in a 10-ml plastic beaker, mixed, and sampled. The remaining eluent was recycled through the membrane a certain number of times and the eluate was assayed to determine if increasing the contact time aided recovery of attached virus.

4.8.4 Direct Plating

One ml of the virus-solids suspension was added to 2 ml of 2% FCS-water or 2% TSB-water. Ten-fold serial dilutions were then made in this diluent. A 0.5-ml sample of each dilution was plated directly.

4.8.5 Molar Salt Solutions

A two-molar salt solution was prepared in purified water and adjusted to pH 7. Five ml of the virus-solids suspension were added to 5 ml of the salt solution and vortex mixed for 2 minutes. This mixture was then immediately diluted in 2% TSB-water.

4.8.6 Sonication

Ten-ml volumes of the virus-solids suspension were chilled and placed in thick-walled glass tubes and sonicated at 20 khz for 30 seconds by a Bronwill Biosonic BP-III sonicator. After sonication, samples were

immediately diluted in 2% TSB-water diluent.

4.9 Association of Virus with Organic Solids

Suspensions of selected organics at neutral pH were prepared in either purified water or salt solutions made in purified water. Viruses were diluted from stock ampules in purified water, and 1 ml of the appropriate dilution was added to 99 ml of the humic acid suspension which was in a number 146401 reaction flask. Contents of the flask were stirred by magnetic mixing bar at about 200 rpm. Temperatures were within the range of 22-24°C.

Sixty minutes were allowed for equilibration of adsorbed and non-adsorbed viruses. At the end of this time, 25 ml of the virus-solids suspension was withdrawn by pipet and added to a heavy-walled plastic centrifuge tube. Tube contents were sedimented at 5800 g for 15 minutes in a Servall RC-2B. As a control, 1 ml of the diluted stock virus was also added to 99 ml of purified water, without salt, stirred for 60 minutes at 200 rpm, and centrifuged. Supernatants were sampled and discarded. The pellet was resuspended in 25 ml of salt solution and centrifuged as before. After this second supernatant was sampled and plated, it was discarded. The pellet was either stored at 4°C or resuspended for immediate testing.

4.10 Association of Virus with Preformed Aluminum Flocs

Two 200-ml flasks were equipped with magnetic stirring bars. To the control flask was added 99 ml of dechlorinated tapwater and 1 ml of diluted stock virus. Flask contents were continuously stirred at 200 rpm. To the test flask was added sufficient dechlorinated tapwater and either stock aluminum chloride or aluminum sulfate to attain 98 ml of fluid. The

pH was immediately recorded, and the pH probe was allowed to remain in the stirring flask. Dropwise addition of 1 M Na_2CO_3 was then made until pH 7 was reached. The volume of Na_2CO_3 added was recorded and sufficient dechlorinated tapwater was added to 100 ml. After stirring for 60 minutes, the flask contents were poured into a 100-ml centrifuge tube and spun down at 5800 g for 15 minutes. Typical floc formation data are given in Table 4-1.

Recovery of virus from pelleted flocs was accomplished by resuspending the pellet formed from the 100-ml sample in 5 ml of purified water. One ml of this suspension was then mixed with 1 ml of Armour fetal calf serum and appropriately diluted in 2% FCS-water or 2% TSB-water before plating.

4.11 Chlorine Demand of Virus-Associated Organics

These tests were performed to ascertain if organics accompanying the bacteriophages would exert an appreciable demand for chlorine and at what dilution this effect would disappear. Ten-fold dilutions of stock phage-in-broth were made in sterile purified water. To chlorine-demand free tubes containing 2 ml of an approximate 1 mg/l chlorine solution were then added 1 ml of the diluted phage suspension. Tube contents were vortex mixed and allowed to react for 1 minute at 24°C. To each of the tubes, 0.1 ml of orthotolidine chromogenic reagent was added at the end of the reaction period and the color was allowed to develop for 2 minutes. Developed color was read at 435 nm on the Varian 635 spectrophotometer. Absorbances for the test reactions were compared to control tubes containing 2 ml of purified water, 1 ml of the appropriate phage-broth dilution, and

Table 4-1. Formation of Aluminum Flocs in Tapwater

Salt	Molarity	Al, mg/l	Initial pH	ml/100 ml of Na_2CO_3 added to attain pH 7
$AlCl_3 \cdot 6\ H_2O$	0.0005	13	6.52	0.01
	0.0015	39	4.83	0.10
	0.0045	117	4.20	0.70
	0.0135	351	3.97	2.70
$Al_2(SO_4)_3 \cdot 18\ H_2O$	0.00024	13	7.10	0.00
	0.00072	39	4.80	0.11
	0.00216	117	4.20	0.74
	0.00648	351	4.00	2.70

Tapwater: pH 8.2, 81 mg/l hardness ($CaCO_3$), 201 mg/l alkalinity ($CaCO_3$).

0.1 ml of orthotolidine reagent.

4.12 Batch Reaction Tests for Freely Suspended Virus

Both the test and control reactors were Corning, number 5900, 150-ml reaction flasks. To the control flask was added 99 ml, pH 6, 0.05 M $MgCl_2$ made in purified water. Stirring was accomplished by magnetic stir bars at a rate of 250 rpm. To the reaction flask was added 104 ml of the $MgCl_2$ solution.

To the control flask was added 1 ml of diluted stock virus. After conclusion of the inactivation test a 1-ml sample was withdrawn from the control flask and plated as a control for the starting virus titer.

Microliter volumes of stock chlorine were added to the test flask to yield desired final concentrations. An equilibrium time of 30 seconds was allowed after addition of chlorine before taking a 5-ml sample for chlorine measurement. Within 10 seconds after adding the chlorine sample to the chromogenic reagent, 1 ml of the diluted virus was added. The timer was started so that zero time commenced with addition of the virus. One-ml samples were withdrawn and neutralized in 2 ml of a 6 mg/l sodium thiosulfate solution made in Tris buffer. The elapsed time at each sample neutralization was recorded by an assistant. In this way, remaining viable virus could be followed as a function of contact time.

4.13 Batch Reaction Tests for Solids-Associated Virus

Stock virus-solids suspensions were stored at 4°C in plastic vials. Before each test, the solids were diluted in 0.05 M $MgCl_2$ and spun down at 2500 g for 10 minutes. The supernatant was discarded; the pellet was resuspended in an appropriate volume of 0.05 M $MgCl_2$. To the control

flask containing 99 ml, pH 6, 0.05 M $MgCl_2$ was added 1 ml of the virus-solids stock suspension. After 1 minute of mixing by a magnetic bar at 250 rpm, 25 ml was withdrawn and the JTU value was determined. Absorbance of the suspension at 530 nm was recorded.

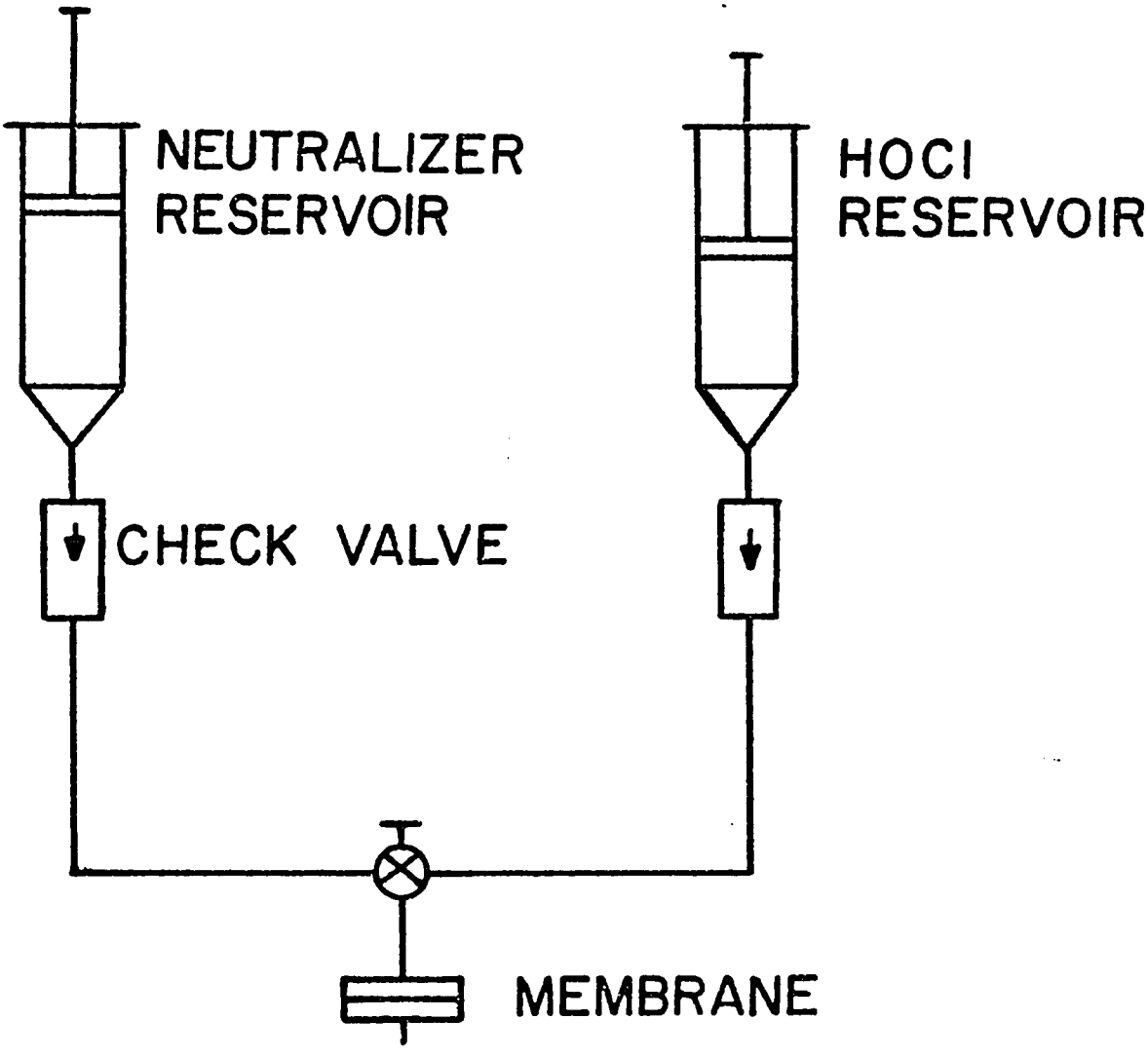
Microliter volumes of stock chlorine were added to 0.05 M $MgCl_2$, pH 6, in the test flask. The initial volume in the test flask was 105 ml; this allowed a 5-ml sample to be withdrawn, prior to adding the virus, for determination of chlorine. An assistant recorded the absorbance of the DPD color-developed 5-ml sample; the investigator immediately added 1 ml of the virus-solids suspension to the test reactor; the timer was started; and 2-ml samples were withdrawn and neutralized in 2 ml of a 50% TSB solution containing 6 mg/l $Na_2S_2O_3$. Elapsed time at neutralization was recorded by an assistant. At the end of the inactivation test, the control flask was sampled.

In investigating the loss of free chlorine as a result of adding either clay or aluminum hydroxide gels to the reactor flask, it was necessary to account for 530 nm absorbance contributed by the solids. The 530 nm absorbance of the control suspension, usually less than 0.025, was subtracted from the 530 nm absorbance of the chlorine detection developed color. This corrected absorbance was then used to calculate the chlorine concentration of the solids-containing test flask.

4.14 Immobilized Disinfection Tests

In order to efficiently treat immobilized virus with chlorine and then neutralize the disinfectant with sodium thiosulfate, the apparatus shown in Figure 4-1 was designed and used. Two 10-ml disposable syr-

Figure 4-1. Disinfection and neutralization apparatus for inactivation of immobilized viruses.


NEUTRALIZER
RESERVOIR
HOCI
RESERVOIR
CHECK VALVE
MEMBRANE

inges were connected to a Millipore micro syringe holder, 25-mm, by tubing containing one-way check valves. These tubing leads met at a three-way valve which then had a common lead to the syringe holder. Thus, chlorine could be infiltrated into the syringe holder containing immobilized virus and, at a time required by the test parameters, the three-way valve could be closed to the chlorine and thiosulfate contained in the other syringe could be injected into the system to immediately stop the disinfection activity.

4.15 Chlorine Demand of Clarified Sewage Constituents

Stock chlorine was prepared by adding 0.2 gm of Olin "HTH" to 100 ml of purified water followed by adjustment to pH 6. A chlorine-demand-free 146401 reaction flask containing 100 ml of sample was mixed magnetically at 200 rpm. All tests were conducted within the temperature range of 22-24°C. Reaction times of 1 minute were allowed after addition of 100-μl increments of stock chlorine. At the end of each reaction time, 5 ml was withdrawn and the residual free chlorine was determined by the Lamotte-Palin DPD method. Before addition of another 100 μl of stock chlorine, 5 ml of the stock sample was added to the flask to maintain a reactor volume of 100 ml.

4.16 Recovery of Coliphages from Clarified Sewage

Five gallons of clarified sewage were obtained from the Southwest Treatment Plant, Houston, Texas, by lowering a 5-gallon stainless steel Millipore pressure vessel by a nylon rope into the exit stream of the circular clarification tank which is nearest the chlorine contact chamber. Five gallons of chlorinated effluent were collected by lowering an identical ves-

sel into the chlorine contact chamber at the point where the fluid exits over the wier. To each of the vessels 50 ml of a 6000 mg/l $Na_2S_2O_3$ solution was added.

A 142-mm, 0.45-μ, disc-shaped HA membrane filter was prepared by mounting it in a stainless steel holder and washing with 1 liter of purified water. The clarified sewage was then thoroughly mixed by bubbling with air. Two gallons were withdrawn and placed in a 2-gallon pressure vessel and 2000 ml were driven through the disc under 5 psi. The filtrate was collected, sampled for freely suspended virus, and stored in a walk-in freezer. One liter of purified water was driven through the disc filter to remove any freely suspended virus trapped atop the filter or within the filter holder. After the membrane was removed, the solids were scraped with a spatula into a 50-ml tube containing 5 ml of trypticase soy broth. Dispersion was accomplished with vortex mixing. The suspension was then sampled and frozen. The chlorinated effluent was processed in exactly the same manner. Sound-disruption of these samples was carried out by chilling and then suspending a 2-ml volume in an ice bath while subjecting the sample to sonication by a Bronwill Biosonic III probe vibrating at 20,000 Hz for 30 seconds.

5. Experimental Results and Discussion

5.1 The Role of Solids in Virus Inactivation by Chlorine

Berg (1974) and Culp (1974) have called for an investigation into the role of solids in virus disinfection by chlorine. An appraisal of the effect of solids was performed by observing the inactivation of a specific group of naturally occurring coliphages as they passed through the chlorination chamber of a municipal sewage treatment plant. E. coli host cell was American Type Culture Collection number 15597, which is a genetic donor possessing F-pili capable of accepting both icosahedral and filamentous phages. Of course, phages specific for this strain of E. coli account for only a fraction of the total virus population present; yet, it is reasonable to assume that the effect of solids on the fate of this heterogeneous subset is analogous to the effect of solids on the fate of the entire virus population.

The virus assays listed in Table 5-1 indicate that about 12% of these coliphages are associated with solids. Although coliphages are extremely sensitive to chlorination, association with solids afforded protection; the titer of solids-associated virus was only reduced by 99%. Sonication at a level capable of disrupting flocs but not sufficient to inactivate viruses did not increase the virus titer; serum treatment, resuspension, and freezing must have released bound virus as effectively as sonication.

This observation of coliphage survival in solids demonstrates that solids-associated viruses are protected during chlorination; but, how they are protected is not known. Research must be conducted which will answer a number of questions. Do the solids protect by exerting chlorine demand? Are viruses that are adsorbed to the periphery of solids protected? Are

Table 5-1. Role of Solids in Virus Inactivation by Chlorine

Virus state, titer	Disinfection chamber	
	Influent	Effluent
Freely suspended, PFU/ml	6	0
Solids-associated, PFU/2000 ml	1380	18
Solids-associated (after sonication), PFU/2000 ml	1350	15

Site: Southwest Sewage Treatment Plant, Houston, Texas.

viruses that are enmeshed within solids protected better than surface-adsorbed viruses? How do the inactivation rates compare for freely suspended viruses and solids-associated viruses?

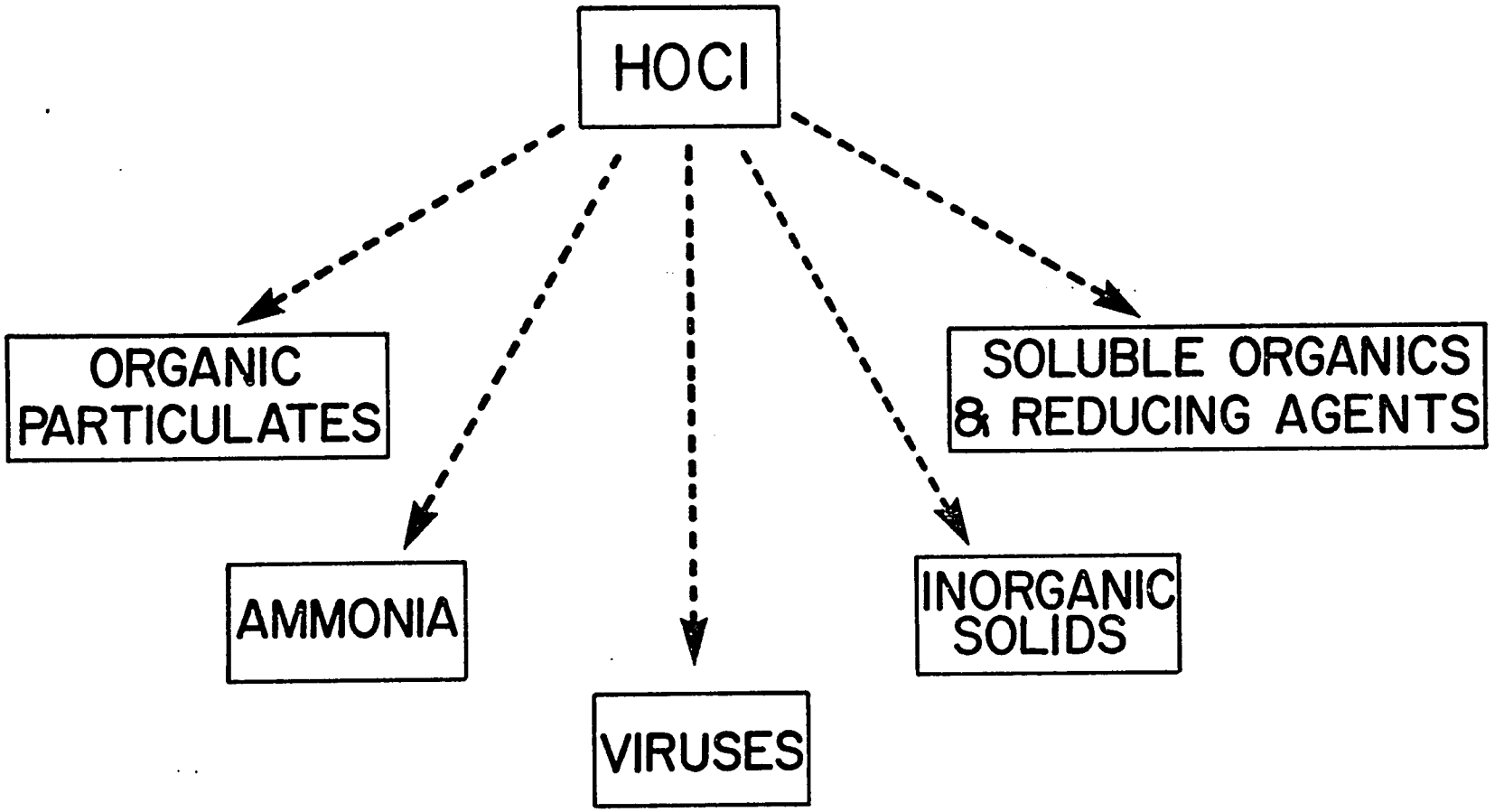
5.2 The Reaction Milieu of the Chlorine Contact Chamber

Texas Water Quality Board standards for wastewater disinfection require a chlorine residual of 2.0 mg/l after a 30-minute contact time. Chlorine residual is determined by the ortho-tolidine method, which indicates both free and combined forms of chlorine.

The Southwest Treatment Plant, Houston, Texas, applies $Na^{+}(OCl^{-})$ to the clarified effluent: chlorine is piped to a point above the entrance to the baffled contact tank; the chlorine then drops from the open end of the pipe to the chamber below. No mechanical mixing of any kind occurs. Flows are controlled so that the chlorinated effluent remains in the tank for 30 minutes before exiting over a wier.

Free chlorine will appear chemically either as hypochlorite ion, OCl^{-}, or as hypochlorous acid, HOCl; relative concentrations of these species depend upon the hydrogen ion concentration of the wastewater. Hypochlorous acid, the most virucidal of the chlorine species, will combine with ammonia, hydrogen sulfide, divalent iron and manganese, suspended solids, phenols, and organic compounds with unsaturated linkages. These compounds compete for the free chlorine, and significant viral disinfection is not achieved until the demand of the extraneous compounds is met. All these chlorine-demanding constituents, along with the viruses themselves, comprise what may be called the reaction milieu, shown diagrammatically in Figure 5-1.

Figure 5-1. The reaction milieu of the chlorine contact chamber.


HOCl
ORGANIC PARTICULATES
AMMONIA
VIRUSES
INORGANIC SOLIDS
SOLUBLE ORGANICS & REDUCING AGENTS

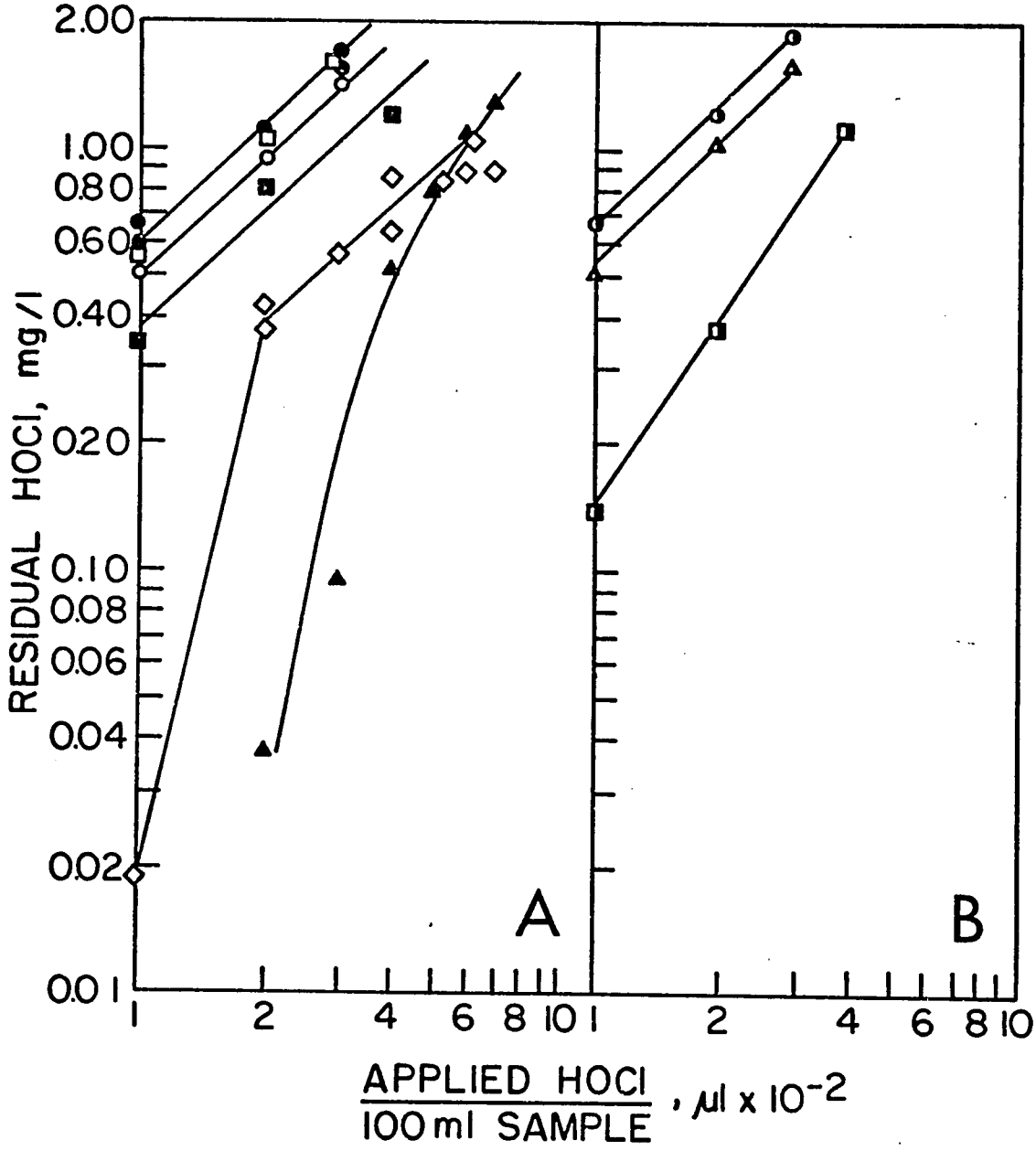
Chlorine demand of each constituent of a typical activated-sludge clarified-effluent was determined by comparison of the free chlorine applied to a sample, until a free residual of 1 mg/l was reached, to an identical reaction carried out in purified water. Each incremental addition of free chlorine was allowed to react for 1 minute before determination of the free residual. Total reaction times were about 6 minutes for addition of chlorine to purified water and from 6 to 10 minutes for treated sewage-constituent reactions.

Kott et al. (1974) have found that sewage effluents contain an average total coliphage and enterovirus titer of 1×10^4 PFU/ml. This virus titer was prepared in purified water and subjected to incremental additions of free chlorine; no detectable chlorine demand was seen. Results of this demand test and tests for other constituents are illustrated in Figure 5-2 (A, B).

Suspensions of bentonite were used to model inorganic suspended solids. Two clay concentrations at 3 and 5 JTU were prepared in purified water and reacted with free chlorine; even at 5 JTU, a chlorine demand of only 0.1 mg/l was observed.

Organic particulates consist of dispersed bacteria, clays with surface-bound organics, and non-settled floc particles. Suspensions of organic particulates were obtained from the Southwest Treatment Plant, Houston, Texas, and washed in purified water. Suspensions were prepared at 3.5, 6.7, and 9.5 JTU in purified water. Chlorine demands for these solids concentrations were 0.4, 0.6, and 1.4 mg/l HOCl.

Figure 5-2. Chlorine demand tests on treated sewage constituents: purified water (●); 10^4 PFU/ml virus (□); 5 JTU clay (○); 16X non-ammonia soluble compounds (■); 16X total soluble compounds (▲); 16X 10.3 mg/l NH_3 (◇); purified water (◑); 3.5 JTU organic particulates (◭); 9.5 JTU organic particulates (◨).


2.00
1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
0.01
RESIDUAL HOCl, mg/l
A
B
1
2
4
6
8
10
APPLIED HOCl / 100 ml SAMPLE, µl x 10⁻²

Soluble constituents of the effluent were obtained by filtration through 0.45-μ membranes. This filtrate was analyzed by the ultraviolet absorbance method of Dobbs et al. (1972) and found to contain 7 mg/l total organic carbon (TOC); and, by the Nessler method, 10.3 mg/l NH_3 was present. After a 16-fold dilution of this filtrate, chlorine demand was determined; on an undiluted basis, 56.3 mg/l HOCl must be applied before attaining a free residual of 1 mg/l.

Where does this soluble chlorine demand originate? By preparing a stock solution of ammonia at 10.3 mg/l, equivalent to that concentration of the sewage effluent, and then diluting this stock 16-fold prior to testing for chlorine demand, it was found that on an undilute basis some 52.6 mg/l HOCl must be applied before attaining a free residual of 1 mg/l. Thus, most of the chlorine demand in the soluble portion of treated sewage is contributed by ammonia. This finding was substantiated in this manner: (1) reduction of the ammonia in the soluble portion from 10.3 to 0.3 mg/l by passage through a calcium-charged clinoptilolite column; soluble organics were not lost as a result of this treatment; (2) dilution of the zeolite-treated solution by 16-fold; and (3) chlorination to a free residual of 1 mg/l. On an undilute basis, soluble compounds other than ammonia account for only 8.5 mg/l of the 56.3 mg/l total soluble chlorine-demand. Laubusch (1971) has stated that the time required to complete most reactions of chlorine with organics usually is considered to be a matter of hours.

These results indicate that neither organic nor inorganic particulates contribute significantly to the total chlorine demand. It is likely that components of the particulates which react with chlorine are those that are

along the surface; and, once these reactions are completed, the chlorine does react with interior constituents.

A listing of the percentage chlorine-demands of each of the milieu constituents of a typical treated sewage effluent is given in Table 5-2.

5.3 Association and Dissociation of Virus with Solids

5.3.1 Determination of Virus Dispersion

To determine if MS-2 bacteriophages were present in large aggregates, a membrane passage test was performed similar to that of Ver et al. (1968) using 0.22-μ porosity GS membranes.

Titer of the challenge was 2.4×10^4 PFU/ml, titer of the first filtrate was 2.4×10^4 PFU/ml, and titer of the second filtrate was 2.0×10^4 PFU/ml. These counts are indicative of monodispersed phage; should any aggregates be present, they are surely small ones consisting of only two or three phages capable of traversing the tortuous channels of the membrane.

Milman et al. (1966) have observed aggregates in a purified preparation of R17, a phage closely related to MS-2. Large aggregates of MS-2 in the host bacterium have been seen by Meijvisch (1973). Since stock phage is prepared by allowing the phage to diffuse into trypticase soy broth from lysed cells, it is likely that such aggregates are dispersed by the broth organics; the agitation of the dilution procedure also dissociates the aggregates.

5.3.2 Virus pH Sensitivity

The methods described by Wallis and Melnick (1967) and Wallis et al. (1972) have involved adjustments in hydrogen ion concentration for

Table 5-2. Chlorine Demand of Reaction Constituents

Constituent	Concentration	Chlorine demand, mg/l	Percent
Viruses	10,000 PFU/ml	0.0	0.0
Activated sludge solids	3.5 JTU	0.4	0.6
Soluble organics and reducing agents	7 mg/l TOC	8.5	13.8
Ammonia	10.3 mg/l	52.6	85.6

poliovirus adsorption to and elution from cellulose membranes; therefore, the sensitivity of MS-2 phage to pH variations was evaluated in batch reaction survival tests conducted through a range of pH values.

Results indicate that MS-2 is both acid and base labile. After 5 minutes at pH 1, only 6% viable phage remained; at pH 3, 48% remained; and at pH 5, 69% remained. At pH 11 approximately 70% of the phage were viable after 5 minutes. The survival curve (Figure 5-3) indicates that the inactivation rate is not constant throughout the contact time.

5.3.3 Adsorption of Virus to Cellulose Nitrate Membranes

5.3.3.1 Effects of pH and Salts

Adsorption tests were performed in which hydrogen ion concentration, salt identity, and salt concentration were varied. Table 5-3 is a composite of experimental results. Virus titer was determined both before and after passage through the membrane. The percent passing is the ratio of filtrate virus titer to challenge virus titer expressed as a percentage.

For viruses suspended in 0.05 M glycine at pH values between 6 and 7, no adsorption occurred. Lowering the pH to 3.5 increased adsorption dramatically. This effect may be due to the virus carrying a net positive charge since the reported isoelectric point (Chaudhuri and Engelbrecht, 1969) is 3.9. Of course, hydrogen ion concentrations in this range are deleterious to the virus. Challenge controls taken immediately before adsorption and immediately after neutralization of the filtrate indicated a substantial loss of titer.

Homma et al. (1973) presented evidence for adsorption of animal viruses to membranes with 0.0005 M $AlCl_3$ at pH 3.5 in sewage organics.

Figure 5-3. Sensitivity of bacteriophage MS-2 to pH variation.

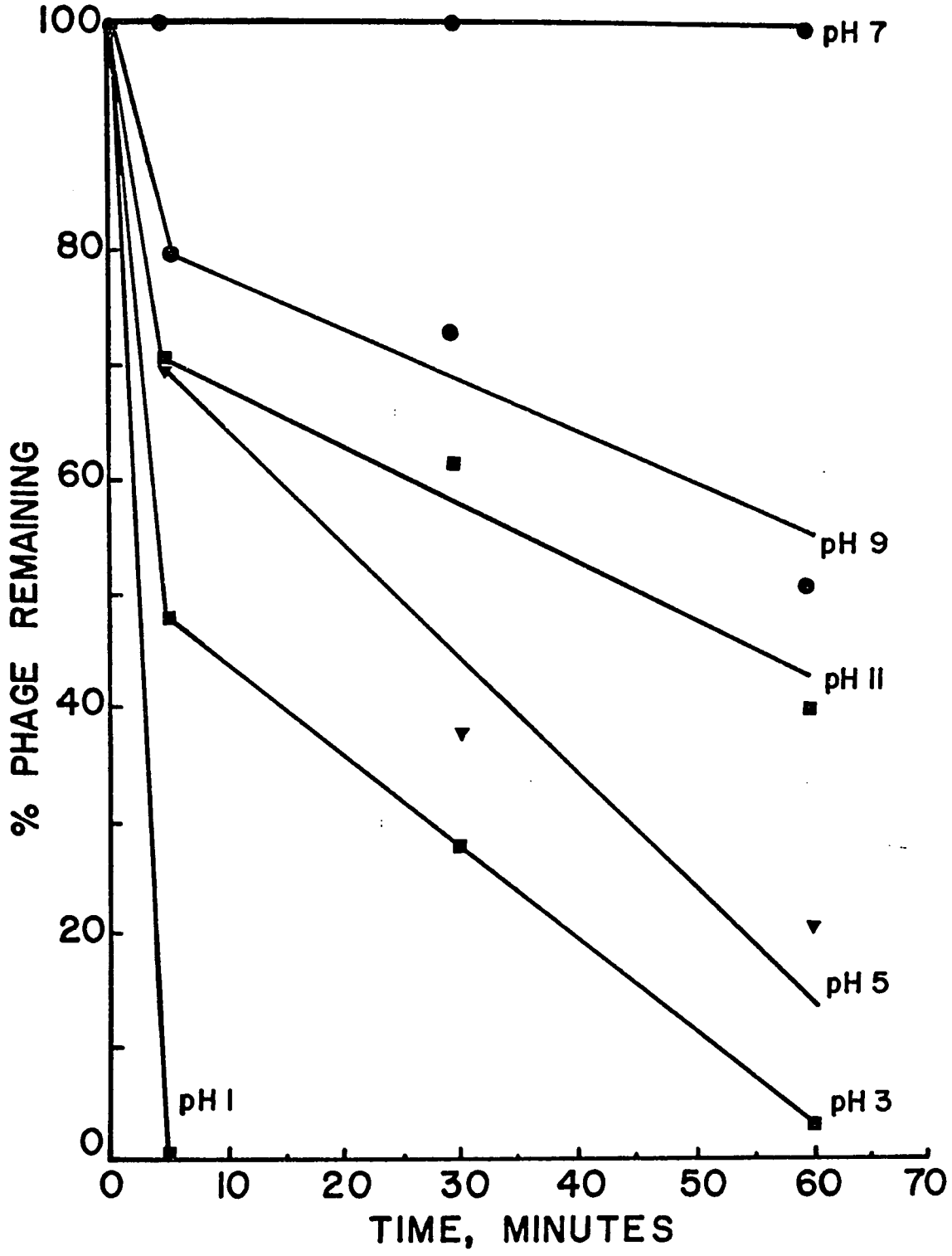

100
80
60
40
20
0
% PHAGE REMAINING
pH 7
pH 9
pH 11
pH 5
pH 3
pH 1
0
10
20
30
40
50
60
70
TIME, MINUTES

Table 5-3. Enhanced Adsorption of Virus to Membranes by Cations

Salt	pH	Percent virus passing
None	6-7	100
None	3.5	<1
.10 M NaCl	6-7	36
.05 M $MgCl_2$	6-7	<1
.05 M $CaCl_2$	6-7	<1
.0005 M $AlCl_3$	3.5	<1

Aluminum cation may aid MS-2 adsorption, but it is not possible to differentiate the influence of the trivalent cation from the effects of reducing the pH and rapid acid lability. However, experiments did demonstrate that MS-2 was more stable at low pH in the presence of salts. Thirty to fifty percent of infective titer was recoverable in eluates of aluminum adsorbed viruses.

Two divalent cations, magnesium and calcium, were evaluated at pH values between 6 and 7. At a concentration of 0.05 M, both adsorbed essentially all of the influent virus. Orientation experiments indicated that bacteriophage MS-2 adsorbed with calcium was more difficult to elute than magnesium-bound virus. Magnesium was therefore the divalent cation selected as adsorbent aid.

Lodish and Zinder (1965) reported that bacteriophage f-2 could be adsorbed to cellulose nitrate membranes if they were suspended in a 0.15 M sodium chloride solution. The adsorption process was also reversible by organic eluents. At neutral pH, 0.1 M NaCl allowed 36% of influent virus to pass the membrane.

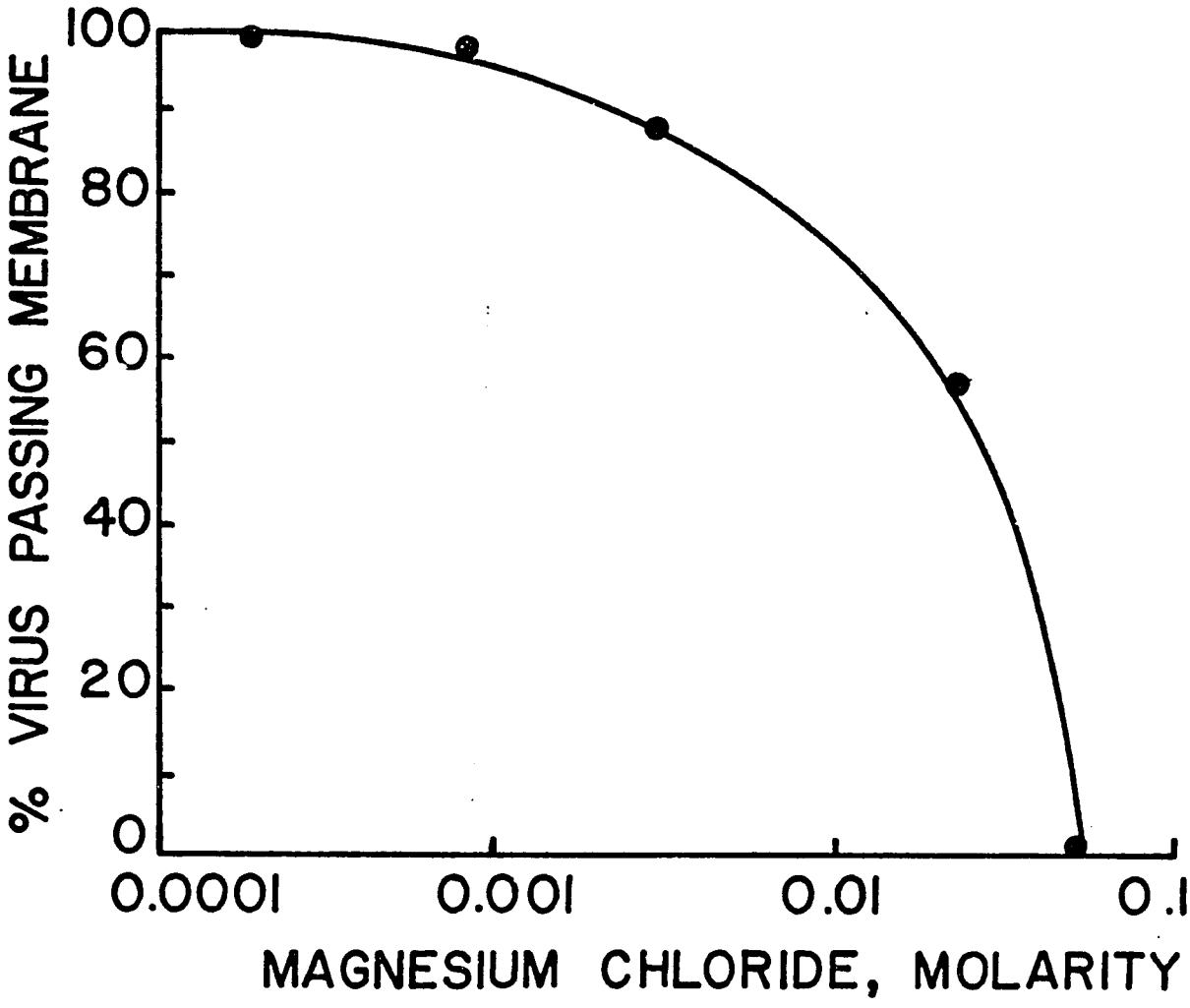
5.3.3.2 Effect of Magnesium Chloride

The role of magnesium ion concentration was explored further by performing adsorption tests through a range of concentrations. Results of this experiment are shown in Table 5-4. Figure 5-4 illustrates that insignificant numbers of phages were adsorbed to the membrane in 0.001 M $MgCl_2$ or less; at 0.05 M $MgCl_2$, 100% were adsorbed. These data are closely related to poliovirus adsorption as reported by Wallis et al. (1972).

Table 5-4. Effect of $MgCl_2$ on Adsorption of Virus to Cellulose Nitrate

$MgCl_2$, M	Virus, PFU/ml			Percent adsorbed
	Control	Filtrate	Adsorbed	
0	9,600			
2×10^{-4}		9,600	0	0
8×10^{-4}		9,400	200	2
3×10^{-3}		8,500	1,100	11
1.2×10^{-2}		5,700	3,900	41
5×10^{-2}		2	9,600	100

Figure 5-4. Effect of $MgCl_2$ concentration on virus adsorption to cellulose nitrate membranes.


100
80
60
40
20
0
% VIRUS PASSING MEMBRANE
0.0001
0.001
0.01
0.1
MAGNESIUM CHLORIDE, MOLARITY

Valentine and Allison (1960) found that for virus adsorption to non-biological surfaces, straight lines are obtained when the logarithm of the adsorbed virus is plotted against the logarithm of the concentration of ions. For this case, their finding nearly holds as is seen in Figure 5-5. However, a smooth curve which begins to level off fits the data nicely and indicates that viral and membrane adsorption sites are becoming saturated with magnesium ions.

5.3.3.3 Pressure and Flow Rate Relationship

Pressure-flow rate characteristics for flow of purified water through the membranes are shown in Figure 5-6. At very low flow rates, less than 0.2 ml/min, the flow rate versus differential pressure curve was not linear. For higher flow rates, the curve was linear as expected.

5.3.3.4 Effect of Flow Rate

Viruses, with and without adsorption aiding salt, were passed at various flow rates through cellulose nitrate membranes. Results are listed in Table 5-5. Flow rates in excess of 6 ml/min yielded viruses in the filtrates. The critical flux is approximately 1.5 ml/min/cm^2. It seems reasonable to suggest that there is a minimum contact time requirement for an electrostatic bond to form between the virus and adsorption site; or, stated in another way, forces tangential to internal membrane surfaces arising from fluid velocity and shear stress distributions must not be greater than adsorptive forces holding the virus particles to the surface.

Figure 5-5. Adsorbed virus as a function of $MgCl_2$ concentration.

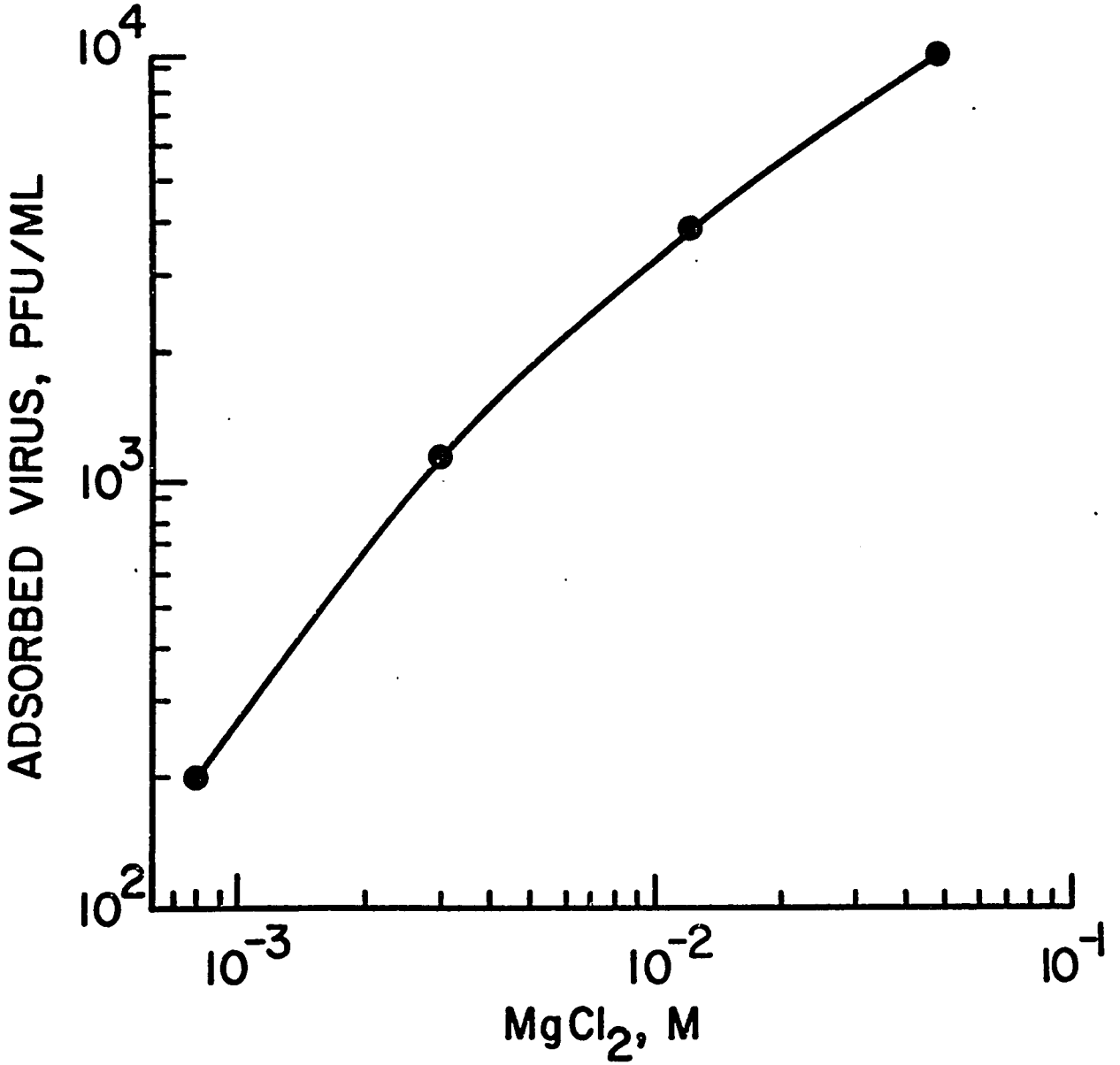

ADSORBED VIRUS, PFU/ML
10^4
10^3
10^2
10^{-3}
10^{-2}
10^{-1}
$MgCl_2$, M

Figure 5-6. Pressure-flow rate characteristics of 25-mm membranes: membrane A (●); first trial membrane B (▲); second trial membrane B (O); and membrance C (Ø).

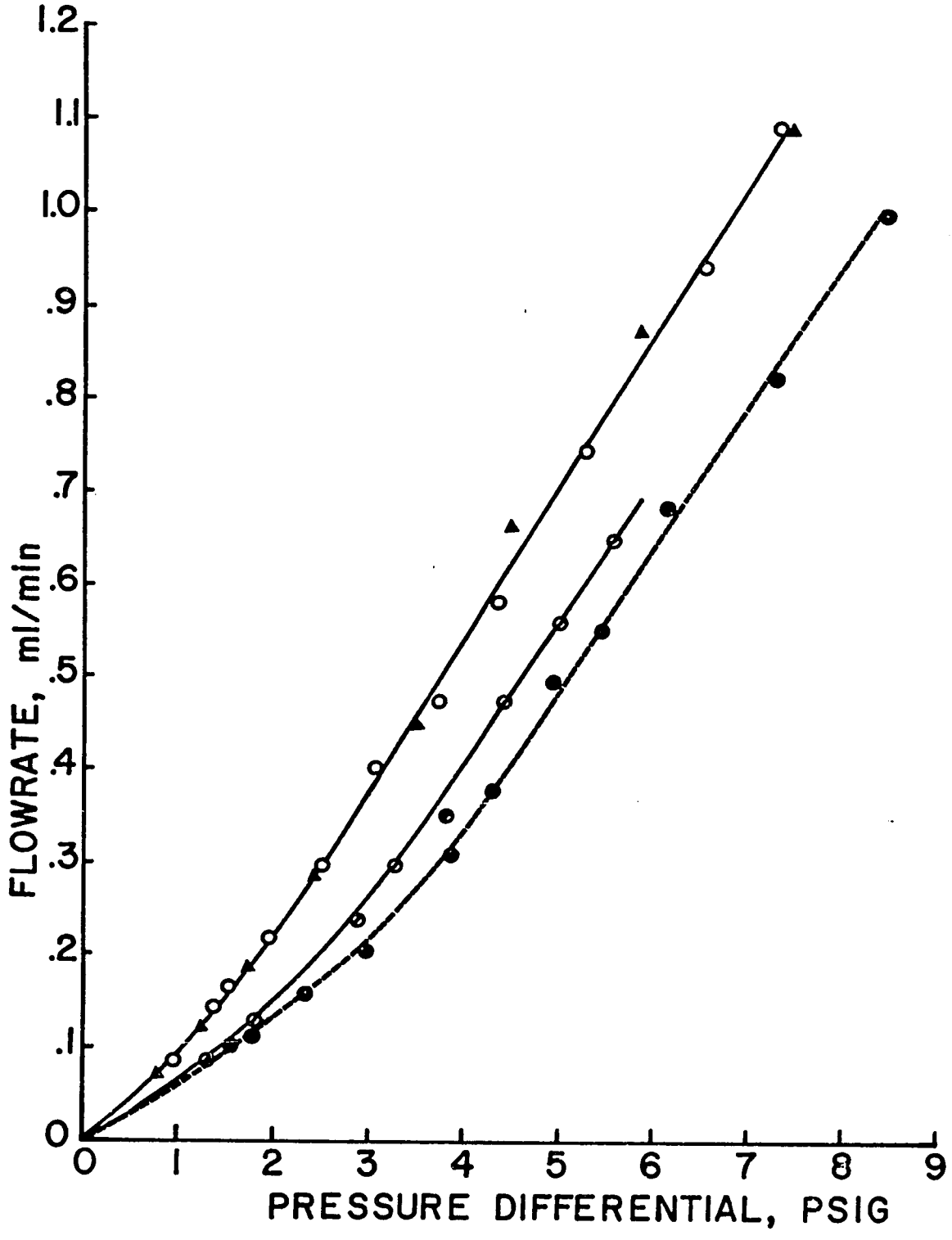

1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
FLOWRATE, ml/min
0
1
2
3
4
5
6
7
8
9
PRESSURE DIFFERENTIAL, PSIG

Table 5-5. Effect of Flow Rate on MS-2 Adsorption to Membranes

Adsorbent aid	Flow rate, ml/min	Percent virus passing
None	0.02	100.0
	0.10	100.0
	0.17	100.0
	0.42	100.0
	1.18	100.0
0.05 M $MgCl_2$	1.06	$\leq$1.0
	1.40	$\leq$1.0
	2.40	$\leq$1.0
	3.20	$\leq$1.0
	5.26	$\leq$1.0
	6.60	$\leq$1.0
	12.50	2.7
	17.00	13.0
	27.50	13.2
	29.50	17.7

5.3.3.5 Effect of Oxidation of Membrane

The existence of chemically alterable membrane adsorption sites was evidenced by a reduction in virus adsorption capacity after pre-treatment with chlorine. As Figure 5-7 and Table 5-6 indicate, the site alteration effect increased with increasing chlorine concentration. Demand for free chlorine was determined by passing a solution of 1 mg/l free chlorine through the membrane and comparing effluent concentration to influent concentration.

Integrity of the oxidized membrane was checked by challenging representative treated membranes with a mixed tapwater bacterial suspension at a concentration of 10,000 CFU/ml, and no bacteria were present in the filtrate.

This adsorption reduction phenomenon should be explored further since it affords a method of preparing preliminary clarifier membranes of extremely low porosity which do not remove viruses by adsorption even with adsorption enhancing salts present.

5.3.4 Elution of Virus from Cellulose Nitrate Membranes

5.3.4.1 Effect of Elevated pH

Wallis et al. (1972) used pH 11.5 glycine-NaOH buffer for elution of poliovirus from Millipore HA membranes. This elution effect was instantaneous. The absence of cations in this eluent immediately dilutes the adsorption enhancing multivalent cations, and the abundance of hydroxyl ions increases the net negative surface charges of the virus and the membrane, allowing repulsive charges to separate the adsorbate and adsorbent.

Figure 5-7. The effect of chlorine preoxidation on virus adsorption. Free chlorine was 70% of total chlorine. A non-exposed membrane served as the control.

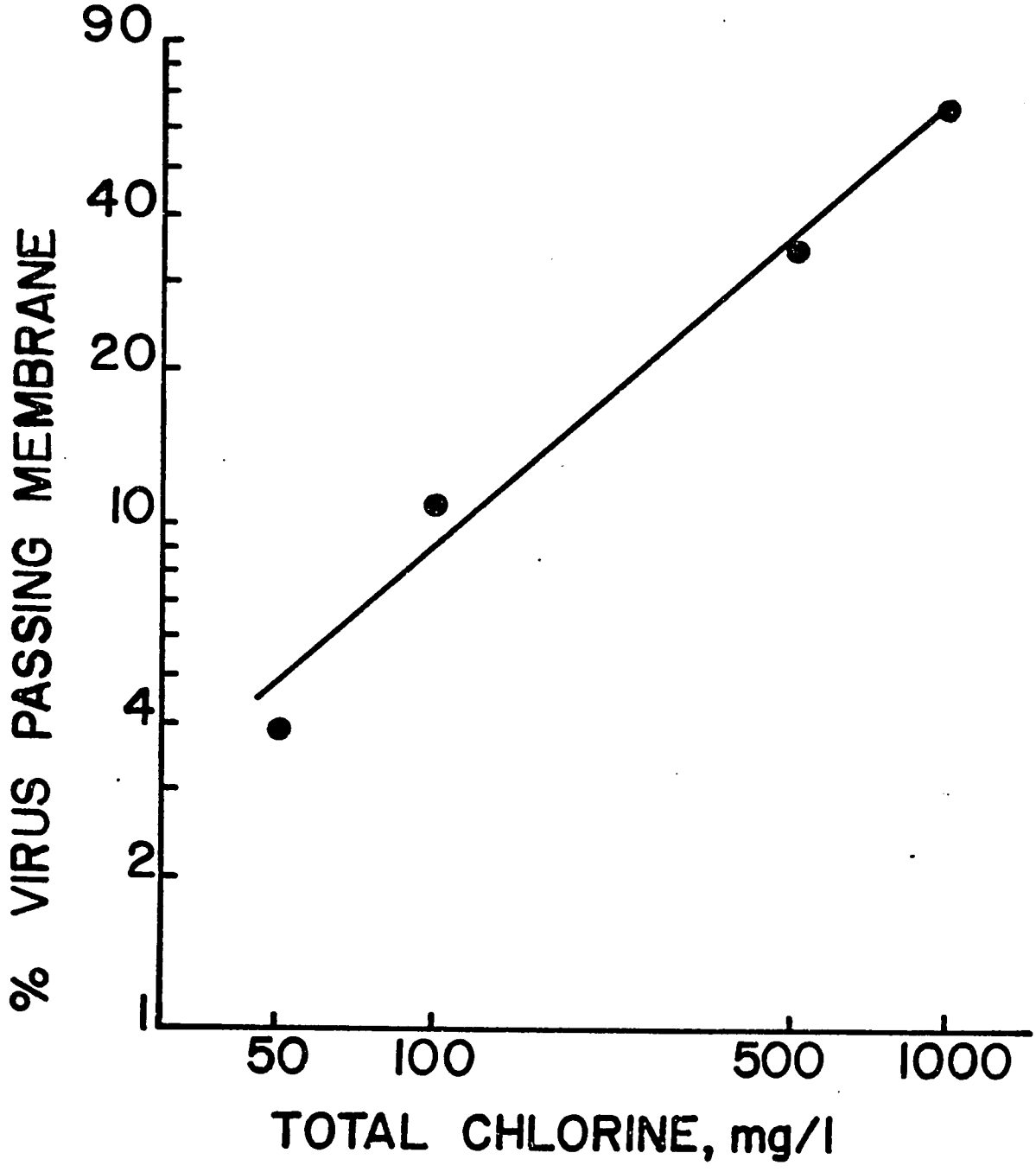

90
40
20
10
4
2
1
% VIRUS PASSING MEMBRANE
50
100
500
1000
TOTAL CHLORINE, mg/l

Table 5-6. Pretreatment of Membranes: Elimination of Chlorine Demand and Reduction of Virus Adsorption Capacity

Free chlorine pretreatment*, mg/l	Virus titer, PFU/ml		Percentage	
	Challenge**	Filtrate	Virus adsorption	Chlorine demand
0	840	0	100	100
35		33	96	<1
70		179	89	<1
350		290	65	<1
700		550	35	<1

* Pretreatment conditions: pH 5.5, 23°C, 22 hours.

** Virus suspended in 0.05 M $MgCl_2$, pH 7.

The effect of elevated pH on elution of MS-2 bacteriophage from cellulose nitrate membranes was determined by adjusting 0.05 M glycine with NaOH to pH 7, 8, 10, 11, and 11.5 and using these eluents to recover virus adsorbed to cellulose nitrate by 0.05 M $MgCl_2$ at pH 7. Variation in recovery as a function of pH is shown in Table 5-7. Glycine adjusted to pH 10 resulted in recovery of about 60% of adsorbed phage.

Less than 10% of adsorbed virus was recovered by pH 11.5 glycine. The possibility that this was due to viral inactivation by hydroxyl ions was checked by performing stirred batch reactions with virus suspended in pH 7, pH 11, and pH 11.5 glycine. Virus suspended in both the basic buffers was inactivated rapidly: 30% of virus was inactivated after 1 minute at pH 11, and 65% of virus was inactivated after 1 minute at pH 11.5. Viral inactivation at these pH levels during a 15-minute test period is shown in Figure 5-8.

5.3.4.2 Effect of Chelating Agent

Dunn and Hitchborn (1964) were able to dissociate magnesium aggregated tobacco mosaic virus suspended in phosphate buffer, pH 7.4, by the addition of excess Na_2EDTA, but this effect was not noticeable at pH 6.

The possibility that addition of EDTA to the glycine eluent would allow elution of membrane-bound virus at pH values nearer neutrality, thereby eliminating the need for neutralization, was investigated. Solutions of 0.01 M EDTA were prepared in 0.05 M glycine buffer at pH values of 7, 8, 10, 11, 11.25, and 11.5. These eluents were then used to elute $MgCl_2$ adsorbed viruses from HA membranes. Figure 5-9 shows that

Table 5-7. Elution by Glycine-NaOH Buffer

Sample	Virus, PFU/ml			Percent recovery
	Challenge	Filtrate (pool)	Eluate	
Control	15,600	0		
pH 7.0			1,080	7
pH 8.0			540	3
pH 10.0			9,000	58
pH 11.0			8,400	54
pH 11.5			780	5

Figure 5-8. High pH lability of MS-2 bacteriophage.

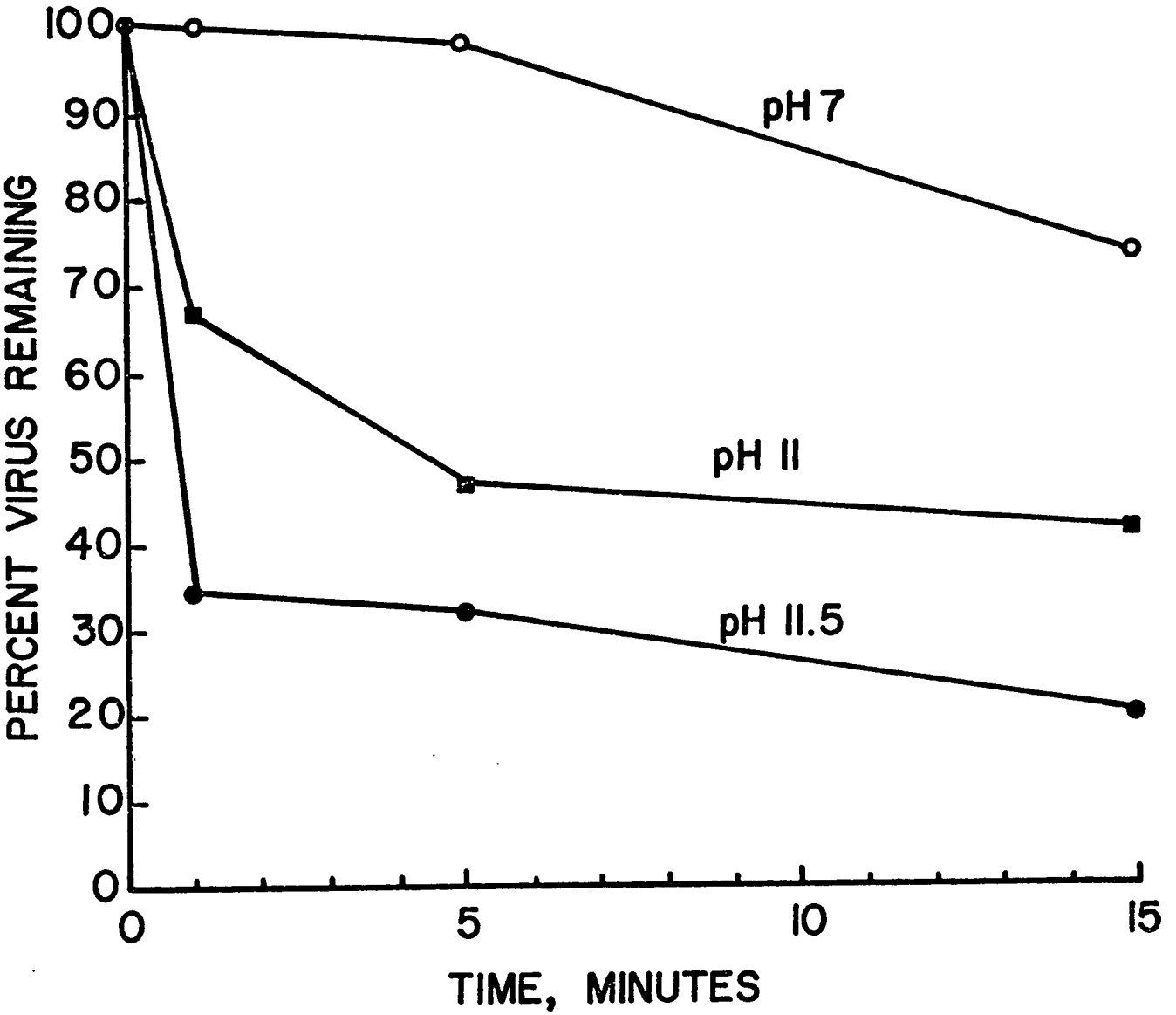
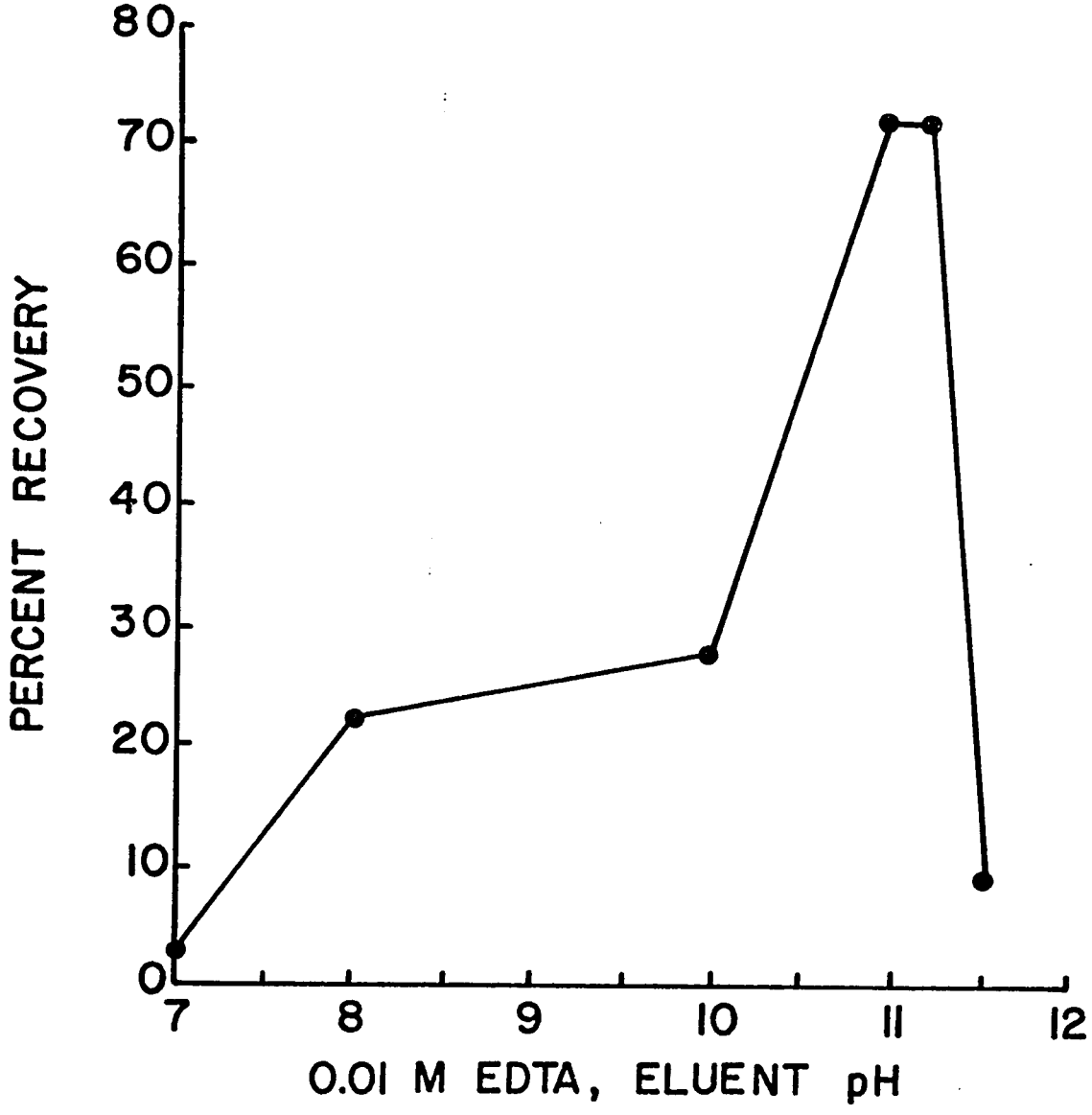

100
90
80
70
60
50
40
30
20
10
0
PERCENT VIRUS REMAINING
pH 7
pH 11
pH 11.5
0
5
10
15
TIME, MINUTES

Figure 5-9. Recovery by EDTA as a function of hydrogen ion concentration.


80
70
60
50
40
30
20
10
0
PERCENT RECOVERY
7
8
9
10
11
12
0.01 M EDTA, ELUENT pH

EDTA at pH 11 is the best eluent and that EDTA does not enhance recovery at lower pH values.

Once again, poor recovery occurred at pH 11.5. Inclusion of the pH 11.25 eluent allowed a demonstration of the sharply defined range of rapid inactivation at high pH. Within the 0.25 pH range of 11.25 and 11.50, recovery dropped from 70% to 10%.

5.3.4.3 Effect of Organic Eluents

Lodish and Zinder (1965) eluted 83% of f-2 bacteriophage from cellulose nitrate membranes by washing with nutrient broth.

This method of elution was tried using trypticase soy broth (TSB), pH 7.2, and fetal calf serum (FCS), pH 7.4, as the organic eluents. Average percent recoveries were 28 with TSB and 34 with FCS, but, as seen in Table 5-8, there was no consistency in these results.

The possibility that by increasing the time of exposure to the organic eluent better recovery may be accomplished was investigated. Time of contact was increased by collecting the eluate and recycling this eluate through the membrane a total of five times. Virus titer of the first eluate and the fifth eluate were determined; the rinse was also assayed to indicate if any virus might be lost at this step. As Table 5-9 shows, increasing the contact time between the adsorbed virus and the organic eluent definitely enhances elution of the virus. These results are indicative of an exchange mechanism in which the organics take the place of the adsorbed virus on the membrane surface.

Table 5-8. Elution by Organics

Eluent	Sample	Virus, PFU/ml			Percentage	
		Challenge	Filtrate	Eluate	Adsorbed	Recovered
TSB	Control	20,100				
	Membrane 1		6	3,600	100	18
	Membrane 2		0	6,300	100	31
	Membrane 3		12	6,900	100	34
FCS	Control	18,900				
	Membrane 1		9	4,500	100	24
	Membrane 2		3	13,000	100	66
	Membrane 3		0	2,700	100	14

Table 5-9. Elution by FCS: Enhanced Recovery by Recycling Eluate

Samples	Virus titer, PFU/ml					Recovery percentage	
	Challenge	Filtrate	Rinse	1st eluate	5th eluate	1st eluate	5th eluate
Control	25,800						
Membrane a		0	3	9,000	17,000	35	67
Membrane b		39	12	11,400	18,300	44	71

5.3.4.4 Synergistic Effect of Basic Organic Eluent

Elution of virus from cellulose nitrate has been shown to occur at neutral pH by a time-dependent exchange mechanism in which organics displace attached virus. Elution also results by an intensification of the repulsion forces between the virus and adsorption site brought about by exposure of the virus-site complex to elevated pH buffers.

A combination of these causal factors would be an organic eluent adjusted to basic pH. Such an eluent combination was prepared by NaOH adjustment of FCS, normally at pH 7.2, to pH 8.5, 10, and 11. Effectiveness of these eluents is shown in Table 5-10. Maximum recovery of virus was achieved at pH 10 and was 77%.

5.3.5 Adsorption of Virus to Clay Particulates

5.3.5.1 Effect of Magnesium Chloride Concentration

The influence of the magnesium ion concentration was explored by varying the concentration of magnesium chloride, with constant virus and clay concentrations, and observing the variation in virus adsorption. Holding constant a virus titer of 1.9×10^5 PFU/ml and clay particulates at 35 mg/l, magnesium chloride was varied from 0 to 0.1 M. Number of unadsorbed viruses for each salt concentration is given in Table 5-11. Counts of unadsorbed viruses decreased sharply from no-salt to 0.01 M; thereafter, additions of salt did not significantly affect the number of viruses adsorbing to the solids. Percentage of viruses adsorbed versus salt concentration is represented in Figure 5-10; the biphasic nature of the relationship is clearly illustrated.

It is interesting to note that viruses adsorbed to the bentonite even without salt in the medium. This behavior may be mediated by sur-

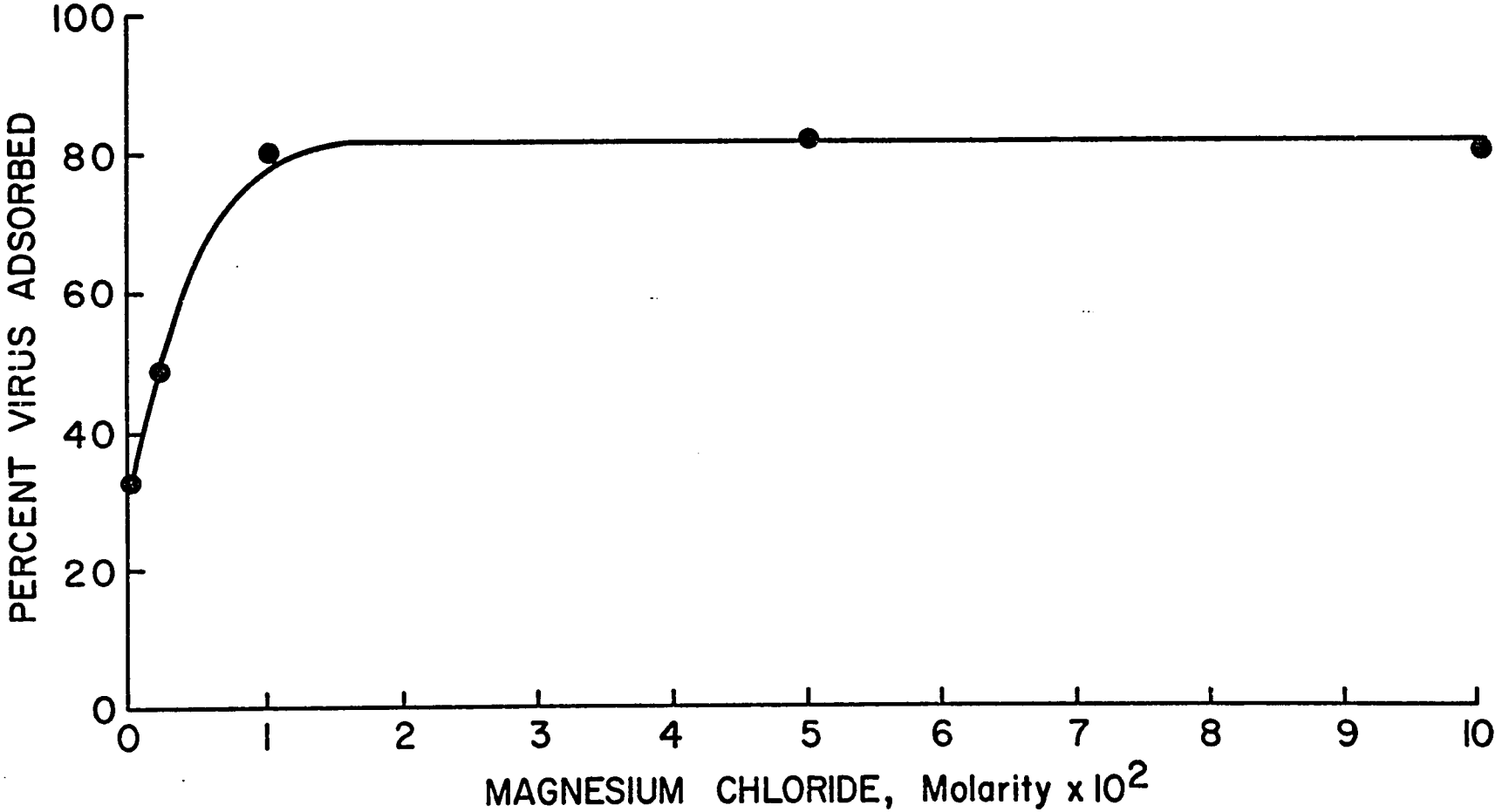
Table 5-10. Elution by Basic FCS

Sample	Virus, PFU/ml			Percent recovery
	Challenge	Filtrate	Eluate	
Control	37,500			
pH 7.2		0	16,500	44
pH 8.5		0	18,300	49
pH 10.0		0	28,800	77
pH 11.0		0	20,100	54

Table 5-11. Effect of $MgCl_2$ on Adsorption of Virus to Bentonite

$MgCl_2$, M	Supernatant virus, PFU/ml		Percent adsorbed
	Without clay	With clay	
0	191,000		
0		131,000	32
0.002		99,600	48
0.010		35,700	81
0.050		34,200	82
0.100		36,900	80

Figure 5-10. Percent virus adsorbed to bentonite as a function of $MgCl_2$ concentration. Test conditions: 35 mg/l bentonite clay, 23°C, 150 rpm, and 50 minutes stirring time.


PERCENT VIRUS ADSORBED
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
MAGNESIUM CHLORIDE, Molarity x 10²

face-bound cations, since, according to Moll (1975), such cations are present and are exchangeable.

5.3.5.2 Time for Adsorption-Desorption Equilibrium

The time necessary to achieve equilibrium between adsorbed and nonadsorbed virus was found by following the percent of virus adsorbed versus the time of contact with the clay particulates.

It was possible to determine the titer of unadsorbed virus by withdrawing 5 ml from the shaking suspension and immediately passing the sample through a non-virus-adsorbing membrane which held back all particulates and any viruses attached to them but allowed free viruses to pass into the filtrate. Table 5-12 lists the filtrate assay versus contact time. After 50 minutes, 90% of the viruses were associated with solids, and only 8% more attached within the next 25 minutes. A contact time of 50 minutes was used in all subsequent tests.

Figure 5-11 is a semilogarithmic plot of unadsorbed virus as a function of contact time. The straight line is indicative of a first-order reaction with respect to unadsorbed virus. This relationship can be expressed mathematically as

$$V_u = V_{uo}e^{-kt} \qquad (5\text{-}1)$$

where V_u = unadsorbed virus at time t;

V_{uo} = initial unadsorbed virus; and,

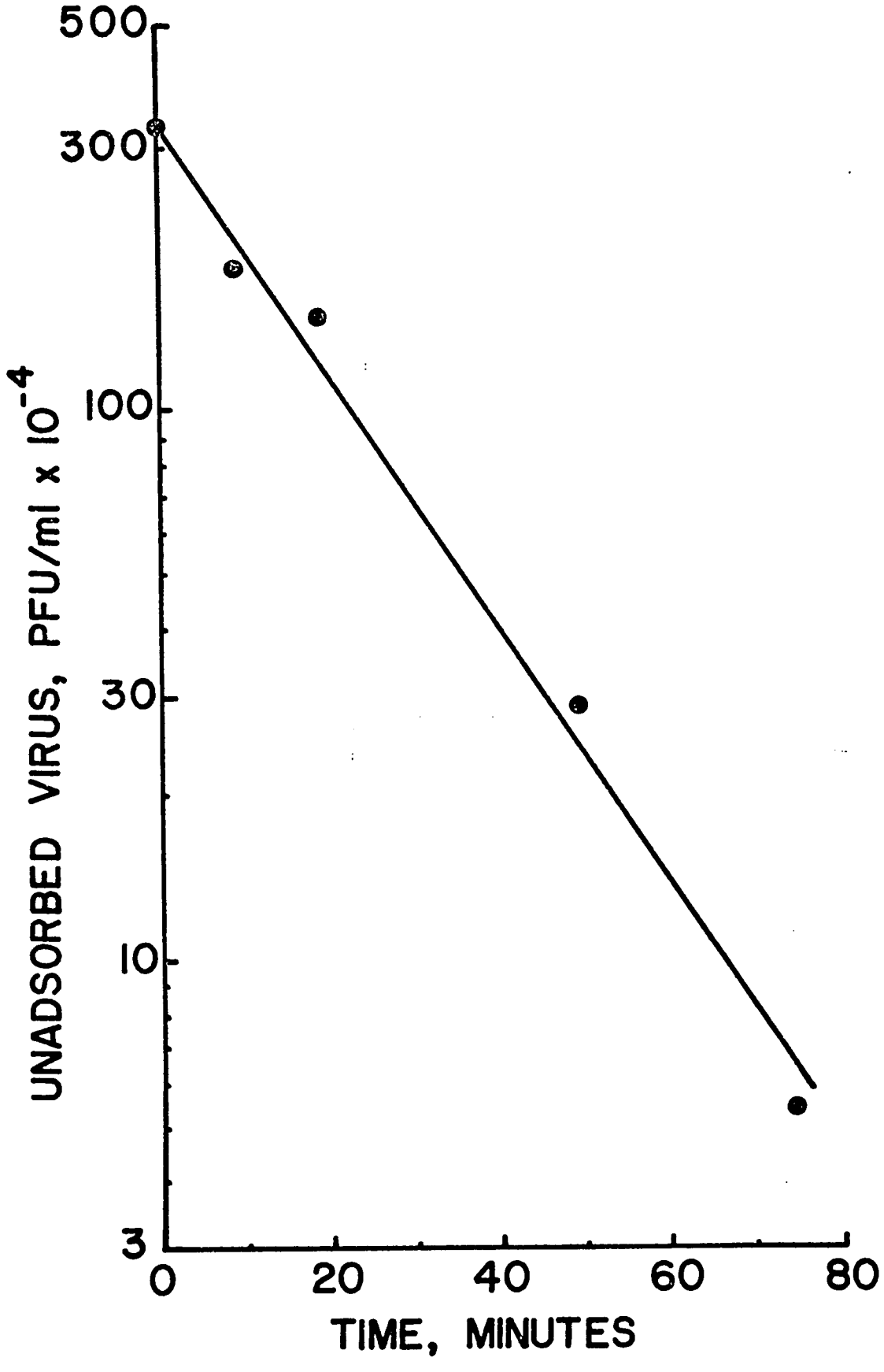
k = reaction rate constant.

Table 5-12. Equilibration Time for Virus Adsorption to Bentonite

Sample	Virus, PFU/ml	Percent adsorbed
Control	3.36×10^6	
Control filtrate	3.60×10^6	
10 min filtrate	1.80×10^6	46.5
20 min filtrate	1.46×10^6	56.6
30 min filtrate	1.06×10^6	68.4
50 min filtrate	2.85×10^5	91.5
75 min filtrate	5.40×10^4	98.4

Test conditions: Bentonite, 75 mg/l; pH 6.9; 0.05 M $MgCl_2$; 23°C; 150 rpm.

Figure 5-11. Unadsorbed virus as a function of contact time.


500
300
100
30
10
3
UNADSORBED VIRUS, PFU/ml x 10^{-4}
0
20
40
60
80
TIME, MINUTES

The value of k then can be determined from

$$k = \frac{-2.3}{t} \log_{10} \frac{V_u}{V_{uo}} \quad ; \text{ and,} \qquad (5\text{-}2)$$

for these conditions, $k = 0.04 \text{ min}^{-1}$.

5.3.5.3 Effect of Clay Concentration on Equilibrium

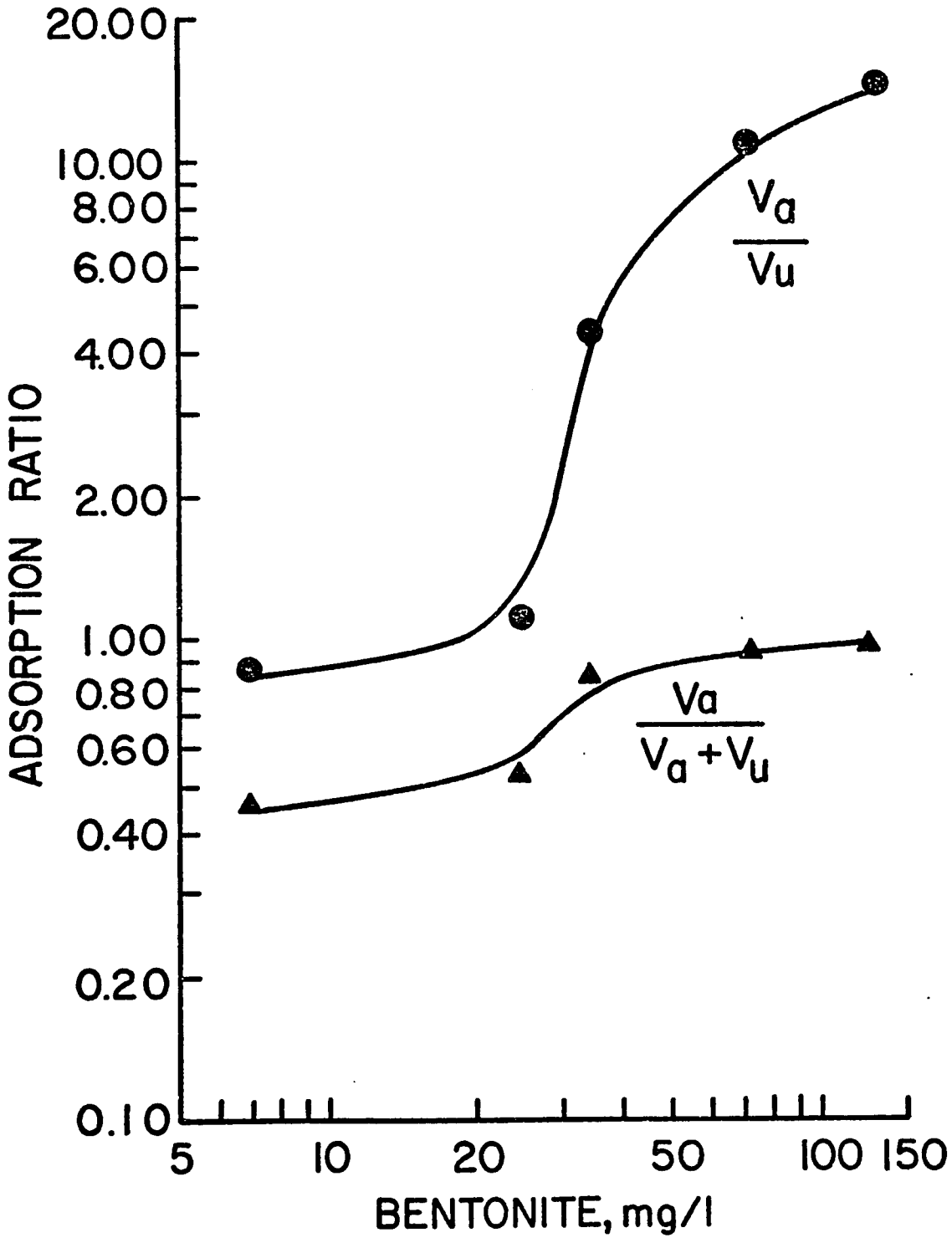
For a clay concentration of 75 mg/l it was found that 90% of the initial virus titer had been adsorbed after 50 minutes. Adsorption of virus to suspended solids is, of course, dependent upon collision of the virus with the particulates; it was therefore necessary to ascertain through what range of clay concentrations 50 minutes would be sufficient to achieve 90% adsorption of the initial virus titer.

Results of adsorption tests in which the bentonite concentration varied from 7 mg/l to 130 mg/l are depicted in Figure 5-12. The ratio of adsorbed virus to unadsorbed virus, V_a/V_u, increases with increasing clay concentration in a sigmoidal manner. Percentage of virus adsorbed to the solids, $V_a/(V_a + V_u)$, is at least 90% within 50 minutes for bentonite concentrations greater than 50 mg/l and agitated at 150 rpm.

5.3.5.4 Effect of Agitation on Adsorption Rate

Attachment of virus to suspended matter is dependent upon (1) transport of the viruses and particulates toward one another and (2) formation of an attractive force which binds the virus to the solid. Virus and particulate must collide before attachment can occur. Agitation of a virus-clay suspension should influence the rate of adsorption because the number of collisions per unit time is proportional to the agitation speed.

Figure 5-12. Effect of clay concentration on adsorption ratios.


20.00
10.00
8.00
6.00
4.00
2.00
1.00
0.80
0.60
0.40
0.20
0.10
ADSORPTION RATIO
Va / Vu
Va / Va + Vu
5
10
20
50
100
150
BENTONITE, mg/l

To explore the role of agitation speed, adsorption tests with identical solids concentrations were conducted at 23°C with rotation rates of 50, 100 and 200 rpm. Titer of unadsorbed virus was followed as a function of stirring time by using the FCS-pretreated membrane method. Results of these tests are tabulated in Table 5-13.

Rate of virus adsorption to clay is definitely dependent upon agitation speed. This indicates that the adsorption phenomenon is diffusion limited. After 50 minutes, clay suspensions stirred at 50, 100 and 200 rpm had adsorbed 42, 58 and 93% of the initial virus titer, respectively. Fraction of unadsorbed virus against time is depicted in Figure 5-13 for the 100 and 200 rpm tests. Adsorption rate constants have been calculated at 50, 100, 150, and 200 rpm and are listed in Table 5-14.

5.3.5.5 Effect of Temperature on Adsorption Rate

To ascertain the effect of temperature upon the rate of virus adsorption to bentonite particulates, two suspensions were prepared having identical solids concentrations; however, one suspension was maintained at 5°C whereas the other was kept at 37°C. Test results are given in Table 5-15. Adsorption data at both temperatures plot as straight lines of negative slope, as illustrated in Figure 5-14.

The rate of virus adsorption to the clay particulates increased with increasing temperature. At 5°C the reaction rate constant is 0.028 min^{-1}; at 37°C the value of the reaction rate constant is 0.034 min^{-1}. Oza and Chaudhuri (1975) have reported that adsorption of bacteriophage T4 to Giridih coal increases with temperature elevation from 28°C to 39°C.

Table 5-13. Unadsorbed Virus as a Function of Agitation Rate

Rotational speed, rpm	Virus titer, PFU/ml x 10^{-3}				Percentage adsorbed after 50 minutes
	Time, minutes				
	0	10	30	50	
50	282	200	162	162	42
100	291	221	138	122	58
200	182	99	42	13	93

Test conditions: Bentonite, 70 mg/l; pH 7; 23°C; 0.05 M $MgCl_2$.

Figure 5-13. Fraction of unadsorbed virus as a function of stirring speed.

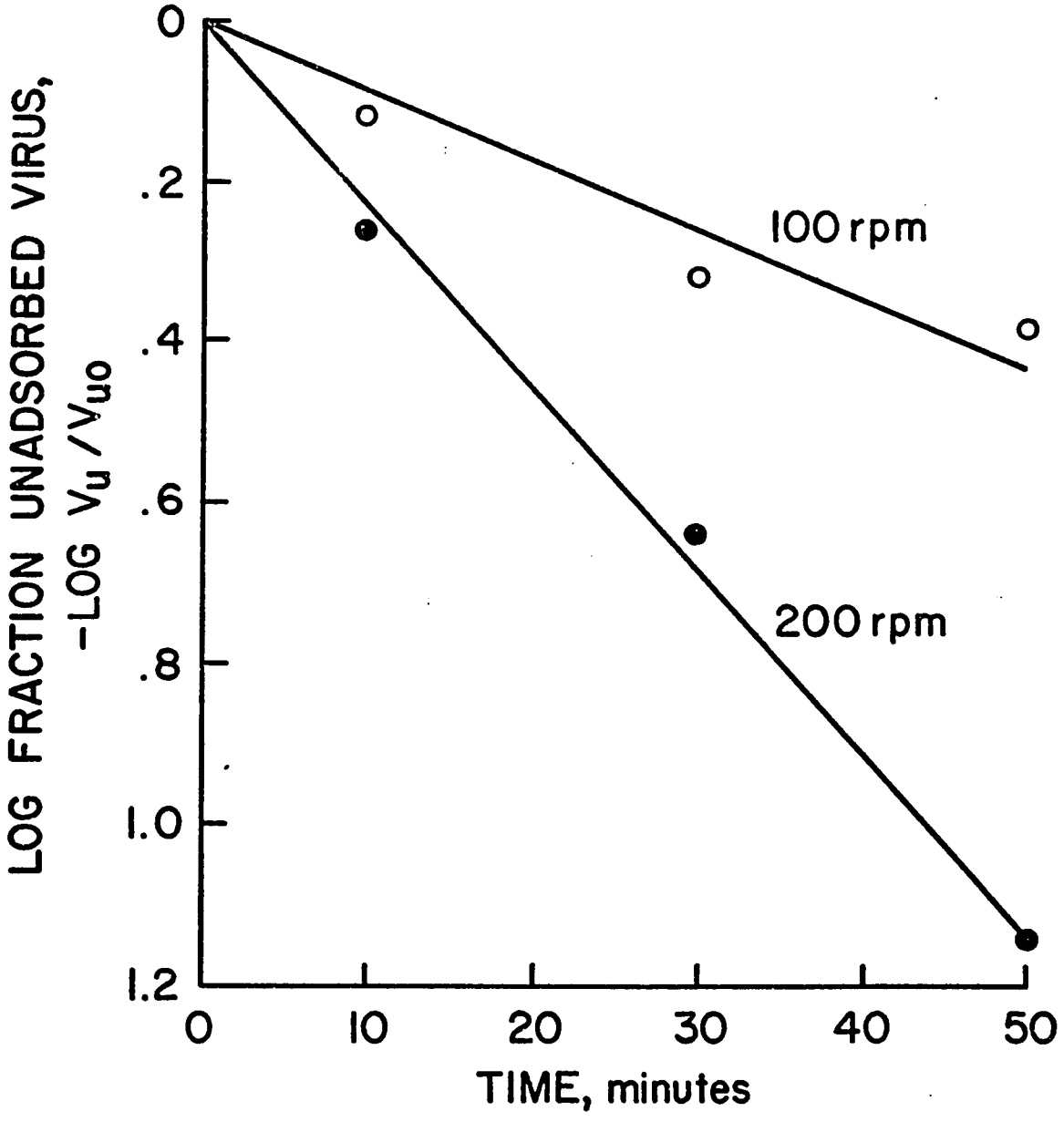

LOG FRACTION UNADSORBED VIRUS,
-LOG V_u / V_{uo}
0
.2
.4
.6
.8
1.0
1.2
0
10
20
30
40
50
TIME, minutes
100 rpm
200 rpm

Table 5-14. Adsorption Rate Constant as a Function of Stirring Speed

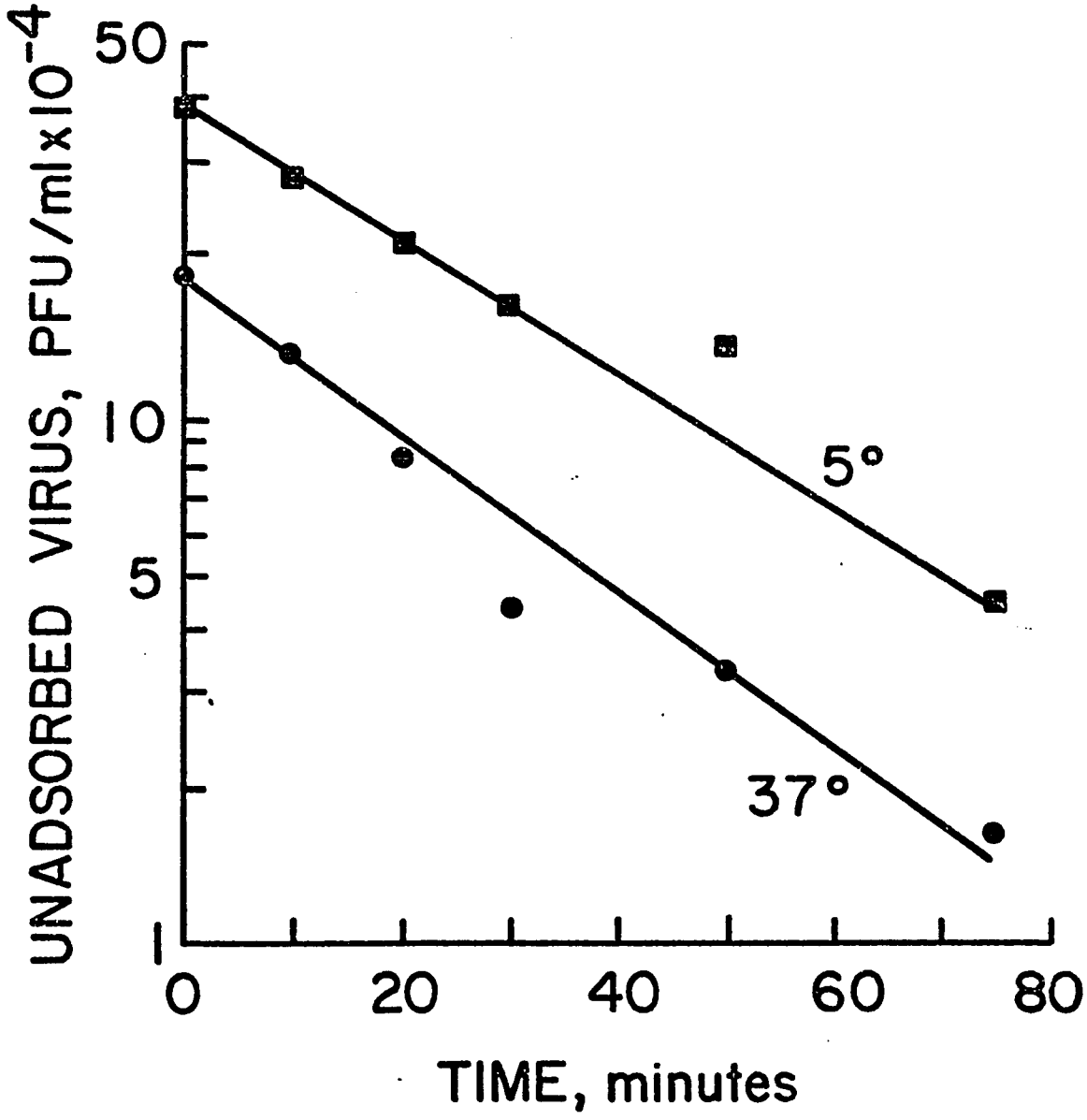
Rotational speed, rpm	Unadsorbed virus, PFU/ml		Adsorption rate constant, min^{-1}
	Time, minutes		
	0	30	
50	2.82×10^5	1.62×10^5	0.019
100	2.91×10^5	1.38×10^5	0.025
150	3.36×10^6	1.06×10^6	0.038
200	1.82×10^5	4.20×10^4	0.049

Table 5-15. Adsorption as a Function of Temperature

Sample	Unadsorbed virus, PFU/ml	
	5°C suspension	37°C suspension
Control	495,000	267,000
Control filtrate	378,000	186,000
10 min filtrate	279,000	135,000
20 min filtrate	210,000	82,800
30 min filtrate	162,000	43,050
50 min filtrate	138,000	33,900
75 min filtrate	45,000	16,200

Test conditions: Bentonite, 75 mg/l; pH 7.0; 0.05 M $MgCl_2$; 90 rpm.

Figure 5-14. Effect of temperature on adsorption rate.


UNADSORBED VIRUS, PFU/ml x 10⁻⁴
50
10
5
1
0
20
40
60
80
TIME, minutes
5°
37°

Cookson (1966) adsorbed T4 to granular 26/35 carbon and found a rate constant of 0.004 min^{-1} at 23°C and also at 33°C.

The kinetic rate constant varies with temperature. The Arrhenius relationship is as follows:

$$\frac{d\ln k}{dT} = \frac{E}{RT^2} \qquad (5\text{-}3)$$

Integration of this equation and solution for the apparent activation energy E with k_1 = 0.028 min^{-1} at 278° absolute and k_2 = 0.034 min^{-1} at 310° absolute yields an apparent activation energy of 1 kcal/mol. This supports the conclusion that virus adsorption to clay is a diffusion-limited process since such values of activation energy have been found in studies of the effect of bulk temperature changes on the diffusion coefficient.

5.3.5.6 Adsorption Isotherm

Removal of solute molecules from the liquid phase of a solid-liquid system will continue to occur until an equilibrium between adsorbed and free solute molecules is attained. This equilibrium state has traditionally been portrayed by plotting the amount of adsorbed solute per weight of solid versus the concentration of free solute. A graphical display of this type is called an adsorption isotherm.

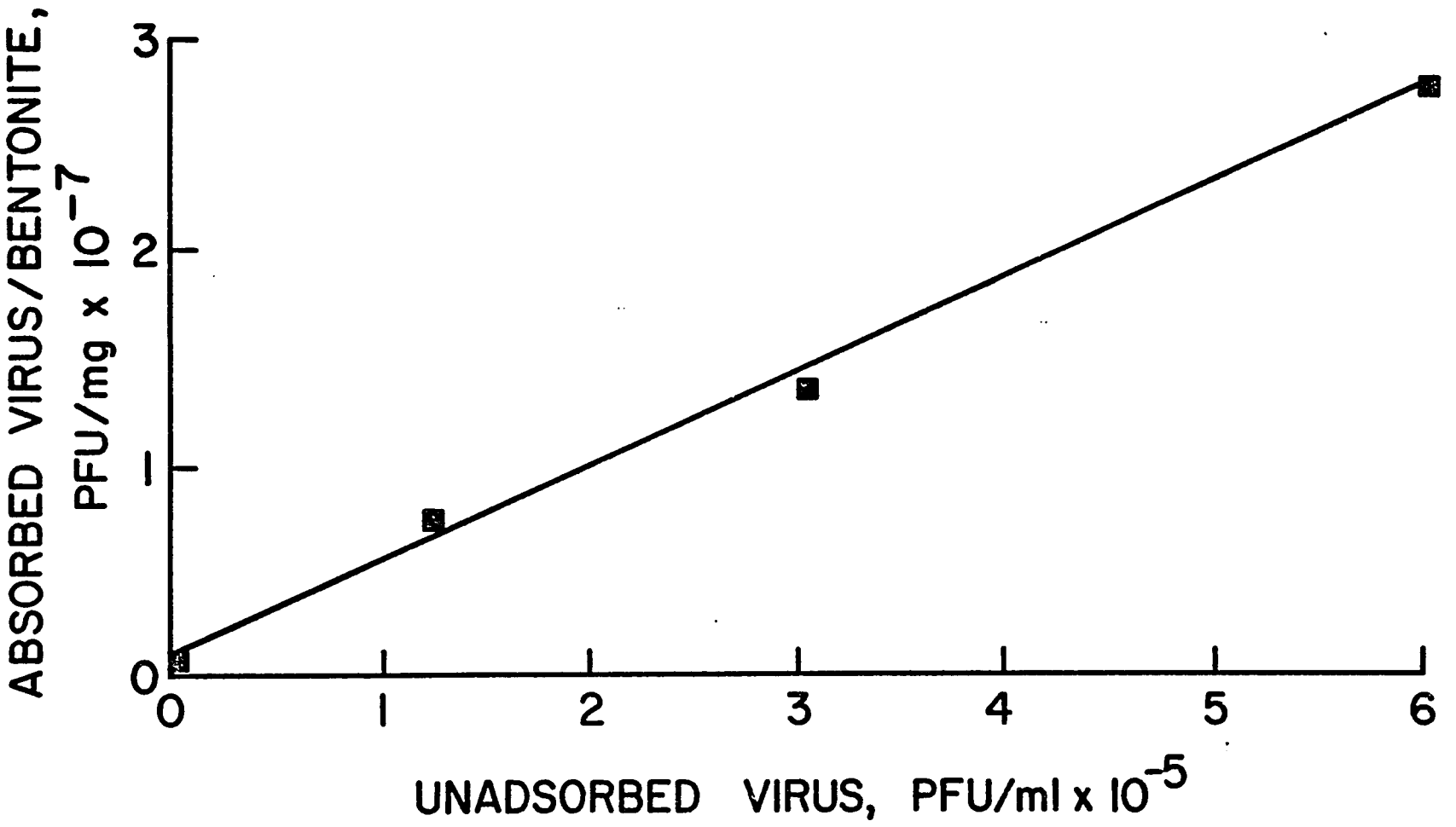
Although viruses suspended in water physically exist as biocolloids and not as solubilized molecules, adsorption of waterborne viruses to solids is amenable to depiction by adsorption isotherms. The data of Table 5-16 was plotted in Figure 5-15. Adsorbed virus per unit weight of solids increases linearly with the concentration of free virus. Giles et al. (1960) have classified dilute solution isotherms according to shape.

Table 5-16. Low Coverage Adsorption

Virus, PFU/ml		Bentonite, mg/l	Adsorbed virus, PFU / Bentonite mg
Unadsorbed	Adsorbed		
1.00×10^3	1.40×10^4	0.130	1.08×10^5
1.20×10^5	6.70×10^5	0.075	7.33×10^6
3.00×10^5	1.03×10^6	0.075	1.37×10^7
6.06×10^5	2.05×10^6	0.075	2.74×10^7

Test conditions: 23°C; 150 rpm; 0.05 M $MgCl_2$.

Figure 5-15. Low coverage adsorption isotherm.


ABSORBED VIRUS/BENTONITE,
PFU/mg x 10^-7
0
1
2
3
0
1
2
3
4
5
6
UNADSORBED VIRUS, PFU/ml x 10^-5

The linear behavior evident in Figure 5-15 is normally seen at low coverages, that is, when very few of the possible adsorption sites are occupied. Under these test conditions, there are about 10^8 clay particles per ml, but at most only about 10^6 adsorbed viruses per ml. It is likely, then, that only one of every 100 clay particles has an attached virus.

5.3.5.7 Effect of Sewage Organics

Carlson et al. (1968) demonstrated that egg albumin, bovine albumin and raw wastewater compete with bacteriophage T2 for adsorption sites on kaolinite.

Since this study is concerned with chlorination of solids-associated virus suspended in clarified effluent, it is useful to evaluate the effect of soluble sewage-effluent organics on adsorption of virus to the model solids. These organics were tested as site-competitors with bacteriophage MS-2 for bentonite under the conditions of maximal adsorption. Virus titers and test data are listed in Table 5-17. The presence of 3 mg/l TOC reduced adsorption of virus from 97% to 35%. It is evident, then, that freely suspended virus will associate with clay solids even in the presence of soluble organics; however, the extent of adsorption is much less than that occurring without organics present.

It has been demonstrated that viruses will bind to bentonite without the mediation of salts; however, the percentage of viruses binding under this condition for equivalent times is much less than that for adsorption occurring in the presence of salt. Reduction in virus adsorption caused by sewage organics added to a salt-less suspension of clay could not be as clearly demonstrated as performing the site competition experiment

Table 5-17. Effect of Sewage Organics on Adsorption of Virus to Clay

Bentonite, mg/l	Organics (TOC), mg/l	Supernatant virus, PFU/ml	Percent adsorbed
0	0	690,000	
110	0	23,700	97
110	3	450,000	35

Test conditions: 22°C; 150 rpm; 0.05 M $MgCl_2$; 0.45-μ filtered clarified effluent diluted 2X in reaction mixture; ABS_{254} = 0.0465.

at maximal adsorption conditions.

5.3.6 Elution of Virus from Clay Particulates

5.3.6.1 Evaluation of Candidate Eluents

Malina (1975) found that phage f2 did not elute easily from bentonite. The eluent here was borate saline adjusted to pH 9. After adsorption of the virus to bentonite, the pellet was resuspended in the eluent for 15 minutes with periodic shaking; however, less than 15% of the adsorbed virus could be recovered.

An evaluation of various candidate eluents was done in which virus adsorbed to bentonite in the presence of 0.05 M $MgCl_2$ was exposed to eluent for 1 minute under vortex mixing. Results of this eluent scan indicate that soy organics, serum organics, and detergent are the best of the eluents evaluated. Purified water, neutral 0.05 M glycine, and pH 11 glycine are less capable of eluting adsorbed virus. Although only a 4 or 5% recovery was achieved by the organics, this was sufficient to warrant further studies of such organics as eluents. Table 5-18 shows the percentage recoveries elicited by the various eluents and the adsorption samples upon which recovery percentages were calculated.

5.3.6.2 Effect of Time on Elution by Organics

The scan of candidate eluents indicated that fetal calf serum elicited recovery of about 5%. Time of exposure to this eluent was extended to determine if this might augment elution. As Table 5-19 attests, numbers of eluted viruses did increase with time but only insignificantly.

Table 5-18. Evaluation of Candidate Eluents

Sample	Virus, PFU/ml	Percentage	
		Adsorbed	Recovered
Control	1,740,000		
First supernatant	300,000		
Second supernatant	10,000		
Total supernatant	310,000		
Total adsorbed	1,430,000	82	
Eluents			
Purified water	350		0.02
Glycine, pH 7	1,500		0.10
Glycine, pH 11	300		0.02
TSB	55,000		3.80
50% TSB	57,000		3.90
FCS	77,000		5.40
0.1% Tween 80	52,000		3.60

Table 5-19. Effect of Time on Elution by Fetal Calf Serum

Sample	Virus, PFU/ml	Percentage	
		Adsorbed	Recovered
Control	191,000		
Supernatant	34,200		
Adsorbed	156,800	82	
Minutes of exposure to eluent			
Serum:			
5	3,780		2.4
40	4,320		2.8
80	6,480		4.1
Purified water:			
80	540		0.3

5.3.6.3 Effect of Immobilization followed by Elution at Rapid Flow Rate

Adsorption of virus to cellulose nitrate membranes was found to be dependent upon flow rate. Data of Table 5-3 indicate that attachment of the virion to an adsorption site is impeded by shearing stresses. Might these stresses enhance desorption of viruses from clay either by forcing the eluent molecules to protected attachment sites or by physically shearing the virus-clay bond?

To test this desorption scheme, viruses were first adsorbed to clay particulates. These particulates, along with their attached viruses, were then immobilized onto Millipore HA, 25-mm, FCS-treated membranes. Eluent was then passed over these particulates and through the membrane at approximately 3 ml/minute/cm^2. After collection of the eluate, it was recycled through the membrane five times to increase contact time. Experimental data are given in Table 5-20.

Attached viruses were desorbed by shearing stresses. Even the procedure of immobilization resulted in release of about 3% of the attached viruses. One passage of purified water through the membrane eluted about 3% also; but with five passages through the membrane, recovery percentage increased to 6%. Rotary shaking in purified water at 100 rpm for 80 minutes (see Table 5-19) had only resulted in a recovery of 0.3%.

At the first passage, fetal calf serum adjusted to pH 10 eluted about 6%: twice as many viruses than did pH 7.2 FCS in one passage; however, after five passages, both eluents had released about 11% of their attached viruses.

Table 5-20. Elution of Immobilized Solids-Associated Virus

Samples	Virus, PFU/ml	Percentage	
		Adsorbed	Recovered
Control	28,500		
First supernatant	8,970		
Second supernatant	762		
Total supernatant	9,730		
Total adsorbed	18,700	66.0	
Pooled filtrate	480		2.6
Eluates			
Purified water (a)	501		2.7
Purified water (b)	1,170		6.4
pH 7 FCS (a)	570		3.1
pH 7 FCS (b)	1,989		10.9
pH 10 FCS (a)	1,090		5.9
pH 10 FCS (b)	2,020		11.1
pH 11 EDTA (c)	1,080		5.9

(a) one passage; (b) five passages; and (c) two passages.

A 0.01 M Na_2EDTA solution at pH 11 was no better than a single passage of pH 10 FCS.

5.3.6.4 Direct Plating

Gerba (1973) was able to plate a T2-kaolinite suspension directly; Boardman and Sproul (1975) plated a T7-kaolinite suspension directly. This method was evaluated for its capacity to elute bacteriophage MS-2 from bentonite. Results of this evaluation are listed in Table 5-21. Average recovery percentage was 11% with TSB diluent and 15% with FCS diluent. The (MS-2)-bentonite bond is much stronger than that of the T2- or T7-kaolinite bond.

5.3.6.5 Molar Salt Solutions

Fildes and Kay (1963) found that bacteriophage adsorption to kaolinite was maximized at 0.05 M NaCl, and that with increases in NaCl concentration, fewer and fewer viruses adsorbed to the solids. High salt concentrations (1 molar) of NaCl and $CaCl_2$ were tested as eluents. From Table 5-22 it is evident that this technique does not yield increased recoveries; average recovery for both 1 M NaCl and 1 M $CaCl_2$ was about 9%.

5.3.6.6 Sonication

An attempt was made to disrupt the virus-bentonite bond by high-frequency sound waves. Sonication was carried out in 0.05 M $MgCl_2$, 1 M NaCl, and 1 M $CaCl_2$; but, only about 10% of the viruses were desorbed. Sonication did not inactivate viruses. Experimental data appear in Table 5-23.

Table 5-21. Elution by Direct Plating

Samples	Virus, PFU/ml	Percentage	
		Adsorbed	Recovered
Control	99,500		
First supernatant	56,400		
Second supernatant	738		
Total adsorbed	42,300	42.5	
Plating diluents			
2% TSB-H_2O	5,380		12.7
2% TSB-H_2O	4,140		9.8
2% FCS-H_2O	7,800		18.4
2% FCS-H_2O	4,900		11.8

Table 5-22. Elution by Molar Salt Concentrations

1 M salt	Virus, PFU/ml		Average percent* recovery
	Trial 1	Trial 2	
NaCl	4140	3540	9.1
$CaCl_2$	3192	3900	8.4

* Titer of adsorbed virus was 42,300 PFU/ml.

Table 5-23. Elution by Sonication

Sonication medium	Virus, PFU/ml		Average percent* recovery
	Trial 1	Trial 2	
0.05 M $MgCl_2$	3450		8.2
1 M NaCl	4440	3900	9.9
1 M $CaCl_2$	3540	3780	8.7

* Titer of adsorbed virus was 42,300 PFU/ml. Control titer: 99,500 PFU/ml; sonicated control titer: 98,400 PFU/ml.

5.3.7 Association of Virus with Neutral Aluminum Flocs

Viruses can be removed by entrapment within forming aluminum hydroxide flocs or by adsorption to the periphery of formed flocs. Lability of the model virus at pH extremes limited these association tests to preformed neutral flocs made in dechlorinated tapwater. Nevertheless, entrapped viruses can be produced by pelleting the flocs with peripherally attached viruses at high centrifugal force to fuse floc particles by compacting them in the pellet; then, dispersion of the pelleted floc results in new particles with entrapped viruses.

Removal of suspended virus by adsorption to flocs is strikingly dependent upon concentration of aluminum and is somewhat influenced by the identity of anions. In Table 5-24 it can be observed that the best association occurs at about 100 mg/l Al, regardless of the aluminum salt used; however, at lower concentrations, aluminum sulfate performs better than aluminum chloride. Both association curves are trough-shaped (see Figure 5-16) with removal percentages rapidly decreasing with increasing aluminum concentrations past the maximal concentration. York and Drewry (1974) have reported a small upturn in the association curve of bacteriophage f2 for coagulation jar tests with aluminum sulfate and ferric sulfate.

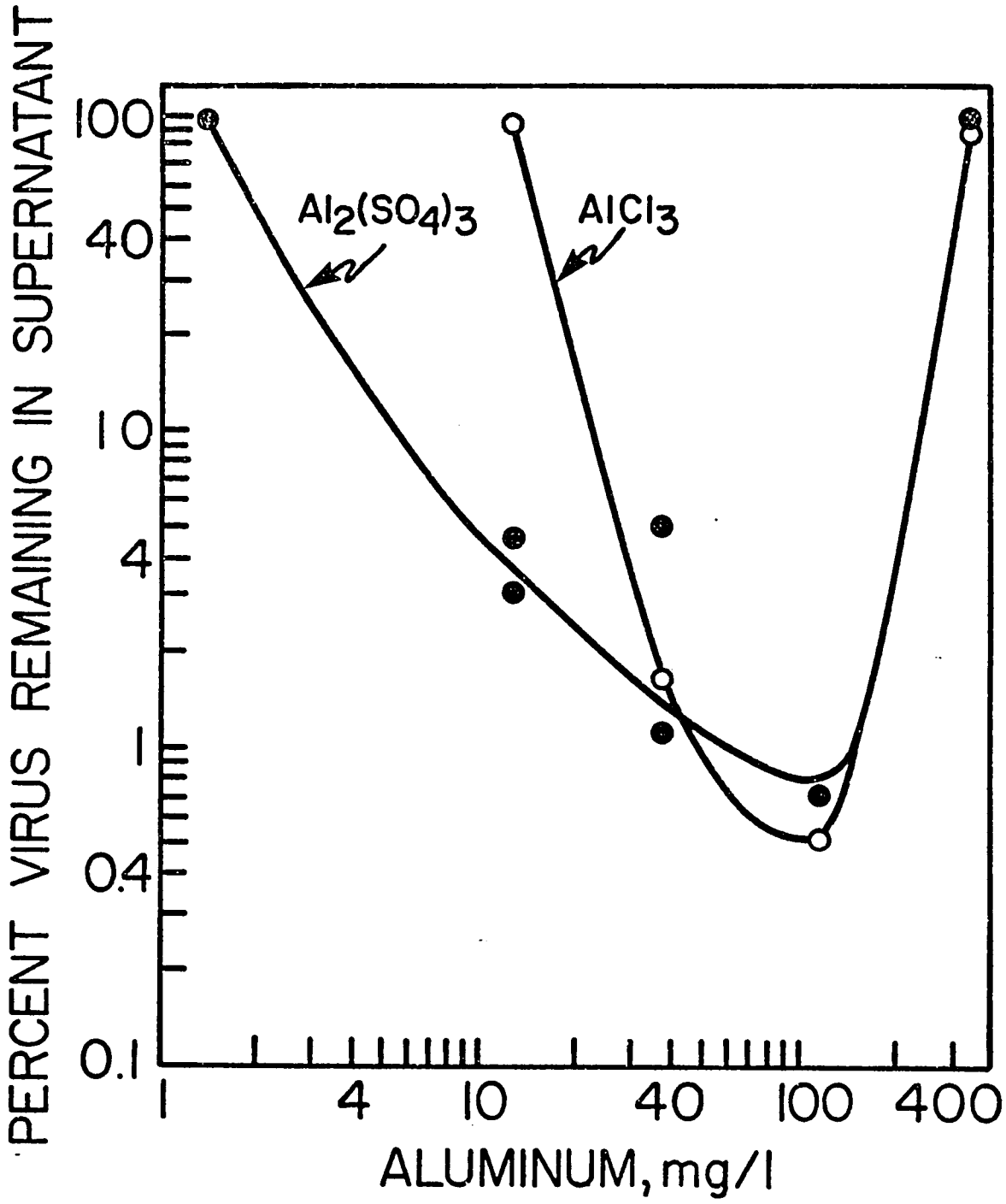
5.3.8 Recovery of Virus from Flocs

Excellent recovery of virus from $AlCl_3$ formed flocs was achieved by simply mixing the pelleted flocs with fetal calf serum. The floc matrix is disturbed by the organics and the viruses are released in a viable state.

Table 5-24. Variation in Virus Removal with Aluminum Concentration

Salt	Al, mg/l	Supernatant virus, PFU/ml	Percent removal
$AlCl_3 \cdot 6 H_2O$	0.0	19,900	0.0
	13.0	19,300	3.1
	39.0	315	98.4
	117.0	96	99.5
	351.0	18,900	4.5
$Al_2(SO_4)_3 \cdot 18 H_2O$	0.0	23,700	0.0
	13.0	1,050	95.5
	39.0	261	98.9
	117.0	171	99.3
	351.0	24,000	0.0
	0.0	54,000	0.0
	1.4	52,200	3.3
	4.3	18,300	66.1
	13.0	3,900	92.7
	39.0	2,700	95.0

Figure 5-16. Variation of virus removal with aluminum concentration.


PERCENT VIRUS REMAINING IN SUPERNATANT
100
40
10
4
1
0.4
0.1
$Al_2(SO_4)_3$
$AlCl_3$
1
4
10
40
100
400
ALUMINUM, mg/l

Recovery of more than 100% may be explained by slight inactivation or aggregation of the virus in the control flasks, since these samples were taken at the end of the association tests. The lower concentrations of aluminum sulfate do not stabilize the viruses in the flocs as well as aluminum chloride. As listed in Table 5-25, recovery of more than 80% of floc-associated virus is attained for aluminum concentrations of 117 mg/l.

5.3.9 Association of Virus with Organic Solids

5.3.9.1 Humic Acid

Humic compounds are chemically complex substances which range in molecular weight from a few hundred to several thousand daltons. They are acidic, dark colored, and partially aromatic. The presence of carboxyl and phenolic hydroxyl groups provides humic acids with a considerable ion exchange capacity. Humic acid is soluble in alkali but insoluble in acid. Narkis and Rebhun (1975) have found that humic acid interacts with montmorillonite to increase the negative mobility of these clay solids. Farrah et al. (1976) have complexed the LSc strain of poliovirus with humic acid flocs formed at pH 2.5. Viable virus was recovered by solubilization of the flocs under alkaline conditions.

The influence of $MgCl_2$ concentration upon association of viruses with humic acid at neutral pH was investigated by conducting association tests at various $MgCl_2$ molarities. Percentage of viruses associated with organics versus salt concentration is given in Table 5-26. Although the percent of humic acid removed with the floc increased with $MgCl_2$, only about 10% of the viruses were removed at any $MgCl_2$ concentration. Com-

Table 5-25. Recovery of Virus from Flocs with Fetal Calf Serum

Salt	Al, mg/l	Virus, PFU/ml			Percent recovery
		Supernatant	Pellet	Total	
$AlCl_3 \cdot$	0	19,900	0	19,900	
$6\ H_2O$	13	19,300	720	20,000	>100
	39	315	22,200	22,500	>100
	117	96	28,300	28,400	>100
	351	18,900	3,220	22,100	>100
$Al_2(SO_4)_3 \cdot$	0	23,700	0	23,700	
$18\ H_2O$	13	1,050	10,100	11,200	47
	39	261	3,630	3,890	16
	117	171	19,700	19,900	84
	351	24,000	1,810	25,800	>100

Table 5-26. Effect of $MgCl_2$ on Formation of Organic Solids and Association of Virus with the Forming Solids

$MgCl_2$, M	Humic acid, mg/l		Virus, PFU/ml		Percent removed	
	Control	Supernatant	Control	Supernatant	Humic acid	Virus
0	10.0	9.3	10,200	10,200	7	0
0.001		8.4		9,300	16	9
0.010		5.3		9,300	47	9
0.050		3.8		9,000	62	12

Test conditions: 22°C; pH 7; 200 rpm stirring; 60 minutes; centrifugation, 2100 g for 5 minutes.

parable concentrations of NaCl do not mediate the formation of a floc; this finding is given in Table 7-2 of the Appendices.

A kinetic association test was also performed using 3 mg/l humic acid and 0.05 M $MgCl_2$ at neutral pH. The titer of remaining free virus was followed by passing 5-ml volumes of the stirred suspension through non-adsorbing membranes. Filtrate assays are listed in Table 5-27, and it is evident that little association of virus with humic acid occurred through time.

5.3.9.2 Activated Sludge Particulates

Activated sludge consists of flocculent masses containing microbial cells, organic debris and various inorganic solids such as clays. These flocs are formed as a result of the mentioned solids becoming entrapped in a gelatinous mucilage. Nishikawa and Kuriyama (1968) have identified DNA and RNA as components of this mucilage. Pavoni et al. (1972) have found that the mucilage consists primarily of four types of polymers: polysaccharide, protein, RNA and DNA.

Moore et al. (1975) reported that poliovirus, T7, T2, and f2 do not associate with raw wastewater suspended solids or final effluent solids from an activated sludge treatment plant. These tests were conducted in autoclaved samples which contained soluble organics.

The solids in this investigation were centrifuged and then resuspended in purified water so that no interference by organics occurred. Results of model virus adsorption to prepared activated sludge particulates are given in Table 5-28. The results are interesting: even without salts

Table 5-27. Association of Virus with Humic Acid as a Function of Time

Time, minutes	Virus, PFU/ml	Percent virus associated
Control	16,800	
6	17,000	0
10	13,900	17
20	13,200	21
40	13,000	23
60	14,700	12

Test conditions: 22°C; pH 7; 200 rpm stirring; 0.05 M $MgCl_2$.

Table 5-28. Association of Virus with Washed Activated Sludge Solids

Salt, M	Initial JTU	Supernatant virus, PFU/ml	Percent virus removal
0	0	342,000	
0	6	318,000	7
$MgCl_2$, 0.05	6	282,000	18
$AlCl_3$, 0.0005	6	30	>100

Test conditions: 24°C; pH 7; 200 rpm stirring for 60 minutes; centrifugation, 5800 g for 15 minutes.

about 7% of the viruses attached to the solids; and, in the presence of 0.05 M $MgCl_2$, the adsorption percentage increased to 18%. Attachment of virus to suspended sewage solids is impaired by the presence of soluble organics; and, in the light of the results of section 5.3.5.7, attachment is probably only occurring onto suspended mineral solids. Camp and Meserve (1974) have found that about 30% of suspended sewage solids are mineral; the remainder of the solids are organic in nature.

It is reasonable, then, to state that freely suspended viruses will associate with suspended mineral solids even in the presence of organics; these viruses will not associate with the suspended organic solids; divalent cations enhance the association phenomenon; and, therefore, the model solid selected for inactivation tests should be mineral in nature.

Formation of aluminum hydroxide flocs removed both viruses and the activated sludge solids. Flocs, whether biological or chemical, which remain suspended will contain enmeshed viruses along with solids.

5.4 Chlorination of Freely Suspended Virus

5.4.1 Development of Expression for Virus Reaction Order

During isothermal batch reactions there are several variables influencing the rate of disinfection: the concentration of disinfectant, the number of remaining viable viruses, and the contact time. A power-law model for virus inactivation rate, in the case of a homogeneous phase

reaction, takes this form:

$$r_v = \frac{dC_v}{dt} = -k C_v^n C_c^m \qquad (5\text{-}4)$$

where r_v = virus inactivation rate, PFU/sec;

C_v = concentration of viable viruses, PFU/ml, remaining at any time t;

t = time of contact, sec;

k = inactivation rate constant;

C_c = chlorine concentration, mg/l;

n = virus reaction order; and

m = chlorine reaction order.

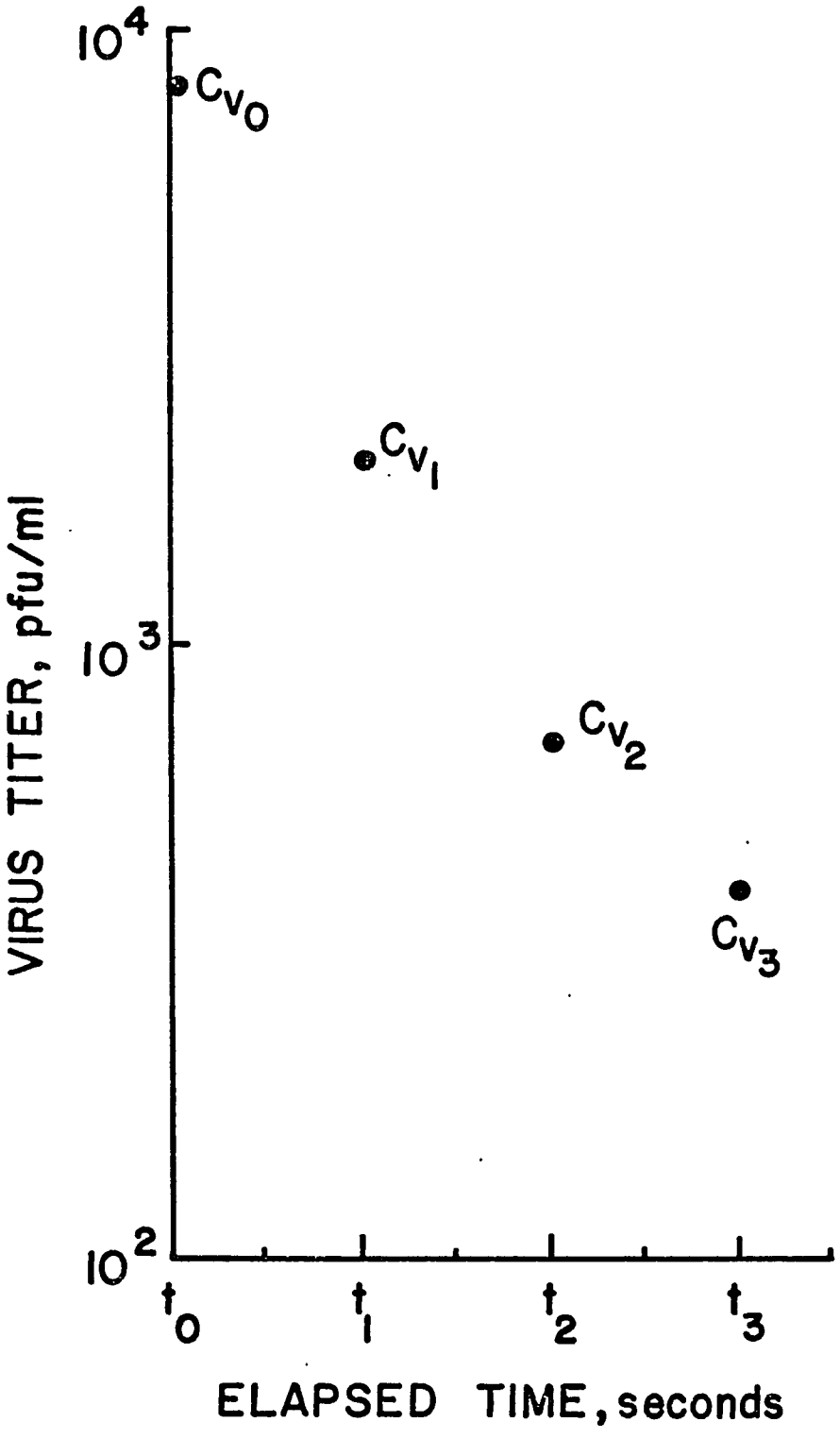
Should the concentration of chlorine remain constant throughout the reaction and be of much greater concentration than that of the viruses, equation 5-4 can be rewritten as

$$r_v = \frac{dC_v}{dt} = -k' C_v^n \qquad (5\text{-}5)$$

in which the chlorine concentration term has been grouped with the true reaction rate constant, and this new reaction constant is denoted by k'.

For an irreversible, isothermal, constant density batch reaction, Levenspiel (1972) has developed a method for obtaining reaction order with respect to the limiting reactant. Application of this method to virus inactivation entails first plotting the remaining viable virus, C_v, as a function of contact time, t. A typical representation of this functional relationship is shown in Figure 5-17.

Figure 5-17. Reduction in viable viruses as a function of contact time.


10^4
C_{V_0}
C_{V_1}
10^3
C_{V_2}
C_{V_3}
10^2
VIRUS TITER, pfu/ml
t_0
t_1
t_2
t_3
ELAPSED TIME, seconds

The inactivation rate, $\frac{dC_v}{dt}$, can be approximated by an expression of this form:

$$\frac{\Delta C'_{v_i}}{\Delta t_i} = \frac{C_{v_i} - C_{v_{i-1}}}{t_i - t_{i-1}} \qquad (5\text{-}6)$$

which is the inactivation rate at a viable virus concentration of

$$C'_{v_i} = \frac{C_{v_i} + C_{v_{i-1}}}{2} . \qquad (5\text{-}7)$$

In this approximate form, equation 5-6 becomes

$$\frac{\Delta C'_{v_i}}{\Delta t} = -k' C'^{\,n}_{v_i} . \qquad (5\text{-}8)$$

The logarithmic form of the previous equation is

$$\ln\left[\frac{-\Delta C_{v_i}}{\Delta t}\right] = \ln(k') + n \ln(C'_{v_i}) \qquad (5\text{-}9)$$

which is a linear equation with intercept at ln (k′) and a slope of n. A plot of inactivation data in this form would yield virus reaction order by graphical determination of the slope.

5.4.2 Development of Expressions for Chlorine Reaction Order

Equation 5-4 was the general power law equation for virus inactivation rate:

$$\frac{dC_v}{dt} = -k C_v^{\,n} C_c^{\,m} . \qquad (5\text{-}4)$$

Collection of like terms and then integration for $n \neq 1$ results in

$$\frac{1}{1-n}\left\{C_v^{1-n} - C_{v0}^{1-n}\right\} = -k C_c^{\,m} (t - t_0); \qquad (5\text{-}10a)$$

and, for n = 1,

$$\ln \left[\frac{C_v}{C_{v_0}}\right] = -k C_c^{\,m} (t - t_0). \qquad (5\text{-}10b)$$

The value of n can be determined experimentally; the ratio of C_v/C_{v_0} can be selected arbitrarily to be that for 99% inactivation, that is, $C_v/C_{v_0} = 0.01$ for which $t = t_{99}$. C_{v_0} and n are thus known; C_v is simply $0.01\ C_{v_0}$; t_{99} can be determined graphically from the survival curve. For any experiment then, the left-hand side of equation 5-10 is calculable; however, k and m are not known. In all cases $t_0 = 0$.

The logarithmic forms of equations 5-10a, b are linear:

$$\ln t_{99} = \ln \left[\frac{1}{n-1}\left[C_v^{\,1-n} - C_{v_0}^{\,1-n}\right]\right] - \ln k - m \ln C_c \qquad (5\text{-}11a)$$

and

$$\ln t_{99} = \ln \left[\ln \left\{\frac{C_{v_0}}{C_v}\right\}\right] - \ln k - m \ln C_c. \qquad (5\text{-}11b)$$

A plot of $\ln t_{99}$ versus $\ln C_c$ would yield the chlorine reaction order m. With the value of m determined, k can be calculated from equations 5-11a, b.

5.4.3 Chlorine Demand of Virus-Associated Organics

Stock phage is obtained by adding 5 ml of trypticase soy broth over a plaque-confluent assay plate. Because of this organic load accompanying the virus, it was necessary to determine the dilution needed to overcome chlorine-demand interference by these organics.

Table 5-29 gives the results of tests performed at two pH levels: 5.5 and 11.1. At pH 5.5 the free chlorine is in the form of hypochlorous acid, HOCl; at pH 11.1, the free chlorine is in the anionic form of hypo-

Table 5-29. Chlorine Demand of Extraneous Organics

Reaction pH	Log_{10} dilution	OT chlorine, mg/l		Percentage lost
		Control	Phage-broth	
5.5	-2.3	1.54	0.09	94
	-3.3	1.61	0.54	67
	-4.3	1.60	1.39	13
	-5.3	1.58	1.55	2
	-6.3	1.57	1.50	5
	-7.3	1.62	1.59	2
11.1	-2.3	0.98	0.01	99
	-3.3	0.99	0.39	61
	-4.3	0.99	0.80	19
	-5.3	1.01	0.95	6
	-6.3	0.97	0.98	0
	-7.3	0.99	0.97	2

chlorite, OCl^-.

At both pH levels a dilution of at least 1×10^6 fold will ensure that chlorine-demand by the extraneous organics is not significant.

5.4.4 Validity of Batch Reactions for Free Chlorine

Batch reactions were performed to determine those conditions under which there is no appreciable loss of free chlorine throughout the contact time, and if the viruses exerted a significant demand for free chlorine. These evaluation tests were performed both with and without addition of viruses. Reactions were carried out in 0.05 M $MgCl_2$ at pH 6 so that essentially all free chlorine was in the form of hypochlorous acid.

Results are shown in Figure 5-18. Zero time indicates the addition of microliter volumes of free chlorine. The star symbols indicate addition of 1 ml of diluted stock virus at a titer of approximately 3×10^4 PFU/ml.

Considering first those tests performed without virus, it may be observed that there is a rapid loss of free chlorine in the first 20 seconds followed by a stable period in which there is little chlorine dissipation. Presumably the initial loss is due to reactions with the reactor wall or with trace organics.

The addition of viruses at the titers used for inactivation tests does not appreciably increase the loss of free chlorine for concentrations greater than about 0.20 mg/l. Percentage loss is greater for the smaller starting chlorine concentrations. Table 5-30 displays free chlorine concentration at the time of the addition of viruses (start) and chlorine concentration after approximately 30 seconds (end). Percentage losses are

Figure 5-18. Free chlorine concentration as a function of time in stirred batch reactions, for reactions without addition of virus (■) and with addition of virus (●) at the time indicated by (★).

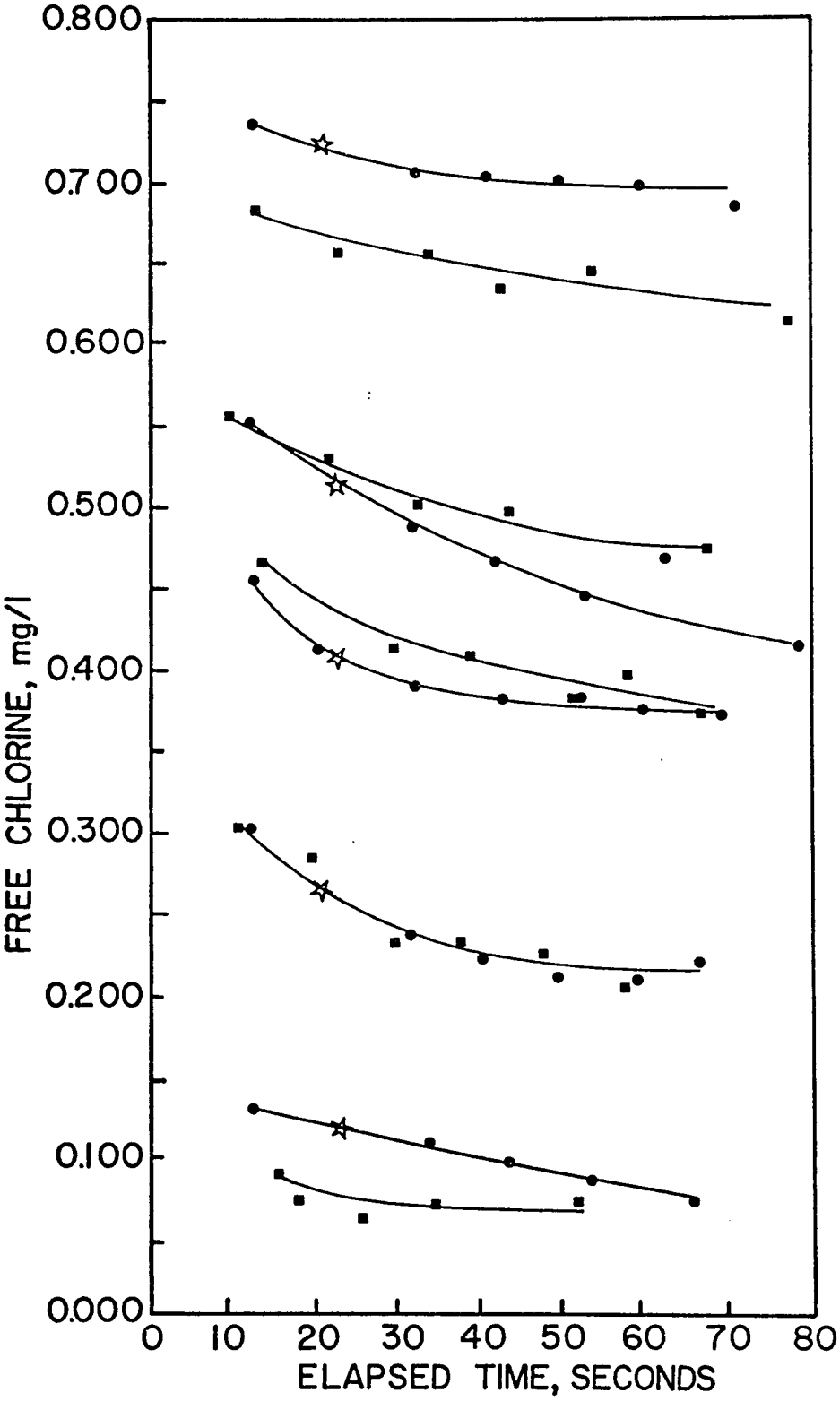

0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
FREE CHLORINE, mg/l
0
10
20
30
40
50
60
70
80
ELAPSED TIME, SECONDS

Table 5-30. Percentage Loss of Free Chlorine During Stirred Batch Reactions

Expt. no.	Temp., °C	Time, sec	Free chlorine, mg/l				Percent loss
			Without virus		With virus		
			Start	End	Start	End	
I-12b	21.5	28.0	1.06	1.05			1.0
I-14b	22.5	27.0			0.71	0.70	1.2
I-14a	22.5	30.6	0.66	0.65			2.0
I-12a	21.5	35.0	0.50	0.46			5.6
I-13b	23.0	31.2			0.49	0.47	4.2
I-13a	23.0	30.6	0.41	0.38			7.1
I-14e	24.0	27.6			0.39	0.37	4.2
I-14g	24.5	28.2	0.29	0.22			22.5
I-14f	24.5	27.0			0.24	0.21	12.0
I-14d	23.0	32.4			0.11	0.07	33.0
I-14c	23.0	19.2	0.09	0.07			20.0

also shown for similar reactions without addition of viruses.

As a result of these tests, several conclusions concerning virus inactivation by free chlorine in batch reactors can be drawn: after addition of chlorine a stabilization period of 30 seconds should be allowed before taking chlorine measurements; immediately after adding chlorine sample to chromogenic reagent, add virus and start time recorder; for inactivation tests concluding in 30 to 40 seconds, only the initial chlorine measurement is necessary.

5.4.5 Virus Reaction Order

Virus reaction order was determined by performing two inactivation tests at a hypochlorous acid concentration of 0.26 mg/l and displaying the data graphically by equation 5-9:

$$\ln\left(\frac{-\Delta C'_{V_i}}{\Delta t}\right) = \ln (k') + n \ln (C'_{V_i}). \qquad (5\text{-}9)$$

Survival-ratio curve for these two tests is shown in Figure 5-19. Table 5-31 lists the experiment number, chlorine concentration, contact times, and surviving viruses. Reaction temperatures were 22-24°C and the viruses were suspended in 0.05 M $MgCl_2$ at pH 6. Calculated terms for equation 5-9 are shown in Table 5-32. Virus reaction order n is found graphically by plotting equation 5-9 as shown in Figure 5-20; the slope is approximately 1:1, which indicates n = 1. The intercept is not well defined; that is, it is located somewhere between -1 and -2, depending upon where the straight line fitting the three pairs of calculated points is drawn.

Figure 5-19. Survival ratio for virus at a free chlorine concentration of 0.26 mg/l HOCl: experiments I-15b (▲) and I-15c (■).

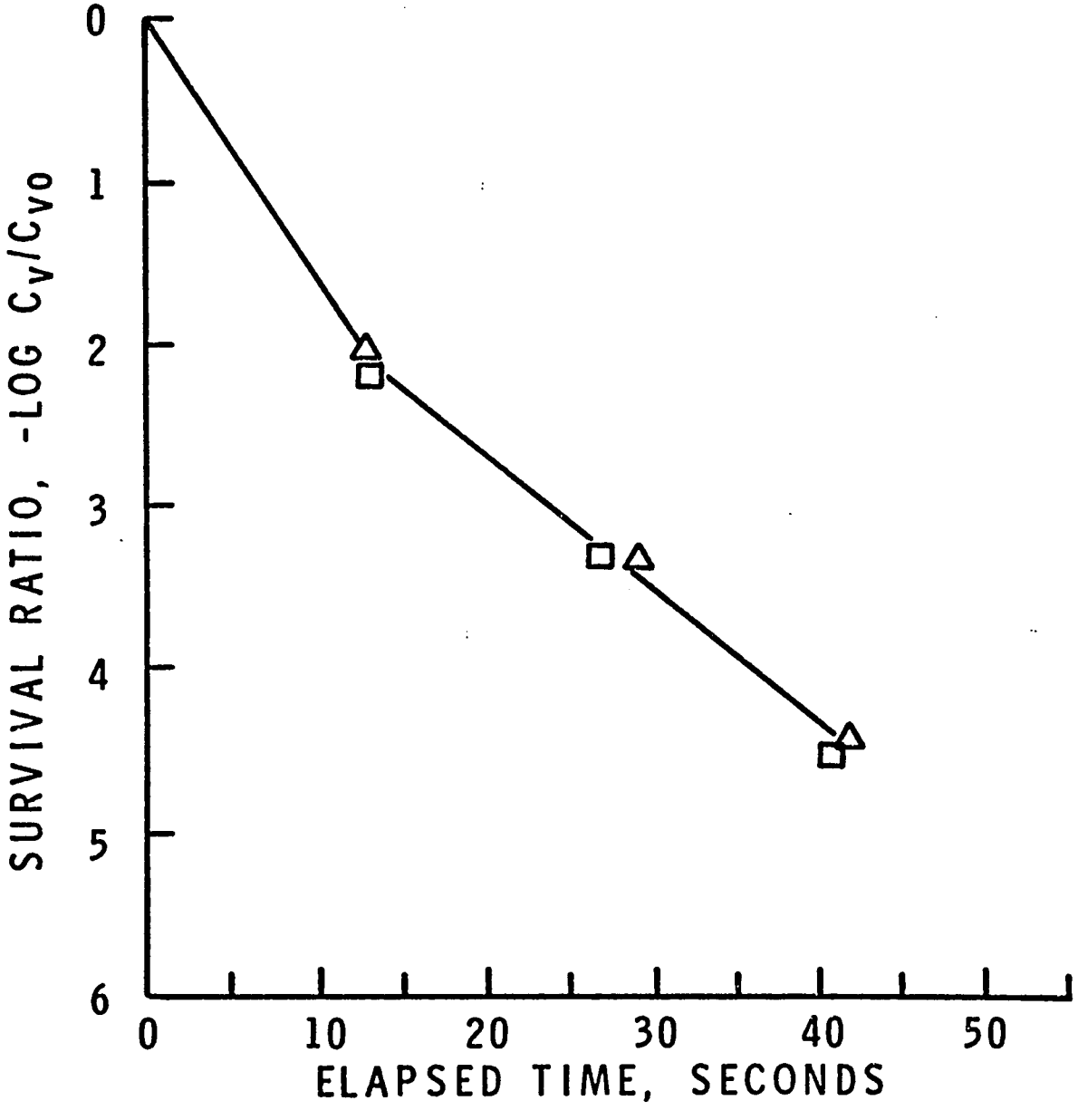

SURVIVAL RATIO, -LOG C_V/C_{VO}
0
1
2
3
4
5
6
0
10
20
30
40
50
ELAPSED TIME, SECONDS

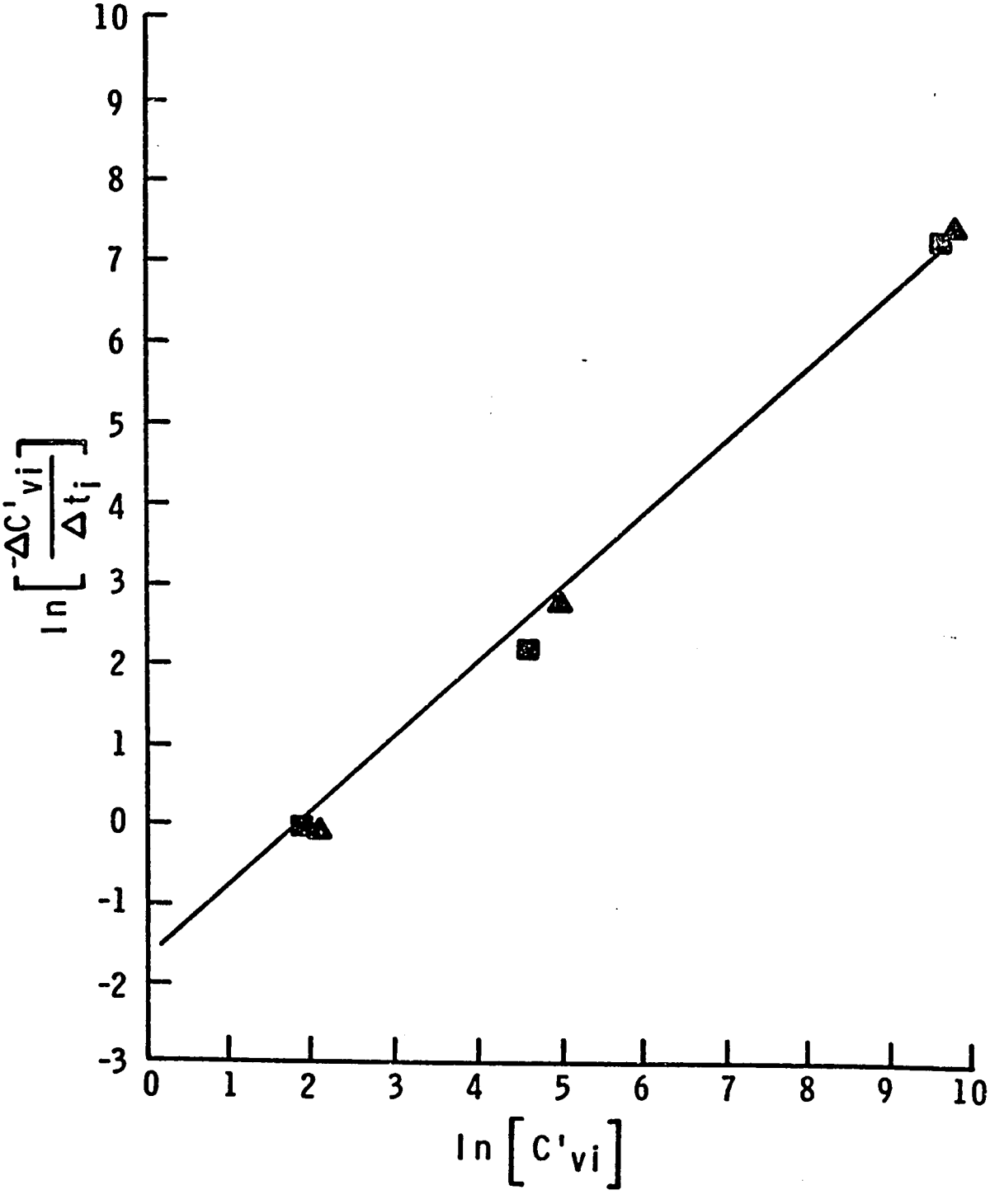
Table 5-31. Survival Curve Data for Freely Suspended Virus

Expt. no.	HOCl, mg/l	Average titer, PFU/ml				Contact time, seconds				t_{99} seconds
		C_{v_0}	C_{v_1}	C_{v_2}	C_{v_3}	t_0	t_1	t_2	t_3	
I-10a	0.30	23,000	180	0	0	0	15	36	61	14
I-10b	0.37	22,000	40	0	0	0	20	40	61	11
I-15b	0.26	30,300	249	15	0	0	14	28	42	13
I-15c	0.26	24,800	162	12	0	0	14	30	43	13
I-16a	0.20	15,700	66	9	0	0	14	28	47	12
I-16b	0.20	13,500	9	0	0	0	15	31	48	9
I-16c	0.21	3,720	9	3	0	0	15	30	46	11
I-62a	0.12	19,600	282	44	6	0	15	28	41	17
I-62b	0.09	19,600	286	86	20	0	15	27	41	19
I-62c	0.27	11,600	2	0	0	0	17	32	46	9
I-62d	0.31	11,600	132	10	0	0	16	28	40	17
I-62e	0.07	4,400	96	34	12	0	15	26	40	23
I-62f	0.10	4,400	88	24	4	0	14	26	38	21
I-63a	0.06	56,000	658	108	4	0	14	25	36	16
I-63b	0.26	56,000	12	0	0	0	13	24	36	8
I-63c	0.60	56,000	2	0	0	0	11	23	34	7
I-63d	0.90	56,000	0	0	0	0	12	20	37	≤ 7

Table 5-32. Calculated Terms for Equation 5-9

Expt. no.	PFU/ml			PFU/ml/sec		
	C'_{v_1}	C'_{v_2}	C'_{v_3}	$\frac{-\Delta C'_{v_1}}{\Delta t_1}$	$\frac{-\Delta C'_{v_2}}{\Delta t_2}$	$\frac{-\Delta C'_{v_3}}{\Delta t_3}$
I-15c	12,481	87	6	1,785	9.2	1.0
I-15b	15,275	132	8	2,178	16.9	1.1

Figure 5-20. Graphical display of equation 5-9 for determination of virus reaction order n.


10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
8
9
10
ln [-ΔC'vi / Δti]
ln [C'vi]

Fortunately, since the reaction is first-order, there is another method of estimating the reaction rate constant. For any one chlorine concentration, changes in the initial virus titer do not alter the time necessary to achieve 99% inactivation. An explicit expression for the observed rate constant can be obtained by integration of equation 5-5:

$$k' = \frac{-1}{t_i} \ln\left[\frac{C_{v_i}}{C_{v_0}}\right]. \qquad (5\text{-}12)$$

For experiments I-15b, c the initial virus titer was reduced by 99% after 13 seconds. The observed rate constant k' was calculated by equation 5-12 and found to be 0.35 sec^{-1}.

5.4.6 Hypochlorous Acid Reaction Order

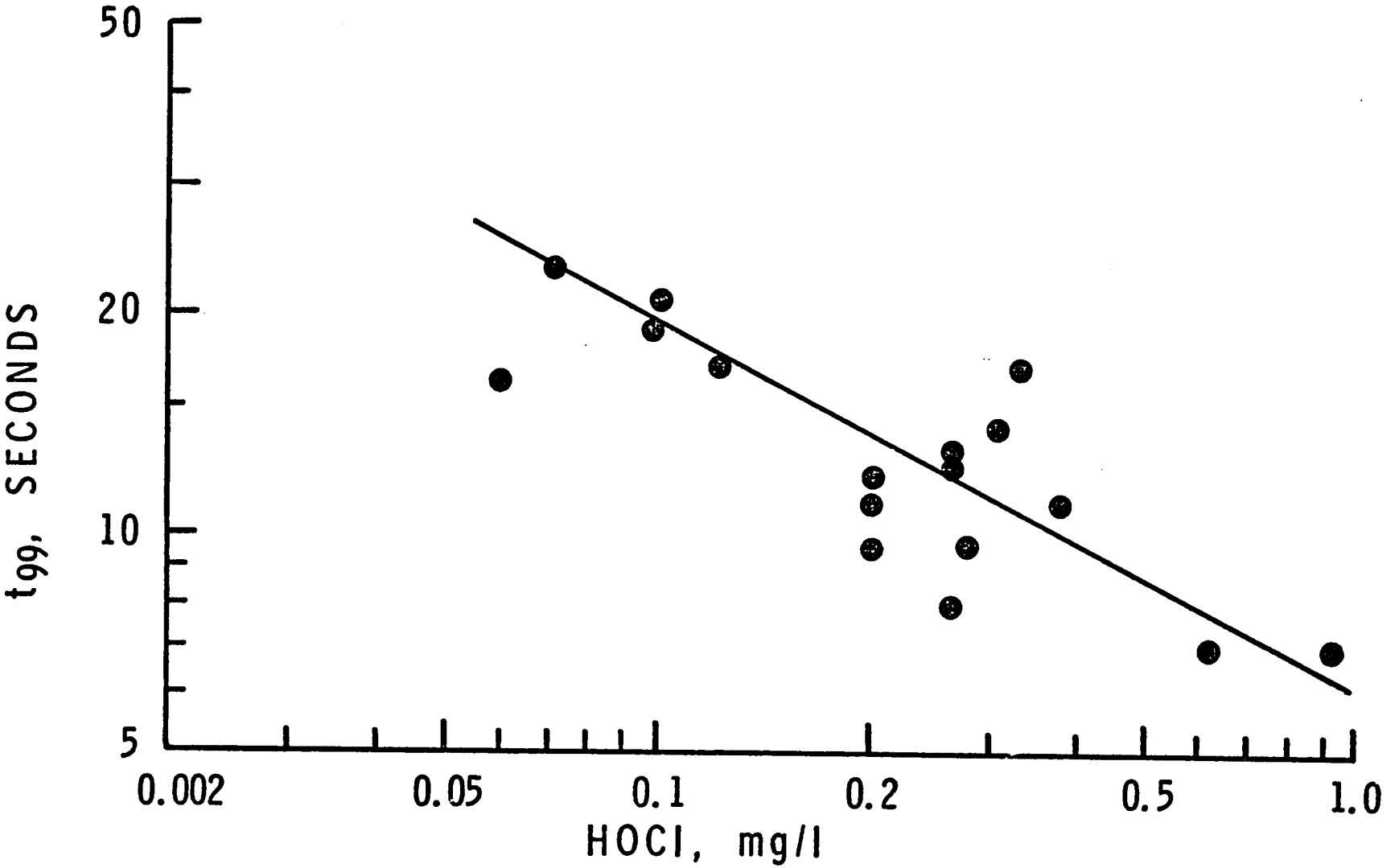
Logarithm of the time required for inactivation of 99% of the starting virus titer versus logarithm of the chlorine concentration is plotted in Figure 5-21. From equation 5-11, the reaction order m for chlorine is known to be the slope of the $\log(t_{99})$ versus $\log(C_c)$ line. Graphical determination of the slope yields a value for m of 0.50. The true inactivation rate constant k can be calculated by substitution of m into equation 5-11b; from this calculation, k has the value 0.69 $(mg/l)^{-0.5}$ $(sec)^{-1}$.

5.4.7 Rate Equation for Freely Suspended Virus

The final form of the rate equation for inactivation of freely suspended virus subjected to hypochlorous acid in 0.05 M $MgCl_2$ at pH 6 is:

$$\frac{dC_v}{dt} = -0.69\ (mg/l)^{-0.5}\ (sec)^{-1.0}\ C_v^{1.0}\ C_c^{0.5}. \qquad (5\text{-}13)$$

Figure 5-21. Time to inactivate 99% of freely suspended virus as a function of HOCl concentration.


50
20
10
5
t_{99}, SECONDS
0.002
0.05
0.1
0.2
0.5
1.0
HOCl, mg/l

Such non-integer reaction orders indicate that the inactivation process is complex. The virus reaction order is very close to first order, which is expected; however, the fractional value of m suggests that several steps are necessary for inactivation to occur or there are multiple pathways for inactivation.

There are no published reports of rate equations developed for chlorination of virus; there is, however, a report by Majumdar, Ceckler and Sproul (1972) for ozonation of poliovirus in which a rate expression was developed. For inactivation of poliovirus by ozone in distilled water, the rate equation developed was:

$$\frac{dC_v}{dt} = -K_1 C_v^{1.80} C_o^{0.61}. \qquad (5\text{-}14)$$

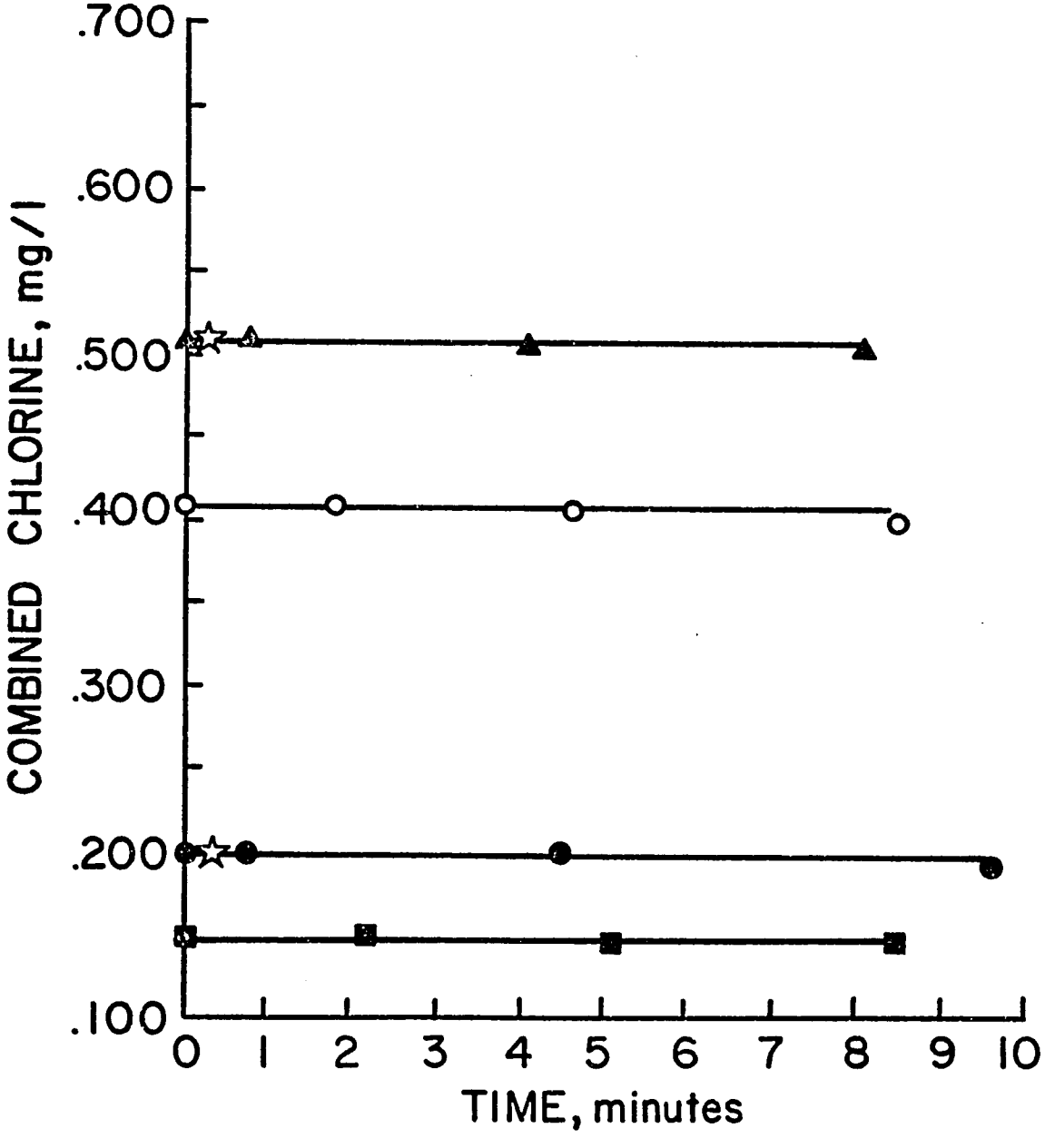
5.4.8 Inactivation of Virus by Combined Chlorine

Batch reactions were performed to determine those conditions under which there is no appreciable loss of combined chlorine forms throughout the contact time and if the viruses exert significant demand for combined chlorine. Evaluation tests were performed with and without addition of viruses.

Results of these tests are shown in Figure 5-22. Zero time indicates the addition of small volumes of stock combined chlorine. Star symbols indicate addition of 1-ml volumes of diluted stock virus at a titer of approximately 2×10^6 PFU/ml to 99 ml combined chlorine.

With combined chlorine forms, in this case 93% monochloramine and 7% dichloramine, at pH 6, there is little loss whether viruses are present or absent.

Figure 5-22. Concentration of combined chlorine during stirred batch reactions.


.700
.600
.500
.400
.300
.200
.100
COMBINED CHLORINE, mg/l
0
1
2
3
4
5
6
7
8
9
10
TIME, minutes

Several conclusions concerning virus inactivation by combined chlorine in batch reactors can be drawn: after addition of chlorine there is little loss of the initial concentration even after 10 minutes; addition of the viruses does not lower the concentration of combined chlorine; maximum loss of combined chlorine during an 8-minute reaction period was only 4.8%. Percentage losses for four tests are shown in tabular form in Table 5-33.

Two stirred batch reaction tests (Table 5-34) were performed with combined chlorine in the form of mono- and dichloramine. At combined chlorine concentrations of 0.220 and 0.504 mg/l there is essentially no loss of viable virus even after 5 minutes of contact time. This finding is in accord with that of Lothrop and Sproul (1967), who reported that T2 phage was more susceptible to free chlorine than combined chlorine.

Higher concentrations of combined chlorine were not evaluated because of the presence of small concentrations of residual free chlorine at higher combined chlorine concentrations. Unless there is a sensitive means for detecting free chlorine, whatever viral inactivation observed at combined chlorine concentrations of 1 mg/l or more may very well be due to the small free chlorine residuals.

The lack of noticeable inactivation of this bacteriophage by combined chlorine precludes the usefulness of combined chlorine in comparative inactivation tests of freely suspended and adsorbed viruses.

Table 5-33. Percentage Loss of Monochloramine during Stirred Batch Reactions

Expt. no.	Temp., °C	Time, min	Monochloramine, mg/l				Percent loss
			Without virus		With virus		
			Start	End	Start	End	
I-22a	22.7	8.54	0.147	0.140			4.8
I-22b	24.0	9.60			0.196	0.192	2.1
I-22c	23.5	8.70	0.418	0.402			3.9
I-22d	23.5	8.10			0.504	0.504	0.0

Table 5-34. Inactivation by Combined Chlorine

Chloramine, mg/l	Time, min	Virus titer (PFU/ml x 10^{-3})
0.220	0.00	76
	0.43	62
	0.99	64
	2.25	59
	5.56	61
0.504	0.00	85
	0.25	92
	0.60	91
	1.35	90
	3.15	66

5.5 Chlorination of Inorganic Solids-Associated Virus

5.5.1 Form of the Rate Expression

Heterogeneous reactions involving adsorbed molecules are described by Langmuir-Hinshelwood kinetics. The basic assumption is that the reaction rate is proportional to the coverage. Since viruses cannot be prepared in concentrations sufficient to achieve coverage ratios applicable to the Langmuir-Hinshelwood model, it is appropriate then to describe the inactivation of solids-associated virus by a pseudo-homogeneous expression of this type:

$$r_v = \frac{dC_v}{dt} = -k_p C_v^n C_c^m \qquad (5\text{-}15)$$

where, except for k_p, all terms have been defined in section 5.4.1. The kinetic rate constant k_p is termed the pseudo-homogeneous rate constant.

For these reactions involving inactivation of a surface-attached virus, it is important to recall from section 5.3.5.6 that only one of every 100 clay particles has an attached virus. The term pseudo-homogeneous aptly describes these reactions: the state of any one virus is not influenced by any other virus; all reaction parameters are identical to the homogeneous case; the viruses are identical to the homogeneous case; every virus is bound to the surface of a solid; and, any rate differences observed are due solely to the physical immobility of the virus on the clay surface and to whatever effect the clay particles may have on the passage of disinfectant molecules from solution to the virus.

5.5.2 Validity of Batch Reactions

Batch reactions were performed to determine if any significant loss of free chlorine occurred as a result of the addition of bentonite clay to the stirring reactor. Following the addition of stock chlorine, a 30-second equilibrium period was allowed before addition of bentonite. Immediately before adding the clay, a sample was withdrawn for free chlorine measurement; the bentonite was added; and, after about 1 minute, another sample was withdrawn for chlorine measurement. Table 5-35 lists the pertinent data: throughout a range of clay and initial chlorine concentrations, average loss was less than 10%.

5.5.3 Virus Reaction Order

Survival curve data for Expt. I-61b were reduced to fit the form of equation 5-9; these reduced data are listed in Table 5-36. The slope of this linear form, which appears in Figure 5-23, is approximately (1.1):(1.0), which indicates that n = 1.1.

5.5.4 Hypochlorous Acid Reaction Order

The slope of $\log(t_{99})$ versus $\log(C_c)$ was found by selecting the straight line which best fitted the data. This negative slope was -0.36; that is, the value of the chlorine reaction order m is approximately 0.4. Since n = 1.1, equation 5-11a, Expt. I-61b, and Figure 5-24 must be used to calculate the true rate constant. With these substitutions, the value of k_p is $0.18\ (\text{mg/l})^{-0.36}\ (\text{PFU/ml})^{-0.10}\ (\text{sec})^{-1.0}$.

Table 5-35. Percentage Loss of Free Chlorine during Stirred Batch Reactions with Bentonite Particulates

Expt. no.	JTU	Temp., °C	Time, sec	Free chlorine, mg/l HOCl		Percent loss
				Start	End	
I-44a	1.9	23.2	48	.64	.64	0
I-44b	1.2	24.0	60	.20	.18	12
I-45a	4.4	24.1	56	.15	.14	8
I-45b	4.5	25.0	48	.50	.48	5
I-45c	4.8	24.0	60	.18	.17	7

Table 5-36. Survival Curve Data for Virus Attached to Clay

Expt. no.	HOCl, mg/l	Average titer, PFU/ml				Contact time, seconds				t_{99} seconds
		C_{v0}	C_{v1}	C_{v2}	C_{v3}	t_0	t_1	t_2	t_3	
I-61a	0.03	18,300	760	158	62	0	23	43	60	40
I-61b	0.15	18,300	214	34	8	0	17	34	54	20
I-61c	0.25	18,300	206	36	12	0	18	34	48	20
I-61d	0.35	18,300	348	102	22	0	17	31	46	24
I-63e	0.30	480	0	0	0	0	16	30	43	$\leq$16
I-63f	0.55	480	4	4	0	0	13	24	37	12
I-63g	0.12	480	40	8	0	0	13	27	40	30
I-63h	0.90	480	0	0	0	0	14	28	41	$\leq$14

Figure 5-23. Survival ratio curve for experiment I-61b.

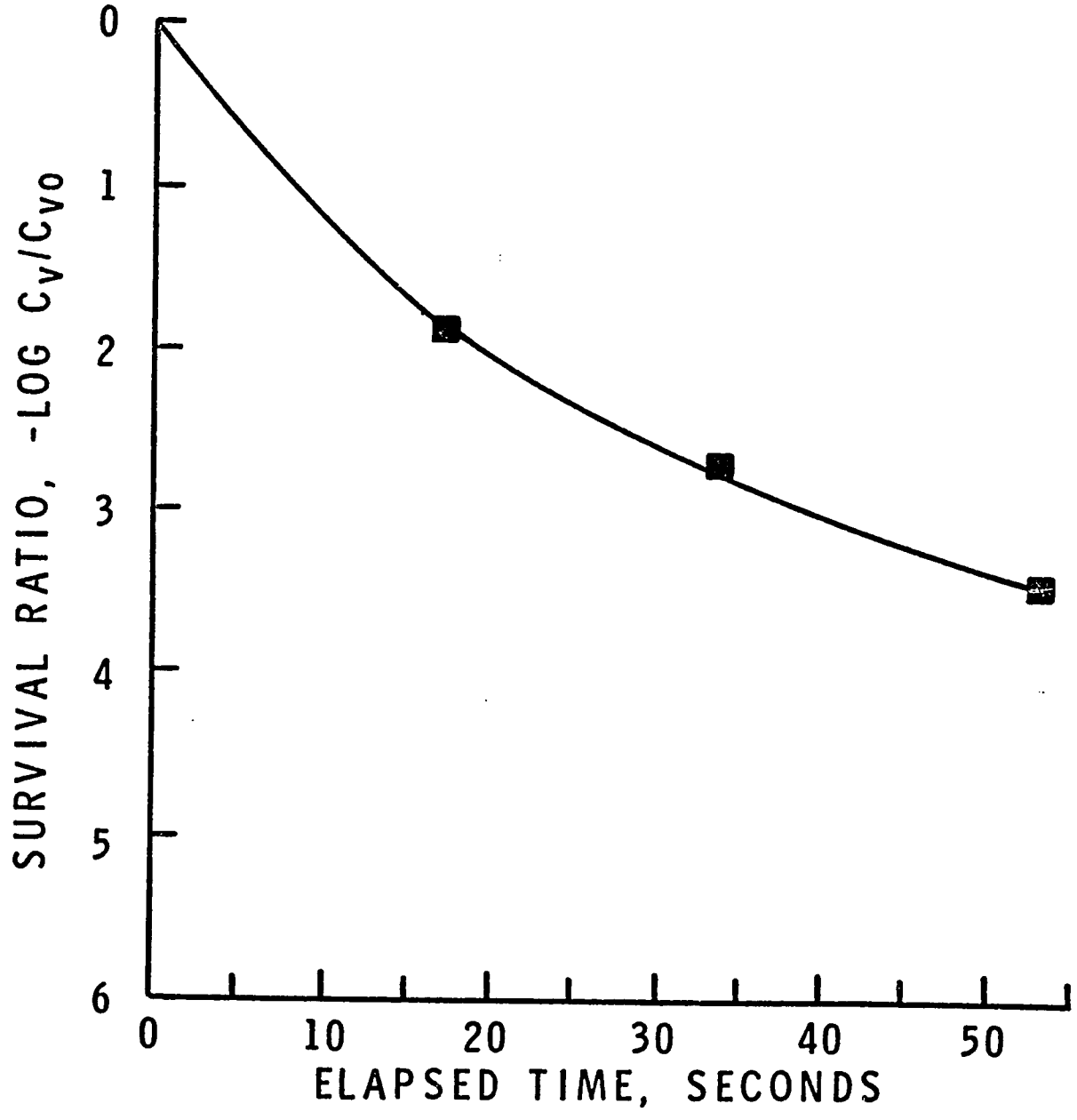

SURVIVAL RATIO, -LOG C_V/C_{VO}
0
1
2
3
4
5
6
0
10
20
30
40
50
ELAPSED TIME, SECONDS

Table 5-37. Calculated Terms for Equation 5-9

Expt. no.	PFU/ml			PFU/ml/sec		
	C'_{v_1}	C'_{v_2}	C'_{v_3}	$\frac{-\Delta C'_{v_1}}{\Delta t_1}$	$\frac{-\Delta C'_{v_2}}{\Delta t_2}$	$\frac{-\Delta C'_{v_3}}{\Delta t_3}$
I-61b	9260	124	21	1060	10.5	1.3

Figure 5-24. Graphical display of equation 5-9 for determination of virus reaction order n.

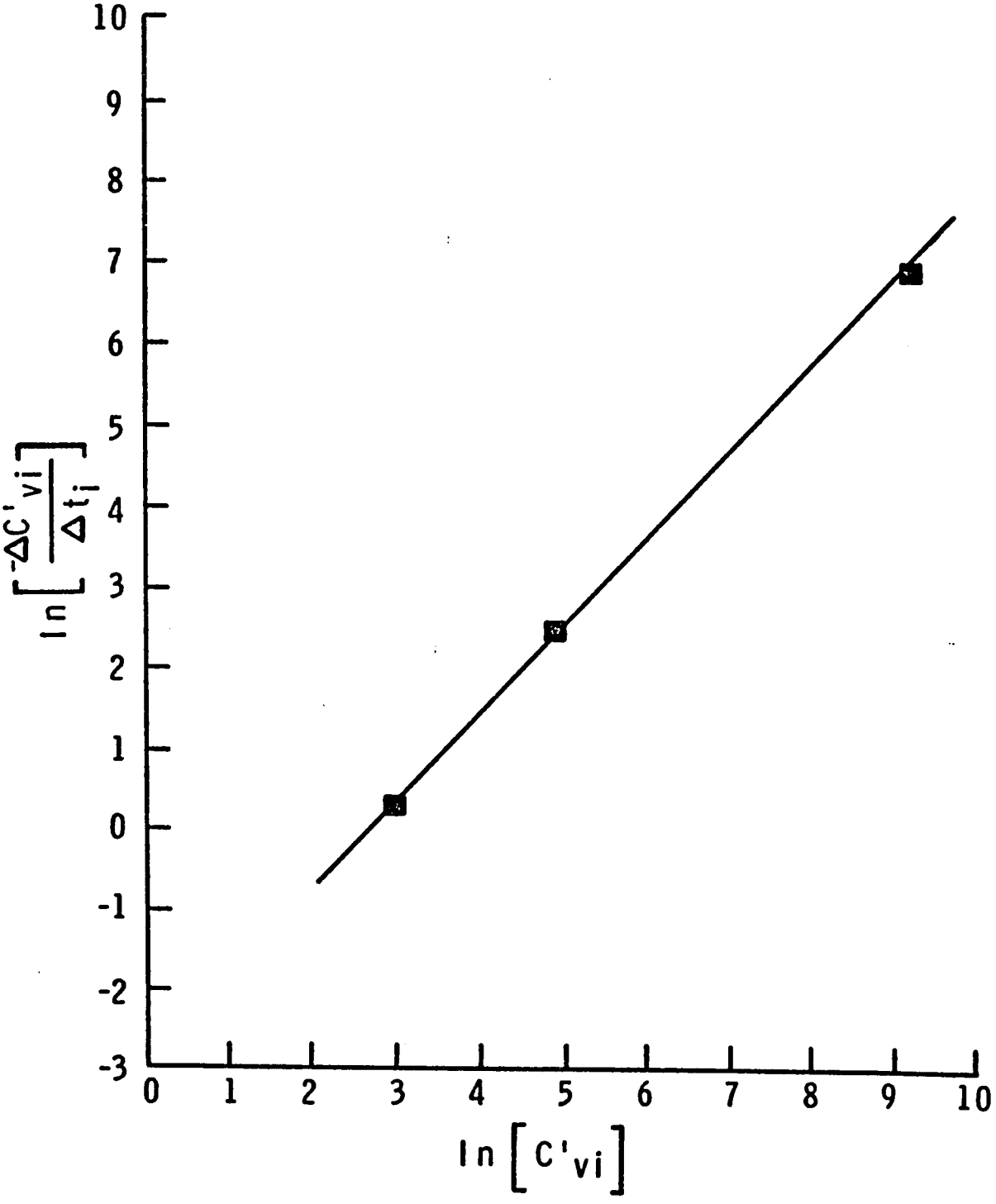
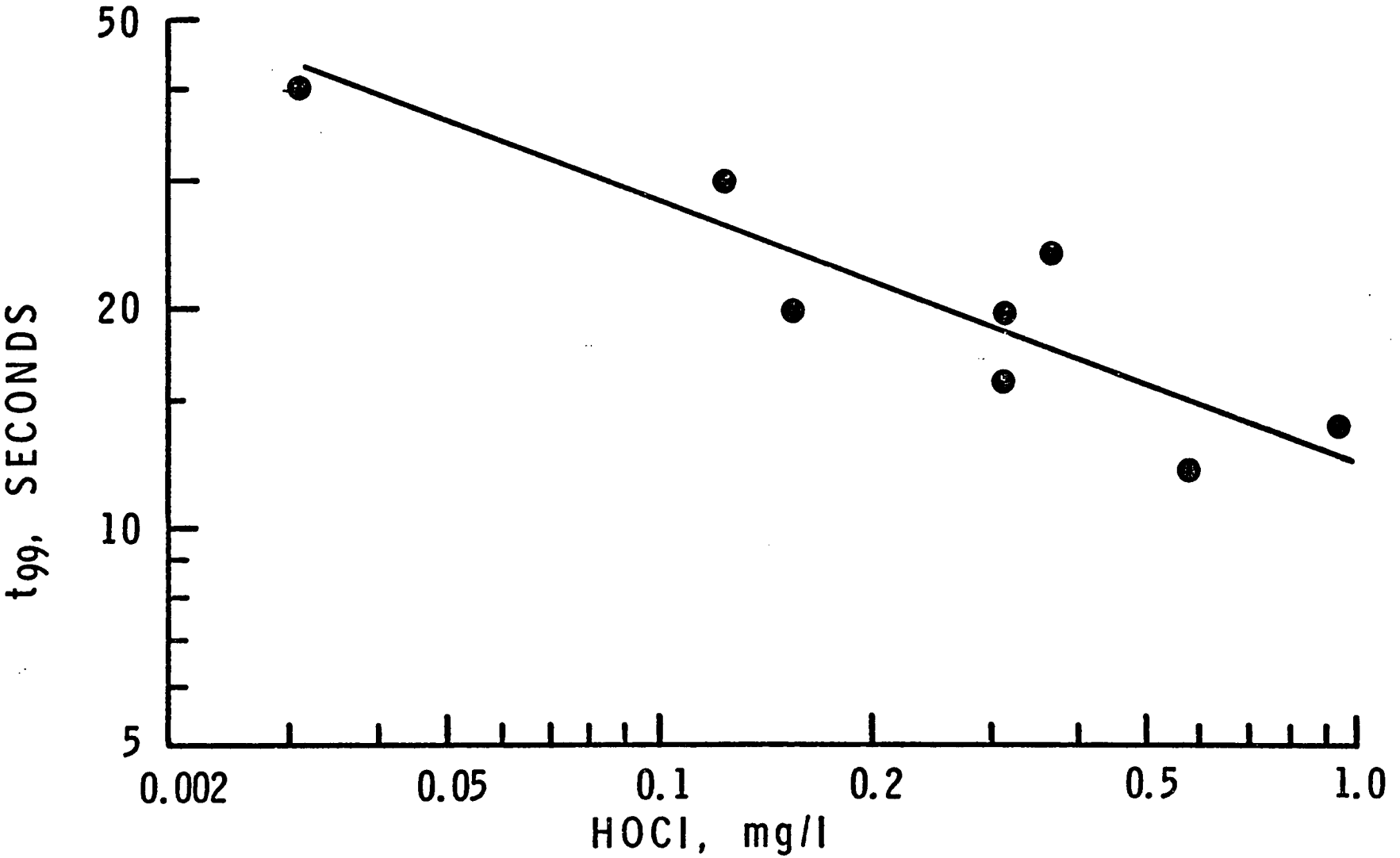

10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
8
9
10
ln [-ΔC'vi / Δti]
ln [C'vi]

Figure 5-25. Time to inactivate 99% of clay-attached virus as a function of HOCl concentration.


50
20
10
5
t_{99}, SECONDS
0.002
0.05
0.1
0.2
0.5
1.0
HOCl, mg/l

5.5.5 Rate Equation for Inorganic Solids-Associated Virus

The final form of the rate equation for inactivation of clay-attached virus by hypochlorous acid in 0.05 M $MgCl_2$ at pH 6 is:

$$\frac{dC_v}{dt} = -0.18\ (mg/l)^{-0.4}\ (PFU/ml)^{-0.1}\ (sec)^{-1.0}\ C_v^{1.1}\ C_c^{0.4}. \qquad (5\text{-}16)$$

5.6 Chlorination of Floc-Associated Virus

5.6.1 Form of the Rate Expression

The selected form of the rate equation is of the pseudo-homogeneous type:

$$r_v = \frac{dC_v}{dt} = -k_g\ C_v^n\ C_c^m, \qquad (5\text{-}17)$$

where k_g is the inactivation rate constant for virus enmeshed in aluminum hydroxide gels.

5.6.2 Validity of Batch Reactions

Batch reactions were performed to ascertain if significant losses of chlorine occurred during the reaction. Data for 30-second tests are given in Table 5-38. Average loss was less than 10%.

5.6.3 Virus Reaction Order

Survival curve data for Expt. I-65a were reduced to fit the form of equation 5-9; these reduced data are listed in Table 5-40. The slope of the linear form, which appears in Figure 5-24, is approximately (1.2):(1.0), which indicates that n = 1.2.

5.6.4 Hypochlorous Acid Reaction Order

Slope of $\log(t_{99})$ versus $\log(C_c)$ was found by selecting the straight line which best fitted the data. This negative slope was -0.83; that is, the value of the chlorine reaction order m is about 0.8. Since n = 1.2, equation

Table 5-38. Percentage Loss of Free Chlorine during Stirred Batch Reactions with Aluminum Hydroxide Gels

Expt. no.	JTU	Temp., °C	Time, sec	Free chlorine, mg/l HOCl		Percent loss
				Start	End	
I-65a	2.2	23.5	30	0.50	0.48	4
I-65b	4.4	23.5	30	0.13	0.11	15
I-65c	3.5	23.5	30	0.70	0.70	0
I-66a	6.4	22.0	30	1.00	0.90	10
I-66b	11.8	22.8	30	1.30	1.20	8

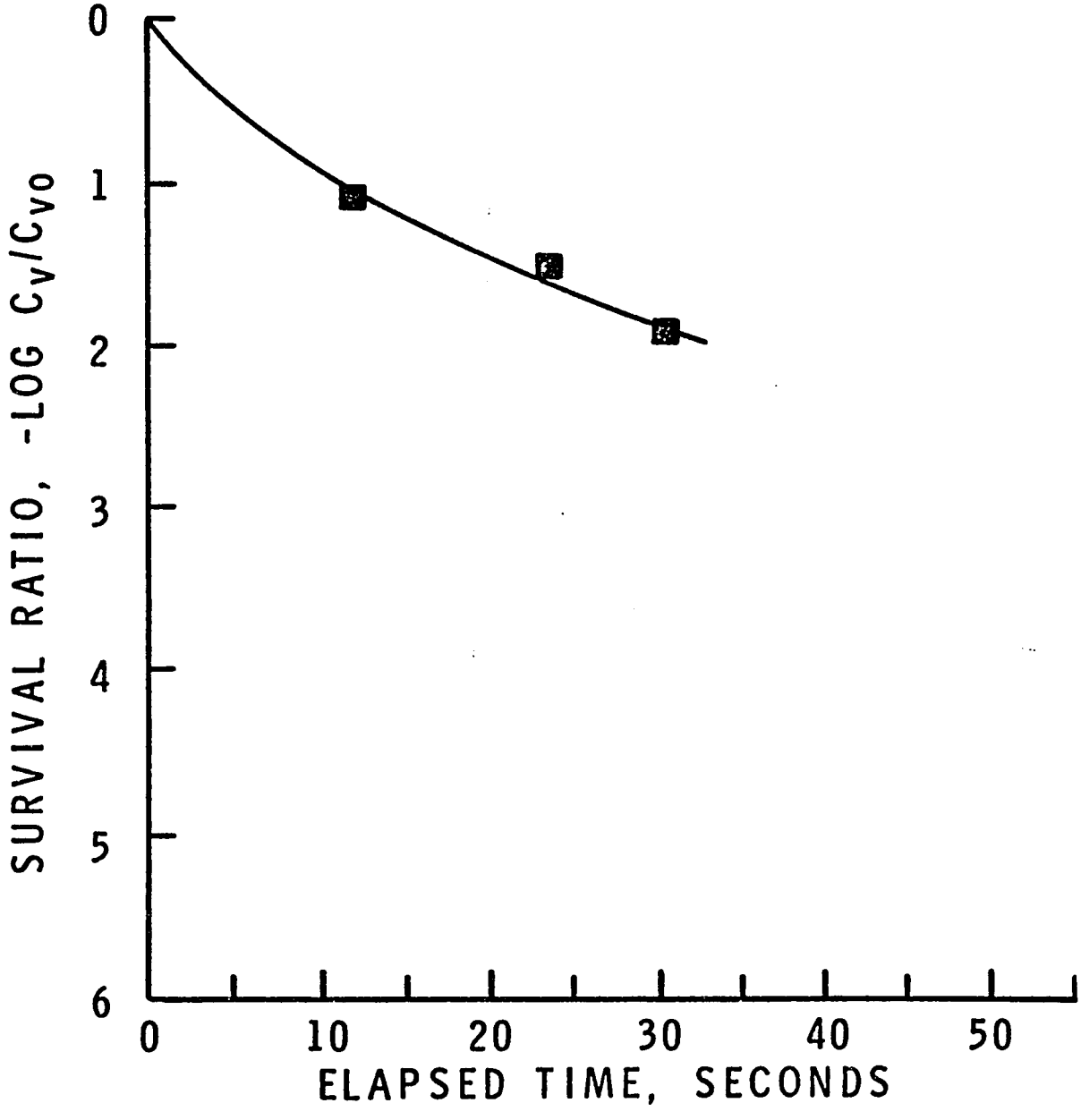
Table 5-39. Survival Curve Data for Virus Enmeshed in Flocs

Expt. no.	HOCl, mg/l	Average titer, PFU/ml				Contact time, seconds				t_{99} seconds
		C_{v_0}	C_{v_1}	C_{v_2}	C_{v_3}	t_0	t_1	t_2	t_3	
I-65b	0.03	112,000	33,200	20,000	12,000	0	13	24	35	>50
I-65a	0.12	112,000	10,600	4,680	1,830	0	12	23	30	35
I-65c	0.30	111,000	3,840	437	318	0	10	19	31	15
I-65d	0.52	111,000	790	38	0	0	11	22	31	10
I-65e	0.90	111,000	204	0	0	0	10	20	30	7

Table 5-40. Calculated Terms for Equation 5-9

Expt. no.	PFU/ml			PFU/ml/sec		
	C'_{v_1}	C'_{v_2}	C'_{v_3}	$\frac{-\Delta C'_{v_1}}{\Delta t_1}$	$\frac{-\Delta C'_{v_2}}{\Delta t_2}$	$\frac{-\Delta C'_{v_3}}{\Delta t_3}$
I-65a	61,300	7,640	3,300	8,450	538	407

Figure 5-26. Survival ratio curve for experiment I-65a.


SURVIVAL RATIO, -LOG C_V/C_{VO}
0
1
2
3
4
5
6
0
10
20
30
40
50
ELAPSED TIME, SECONDS

5-11a, Expt. I-65c, and Figure 5-27 must be used to calculate the true rate constant. With these substitutions the value of k_g is $0.02\ (mg/l)^{-0.83}\ (PFU/ml)^{-0.20}\ (sec)^{-1.0}$.

5.6.5 Rate Equation for Gel-Enmeshed Virus

The final form of the rate equation for inactivation of gel-enmeshed virus by hypochlorous acid in 0.05 M $MgCl_2$ at pH 6 is:

$$\frac{dC_v}{dt} = -0.02\ (mg/l)^{-0.8}\ (PFU/ml)^{-0.2}\ (sec)^{-1.0}\ C_v^{1.2}\ C_c^{0.8}. \qquad (5\text{-}18)$$

5.7 Chlorination of Immobilized Virus

In the last ten years considerable attention has been given to reactions involving the passage of substrate molecules through a tubular reactor containing enzymes attached to the surfaces of granular or fibrous solids. An important advantage to this scheme is that diffusional limitations are not important since the substrate molecules approach the immobilized enzymes with the bulk velocity of the fluid.

An analogous reaction system was devised in which viruses were immobilized onto and within cellulose nitrate membranes. Chlorine was then passed through the membrane for any desired length of time. Elution of viruses from membranes exposed for different contact times allowed construction of a survival curve. A diagram of system components is illustrated in Figure 5-29.

Reaction rates at chlorine concentrations used in the batch reactions of this study were far too fast to follow in immobilized tests. It was necessary to reduce free chlorine concentrations to $\leq$ 0.01 mg/l HOCl

Figure 5-27. Graphical display of equation 5-9 for determination of virus reaction order n.

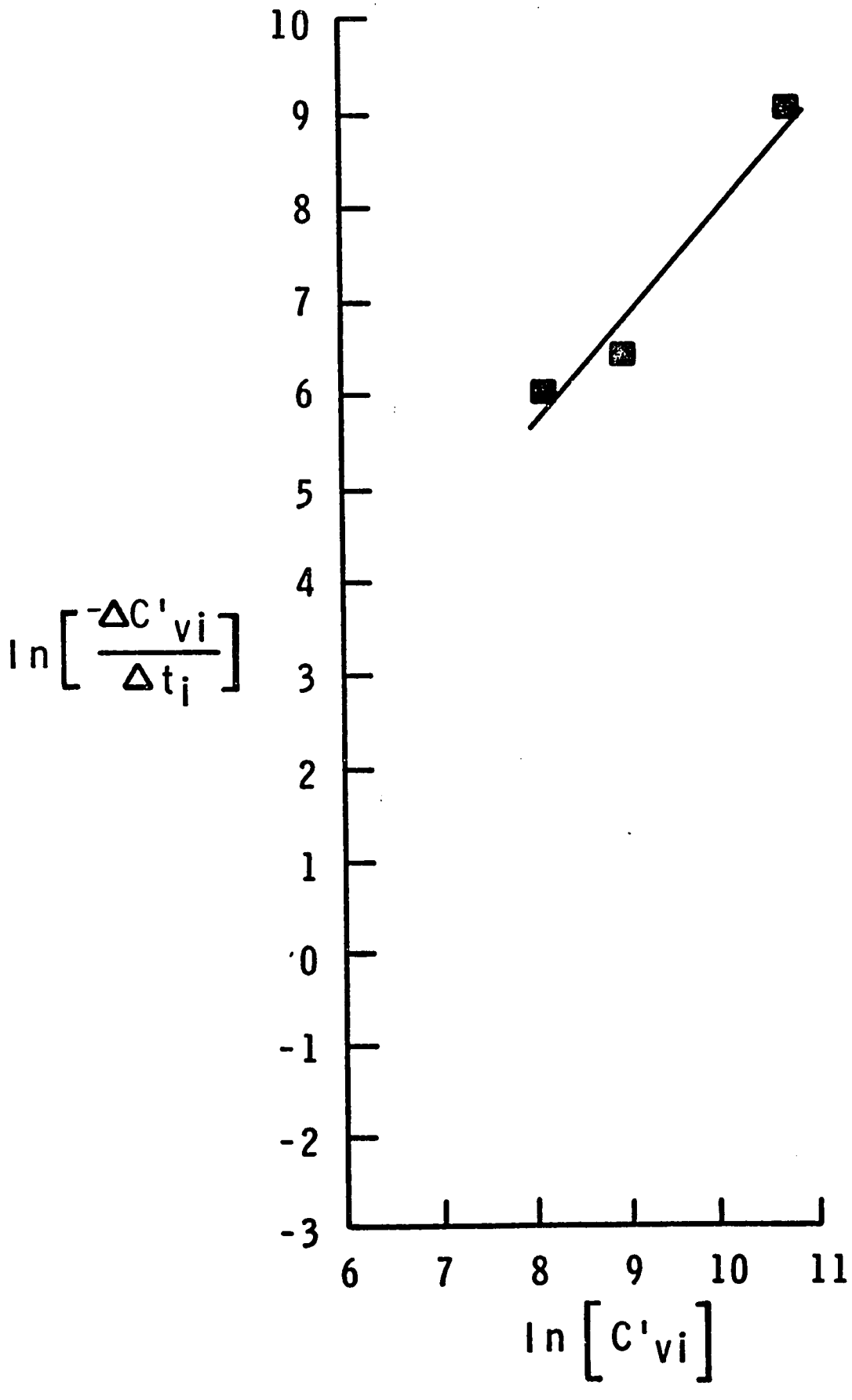

10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
6
7
8
9
10
11
$\ln\left[\frac{-\Delta C'_{vi}}{\Delta t_i}\right]$
$\ln\left[C'_{vi}\right]$

Figure 5-28. Time to inactivate 99% of gel-associated virus as a function of HOCl concentration.

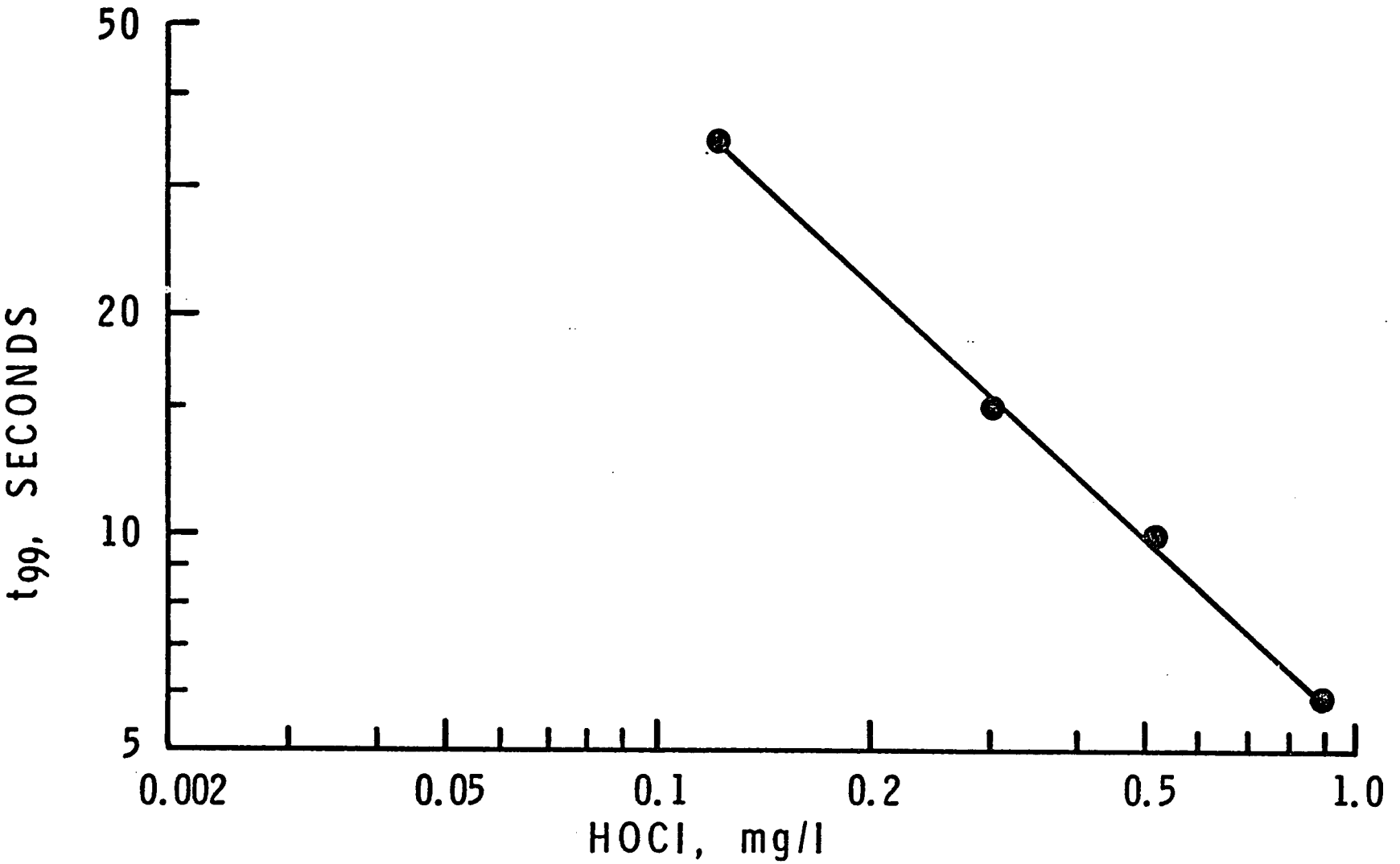
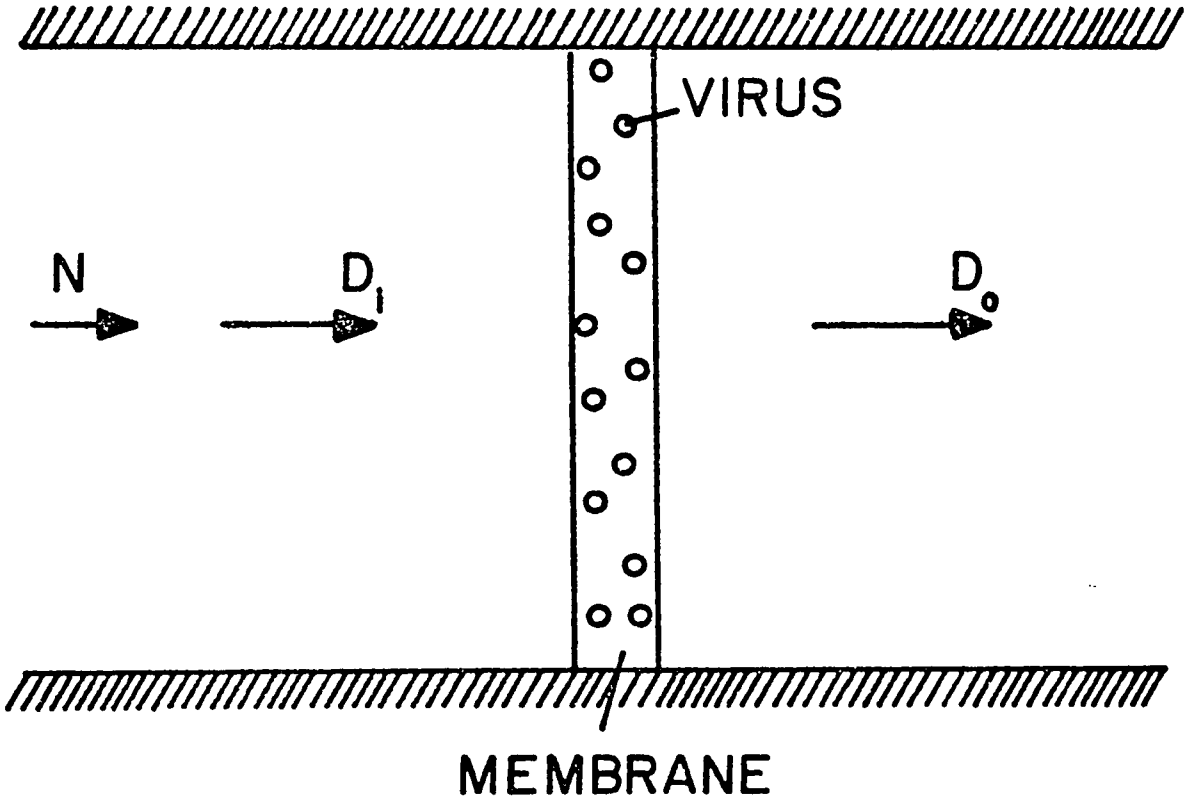

50
20
10
5
t_{99}, SECONDS
0.002
0.05
0.1
0.2
0.5
1.0
HOCl, mg/l

Figure 5-29. Inactivation of immobilized virus: influent disinfectant concentration (D_i); effluent disinfectant concentration (D_o); neutralizer concentration (N).


VIRUS
N
D_i
D_o
MEMBRANE

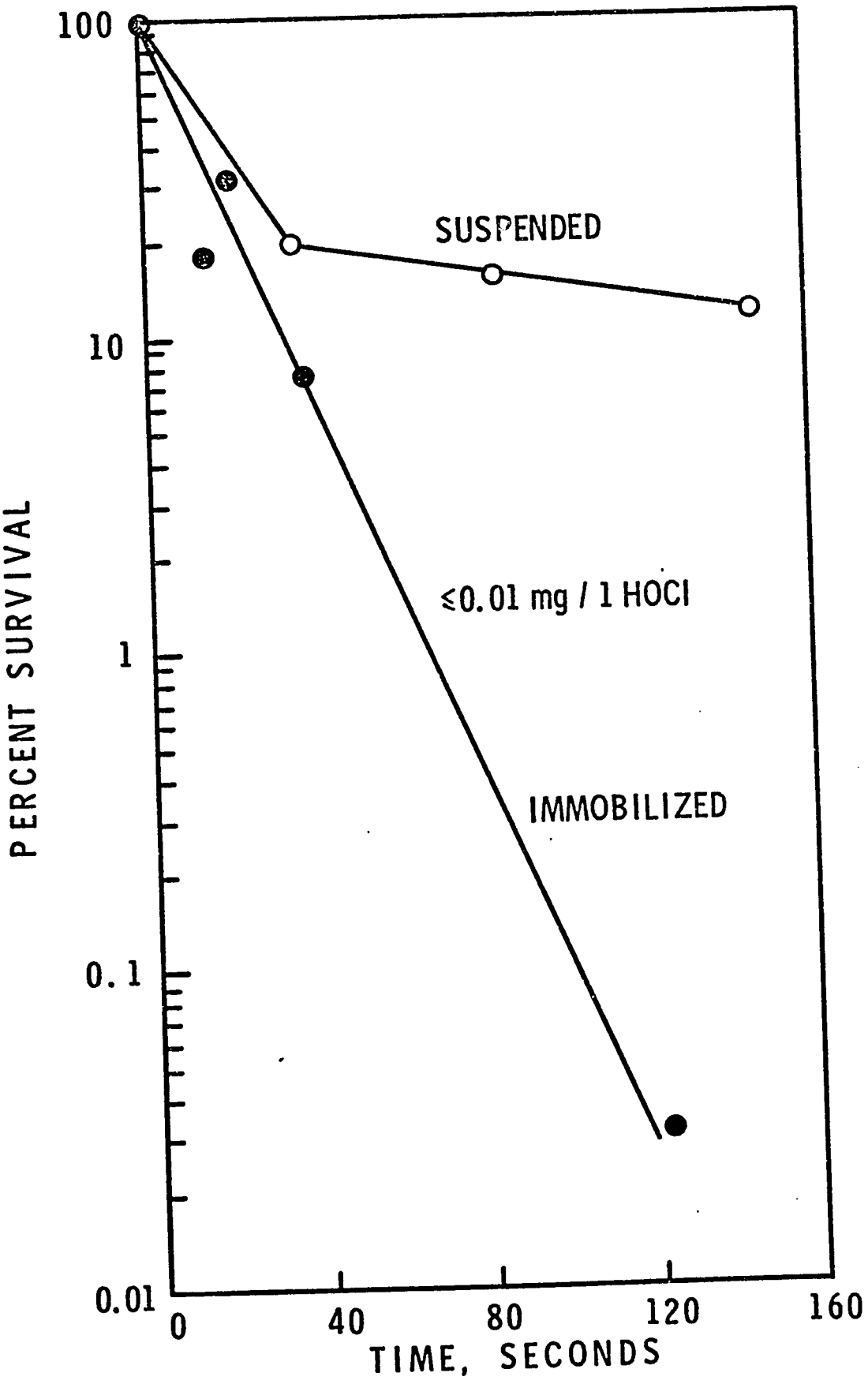
before inactivation could be followed. Parallel batch and immobilized tests were conducted at these low levels of free chlorine. Survival curves for these reactions are shown in Figure 5-30. The biphasic nature of the batch-reaction curve is probably the result of depletion of free chlorine. Yet, for immobilized virus, inactivation occurred rapidly and was first-order throughout the contact time.

A virus suspended in a batch reactor is exposed to fewer and fewer chlorine molecules as elapsed time increases. Whether neighboring viruses have been inactivated or not, they exert chlorine demand which lowers the number of chlorine molecules available for reaction with the virus in question. Any decrease in the bulk concentration of chlorine diminishes the rate at which chlorine molecules approach the virus surface since diffusion velocity is directly proportional to the bulk concentration of chlorine.

In contrast, an immobilized virus is exposed to a constant bulk-concentration of chlorine throughout the reaction time. For long tubular reactors there will be a "moving front" of constant chlorine concentration; but, for these thin disc filters, the reactor length is not significant. Also, the velocity at which disinfectant molecules approach the immobilized viruses is equivalent to the bulk fluid velocity which is many times greater than diffusion velocities.

Other than demonstrating the importance of diffusion in virus inactivation, this immobilization scheme may be helpful in the development of completely "killed vaccines." The Salk (1960) polio vaccine was prepared

Figure 5-30. Comparison of batch and immobilized reactions.


100
10
1
0.1
0.01
PERCENT SURVIVAL
SUSPENDED
≤0.01 mg / 1 HOCl
IMMOBILIZED
0
40
80
120
160
TIME, SECONDS

by formaldehyde treatment in batch reactors; because this preparation resulted in a residual fraction of viable viruses, a risk was inherent in its use. Preparation of totally killed vaccines in immobilized-virus reactors may insure safe vaccines composed of non-viable viruses without risk of infection.

6. Conclusions

6.1 Association and Dissociation of Virus with Solids

Adsorption of bacteriophage MS-2 to cellulose nitrate membranes is influenced by hydrogen ion concentration, cation identity and concentration, and the velocity of the virus past the attachment site. Maximal adsorption conditions at pH 7 are 0.05 M $MgCl_2$ and a flux no greater than 1.5 ml/min/cm^2. Oxidation of the membrane by hypochlorous acid reduces the capacity of the cellulose nitrate to adsorb virus.

Virus will attach to bentonite without the presence of salt cations at pH 7. Numbers of viruses adsorbing increases with increasing $MgCl_2$ concentration from no salt to 0.01 M. No increase in virus attachment is noticeable for higher concentrations of salt. The rate of virus attachment to clay is dependent upon both virus and clay concentration. For clay concentrations of at least 50 mg/l, the virus adsorption rate is first-order with respect to remaining free virus and zero-order with respect to clay concentration. The kinetic rate constant is 0.025 min^{-1} at a stirring speed of 100 rpm; the constant varies from 0.019 min^{-1} at 50 rpm to 0.049 min^{-1} at 200 rpm. Variation of the rate constant with stirring speed indicates that the adsorption process is diffusion limited, and, calculation of an apparent energy of activation of 1 kcal/mol substantiates this conclusion. Adsorbed virus per unit weight of solids increases linearly with the concentration of free virus; this indicates that few of the possible adsorption sites are occupied. The ratio of clay particulates to adsorbed virus is about 100:1 for all experiments in this

study. Treated sewage effluent organics inhibit attachment of virus to clay.

Virus can be removed from suspension by forming aluminum hydroxide flocs or by adsorption to the periphery of formed flocs. Virus removal percentage is dependent upon the concentration of aluminum and the identity of the aluminum-salt anion. Aluminum concentrations greater than the concentration which maximizes virus association produce flocs which repel viruses and inhibit association of viruses with the flocs.

Virus recoveries from cellulose nitrate of 60% or greater can be accomplished by several eluents: alkaline buffer at pH 10, EDTA at pH 11, fetal calf serum at pH 7, and fetal calf serum at pH 10. Maximum recovery is 80% and is achieved with pH 10 fetal calf serum. Recovery by organics is an exchange process because recovery percentage increases with increasing exposure time. The virus:clay bond is stronger than the virus:cellulose nitrate bond. The best eluent for recovery from clay is fetal calf serum which yields 15% of attached viruses in the eluate. Complete recovery from $AlCl_3$ formed flocs is achieved by mixing pelleted flocs with fetal calf serum.

6.2 Chlorination of Solids-Associated Virus

Bacteriophage MS-2 is readily inactivated by hypochlorous acid; however, this phage is not susceptible to chloramines. The rate of inactivation is dependent upon the physical state of the virus and the concentration of hypochlorous acid.

Freely suspended viruses are most susceptible to HOCl; as listed in Table 6-1, 99% of the initial virus titer is lost within 20 seconds for all measurable concentrations of HOCl. Attachment of virus to clay or enmeshment within aluminum hydroxide flocs protects the associated virus by reducing the rate of inactivation. This protective effect most likely results from physical interference with the transport of HOCl molecules toward the virus. For chlorine concentrations from 0.4 to 1.0 mg/l, the protective effect of the aluminum hydroxide gel is not as apparent as that of clay; HOCl concentrations in this range must overcome the resistance of the gelatinous films.

Rate equations were developed for inactivation of viruses in each physical state. These rate expressions are given in tabular form in Table 6-2. The fractional chlorine reaction order indicates that the inactivation phenomenon is complex and may involve a number of steps for completion.

Table 6-1. Effect of Physical State of the Virus on the Time to Inactivate 99% of the Initial Virus Titer

HOCl range, mg/l	Average time, seconds		
	Freely suspended	Clay attached	Within gel
0.03 - 0.10	20	35	>50
0.10 - 0.40	12	20	25
0.40 - 1.00	7	15	8

Table 6-2. Rate Expressions

Physical state of the virus	Inactivation rate constant	Reaction orders	
		n	m
Freely suspended	$0.69 \left(\frac{mg}{l}\right)^{-.5} sec^{-1}$	1.0	0.5
Clay attached	$0.18 \left(\frac{mg}{l}\right)^{-.4} \left(\frac{PFU}{ml}\right)^{-.1} sec^{-1}$	1.1	0.4
Gel enmeshed	$0.02 \left(\frac{mg}{l}\right)^{-.8} \left(\frac{PFU}{ml}\right)^{-.2} sec^{-1}$	1.2	0.8

7. Appendices

Table 7-1. Surface Characteristics of the Major Alumino-Silicate Clay Minerals*

Characteristics	Montmorillonite	Vermiculite	Illite	Kaolinite
Lattice stacking units (Si tetrahedral layers: Al octahedral layers)	2:1	2:1	2:1	1:1
Total specific surface area ($m^2\ g^{-1}$)	700-800	700-800	100-200	25-50
Percentage of internal surface	10 to 90%	80 to 90%	0	0
Cation exchange capacity ($meq\ g^{-1}$)	0.8-1.2	1.2-2.0	0.15-0.40	0.02-0.10
Average surface charge density ($esu\ cm^{-2}$)	$3.3\text{-}4.3 \times 10^4$	$5.0\text{-}7.2 \times 10^4$	$4.3\text{-}5.8 \times 10^4$	$2.3\text{-}5.9 \times 10^4$
Area per elementary charge ($\mathring{A}^2$)	110-150	60-100	80-110	180-200
Free swelling in water ($cm^3\ g^{-1}$)	0.5-20**	0.5-15**	0.1-1.2	0-0.3
Range of interlammelar expansion	6-240 Å**	6-200 Å**	—	—

* Anderson and Banin (1975).

** Depending upon exchangeable cation.

Table 7-2. Failure of NaCl to Mediate Humic Acid Floc Formation

NaCl, M	Humic acid, mg/l		Percentage humic acid removed
	Control	Supernatant	
0	10.0		
0		9.0	10.0
0.002		8.4	16.0
0.020		8.7	13.0
0.100		8.5	15.0

Test conditions: pH 7; 200 rpm, 1 hr; centrifugation, 2100 g, 5 minutes.

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