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STUDIES ON THE QUANTITATIVE ENUMERATION OF VIRUSES IN WATER

by

FREDERICK CARLTON PEARSON III

B.A. Nasson College, 1966

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Microbiology

July, 1971

This thesis has been examined and approved.

Theodore G. Metcalf, Professor of Microbiology, and
Thesis Director

Lawrence W. Slanetz, Professor of Microbiology

William R. Chesbro, Professor of Microbiology

Miyoshi Ikawa, Professor of Biochemistry

Edward J. Herbst, Professor of Biochemistry

April 26, 1972
Date

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ABSTRACT

STUDIES ON THE QUANTITATIVE ENUMERATION
OF VIRUSES IN WATER

by

FREDERICK CARLTON PEARSON III

A number of virus-collecting surfaces were examined for their ability to quantitatively recover enteric viruses from water. Magnetic iron oxide was found to be highly effective for recovery of virus from water. Salts, organic matter and pH influenced virus recovery. Hydrogen bonding was believed to be responsible for binding of virus to iron oxide surfaces.

Recovery of virus in semi-quantitative amounts was best accomplished upon thin layers of iron oxide contained in a membrane filter holder.

INTRODUCTION

One of the more important challenges in environmental virology is the quantitative enumeration of virus particles found in surface waters. A variety of methods have been advanced for this purpose, but only two have been tested adequately enough to permit insight into the problems involved.

Several parameters must be considered in establishing a system for the quantitative enumeration of virus. An important consideration is the virus retaining surface used to collect virus from large amounts of water. Enteroviruses and possibly other viruses can then be detected in reservoirs, drinking water, swimming pools, recreational waters, river and marine waters. Another factor is the recovery of virus in a volume of eluate permitting accurate measurement of numbers.

Determination of an appropriate virus-collecting substance includes the ability to elute, as well as collect virus. The virus-collecting substance must be incorporated into equipment permitting the examination of large volumes of water. The most practical system is one in which influent is delivered to the virus-collecting surface under the influence of positive pressure. Removal of sediment or suspensoids must be done to avoid clogging of the virus-collecting surface. Removal of particulates prior to recovery of virus upon a collecting surface must be achieved without concomitant loss of virus.

Objectives of this study were: (1) to develop equipment and determine the identity of virus-collecting substances permitting the quantitative recovery of virus from water and (2) to offer an explanation for the basis of recovery of virus by a collecting substance.

LITERATURE REVIEW

A variety of substances have been employed as collecting surfaces for the concentration and enumeration of virus particles. One of the first substances used was gauze, which is extremely simple and inexpensive. Moore (1948) introduced gauze padding by using it for the successful isolation of the typhoid bacillus from raw sewage. Subsequently, he was able to identify paratyphoid carriers in towns by examination of town sewage. Later (1952) Mac Callum et al. adopted the method of Moore and discovered that increased viral isolations could be realized by using gauze padding rather than by collecting "grab" samples or "bulk" samples and assaying the sample directly. The gauze pads were prepared for field use by folding strips of cotton gauze into the desired size. Ultimately, the pads were suspended in raw sewage for three days, then collected, and transported to the laboratory where the fluid was mechanically expressed by placing them in a juice press. The samples were then treated with ether and assayed for viral content.

During the next year, Kelly (1953) extended Moore's concept to cheesecloth swabs which were suspended in sewage for 24-48 hours. This procedure proved conclusively that more coxsackie virus could be isolated by using gauze swabs than by testing of "grab" samples. "Grab samples" were taken during the 24-48 hour period through which the gauze swabs were suspended in sewage and the swabs gave markedly more

viral isolations. Melnick et al. (1954) corroborated this finding by obtaining more viral isolations from gauze pads than grab samples. The pads were extracted by mechanical expression of their fluid at a pH 8.0 which was adjusted with 1 N NaOH. The virus was then precipitated with ammonium sulfate and subjected to ultracentrifugation.

Bloom et al. (1959) used gauze pads which were similar to those described by Moore. Adsorbent cotton was placed between two layers of cheese cloth 4 by 4 inches square, which were sewed together around the periphery. The pads were then placed directly into the flow of sewage for 24-72 hours. One hundred and fifty isolations were made from 1,018 sewage samples examined. Thirty-one isolates were echoviruses, four were polioviruses and seventy six were coxsackieviruses. Gravelle (1960) also used a modified technique employing gauze swabs based on Moore's original discovery in which the major representatives of the enteroviral group were found in sewage. Fluid was expressed from the swabs and assayed either in unconcentrated form, or subjected to ultracentrifugation, or placed on Dowex 1-X-8 ion exchange resin. Ultracentrifugation was done at 39,000 r.p.m. for one hour with 2.4% gelatin being added to each 40 ml sewage sample. A total of 50 ml of clarified sewage was mixed with 5 g of Dowex ion exchange resin in a flask and placed on a rotary shaker for 5 minutes. The virus was eluted with disodium phosphate at pH 8.5.

Ultracentrifugation of the gauze swab eluate was found to give more viral isolation than the unconcentrated fluid or that subjected to Dowex ion exchange treatment.

Lamb et al. (1964) used three yards of surgical gauze to make a gauze pad that measured approximately 12x4x1 inches. This was then inserted in a stockinette and knots tied at each end to encase the pad. The finished product was then suspended in the water for seven days. The virus was eluted at pH 8.0 and gelatin added to eluate prior to differential centrifugation. Centrifugal speeds up to 29,000 r.p.m. for one hour were used. Sedimented virus was then resuspended in 2.0 ml of phosphate buffer.

Liu et al. (1970) investigated a flow through system in which gauze was placed in a chamber into which water may be continuously pumped. Recently Liu reported 2% of seeded virus recoverable from tap water, and 15-19% of virus recoverable from sea water following pumping of sample through a sampling device. When appropriate amounts of sodium chloride were added to virus seeded tap water, recoveries rose to approximately 50%. Elution was carried out using small amounts of serum at pH 8 - 9.

It has long been known that clays and silts in a finely divided state have adsorptive surfaces. Oker - Bloom (1953) purified a number of viruses from crude harvest material by the use of bentonite. Bartell, Pierzchala, and Tint (1960) adsorbed a spectrum of enteroviruses to attapulgite which is a hydrated magnesium aluminum silicate clay. One hundred milligrams of the attapulgite clay was added to Hank's balanced salt solution (BSS) and then any one of a number of

enteroviruses was added to the system. Subsequently, the virus-clay preparation was centrifuged at 100 r.p.m. and a reduction of 99.9% of poliovirus 1 and poliovirus 2 was noted in the supernatant fluid. Less impressive reductions in titer were found in the supernatant fluids in which poliovirus 3, echovirus 9, and coxsackievirus B3 were used. Adsorption of virus to clay was unaffected by temperatures of 4, 25, and 37° C indicating the absence of any chemical reaction. Adsorption was efficient through a pH range of 3.0 - 9.0. It is interesting that adsorption was unaffected above pH 8.0 as most viral adsorption to clay is less efficient above pH 8.0 and markedly less efficient above pH 8.5.

Carlson _et al_. (1966) studied inactivation of T2 bacteriophage in natural waters and found up to 99% adsorption to the clay when sodium and calcium salts were used at a concentration of 0.02 M. Bacteriophage adsorption was impressive with kaolin, montmorillonite, and illite. Adsorption to the clay was demonstrated to occur within five minutes and disassociation of the clay-virus complex took place at lower levels of salts. The association remained stable in sea water, however. Organics were shown to interfere with virus adsorption to clay. The authors conjectured that cations are needed for the association of virus and clay in such a way that ultimately a cationic bridge exists between the

virus and the clay. It was determined that naturally occurring clays in the Missouri River adsorbed virus in a manner similar to pure clays. Cationic bridges linking virus to the clay were postulated.

Carlson (1968) published on the inactivation of virus on clay particles by a system using T_4 and poliovirus 1 (Sabin). Studies were conducted at pH 7 and a contact time of 0.5 hrs. permitted 74% adsorption of poliovirus. Better adsorption was found with 0.01 M C_aCl_2 than with 0.01 M NaCl and was thought to be due to a slight positive charge on the clay rather than the usual negative charge. When as little as 1 mg of bovine albumin was added to the system, virus adsorption to clay dropped from 93% to 53% and when 10 ml of sewage was added to the system virus adsorption fell to 81% from a high of 93%. Adsorption could be reversed by distilled water.

Marshall (1969) studied the relation between Rhizobium and illite or montmorillonite and found that the clays were adsorbed to the surface of the organism rather than the organism being adsorbed to the clay. The adsorption phenomenon was studied by microelectrophoretic technique and electrophoretic patterns were established for the clay and the bacterium independently as well as a composite electrophoretic pattern for virus-clay complex. Small amounts of clay were used (100 mg / ml), but with increasing concentrations of

clays an increase in the shell of material surrounding the cell was noted. Freeze dried preparations of the clay and bacteria were done.

Jakubowski (1969) carried out an extensive study on the adsorption of poliovirus to both natural clays and commercially prepared varieties of clays. He found Purdy clays, collected from Puget Sound, removed 99% of poliovirus from sea water. When a relatively large input of poliovirus was used (5.5×10^5 PFU), 98% of the virus was adsorbed. Significant poliovirus adsorption (40%) occurred with 1 mg of kaolinite clay rising to a maximum of 99% at 25 mg/l. Montmorillonite at a concentration of 1 mg/l adsorbed 57% of the poliovirus, but at 10 mg/l 94% of the poliovirus was adsorbed. In all three cases there was an optimal concentration above which a decrease in virus adsorption was noted. Particle size of the clays as related to viral adsorption did not seem important with the natural occurring Purdy clay. With kaolinite adsorption increased from 21% with $50 - 20_u$ fractions to 97% with fractions smaller than 0.12_u. A similar situation was encountered with montmorillonite. Adsorption to clay was inhibited by 2% fetal bovine serum when added to the system before the virus. Two per cent fetal bovine serum added to the virus-clay complex did not remove the virus from its association with clay. The effect of pH from 3 - 9 seems undramatic as virus adsorption ranged from 94% to 97%.

Salinity required for optimal adsorption for the three clays examined varied from 1 to 10 parts per thousand (ppt). Optimal adsorption of virus to kaolinite took place at 10^{-2} M NaCl, 10^{-3} M $MgCl_2$, and 10^{-5} M $AlCl_3$ showing the higher valent cations to be more efficient in promoting virus adsorption. Limited electronomicrographs were offered of the sheet-like clay particles with bodies adsorbed to the edge of the clay sheets. The bodies appeared to possess polyhedral symmetry and have diameters of 31 mu indicative of poliovirus.

The effective use of charcoal as a collecting surface for virus dates to 1930 and 1931 and the work of Poppe and Pyl respectively. Both workers employed carbon to "adsorb" the virus of foot and mouth disease. When the carbon was injected into guinea pigs, infection followed.

Carlson (1942) reported partial removal of poliovirus from water with only 10 - 50 ppm activated carbon. The extent of virus removal was not reported. Neefe (1947) reported the removal of 40% of infectious hepatitis virus by the use of activated carbon at 25 ppm. This method was used in conjunction with flocculation which was generally considered to be more effective in removing virus than adsorption by charcoal. It was entirely plausible to attribute the 40% removal of virus largely or entirely to flocculation and not adsorption to charcoal. Fair, Chang, and Moore (1948) found that the "adsorption" of Theiler's virus to activated carbon was not an effective process. It was concluded that the dosage of

activated charcoal applied in conventional water treatment is ineffectual in the removal of virus from water. More recently in 1959 Clarke and Chang reported the use of activated carbon for the adsorption of polio virus and infectious hepatitis.

Cookson (1967) studied the adsorption of viruses to activated carbon. Adsorption was found to be reversible and desorption was enhanced by the presence of a competitive adsorbate such as tryptone. Adsorption and desorption did not alter the infective ability of T_4 bacteriophage. Adsorption can be represented by the Langmuir isotherm and suggests a formation of a unimolecular layer. The maximum surface coverage of the adsorbent was estimated to be only 18%. This figure indicates that pore areas are not used as would be expected due to the mutually exclusive size of the T_4 bacteriophage. Application of the Langmuir isotherm yielded a carbon capacity of 1.6 x 10 sites per mg. Adsorption of _Escherichia coli_ bacteriophage T_4 on activated charcoal follows reversible second order kinetics.

Logrippo (1950) was one of the first workers to employ ion exchangers for the purification and concentration of viruses. The Lansing strain of poliomyelitis was adsorbed to a strong base anion exchange, Amberlite XE - 67, and successfully concentrated by elution from the resin with 10% disodium acid phosphate in distilled water. During the same year Muller (1950) purified a number of neurotropic

viruses with emphasis on the deletion of extraneous material from the crude harvest fluid to increase the "purity" of the virus suspension. A cation resin, (XE -64) 140 -180 mesh was used for this purpose and allowed virus to percolate through the column to ultimately be subjected to amberlite, an anion exchange resin for adsorption, elution, and thus further purification. Influenza virus, rabies virus, and arboviruses such as Eastern equine encephalitis virus were purified by this method.

Two years later Logrippo (1952) published a more detailed study on the purification of The Lansing strain of poliomyelitis. A strong cation exchanger, IR - 120, and a strong anion exchanger, IRA - 410, were used to clear one liter of a 1% suspension of polio infected brain tissue. The virus was then adsorbed to a strong anion exchanger, XE - 67, and eluted with 10% disodium acid phosphate. Excess disodium acid phosphate in the virus eluate was then removed by a combination of weak anion and cation exchangers which left the virus in a purified and concentrated state. Muller (1952) also published on the purification of influenza virus (PR8 strain) by the use of Amberlite XE - 64 which adsorbed the virus, but allowed much of the extraneous tissue material to pass through the column. Elution was conducted with high concentrations of sodium chloride and the virus was ultimately concentrated 8 - 16 times.

Puck (1953) found that bacteriophage T_2 would adsorb to a cation exchanger, Nalcite $R^{+}Na^{+}$ at pH 7, but would not adsorb to an anion exchanger, Dowex 1. However, it was demonstrated that in the presence of 0.15 M NaCl that T_2 could be made to adsorb to an anion resin. By labelling with P^{32} and S^{35} it was shown that immediately after T_2 adsorbs to the resin it is split into S^{35} and P^{32} rich components. Influenza PR8 was demonstrated to adsorb to ion exchange resins, not to split into S^{35} and P^{32} fractions, and to elute well. Up to 59% and 25% recoveries of virus were obtained by eluting with 10% NaCl and distilled water respectively. It was also demonstrated that ion exchanges have such an avidity for influenza PR8 that the virus can be stripped from red blood cells to which the virus has been previously adsorbed. Puck suggests that cellular carboxyls and virus amino groups take part in the binding of bacteriophage T_2.

Takemoto (1954) stated that more primary isolations of influenza virus could be realized by subjecting throat washings to ion exchange resins and eluting with 10% NaCl than by direct testing of the throat washings. Creaser (1957) reported the successful use of ECTEOLA-cellulose for the adsorption of bacteriophage. Virus was eluted with increasing salt (NaCl) concentrations.

Shainoff (1956) used column chromatography on Amberlite XE67 for the purification of the Southern Bean mosaic virus.

Recovery of both the nucleoprotein and biological activity of the virus were demonstrated quantitatively.

Taylor (1958) used Dowex 1 anion exchange resin 250 -500 mesh with 10% crosslinking for the purification and concentration of phosphorus labeled poliovirus. Elution was accomplished by adding 0.05M NaCl and collecting 4 ml fractions for assay. Ion exchangers, pH, and the correct salt concentration is a very specific system which can extend high resolution of particular viruses even within rather homogeneous group of viruses such as the polio group. Hodes _et_. _al_. (1960) found DEAE cellulose to bind attenuated LS_c, 2ab strain of type 1 poliovirus much more avidly than it did the neurovirulent type 1 poliovirus. Recovery of Mahoney strain amounted to 94% and much less for the attenuated strain under the conditions employed.

Poliovirus was purified by adsorption to DEAE 'Sephadex' A25 and eluted by 0.15 M phosphate buffer at pH 7.5 by Giron _et_ _al_. (1964). Fractions were collected and assayed for the most concentrated virus.

A carboxylic acid type ion exchanger (CG -50 - 1) was used in the divalent calcium form by Neurath (1967) for the purification of A_2/Taiwan, A/PR 8, and A/ Ann Arbor/ 1/57 influenza virus B. Maryland /1/59 did not adsorb to either the calcium or the sodium form of the resin. Elution was accomplished with distilled water or with 0.3 M disodium ethylenediaminetetracetic acid. It is suggested that water

soluble organic polymers with metallic ions such as calcium which are entrapped in the lattice would be a useful approach to the chromatography of macromolecular substances such as virus.

While working on the phagocytosis of iron oxide in cell cultures Warren et al (1966) found by accident that when iron oxide was dropped into myxovirus laden allantoic fluid that a substantial drop in titer resulted. It was concluded that the virus had adsorbed to the settling iron oxide particles and it was later demonstrated that the iron oxide did not destroy infectivity or antigenicity. A magnet was introduced at the bottom of a glass vessel and permitted a method for the rapid separation of the iron oxide from the supernatant. It was confirmed that separation of concentrated viral antigen from such complexes could be readily accomplished by appropriate treatment. Several strains of myxovirus were "adsorbed" to iron oxide with a resultant drop in fluid titer which was well in excess of 90%, with the single exception encountered with the B/Lee strain of influenza virus. A concentration of 40 mg/ml iron oxide removed nearly 100% of the original 960 PFU of influenza PR 8 in the original suspension. Elution of virus could not be accomplished with borate, citrate or veronal buffers, various quaternary ammonium wetting agents or hypertonic solutions of sodium chloride, acetate or glutamate. Elution was accomplished, however, with 10% sodium phosphate (Na_2HPO_4) and both

viral infectivity and the HA antigen were accounted for on a quantitative basis. Elution was optimal at pH 8.5. Adsorption of virus to iron oxide was not affected adversely between the pH of 5.5 and 8.0.

Kende et al. (1968) used iron oxide as a selective collecting surface for the preparation of respiratory syncytial virus and *Mycoplasma* vaccines. Elution was accomplished with high concentrations of halides of phosphates, carbonates, and sulfates. Temperature was considered a factor affecting adsorption but the efficacy of adsorption was not changed by a wide range of pH. It was concluded that iron oxide is not only useful for concentration of virus, but acts as an adjuvant for parenteral antigens.

Rao et al. (1968) were the first group to test the potential of magnetic iron oxide to adsorb virus quantitatively from water. Magnetic iron oxide was proven to be more efficient than iron oxide for the quantitative enumeration of virus. A flow through system was adopted in which positive pressure was sponsored by nitrogen gas. The virus-containing sample was placed in a pressure vessel and was fed to a magnetic iron oxide column where it was forced through the column and the output collected for assay. Poor elution was obtained using 10% Na_2APO_4 (29%) and saturated $NaHCO_3$ (10%). However, recoveries of 87.5% and 100% were obtained with the use of fetal calf serum and 3% beef extract solution respectively. Removal of 100% of the following viruses was accomplished by the use of magnetic iron oxide: coxsackievirus B-2, echovirus 11,

poliovirus 3, adenovirus 2, SV40, influenza A herpes simplex, reovirus 1 and a pool of the above viruses. Very limited experiments were conducted on the recovery of coxsackievirus A 9 from sewage effluent (93.5%), Ohio River water, (70.6) and tap water (86%). Twenty five grams of magnetic iron oxide completely adsorbed 2×10^{8} Pfu of coxsackievirus A 9. This amount of virus is approximately equivalent to the calculated amount of virus in four million gallons of polluted surface water.

Another means for the concentration and purification of virus is precipitation. Rhoades (1931) was working on immunization with mixtures of poliomyelitis virus and aluminum hydroxide when he discovered that poliovirus was adsorbed to and inactivated by aluminum hydroxide at a pH of 5.5 and 7.0 but not a pH of 8.8. Subsequently, Sabin (1932) reported the use of alumina gel C for the adsorption of poliomyelitis virus. Unlike Rhoades, Sabin was able to accomplish elution of the poliomyelitis virus from the precipitate by the use of an alkaline pH with 0.15 M Na_2HPO_4. A concentration of 21-25 mg of aluminum hydroxide per ml was used.

Another type of precipitation is that of ammonium sulfate and virus. Since this type of precipitation had been used for the purification of other protein substances it was not unlikely to assume that this technique could be applied to the purification of a nucleoprotein or virus. Grad (1940) used 50% saturated solution of ammonium sulfate in a mixture of 40 g / 100 g of sewage. The ammonium

sulfate virus complex was then dialyzed with distilled water. Melnick (1954) used ammonium sulfate precipitation for concentration of virus from both pad extracts and grab samples.

Salk (1941) was the first to use calcium phosphate for the partial purification of virus. When formalinized influenza virus from allantoic fluid was adsorbed to calcium phosphate, it was found that an enhanced immunizing potency was realized. Given impetus by this work, Stanley (1945) published on the precipitation of purified concentrated influenza virus and vaccine on calcium phosphate. Virus was demonstrated to adsorb better when calcium phosphate was precipitated in the presence of virus. Phosphate buffer, sodium hydroxide, and calcium chloride were used in various ratios to define the optimum concentrations of the ingredients.

Miller (1955) used aluminum phosphate for the adsorption of influenza virus. Twenty milliliters of a 16% solution of $Na_3PO_4 \cdot 12\ H_2O$ was diluted to one liter and 20 ml of 10% $AlCl_3 \cdot 6\ H_2O$ (W/V) was added to give a gelatinous precipitate. Adsorption was executed at a pH less than 7.0 and in the presence of 0.063 M buffer. Elution was accomplished by increasing the molarity of the buffer to 0.25 M and the pH to 8.0. Temperatures of $5^{\circ}C$, $36^{\circ}C$ and $56^{\circ}C$ did not change the adsorption of the virus to aluminum phosphate. Five different strains of influenza were tested and by slight changes in the salt concentrations it was possible to elute a strain separately

as well defined fractions.

An extensive study was undertaken by Chang (1958) on the comparative behavior of coxsackievirus and bacteriophage when subjected to flocculation with aluminum sulfate or ferric chloride under different test conditions. The bacteriophage used was specific for Micrococcus pyogenes var. albus. When 40 ppm ammonium sulfate was employed 86.3% of the coxsackievirus was adsorbed, but in the presence of 100 ppm alum 98.7% of the virus was adsorbed. A concentration of 40 ppm alum adsorbed 93.5% of the bacteriophage and 100 ppm adsorbed 99.8% of the phage. Coxsackievirus was adsorbed at a pH of 5.5, 6.2 and 7.2, with adsorption percents being 94.6, 97.1, and 99.0 respectively. Bacteriophage was demonstrated to adsorb poorly at pH 5.5 and 8.2. at pH 5.5 and 6.2 adsorption was well in excess of 90%. Ferric chloride in concentrations of only 20 ppm and 40 ppm resulted in 96.6% and 98.1% adsorption of coxsackievirus respectively. At the same concentrations, 99% of the bacteriophage was adsorbed. Elution was accomplished by using a bicarbonate buffer at pH 8.5. A recovery of 60% was common for the coxsackievirus and 10-25% for the bacteriophage. The low recovery for the bacteriophage could have been due to destruction of the phage rather than an inability to elute. It is of interest to note that the high virus removal attained by Chang (1958) was achieved with low dosages of aluminum sulfate, whereas the poorer success of other workers was with high concentrations of aluminum sulfate.

Chang (1958) also stated that by using aluminum sulfate

to produce a flocculation more than 95% of added virus could be removed with 15-20 ppm of alum (or ferric chloride) in moderately polluted water having a turbidity of less than 260 ppm. When double flocculation was employed with the same concentration of alum or ferric chloride, 99% removal of virus was achieved. The studies also showed that virus removal approximately paralleled the removal of coliforms, total bacteria and turbidity. Again it was demonstrated that the virus was not destroyed by adsorption to the floc, but only concentrated. Chang states that chemical flocculation appears to be the result of a metal cation-protein complex which is formed and then aggregates to form a precipitate. This type of reaction is non-specific and therefore flocculation with alum or ferric chloride should work equally well for all animal viruses.

Later in 1958, Taverne et al. published on the concentration of viruses and virus soluble antigens on calcium phosphate. A 30-100 fold increase was established for influenza PR8 with a recovery of 50%-80% of the total virus input. Similar results were obtained for vaccina, encephalomyocarditis, coxsackie, and poliomyelitis type 3 viruses. Elution was conducted at an alkaline pH with the aid of Na_2HPO_4 and KH_2PO_4.

Wallis and Melnick (1967) reported the successful adsorption and concentration of virus on aluminum and calcium salts. More than 99% of the virus was adsorbed at pH 6, 7, 8, and 9. Adsorption at pH 9 seems unreasonable, but it must be remembered that elution from these substances is not solely dependent on an alkaline pH but on an alkaline pH coupled

with an increased salt concentration. Adsorption of herpesvirus to the precipitate was almost immediate; adsorption occurred equally well at temperatures of 4°C, 25°C, and 37°C. A 0.1% fraction of virus was not adsorbed even in an excess of precipitate, nor was it inactivated by herpesvirus hyperimmune rabbit antiserum, normal rabbit serum, or DNase. Salt was required for complete adsorption. Only acid sensitive viruses such as poxvirus, herpesvirus, myxovirus, arboviruses, and rhinoviruses were adsorbed to aluminum phosphate. All viruses tested were adsorbed to aluminum hydroxide and all but reovirus were adsorbed to calcium phosphate. McLean (1967) also reported on the successful adsorption of virus to calcium phosphate. Initially, a leaf homogenate of the lettuce necrotic yellows virus (LNYV) was subjected to charcoal as an aid in purification. Subsequently, calcium phosphate was employed. During the same year, Burnes (1967) published on the separation of plaque type varients of the encephalomyocarditis virus by chromatography on calcium phosphate. A large plaque virus was eluted at 0.17 M phosphate buffer and a small plaque virus at 0.31 M phosphate buffer. This was established by reading OD at 260 and biological infectivity.

Moore (1968) compared three methods for the concentration and detection of viruses in waste water and found that precipitation with aluminum hydroxide was far superior to the use of Millipore membranes or anion exchanges. The use of Millipore membranes resulted in 3.4%-28.0% recovery of virus, which is roughly in agreement with Metcalf (1961). Ion exchange

technique increased the recovery of virus to 29%-32%. However, with aluminum hydroxide a thousand fold concentration was realized and 100% recovery of virus was obtained. Smirnova (1969) reported the concentration of parainfluenza virus by adsorption to barium sulfate or precipitation with zinc acetate. Samples were concentrated by 1.7 logs over the initial concentration and elution was accomplished by using 0.25 M sodium citrate.

Demonstration that Millipore membrane filters could be used for the collection or concentration of viruses was promulgated by Metcalf (1961). Influenza virus was successfully separated from bacteria and also isolated from clinical specimens of influenza. Recoveries of approximately 5%-20% of the virus was obtained. Shortly thereafter, Hsiung (1965) was able to reduce the ten ill-defined groups of animal viruses to six categories based solely on the size of the virus particle as defined by ultrafiltration through Millipore membranes. Through this effort both DNA and RNA containing viruses were separated into three groups: those greater than 100 mu, those between 50 - 100 mu, and those smaller than 50 mu. Establishment of this physical parameter helped greatly in placing existing viruses into natural groups sharing other common parameters.

Wallis (1966) reported the successful concentration of viruses from sewage by collection on Millipore membranes. The samples were first treated with anion resins to remove extraneous organic material and $MgCl_2$ was found to increase

the adsorption of virus to membrane. Recovery was aided by homogenization of the membrane in a small volume. Poliovirus type 2 and 3, echovirus 9, and coxsackievirus was recovered. Subsequently, a more extensive report followed on the concentration of enterovirus on membrane filters (Wallis 1967). Salts were found to facilitate the adsorption of virus to membrane filters and membrane coating components (MCC) were found to inhibit the adsorption process. Membrane coating components are substances that support viral growth and must be removed before virus can be adsorbed to the membrane. Membrane coating components may be used to elute virus once it has been adsorbed and where elution with cell extracts or serum is efficient. Other substances that lower surface tension such as sodium lauryl sulfate and 0.1% saponin are also capable of causing elution of virus. Optimal adsorption took place at pH 5 and optimal elution was attained at pH 8. Interfering proteins were removed with protamine sulfate and interfering lipids were removed by treatment with chloroform.

The first to report on the removal of virus from water by the use of polyelectrolyte was Johnson <u>et</u> <u>al</u>. (1967). Polymers based on divinylbenzene cross linked styrene/ maleic anhydride copolymer adsorbed 100% tobacco mosaic virus and up to 99.99% of polio virus. Extreme concentrations of salt inhibited the adsorptive phenomenon, but 97% adsorption of virus was accomplished at a concentration of 0.17 M NaCl. Adsorption was generally better when conducted in Hank's balanced salt solution than when conducted in water.

It was also demonstrated that as much as 90% of bovine serum albumin was adsorbed.

Wallis and Melnick (1969) reported on the enumeration of virus by a collecting surface of insoluble cross-linked copolymer of maleic anhydride. Adsorption to polyelectrolytes was compared to the adsorption of virus to aluminum salts and membrane filters. Aluminum salts gave recoveries of approximately 56% and severely limited the amount of water that could be sampled at top adsorptive efficiency. Millipore membranes yielded a recovery of 64%. However, when polyelectrolyte (PE60) was employed 93% recovery of virus was attained. NaOH treated polyelectrolyte at a collection pH of 5.0 preferentially adsorbed virus over organics from a crude tissue culture harvest. However, water washed polyelectrolyte at pH 5.5 - 6.0 was more effective for adsorbing virus from sewage. Only 100 mg of PE60 per gallon of sewage with a contact time of 4 hours or 400 mg of PE60 per gallon of sewage for 1 hour seemed to give optimal virus adsorption. All trials were carried out at room temperature with the aid of mechanical stirring. The suspension of virus and PE60 were filtered and trapped on a 47 mm fiberglass pad and subsequently recovered by a spatula. The material was then eluted in 3 - 5 ml of 10% fetal calf serum at pH 3 in either phosphate buffer or borate buffer. When sewage was subjected to this method for the concentration of virus it was found that naturally occurring virus could be

identified many more times than by either aluminum salts or Millipore membranes. Poliovirus, echovirus type 7, Coxsackievirus A 9, and Coxsackievirus B 3 all were demonstrated to adsorb well. Successful concentration of virus from feces and urine was also demonstrated. When an input of 1000 plaque forming units (PFU) was suspended in 25 gal. a recovery of 80% of the virus was demonstrated (personal communication).

Wesslen and Albertson (1959) were the first to describe a method by which animal viruses could be separated by the use of a two phase polymer system such as polyethylene glycol (PEG) and dextran sulfate. Conventionally, the virus should associate with one phase or the other, and preferably the smaller phase, so that ultimately a true concentration of virus is accomplished.

Soon after this, Albertson and Philipson published on the concentration of bacteriophage T2, adenovirus, and echoviruses 7 and 19 by phase separation using not only PEG and sodium dextran sulfate, but also methylcellulose and polyvinyl alchohol. Sodium dextran sulfate and methylcellulose were more expedient in recovery of larger viruses as the smaller enteroviruses tended to distribute more evenly. Sodium dextran sulfate and PEG could be used for all viruses with the correct salt concentration. All viruses favored the bottom phase with low salt concentration and all viruses favored the top phase with higher salt concentration.

Subsequently, Cliver (1965) concentrated virus by dialysis against polyethyleneglycol and sample volumes were reduced 98% but only 10 - 30% of the original virus was recovered. This method is simple and inexpensive, but would be difficult to apply if a large volume of water were to be sampled. Lund et al. (1966) applied the aqueous polymer phase system to the isolation of enteroviruses from sewage. Only 200 ml of sewage could be accommodated in the system, however. To this volume 20 g of 5 M NaCl, 58 g of 30% PEG and 2.7 g of 20% dextran sulfate was added. This system was shaken for one hour and then placed in a separatory funnel for 24 hrs. at $4^{o}C$ for resolution. Poliovirus 3, Coxsackieviruses B_4, B_5, B_6, and echovirus 32 were successfully isolated.

Grindrod (1969) reported on the detection of seven enteroviruses by the phase separation technique when PEG 6000 and dextran sulfate were employed. Coxsackievirus B_2 and echovirus 6 did not respond to this treatment and influenza virus PR 8 was inhibited. The author warns that "users of this procedure in environmental virology studies should be aware that their results may be biased significantly by the selectivity of the dextran sulfate employed in this technique." Shuval in the same year (1969) reported a 500 fold concentration of enteroviruses and suggested that as few as 1 - 2 infectious virus particles per liter could be recovered by the two phase polymer system when polyethylene glycol and sodium dextran sulfate was employed. Better recoveries of virus

were found with antibiotic treatment rather than ether treatment prior to assay. Through this system a maximum number of 11,184 virus / l were found in raw sewage.

Leberman (1966) described the precipitation of two plant viruses and a bacteriophage using PEG alone. This was a pH dependent phenomenon which varied with the buffer employed. Salt concentrations also were important.

Kanarek (1967) was able to precipitate virus from suspension in balanced salt solution by mixing with PEG at pH 7.4. Increased salt concentration above physiological levels reduced the efficiency of concentration. An optimal concentration of PEG was 7.5%. The components were allowed to react in the system for one hour at $4^{o}C$ and then the precipitate was harvested by centrifugation and subsequently resuspended in phosphate buffered saline. Influenza virus, respiratory syncytial virus, bovine parainfluenza 3, and rubella were concentrated as indicated by both hemagglutination and infectivity. The HA titer of influenza virus A was increased by 64 and its infectivity by 100. With the exception of rubella, both HA titer and infectivity increased by at least 50 times. Volumes up to 17 l of crude lysate have been purified by the rapid sedimentation of virus in the presence of polyethylene glycol alone. (Yamamato and Alberts, 1970) Concentrations from 10^5 to 10^{13} pfu have been achieved and λ, T_4, T_7, P_{22}, fd, ϕx174, and r17 bacterial viruses have been concentrated with 2-10% PEG 6000. The ionic strength and pH are relatively elastic and not critical. Asymmetric

viruses are easier to recover than symmetric ones. The mechanism by which the virus is concentrated is unknown. Although it has been referred to as precipitation, it is more closely related to partition.

Although continuous-flow ultracentrifugation is not a method for the precipitation or adsorption of virus from water, it is a means by which viruses can be concentrated. Anderson (1965) reported on the construction of a continuous-flow ultracentrifuge rotor capable of removing more than 95% of the suspended poliovirus at a flow rate of two liters/hour. This method is extremely costly and since it leads to the loss of some virus, plus its inability to handle large volumes of water, its ultimate effectiveness is open to question.

MATERIALS AND METHODS

Cell Lines

Primary monkey kidney cultures (PMK), human embryonic kidney (HEK), and human amnion (HuAm) were used. The monkey kidney cultures (African green) were prepared from cell suspensions obtained from Flow Laboratories. These cultures were propagated on medium 199 with 20% fetal calf serum and maintained on the same basic medium with 2% fetal calf serum. Medium 199 was supplemented in both cases with yeastolate. Two milliliters of a 5% solution per 100 ml of medium was added.

A stable culture of African Green kidney cells (Vero) was used. This culture was propagated on L-15 (Leibowitz) medium containing 10% fetal calf serum for growth and 2% serum for maintenance.

A stable monkey kidney culture ($LLCMK_2$) was propagated in Difco medium 199 with 2.5% horse serum supplement for growth and 0.5% for maintenance (Flow Laboratories). The cells were obtained in the 207th passage from Dr. Robert N. Hull of The Cell Research Laboratory, Eli Lilly and Company, Indianapolis.

Human amnion[1] (HA) was grown on Eagles medium with 10% fetal calf serum and maintained on 5% fetal calf serum (Flow Laboratories). Human embryonic kidney[1] (HEK) was grown on L-15 Leibovitz medium. A supplement of 10% fetal calf serum and 2% serum was used for growth and maintenance

1 Flow Laboratories, Rockland, Maryland

respectively.

Primary monkey kidney cultures were seeded in 16x125 mm disposable polystyrene tissue culture grade tubes at a rate of 250,000 cells per ml. One ml was inoculated per tube. Vero and $LLCMK_2$ cultures were seeded at 50,000 cells per ml per tube.

Transfer of Cell Cultures

Difco trypsin 1/250 was used at a concentration of 0.25% in Earles Balanced Salt Solution (EBSS) and deionized water. Spent media was decanted from the large Blake bottles housing the mature cell yield and 10 ml of 0.25% trypsin was added to harvest the cell crop. Incubation at 36.5°C for 10 - 20 min. usually released the cells from the glass. The cell yield from each bottle was then centrifuged at 1500 r.p.m. for 10 minutes, the trypsin decanted and the cells resuspended in fresh media. The harvest was split one for two for propagation of cells.

Penicillin[1] and streptomycin[1] were routinely incorporated into tissue cultures. Streptomycin sulfate (1gm) was aseptically rehydrated with 4.5 ml of sterile distilled water to a concentration of 200mg/ml. Buffered potassium penicillin G was rehydrated with 8 ml of sterile water to give 500,000 units per ml. Penicillin and streptomycin were then frozen until used. A dosage of 0.5 ml/1 or 100 units/ml of penicillin was used. Mycostatin[2] was sometimes used as an antifungal agent and was incorporated into tissue culture medium to

1 Eli Lilly and Co., Indianapolis, Indiana

2 E. R. Squibb and Sons, New York, New York

a concentration of 20 units per ml.

All reagents used for tissue culture such as 1N NaOH 7.5% sodium bicarbonate, balanced salt solution, 0.25% trypsin, 1N HCl, and deionized water were filter sterilized by an all glass 47 mm Millipore filter apparatus. A Millipore filter pad of .224 was used. Glassware preparation was done using non-toxic detergents and distilled water rinses with distilled water of a purity conforming to less 0.1 ppm salts read as NaCl.

Polioviruses 1 and 2 were used for the bulk of this work. Coxsackie B3, ECHO 6 and adeno 12 were also used and supplied from the stock virus cultures at The University of New Hampshire. Virus was generated by cultivation on $LLCMK_2$ in large Blake bottles. An inoculum of 0.1 ml of stock virus was used and 30 ml of media to just cover the cells. Subsequent to the satisfactory development of cytopathetic effect (CPE), virus was harvested by centrifugation at 8000 r.p.m. for 0.5 hours. The supernatant was collected and pipetted into glass storage vials in 1 ml quantities and then stored at -90 C in the Revco where a titer of 10^7 - 10^8 $TCID_{50}$/ml was maintained.

Virus Assay

Virus assays were conducted on $LLCMK_2$ monolayers using the unit infecting one-half of the tissue cultures used or the $TCID_{50}$ end point. Some assays were conducted by the most probable number of cytopathic units endpoint (MPNCU) but this assay was not found to be expedient for our purposes.

Bacterial assays were performed by the most probable

number method (MPN) using 5 fermentation tubes of lactose broth for each of 3 test volumes. Studies conducted on the adsorption of bacteria to magnetic iron oxide were assayed by pour plates.

Virus Collecting Surfaces

The following adsorbents were screened for their capacity to adsorb virus from water: DEAE - standard (Schleicher and Schuell Company, Keene, New Hampshire), Sephadex G-25 (Pharmacia, Uppsala, Sweden), Amberlite IRC-50 (Mallinckrodt Chemical Works), Dowex 2-x4 and Dowex 50-W4x (J.T. Baker Chemical Company, Phillipsburg, New Jersey). These chemicals were all in stock at the virology laboratory at The University of New Hampshire. Rexyn AG3 (Fisher Scientific Company, Fair Lawn, New Jersey) was obtained from the Chemistry Department at The University of New Hampshire. Bentonite, Illite, Kaolinite, Feldspar and Quartz were donated to us courtesy of Dr. Allan Prince, Soil and Water Sciences Department, University of New Hampshire. Magnetic iron oxide (Charles Pfizer & Co.), gauze (Sears Roebuck), activated cocoanut charcoal C19A and C190N, both fast filtering, (West Virginia Pulp and Paper Co.) and activated charcoal 50-200 mesh (Fisher) were examined.

Virus Collecting Systems

A total of three independent systems were used to study the collection of viruses by a number of substances.

System 1 was used only to screen a number of substances for their capacity to concentrate virus from water. Pyrex

laboratory glass tubing was made into a series of columns which were 15 inches long by 5/16 of an inch in internal diameter. The columns were slightly constricted at one end and were plugged at the same end with glass wool which held the contents of the column in place and yet permitted the free flow of liquids.

Shredded gauze was packed into three columns to a height of 8 inches and viral adsorption was tested in phosphate buffer at pH 6.5, 7.2 and 8.5. Commonly 10 ml of 10^{-2} or 10^{-3} poliovirus 1 was run through the column until all flow ceased. The effluent was collected and assayed for virus to determine the amount of virus retained by the column material.

One gram quantities of bentonite, illite, kaolinite, feldspar, and quartz were made into slurries and added to the columns by the aid of a glass funnel and a piece of surgical rubber hosing which was attached to the proximal end of the column. The column was secured to a Kahn shaker by a ringstand and the slurry was allowed to trickle into the column while it was in motion on the shaker. This prevented channelling.

Soil impregnated gauze columns were made by mixing a constant amount of soil with a constant amount of shredded gauze in 100 ml of phosphate buffer at pH 6.5, 7.2 and 8.5. The mixture was stirred until the gauze had become well impregnated with soil and the wet gauze was loaded into

the column. Subsequently, the virus suspension was added and treated in a similar manner for assay. Other columns were loaded in the same way with activated charcoal, DEAE, Sephadex, ion exchange resins and magnetic iron oxide.

System 2 was devised and constructed at The Engineering Experiment Station at The University of New Hampshire. This system represented the flow through system concept and was to be a prototype for field monitoring of contaminated surface waters. This system was composed of two interdependent units. The power supply was housed in a grey marine plywood box 17 x 14 x 18 inches and weighed approximately 50 pounds. The power for field studies was derived from two six volt Delco batteries. In the laboratory, power was obtained from a simple 115 volt wall socket and thus in field studies an ATR DC-AC inverter was purchased from the ATR manufacturing company, St. Paul, Minnesota. This system may be reviewed in plate 1.

Input consisted of either 6 or 12 volt DC current and an output of 110 volts with a 40 watt capacity. The electrical plug attached to the pump could be used in a wall socket and in the field could be plugged into the inverter. The inverter was wired so that it could be connected to a positive and negative terminal of the two batteries in a second box. The remaining positive and negative terminals were then connected to complete the circuit. A simple on and off switch was located between the power supply and the inverter.
The second box was rectangular (24 x 20 x 17) and housed

a March model MOX-35 magnetic drive fan cooled pump. The pump contained polypropylene parts 5/8 inch diameter and contained a spindle: Std. 316 stainless.

The system was designed to take samples up to 100 feet through an intake (Buchner funnel) which was equipped with 1/4 inch surgical rubber hosing and could be lowered and cranked manually. The sample entered the pump and was forced through a series of two pyrex glass columns 2 x 16 inches internal diameter (Corning) where the collecting surfaces were stationed. The sample was fed to the top of column 1 and forced through it and to the top of column 2 and then made its exit to the effluent receptacle (Plate 1).

System 3 retained the concept of a flow-through system, but the column of magnetic iron oxide was replaced by a flat bed of iron oxide contained in a 142 mm Millipore filter, which is presented in Plate 2.

Twenty-five g. of magnetic iron oxide was made into a slurry by adding it to 400 ml of deionized water and shaking manually. The oxide was allowed to hydrate overnight and then loaded into the filter with the aid of a glass funnel which was connected to the filter inlet by 1/4 inch surgical rubber hosing. Negative pressure was used to force the oxide slurry into the filter where it was caught on an AP20 Millipore fiberglass filter pad. Subsequently, the filter was dismantled, the iron oxide pad examined, and a second AP20 pad added in order to create an AP20 Fe_2O_3-AP_{20} sandwich.

The filter was reassembled and was ready to filter virus. All pads were treated with Tween 80 (polyoxethylene sorbitan monoleate) by passing 200 ml of 100 mg% solution through six filters simultaneously. All filters had to be trimmed to prevent leakage at high pressure.

The sample of virus suspension was placed in a 5 gal. stainless steel pressure vessel[1]. A 1/4 inch NPT inlet and outlet accommodate special "stepped" connectors for use with 1/4, 3/8, and 1/2 inch I.D. hose.

The vessel is proofed to 200 psi and the head fitted with a manual bleed automatic pressure relief valve set for 125 psi. The sample was forced through the filter by positive pressure supplied by a nitrogen cylinder. Depending on the material being filtered, pressures from 5 psi to 85 psi were used.

Preparation of Magnetic Fe_2O_3

Powdered iron oxide was made into a slurry and then washed three times in distilled water and the floating material decanted. When the iron oxide was washed sufficiently, the water was decanted and the hydrated slurry dried at 100°C for 20 hours, it was then placed in the heat sterilizer at 150°C for 1 hour (Warren and Neal personal communication).

Elution of Virus

A 3% solution of Difco beef extract was conventionally used for elution and was made by dissolving 7.5 g of beef extract in an appropriate amount of distilled water. The

1 Millipore, Bedford, Massachusetts

solution was then autoclaved at 121 C for 20 minutes at 15 pounds pressure. The preparation was then stored at room temperature until used. The pH was adjusted to 8.5 - 8.9 with 1N NaOH. Other percents of beef extract were employed but 3% was generally applied.

Elution of virus from iron oxide was also attempted with Eagle's minimal essential media (MEM) with 20% fetal calf serum. The pH, time, amount of eluate material, and mode of elution were varied to facilitate recovery.

X-ray Diffraction.

Processed and unprocessed magnetic iron oxide was subjected to X-ray diffraction analysis.[1] A total of 9 grains or 0.582 gm. of magnetic Fe_2O_3 was placed in a glass capillary receptacle with an internal diameter of 0.3 mm and a wall thickness of 1/100 mm. The Powder Pattern Technique was employed and a high-resolution X-ray powder diffraction camera was used (57.3 mm diameter Debyre-Scherrer cylindrical model, Siemens and Halske A G, Karlsruhe, Germany). The identification and crystallography of the compound was studied by a consideration of 12 d values for Fe_2O_3 and 8 d values for Fe_3O_4. Ilford type G film was used. Radiation was for the duration of 1 hour at 35 kv and consisted of Fe $K\alpha$.

Collection and Analysis of Sea Water Samples

Several trials were conducted with several sea waters

1 Conducted by Wayne Beasley, Adjunct Professor of Materials Science and Research, Engineering Experimental Station, University of New Hampshire.

using the model system and seeding with poliovirus 1. Initially virus was suspended in Lyman and Fleming sea water at pH 8.1 and salinity 35 ppt. A sample of estuarine water (pH 7.4, salinity 14 ppt) was collected at Adams Point and transported to the laboratory where it was seeded with virus. Open ocean water was sampled for virus at four locations beginning at the mouth of the Piscataqua River, Portsmouth. The second sample was taken one mile seaward from the initial sample and ensuing samples were taken one mile apart culminating at The Isles of Shoals. Samples were collected in 5 gal. Nalgene carboys and transported directly to the laboratory where they were retained at $4^{o}C$ for 12 - 24 hours before processing. The effect of salinity on adsorption and recovery was studied by seeding poliovirus in one liter of Lyman and Fleming sea water which was diluted with deionized water to salinities of 35, 26, 17, and 9 ppt. The effect of salinity on adsorption was also studied in a "tube system" discussed below. A trial was conducted by seeding poliovirus in Instant Ocean[1]. An RBS portable Solu Bridge Salinometer was used to measure salinity (Industrial Instruments).

Adsorption of Virus to Fe_2O_3

A suspension of Fe_2O_3 in distilled water containing 40 mg/ml of Fe_2O_3 was prepared and the following dilutions made: 20, 10, 5, 2.5 and 1.25 mg/ml Fe_2O_3. A total of 10^5 $TCID_{50}$ poliovirus 1 was added to each dilution of 10 ml of

1 Aquarium Systems, Inc.

suspension in 16 x 25 mm screw cap test tubes. The tubes were shaken initially, at 15 and 30 minutes. The tubes were then centrifuged at 1000 r.p.m. for 10 minutes and the supernatant examined for virus.

Salts, Salinity and pH

Ten 16 x 125 mm screw caps test tubes were loaded with 10 ml of 40 mg/ml magnetic Fe_2O_3 and the pH adjusted to give values from 3 to 12. Following a 12 hour standing period at 26 C the pH was readjusted and the experiment conducted by adding 10^5 $TCID_{50}$ poliovirus to each tube and a control, shaking the tubes initially, at 15 minutes, and at 30 minutes to insure optimum collision efficiency. The tubes were centrifuged at 1000 r.p.m. and the supernatant collected for viral assay.

Ten 16 x 125 mm screw cap test tubes were loaded with magnetic iron oxide and the load was hydrated in ten dilutions of Lymen and Fleming sea water ranging from 3.6 to 32.0 ppt. Adsorption and virus controls were included. The pH in each tube was adjusted to 5.6, adsorption was ended at 0.5 hours and samples taken for viral assay.

NaCl, $MgCl_2$, $AlCl_3$, NH_4Cl and LiCl were all tested as to their effect on the Fe_2O_3 - virus complex subsequent to adsorption at pH 5.6. The effect of the salts on elution at pH 8.9 ($AlCl_3$ and NH_4Cl not included), and the effect of the salts on the adsorption of virus to Fe_2O_3 at pH 5.6 was examined and defined as competition or antagonism. The "tube system" was employed with a static amount of Fe_2O_3.

Chemicals, Extracts, and Reagents

The following components were tested as to their capacity to elute virus from iron oxide at pH 6.9 and to antagonize the adsorption of poliovirus to iron oxide at pH 5.6: purines, pyrimidines, vitamins, peptone (Difco), proteose (Difco), amino acids, creatine and creatinine. All amino acids and vitamins were purchased from Nutritional Biochemical Corporation except glycine and alanine which were purchased from Calbiochem. Creatine was purchased from Pfanstiehl Chemical Company. All solutions were prepared in distilled water in 3% concentration. A few amino acids such as valine, isoleucine and phenylalanine were dissolved in a small volume of 4N HCl before addition of distilled water. The vitamins included in the 3% solution were thiamine, biotin, folic acid, pyridoxine, pantothenic acid, riboflavin, and nicotinic acid or nicotinamide.

Streptomycin (60 mg.ml Eli Lilly Co.), 3% basic amino acids (arginine, lysine, histidine), 3% beef extract (Difco), 3% polyamine (spermine and putrescine), 3% glucosamine, 3% glucose, 3% high pKa amino acids (glycine, alanine, valine, proline), MEM with 20% fetal calf serum, Earle's balanced salt solution with 2% fetal calf serum and EDTA (.2gm/l) as Na versenate were employed for studies on the antagonism of virus collection and the elution of virus from the surfaces of magnetic iron oxide.

A standard solution of bovine serum albumin (BSA) was made to contain 200 ug of albumin per ml. A standard curve

was obtained by using 5, 10, 20, 40, 80, and 100 ug of BSA as defined by Lowry protein. A total of 200, 600, 1000, and 1200 ug BSA was loaded into 16 x 125 screw cap tubes containing 0.5 gm. magnetic Fe_2O_3 in 10 ml distilled water pH 6.0. Adsorption and centrifugation were conducted as indicated previously. Readings were taken in Klett units and a Klett Summerson photoelectric colorimeter was used for protein determinations.

Ultracentrifugation and Density Gradient

A total of 200 ml of poliovirus 1 in crude tissue culture harvest was subjected to low speed centrifugation at 9,000 r.p.m. for 0.5 hours to pellet cell debris. The supernatant was collected and loaded into polyallomer 1 x 3½ inch ultracentrifuge tubes with a test torque of 70. A total of 35 ml of clarified virus suspension was placed in each tube by the use of a 10 ml syringe filtered with a canula. Eight tubes were loaded into a Ti 60 head precooled overnight to 4 C. The virus was spun at 35,000 r.p.m. for 3 hours at 5 C.

A pellet was obtained and the supernatant withdrawn with the aid of a syringe and a canula. The pellet was resuspended in 10 ml of 0.02 M Na_2HPO_4 and 0.02 M KH_2PO_4 at pH 7.0. The preparation was placed at 4°C for 18 hours and subsequently resuspended. A composite of all the tubes was again spun at 35,000 r.p.m. The virus pellet finally was resuspended in 12 ml of 0.02 phosphate buffer.

Dry CsCl[1] was added to 12 ml of the concentrated virus

1 Donated by Dr. Fred Hickson, University of New Hampshire, A product of K K Laboratories, 99% pure.

until the refractive index of the suspension was 1.366. An equilibrium density gradient centrifugation determination was made in a Beckman L2 ultracentrifuge using a SW65K swinging bucket head. Virus was spun at 35,000 r.p.m. at 25° C. A model 270 Isco fractionator was employed and 0.25 ml fractions were collected. A model UA3 UV adsorbance apparatus was used and automatically graphed each fraction. An original titer of 10^{6} $TCID_{50}$/ 0.1 ml was demonstrated in fraction 4 of the density gradient purified virus.

Electron Microscopy

Poliovirus purified by differential centrifugation and density gradient was examined in an electron microscope. Virus was stained negatively with 2% phosphotungstic acid at pH 7.0 on formvar coated 300 mesh copper grids[1]. Tungsten shadow cast virus was also examined in the electron microscope.

Magnetic iron oxide was added to 10 ml of distilled water (0.3g/10ml) and 10^{-3} and 10^{-4} dilutions placed on formvar coated grids for examination under the electron microscope.

Concentration of the Eluate

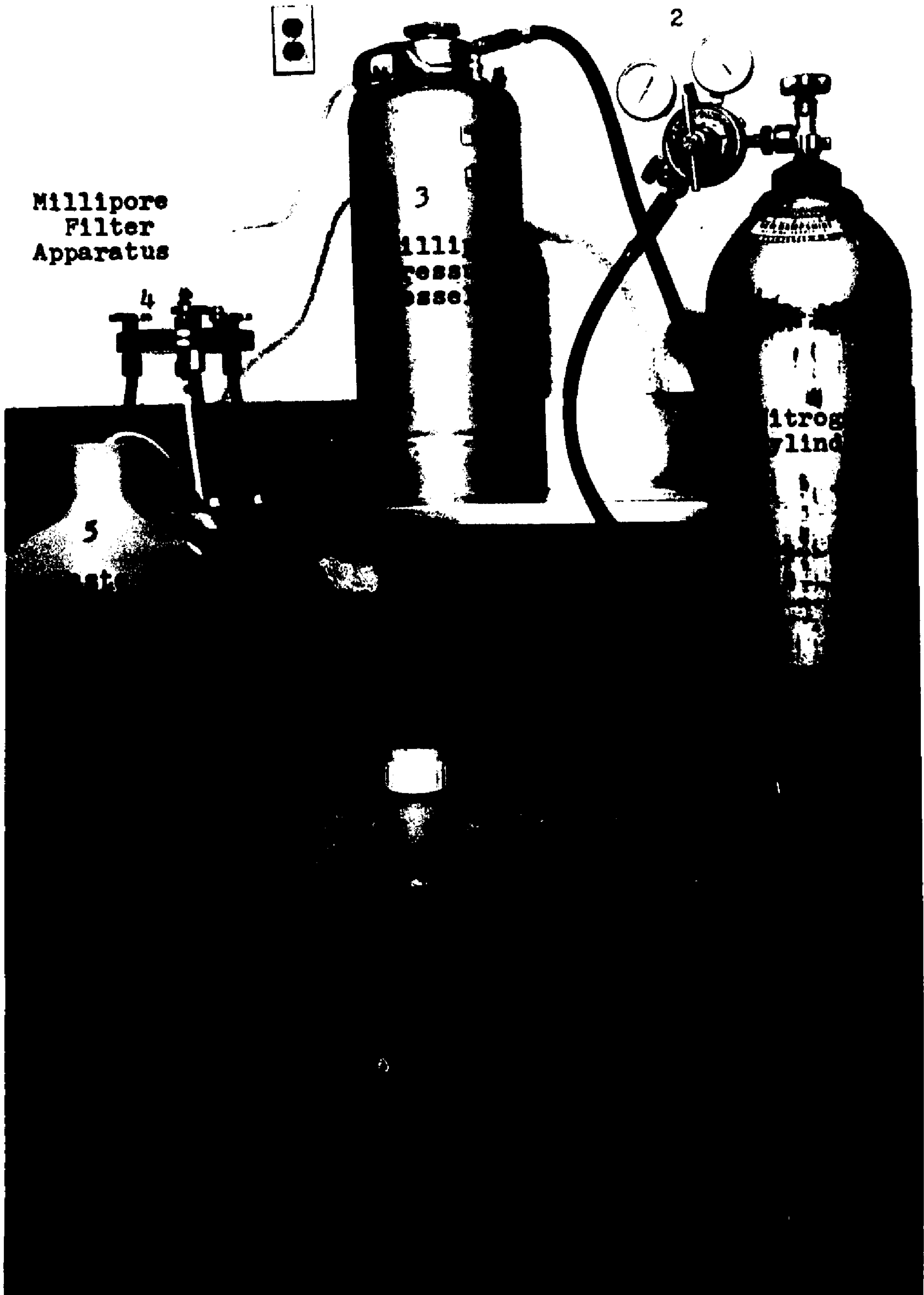
Three procedures were used in an effort to further concentrate virus contained in the 250 ml eluent obtained from elution of the iron oxide collecting surfaces.

1 No. 2214 Earnest F. Fullam, Inc.

1. Precipitation of virus by polyethylene glycol by the method of Yamamoto and Alberts. To the 250 ml eluate 7.3 g NaCl and 25 g PEG was added, then mixed and placed at 4°C for one hour. The mixture was centrifuged at 8000 x g for 20 minutes, the supernatant aspirated and the pellet resuspended in a final volume of 1 ml. A rinse of 5 ml BSS was then employed to remove the residual pellet. The sample was ether treated and assayed as usual.

2. Concentration was also attempted by phase separation techniques using sodium dextran (0.5 g), polyethylene glycol (16.125 g), and sodium chloride (4.38 g) added to the eluate followed by mixing with a magnetic stirrer. The mixture was placed in a 250 ml separatory funnel and stored at 4°C for 18 hours. The bottom layer was drawn off as a 2 ml vol. and the interphase as 2.5 ml. Precipitation was conducted by the addition of 1 ml of 4M KCL (0.8 M final concentration). The sample was then shaken and allowed to stand at room temperature for 15 minutes, then centrifuged at 2000 r.p.m. for 10 minutes. The supernatant volume was 3.0 ml.

3. Several trials were conducted using the thin sandwich tenchique in a 142 mm Millipore filter holder. An eluate was collected, then subjected to ultracentrifugation in a Beckman L-2 ultracentrifuge. The sample was centrifuged at 43,000 r.p.m. for 180 minutes at 5°C. The supernatant was withdrawn with the aid of a canula and syringe and the pellet resuspended in L-15 medium which was serum free but supplemented with 0.1 ml of penicillin and streptomycin per 10 ml of resuspended volume.


Pressure Gauges
2
Millipore
Filter
Apparatus
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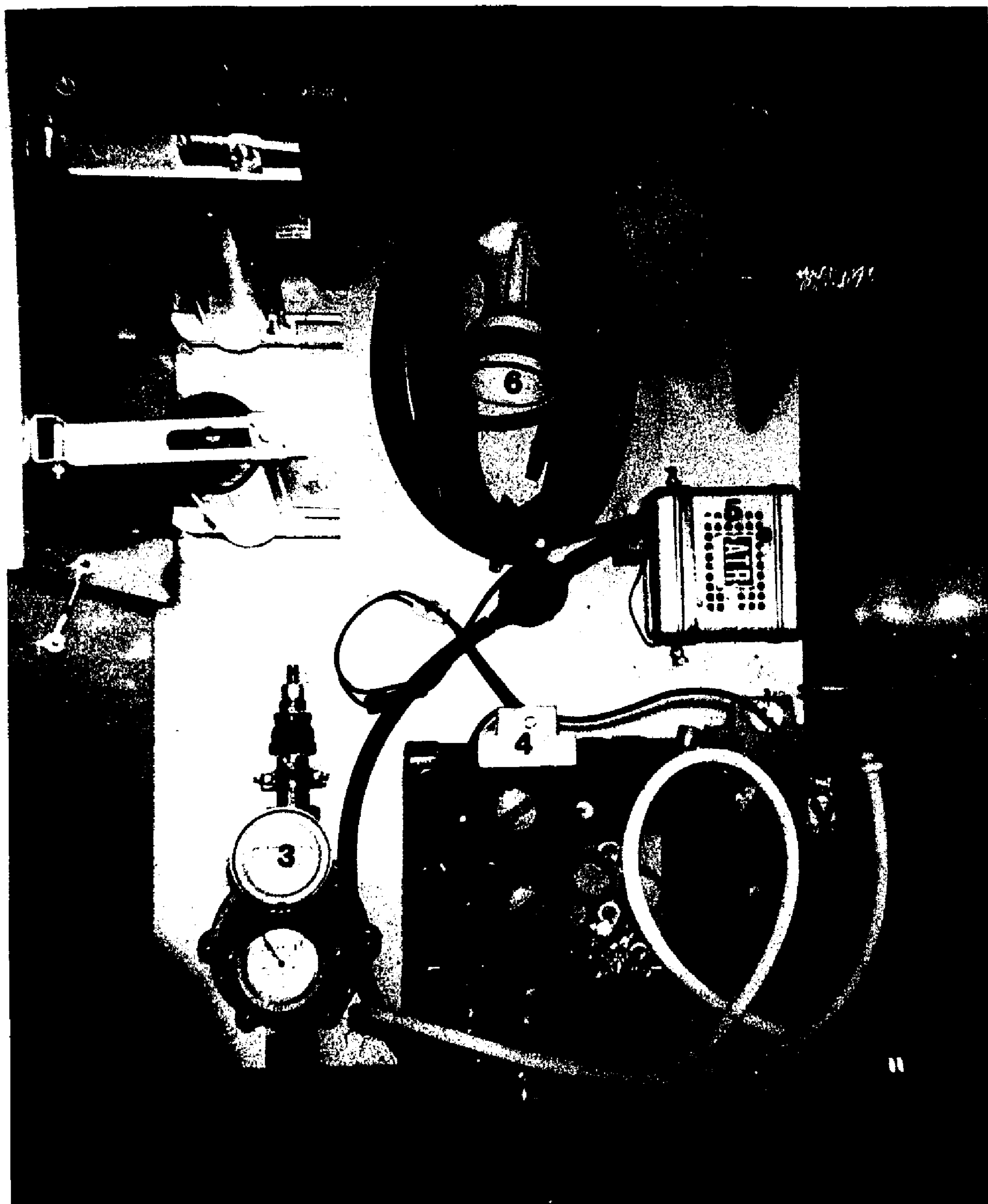


Plate 2. Initial flow through sampler showing: (1) pump, (2) collecting columns, (3) meter, (4) field power supply, (5) inverter, (6) intake hose and funnel, (7) crank for lowering intake.

RESULTS

Virus Collection

Removal of poliovirus 1 from water passing through columns of test substances was used as a means of screening the effectiveness of substances as potential virus collecting surfaces. The results of a series of tests are given in Table 1.

All substances tested were shown to remove virtually all poliovirus added exogenously to water. With the exception of bentonite and quartz, variations in pH had no effect upon the efficiency of virus removal. Examination of exchange resins showed greater variation in virus removal, depending upon resin type and pH of the water. The results are given in Table 2.

Anion exchangers were more effective than their cationic counterparts, removing at least 94% of virus. Anionic exchangers also were influenced less by pH changes. Diethylaminoethyl cellulose (DEAE), Sephadex and activated charcoal all removed virus effectively. Shredded gauze was equally effective in removing virus provided pH values approaching 8.5 were not in existence. Virus removal by gauze was influenced by both column height and sample flow rate. The longer column was more effective for virus removal regardless of the degree of packing obtained. Efficiency of virus removal declined as sample flow rates were increased from gravity flow to 1000 ml/ minute. The data is shown in Table 3.

TABLE 1

The Removal of Poliovirus Added Exogenously to Water by Test Substances

Test Substances	Virus* Input	Virus in Effluent	% Virus Removal
Bentonite	56200	316.0	94+
Illite	56200	100.0	98+
Kaolinite	56200	46.4	99+
Quartz	56200	81.0	99+
Feldspar	56200	46.4	99+
Gauze Impregnated with			
Bentonite			
6.5	56200	0	100
7.2	56200	0	100
8.5	56200	6761.0	88
Illite			
6.5	56200	0	100
7.2	56200	0	100
8.5	56200	144.0	99+
Kaolinite			
6.5	56200	0	100
7.2	56200	0	100
8.5	56200	0	100
Quartz			
6.5	56200	0	100
7.2	56200	0	100
8.5	56200	8100.0	85+
Feldspar			
6.5	56200	0	100
7.2	56200	0	100
8.5	56200	676.1	98+

*Virus in tissue culture infective dose ($TCID_{50}$).

TABLE 2

The Removal of Poliovirus Added Exogenously to Water by Test Substances

Test Substances	Virus Input	Virus in Effluent	% Virus Removal
Shredded Gauze			
pH 6.5	562000	56.2	99
7.2	562000	0.0	100
8.5	562000	4640.0	82
DEAE	562000	464.0	92
Sephadex	562000	676.1	98
Activated charcoal	562000	0.0	100
Amberlite IRC-50			
6.5	112400	67610	40
8.0	112400	100000	11
Dowex 50W-X4			
6.5	112400	10000	91
8.0	112400	100000	9
Rexyn AG3			
6.5	112400	810	99
8.0	112400	6761	94
Dowex 2-X4			
6.5	112400	67.61	99
8.0	112400	0	100

TABLE 3

THE INFLUENCE OF COLUMN HEIGHT AND SAMPLE FLOW RATE

UPON REMOVAL OF POLIOVIRUS FROM WATER

Column 1 (4.5x10 inches)	Column 2 (2x12 inches)	Flow Rate
75*	--	gravity fed
50	--	gravity fed
--	18.4	100 ml/minute
--	5.8	1000 ml/minute

*Expressed in per cent recovery of virus

As a special example of particulate matter, virus, as compared to bacteria was not dependent primarily upon the porosity of gauze columns. This was shown when suspensions of Escherichia coli and poliovirus 2 were passed through gauze columns. Less than 1% of the bacteria were removed compared to 20% of the virus. The data obtained is shown in Table 4.

After the virus collecting capabilities of the test adsorbents had been determined, the ability to recover virus from a collecting surface was examined. It was quickly determined that the case of elution of virus from a collecting surface did not parallel ease of adsorption. For example, when shredded gauze columns impregnated with kaolinite were used for removal of virus from water, 99% removal could be expected. Upon elution of these columns with phosphate-buffered solutions, only 2.4% of the adsorbed virus could be recovered. In the case of activated charcoal, while virtually all virus was adsorbed, none could be recovered following elution with phosphate buffers ranging from 0.01 M to 0.4 M. A similar result was obtained with Dowex 2-X4. As much as 99% of virus was removed from water, but elution with 1 M and 2 M NaCl yielded less than 1% virus recovery. The data is shown in Table 5.

Adsorption of Virus by Magnetic Iron Oxide

Elution of virus from magnetic iron oxide consistently adsorbing greater than 99% of virus from water was attempted in vertical columns and horizontal thin layers held in Millipore filter apparatus. Recovery of adsorbed virus from columns was poor. Elution of virus from thin layers was

TABLE 4

THE REMOVAL OF *ESCHERICHIA COLI* AND POLIOVIRUS
FROM WATER BY MEANS OF
SHREDDED GAUZE COLUMNS IN SAMPLER DEVICE

	INPUT	COLUMN 1	COLUMN 11	EFFLUENT
Virus	1320	17640	4410	978
Bacteria	2×10^{12}	3	700	5500
Per Cent Removal				
Virus	-	16.6	4.2	74.1
Bacteria	-	<0.001	<0.001	0.005

TABLE 5

ELUTION OF VIRUS FROM COLLECTING SURFACES

DISPLAYING HIGH EFFICIENCY OF VIRUS ADSORPTION

Trial	Collecting Surface	Adsorptive Efficiency	Elution Efficiency
Column	Kaolin	99.0**	2.4
Millipore*	Kaolin	96.9	14.1
Column	Dowex 2 x 4	94.0	0.3
Millipore	Dowex 2 x 4	21.0	4.5
Column	Activated charcoal C190A	99.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.0
Column	Activated charcoal C190N	100.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.0
Column	"	100.0	0.1

* Millipore apparatus

** Adsorption efficiency and elutive efficiency in percent

more successful. Minimal essential medium (MEM) and 3% beef extract were used as eluting solutions. Static and shaking conditions were examined for their ability to promote elution. The results of screening tests on elution are shown in Table 6.

Deployment of magnetic iron oxide in columns did not influence adsorption but elution of virus from these columns was poor. Elution of virus from thin "sandwich" layers held in filter holders was more successful. Shaking eluent and iron oxide to promote elution was not successful. When beef extract was used as an eluent, virus recoveries from iron oxide improved greatly.

The use of thin layers of magnetic iron oxide held in filter holders to recover enteric viruses exogenously added to water was studied in a series of repetitive tests to determine the value of the adsorbent and the technique followed. The results of these tests appear in Table 7. Virus recoveries were considered very good, regardless of enteric virus type used. Passage of water in liter volumes through iron oxide layers containing adsorbed virus failed to cause dislodgement of virus.

<u>Adsorption Curve of Virus to Magnetic Iron Oxide</u>

A constant amount of virus ($5.0x10^7$ $TCID_{50}$) was added to increasing amounts of magnetic iron oxide to obtain an adsorption curve. The amount of virus collected on the iron oxide increased rapidly from 2.5 mg/ml to 10 mg/ml iron oxide. From 10-20 mg/ml of iron oxide, a plateau was reached, after which essentially 99-100% of the virus was collected by the magnetic iron oxide

TABLE 6

INFLUENCES AFFECTING ELUTION OF VIRUS ADSORBED TO MAGNETIC IRON OXIDE

Iron Oxide Treatment	Elution Procedure	% Elution of Virus
Gravity column	3% beef extract	0.0
Flow-through sampler	3% beef extract	0.0
Millipore apparatus	Minimal Essential Media (MEM)	25.0
Millipore apparatus	MEM + shaking	0.0
Millipore apparatus	MEM + shaking	8.0
Millipore apparatus	3% beef extract	94.9

TABLE 7

THE RECOVERY OF ENTERIC VIRUS FROM WATER USING MAGNETIC IRON OXIDE AND BEEF EXTRACT BROTH

Trial	Test Virus	Water Samples	Initial	Eluate	Per Cent Recovery
1	Poliovirus I	Distilled Water	2.6×10^{7}	2.5×10^{7}	96.1
2	Poliovirus I	Distilled Water	1.1×10^{8}	9.1×10^{7}	82.7
3	Poliovirus I	Distilled Water	1.0×10^{8}	8.3×10^{7}	83.0
4	Poliovirus I	Distilled Water	1.0×10^{7}	8.4×10^{6}	84.0
5	Poliovirus I	Distilled Water	2.3×10^{6}	1.85×10^{6}	80.6
6	Poliovirus I	Distilled Water	2.16×10^{8}	2.08×10^{8}	96.3
7	Poliovirus I	Distilled Water	2.9×10^{8}	2.5×10^{7}	86.2
8	Poliovirus I	Distilled Water	6.6×10^{7}	6.95×10^{7}	105.3
9	Poliovirus I	Distilled Water	1.7×10^{8}	1.5×10^{8}	88.2
10	Poliovirus I	Distilled Water	6.95×10^{7}	6.6×10^{7}	94.9
11	Echovirus 6	Distilled Water	2.15×10^{8}	2.1×10^{8}	97.6
12	Coxsackievirus B-3	Distilled Water	2.26×10^{6}	1.8×10^{6}	79.9
13	Poliovirus II	Distilled Water	6.9×10^{7}	6.5×10^{7}	94.2
14	Poliovirus I	Tap Water	6.6×10^{7}	6.2×10^{7}	93.9
15	Poliovirus I	Oyster River Water	1.7×10^{8}	1.5×10^{8}	88.2

surfaces. Results are given in Figure 1.

Adsorption of virus to magnetic iron oxide was influenced by pH. Beginning at pH 6.0, the amount of adsorbed virus increased, reaching a maximum value around 9.0, then decreasing rapidly thereafter. The data is shown in Figure 2.

The Effect of Salts on the Iron-Virus Complex

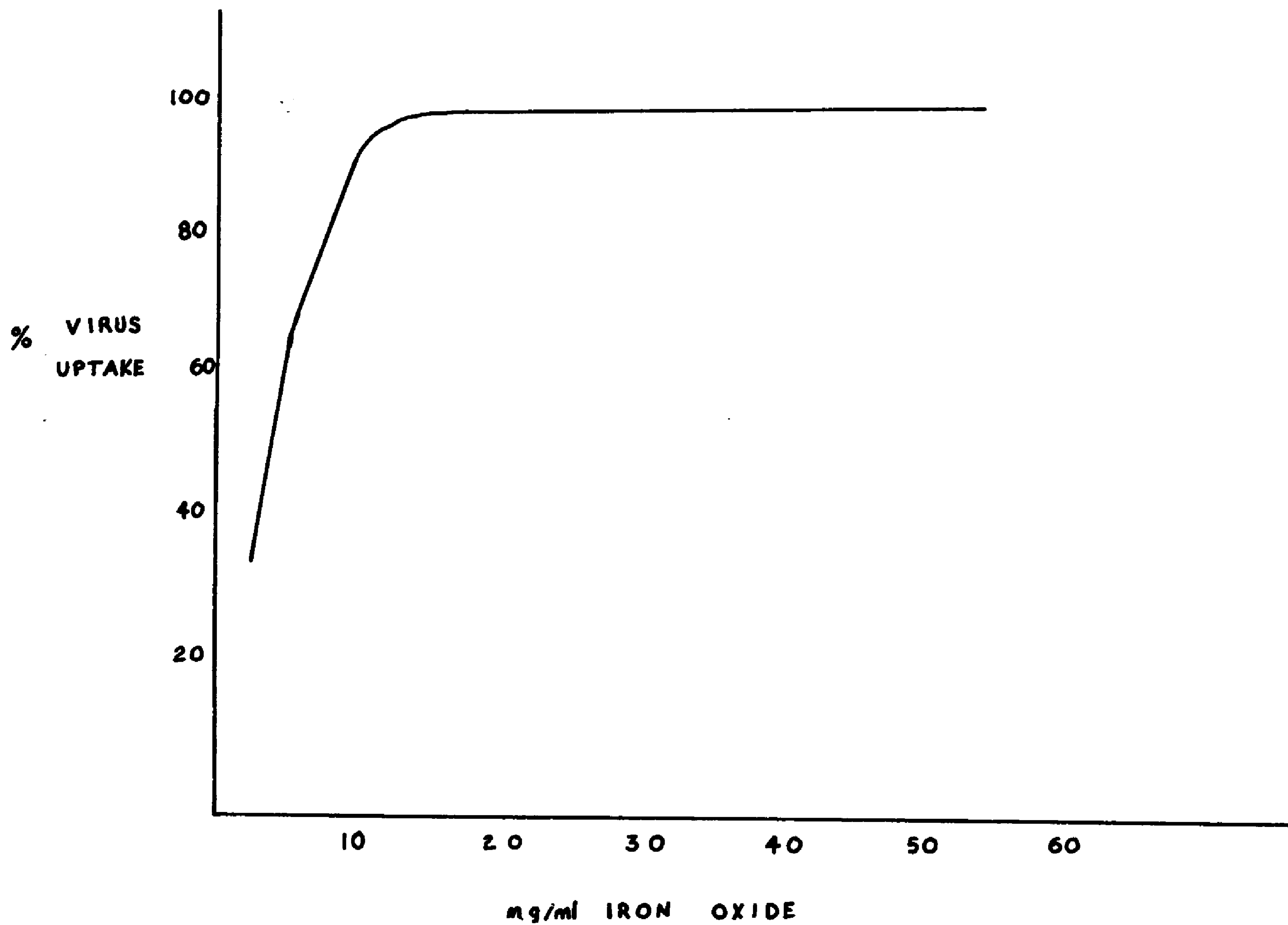
The effect of NaCl on the iron-virus complex was studied between molarities of 0.2 M and 2.0 M. No dissociation of virus and magnetic iron oxide was demonstrated. The effect of a divalent cation was investigated with $MgCl_2$ ranging from 0.6 M to 2.0 M. No effect on dissociation was observed. Several frozen samples were treated with NaCl and $MgCl_2$ at pH 8.5. No dissociation occurred. Samples treated with distilled water at pH 8.5 failed to cause dissociation.

Collection of Bacteriophage and Bovine Serum Albumin by Magnetic Iron Oxide

Addition of bovine serum albumin to magnetic iron oxide in increasing amounts up to 1200 micrograms did not significantly alter adsorptive values. *Escherichia coli* 9637 bacteriophage was adsorbed in a manner similar to enteric virus. Elution of bacteriophage following adsorption was not successful. The data is given in Table 8.

Substances Interfering with Adsorption and Elution of Virus

Proteins interfered with the adsorption of virus to magnetic iron oxide. The individual components of beef extract were studied for their ability to interfere with or antagonize virus adsorption. All components examined except proteose antagonized the adsorption process. Similarly


ADSORPTION OF VIRUS TO IRON OXIDE
100
80
% VIRUS UPTAKE
60
40
20
10
20
30
40
50
60
mg/ml IRON OXIDE

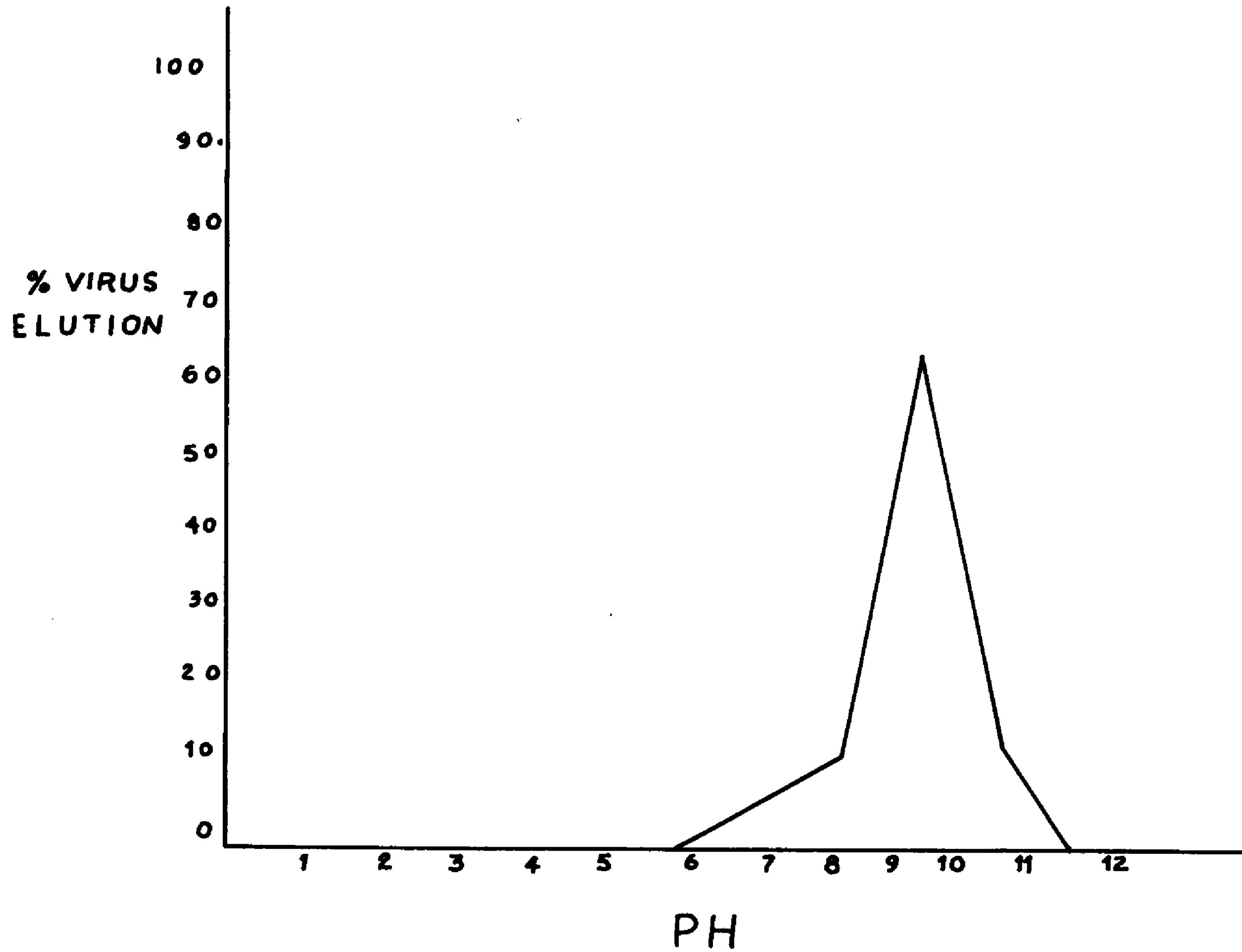

EFFECT OF PH ON THE
ADSORPTION OF VIRUS
% VIRUS
ELUTION
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
PH

TABLE 8

THE ADSORPTION OF *ESCHERICHIA COLI* BACTERIOPHAGE AND BOVINE SERUM ALBUMIN

BY MAGNETIC IRON OXIDE

Bacteriophage			Bovine Serum Albumin		
Initial Number	Number Adsorbed	% Adsorption	Amount Added	Amount Adsorbed	% Adsorption
6.6×10^{7}	9.2×10^{6}	14.4	600	595	99.23
4.0×10^{7}	3.9×10^{7}	98.7	1000	960	96.20
			1200	1143	95.30

all except proteose caused some elution of virus. Beef extract, in concentrations equal to the components studied, exhibited greater antagonism towards virus adsorption than any individual components. It also caused elution of substantially more virus than any of the individual components. Results of the study are given in Table 9.

Peptone and high pKa amino acids were effective antagonists of virus adsorption. They also caused substantial virus elution from magnetic iron oxide. Creatinine, purines, pyrimidines and vitamins were less effective both as antagonists of virus adsorption and inducers of virus elution. Basic amino acids and vitamins fell more or less in the same category.

The correlation found between antagonistic and elutive activities of beef extract components indicated virus adsorption and elution to be different sides of the same coin. If receptor sites could be considered involved in the adsorptive process, they apparently could also be considered important to virus elution.

A spectrum of organic substances with functional amino nitrogen groups were screened for viral inhibitory or competitive properties. Streptomycin failed as both antagonist and elution inducer. Spermine was a moderately effective antagonist as well as eluting substance. Glucosamine was a moderately effective antagonist, but poor elution inducer. Glucose neither antagonized nor eluted virus. The data is presented in Table 10.

Since the alpha amino and carboxyl groups of the amino acids encountered in the protein coat of poliovirus 2 are committed to the peptide bond, it was assumed that the side

TABLE 9

THE INFLUENCE UPON VIRUS ADSORPTION AND ELUTION SHOWN BY

COMPONENTS OF BEEF EXTRACT WITH MAGNETIC IRON OXIDE

Component	Adsorption Antagonism	Elution Effectiveness (log virus)
3% creatinine	$10^{1.0}$	$10^{2.10}$
3% creatine	$10^{4.0}$	$10^{1.50}$
Sat. purines + pyrimidines	$10^{2.50}$	$10^{1.52}$
3% high pKa amino acids*	$10^{3.33}$	$10^{3.52}$
3% basic amino acids	$10^{2.60}$	$10^{2.33}$
3% vitamins	$10^{1.54}$	$10^{2.30}$
3% peptone	$10^{3.61}$	$10^{3.52}$
3% proteose	$10^{3.54}$	0.0
3% beef extract	$10^{3.65}$	$10^{4.26}$

*glycine, alanine, valine, and proline

TABLE 10

THE EFFECT OF NITROGENOUS SUBSTANCES UPON VIRAL ANTAGONISM AND ELUTION WITH MAGNETIC IRON OXIDE

Substance	Adsorption Antagonism (log virus)	Elution Effectiveness (log virus)
3% basic amino acids	$10^{2.60}$	$10^{2.33}$
Streptomycin 60 mg/ml	0.0	0.0
3% spermine	$10^{2.75}$	$10^{1.55}$
2% FCS in EBSS	$10^{4.0}$	$10^{4.33}$
MEM 20% FCS	$10^{4.0}$	$10^{4.65}$
3% purines + pyrimidines	$10^{2.50}$	$10^{1.52}$
3% uracil + Xantheni	$10^{2.65}$	$10^{2.78}$
3% high pKa amino acids*	$10^{3.33}$	$10^{3.52}$
3% Glucosamine	$10^{3.6}$	$10^{1.75}$
3% glucose	0.0	0.0

*glycine, alanine, valine, proline.

groups of the amino acids were involved in the adsorption of virus to magnetic iron oxide. The amino acids present in poliovirus 2 are shown with respective pK and IDE values in Table 11. Changes in charge due to pH change are presented in Table 12.

Only 25% of the sixteen amino acids in the protein coat underwent significant changes in charge from pH 5 of adsorption to pH 9 of elution. Cysteine became negative, histidine lost positivity and the distal amino groups of both arginine and lysine became less positive.

Magnetic iron oxide is thought to serve as an acceptor through its docking sites (OH^- or OH) for either ammonium (NH_3^+) or sulfhydryl groups of the protein coat at pH 5. When the pH is raised to pH 9 for elution, the ammonium becomes an amino group and the sulfhydryl a sulfide radical. Thus dissociation of the ferro-virus complex occurs. The donor and acceptor molecules are presented in Table 13.

Hydrogen bond breakers, urea and triton 100x were toxic in all cell culture systems used and their possible effect upon adsorption or elution could not be determined. Versene (EDTA) treatment of virus adsorbed to magnetic iron oxide led to virtually 100% elution at pH 9.0. The parallel effect by a chelating agent upon elution of virus from iron oxide and release of cells growing upon glass or plastic surfaces pointed to the possibility of cationic or cation-influenced bonding forces occurring in both phenomena.

TABLE 11

PROFILE OF pK AND IED VALUES OF THE AMINO ACIDS IN POLIO VIRUS TYPE II

Amino Acid	pK	pK_2	pK_3	IED
glycine	2.35	9.78		6.1
aspartic	2.09	3.87	9.82	3.0
glutamic	2.19	4.28	9.66	3.2
cysteine	1.96	8.18 SH	10.28	5.07
methionine				
phenylalanine	1.83	9.13		5.48
tyrosine	2.20	9.11	10.1 (OH)	5.7
alanine	2.34	9.69		6.00
valine	2.32	9.62		5.96
isoleucine	2.36	9.68		6.02
proline	1.99	10.60		6.30
threonine				
serine	2.21	9.15		5.68
arginine	2.02	9.04	12.48(guanido)	10.8
histidine	1.77	6.10 (imidazole)	9.18	9.7
lysine	2.18	8.95	10.53 E NH_3	9.7

Reprinted from White, Handler, and Smith Principles of Biochemistry.

TABLE 12

THE AMINO ACIDS OF POLIO VIRUS AS RELATED TO ADSORPTION AND ELUTION

Polio Virus pH 5.0	
Glutamic	COO^-
Aspartic	COO^-
Threonine	$CH-CH_3$ OH
Serine	CH_2OH
Cysteine	CH_2SH
Methionine	$S-CH_3$
Phenalalanine	CH_2 - (ring)
Tyrosine	CH_2 (ring) -OH
Glycine	H
Alanine	CH_3
Valine	$CH-CH_3$ CH_3
Isoleucine	R
Proline	NH
Lysine	$NH_3^+-(CH_2)_4-CH(NH_3^+)-COOH$
Arginine	$NH_3^+-C(=NH)-NH-(CH_2)_3-CH(NH_3^+)-COOH$
Histidine	(imidazole ring)-R, ^+N, NH

Polio Virus pH 9.0	
Cysteine	S^-
Histidine	(imidazole ring)-R, N, NH
Lysine	$NH_2-R-CH(NH_2)-COOH$
Arginine	$NH_2-R-CH(NH_2)-COOH$

TABLE 13

AMINO ACID FUNCTIONAL GROUPS INVOLVED IN HYDROGEN BONDING

Any donor may hydrogen-bond to any acceptor, and vice versa; all combinations are possible, provided only that the bond angles are appropriate for hydrogen bond formation.

Donors	Acceptors
amide hydrogen	amide carbonyl oxygen
carboxylic acid	carboxylic acid and carboxylate
-OH (serine threoine)	-OH
ammonium	amino
phenol	phenolate and phenol
quanidinium	quanidate
imidazolium	imidazol
sulfhydryl	sulfide

Reprinted from Introduction to Biophysical Chemistry by R. Bruce Martin, McGraw-Hill Book Co., New York, N.Y.

X-ray Diffraction Analysis of Magnetic Iron Oxide

An X-ray diffraction pattern of magnetic iron oxide was done and the raw material found to be a gamma spinel of iron oxide (Fe_2O_3) with traces of cubic Fe_3O_4 present. The results of this may be seen in Table 14. The X-ray pattern is shown in Plate 3 and the structure of magnetic iron oxide is depicted in Figure 3. It was suggested that an emission spectra be done for other atomic species that might occur in magnetic iron oxide. The following elements were found in moderate concentrations in terms of parts per million: Si, Cr, and Cu. Larger amounts of Co, Mn, Ni, Zn and Mo were also defined, but were still present in the order of parts per million. The data is given in Table 15.

The unit cell of the gamma spinel was composed of 22 iron atoms distributed randomly among 8 tetrahedra and 16 octahedra. A total of 32 oxygen atoms were in the basic unit, and other metals could substitute randomly in the lattice to replace a small number of the iron atoms as may be seen in Tables 14 and 15. When dry iron oxide was added to water, dissolution of the unit lattice occurred and hydrated iron complexes resulted.

Electron Micrographs of Magnetic Iron Oxide and Poliovirus

Magnetic iron oxide was examined in the electron microscope and found essentially to be a long pleomorphic rectangle with a marked tendency for aggregation which masked the true topography of the basic unit. An easily defined complex is shown in Plate 4.

TABLE 14

X-RAY DIFFRACTION POWDER PATTERN OF MAGNETIC IRON OXIDE

Line	Rel. Int.	Film Readings	0	20	d	Gamma Fe_2O_3	REMARKS	Cubic Fe_3O_4
1	w	170.5	9.5	19.0	5.87	5.95_6		
2	w	172.6	11.6	23.2	4.82	4.84_4	4.85_4	
3	w - m	176.0	15.0	30.0	3.74	3.75_x		
4	w	177.4	16.4	32.8	3.43	3.42_7		
5	w	178.3	17.3	34.6	3.26	3.22_3		
6	m - s_3	180.2	19.2	38.4	2.95	2.95_x+	2.97_7	
7	m_4	181.4	20.4	40.8	2.78	2.80		
8	s_1	183.6	22.6	45.2	2.52	2.52_x+	2.53_x	
9	vw	184.6	23.6	47.2	2.42		2.42_1	
10	w	185.9	24.9	49.8	2.30			
11	m_4	188.7	27.7	55.4	2.08	2.09_x+	2.10_7	
12	vw	192.0	31.0	62.0	1.88			
13	vw	193.1	32.1	64.2	1.82	1.82_2		
14	w	194.0	33.0	66.0	1.78			
15	w - m	195.5	34.5	69.0	1.71	1.70_x+	1.71_6	
16	w	197.4	36.4	72.8	1.63			
17	m - s_3	198.1	37.1	74.2	1.61	1.61_x+	1.61_8	
18	s_2	202.0	41.0	82.0	1.48		1.48_8	

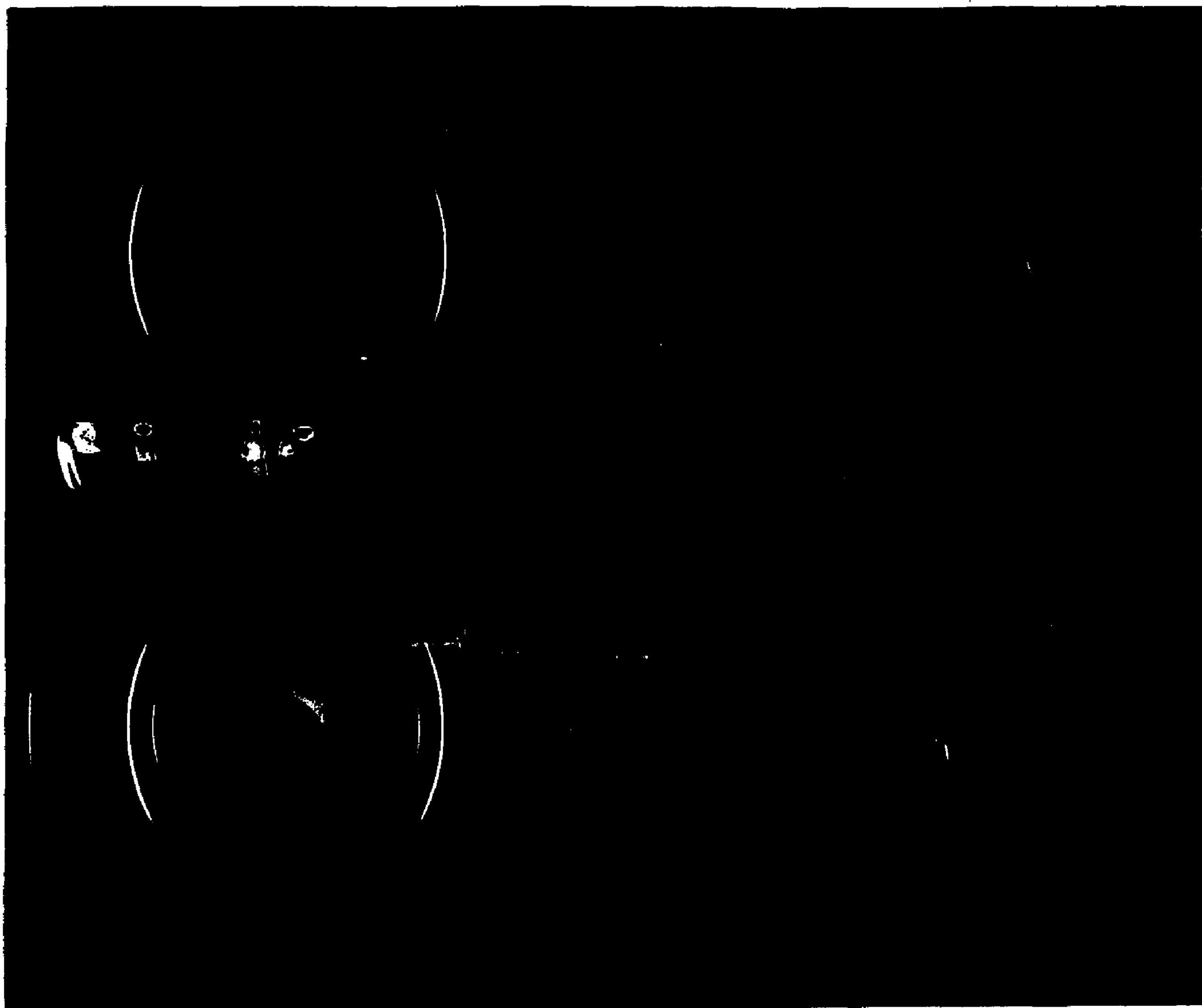


Plate 3. X-RAY DIFFRACTION POWDER PATTERN OF MAGNETIC IRON OXIDE

HYDRATED IRON OXIDE

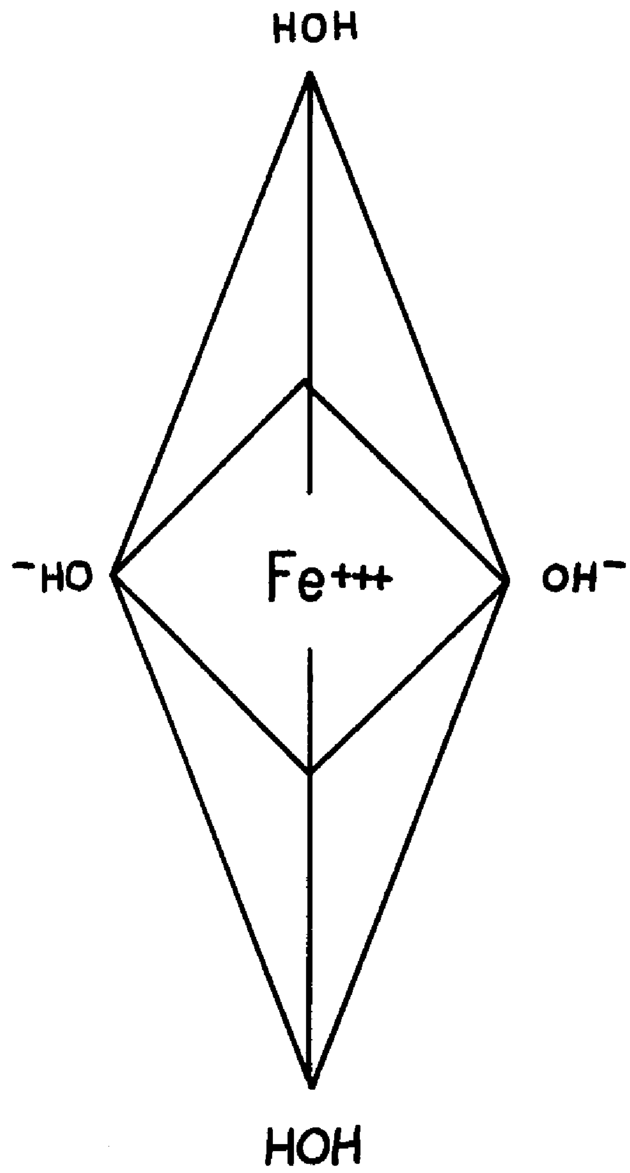


TABLE 15

THE OCCURRENCE OF ATOMIC SPECIES IN MAGNETIC IRON OXIDE

Species	Relative amount present
Si	Medium
Ci	Medium
Co	Large
Ac	Medium
Mn	Large
Ni	Large
Zn	Large
Mo	Large

Relative amounts present represent relative amounts in terms of parts per million.

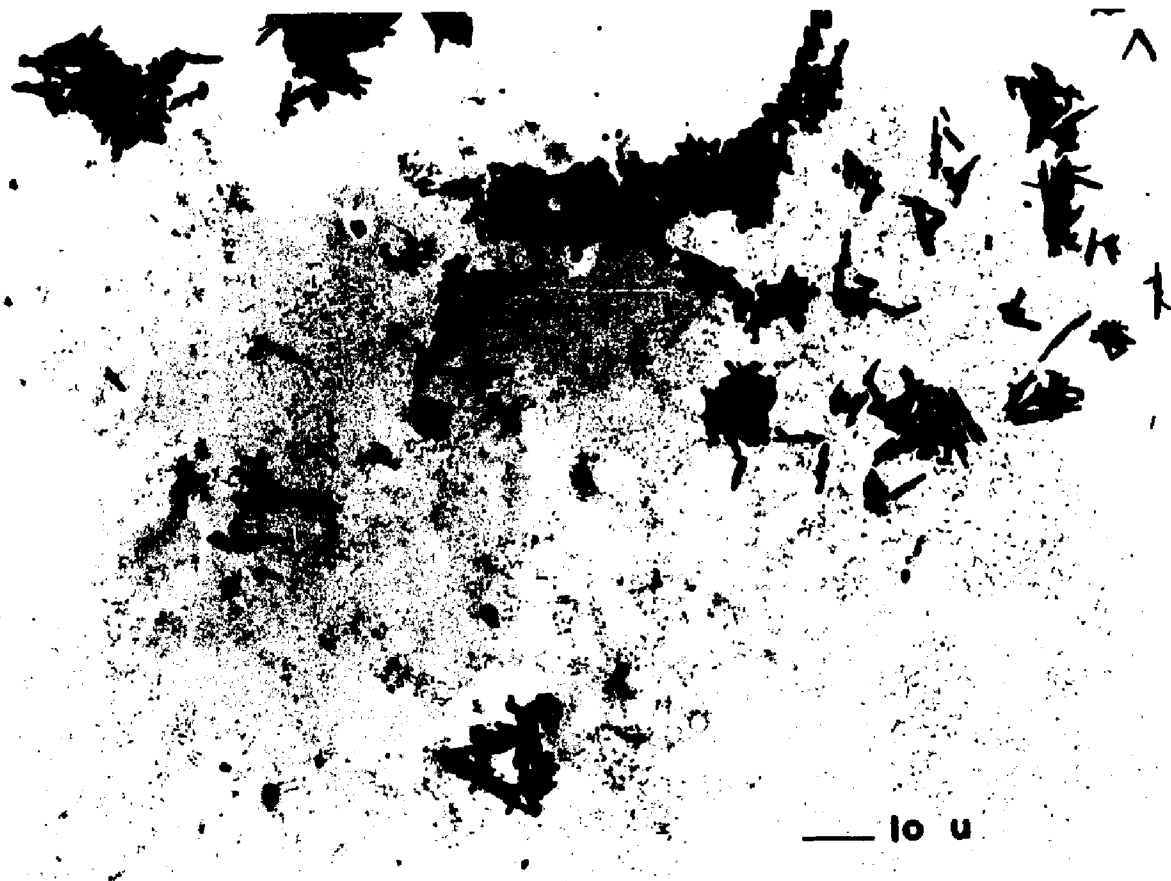


Plate 4. ELECTRONMICROGRAPH OF MAGNETIC IRON OXIDE.

(X 80,000)

poliovirus 2, purified by density gradient centrifugation to a titer of 1×10^{9} $TCID_{50}$ / ml, is shown in Plate 5. The electron micrograph showed a typical enterovirus structure with a size of approximately 25mμ.

Recovery of Poliovirus Added Exogenously to Seawater

Artificial seawater (Instant Ocean) was diluted to give salinities from 10 to 35 parts per thousand (^{o}/oo) in four solutions: 10, 17, 26 and 35°/oo. Virus was added exogenously to each solution and the amount possible to recover following adsorption and elution processes with thin layers of magnetic iron oxide determined. The results are given in Table 16. Adsorption of virus in the presence of salt was excellent. In only one instance was virus detected in the effluent, and then the numbers were quite small. Elution of virus was accomplished with good yields achieved. No significance was attached to fluctuations in amounts of virus recovered in iron oxide eluates.

Plate 5. ELECTRONMICROGRAPH OF PTA STAINED POLIOVIRUS 2.
(X 295,000)

TABLE 16

RECOVERY OF POLIOVIRUS ADDED EXOGENOUSLY TO SEAWATER BY MAGNETIC IRON OXIDE

Salinity (PPT)	Input ($TCID_{50}$)	Effluent ($TCID_{50}$)	Eluate ($TCID_{50}$)	Recovery (%)
10	5.6×10^6	0.0	4.0×10^6	71.5
17	6.3×10^6	0.0	6.25×10^6	99.0
26	3.5×10^6	0.0	2.5×10^6	71.4
35	1.0×10^7	3.9×10^4	6.7×10^7	67.3

DISCUSSION

Quantitative enumeration of virus recovered from surface waters depends upon presentation of a virus collecting surface to which virus will adsorb, followed by removal of virus from the adsorbent. To be successful, the collecting surface initially must demonstrate a high order of attraction for virus, then be persuaded to release all virus previously attracted. These functions must be capable of selective execution in the face of a wide variety of biologic and chemical pollutants found in surface waters, in addition to silts and other suspensoids occurring naturally in these waters. The virus collection process presumes the occurrence of viruses in surface waters in low numbers, necessitating a high order of concentration. A flow-through collection system was sought in the study, permitting the sampling of large volumes of water. A number of virus collecting surfaces were found to be of potential promise for removal of virus from water. Very few of the same surfaces were found to release virus in numbers necessary for their successful adaptation to a flow-through system.

Magnetic iron oxide was found to give reproducible adsorption and elution of virus when used in thin horizontal layers. These processes were notably influenced by pH. Adsorption was favored by a pH of 3 - 5, while elution was most efficient at a pH of 9. Although marked differences in the individual response of different substances to the processes of adsorption and elution occurred, the patterns of response to pH changes were similar. Virus adsorption by magnetic iron oxide was most

successful when the adsorbent was arranged in thin layers. The efficiency of the adsorptive process was shown by the degree of success of virus recovery from samples passing through the iron oxide layer at relatively high flow rates. For elution an amino acid-rich substance such as beef extract, nutrient broth or proteose was required. The behavior of magnetic iron oxide in adsorption and elution phenomena was similar with each of four enteroviruses examined. Bacteria, bacteriophage and bovine serum albumin were all collected by iron oxide, leading to the belief that most biological materials of an appropriate size and charge would be adsorbed.

Elution and antagonism were studied with the idea of determining the kinds of molecules attracted to the collecting surface. Creatine was much more antagonistic to viral adsorption than creatinine. Creatine possesses an amino group not present in creatinine. It furthermore would be positively charged at an acid pH, much like the extra amino groups on the side chains of lysine and arginine and diamino-monocarboxylic acids found in poliovirus coat protein. Purines and pyrimidines competed for available combining sites on iron oxide, as might be expected. Neither was particularly impressive in this respect. By comparison peptone and proteose were active antagonists. Each is rich in basic components, particularly peptone. The amino acids glycine, alanine, proline and valine exhibited marked capability to elute as well as antagonize. This was considered further evidence for the involvement of amino acid nitrogen in the attractive force operating between virus and

magnetic iron oxide. This hypothesis was based on the fact that the positive charge on the NH_3^+ group will be held through an alkaline pH, leading to elutive as well as antagonistic activity in the ferro-virus system. Basic amino acids were not spectacular in antagonizing adsorption or inducing elution. This was not unexpected. The imidazole of histidine has a pK of 6.10 signifying that during adsorption of virus at a pH of 5.6, the histidine residue would not exhibit its full capacity to antagonize virus adsorption. Nor could it exert strong elutive action since all of its positive charge would have vanished at the pH of elution. With histidine representing 33% of the amino acid content of the basic amino acid mixture tested, it was reasonable to assume the high pKa amino acids would prove more active eluters. Application of this principle to vitamin mixtures failed to be substantiated in practice. Beef extract eluted virus more efficiently than any of its components tested singly. As an antagonist, however, it was surpassed by creatine and rivaled by peptone.

Careful examination of beef extract component activity strengthened the idea that amino nitrogen was responsible for the action shown. Serum protein represented amino acids in peptide bonds with effective side groups ionized presumably much like amino acids in virus coat protein. Glucosamine had elutive and antagonistic activities while glucose had neither.

The amino acid content of poliovirus has been examined by Levintow (1960). In an attempt to account for changes

taking place in the protein coat over the pH range 5 to 9, the amino acids were studied individually to account for changes that might be expressed in the behavior of the protein coat. Changes in alpha amino groups and respective carboxyl groups were ignored due to their commitment to the peptide bond. Essentially only the side groups of the amino acids were thought to be important in the adsorption phenomenon. There were sixteen amino acids in the protein coat of the poliovirus, all of which occurred in different frequencies and of these amino acids only 25% of them underwent serious changes in charge from pH 5 (adsorption) and pH 9 (elution). Cysteine became negative ($SH \rightarrow S^-$), histidine became less positive ($N^+ \rightarrow N$) and the distal amino groups of both arginine and lysine became less positive ($NH_3^+ \rightarrow NH_2$).

A covalent bond between the oxide and the virus was impractical to consider as 350k cal/mol were required to break the association between the virus and the oxide. A simple exchange was not thought to be responsible for binding the virus to the oxide such as is encountered in ion exchange due to the inability of a number of salts to either antagonize the collection of virus or induce some elution of virus from the resin. Van der Waals forces were not thought to be involved in the association of the virus with magnetic iron oxide as surface area over which the binding takes place is relatively small in relation to the total size of the virus. Therefore, not enough area is involved to create a solid binding such as is encountered in the system.

Hydrogen bonding may be responsible for the association that virus maintains with magnetic iron oxide. It was impossible to substantiate or refute hydrogen bonding empirically as the agents employed to break hydrogen bonds between the virus and oxide were either toxic to the tissue culture system employed or the virus itself. There is ample opportunity for hydrogen bonding to occur to the iron hydrate complex through the epsilon amino group of lysine, the terminal amino group of arginine or the histidine residue as well as other side groups of the amino acids encountered in the protein coat of virus.

A possible explanation of the association of virus with magnetic iron oxide is through hydrogen bonding which is established between the hydrated portion of the iron complex and any one of the side groups of the protein coat of the virus. Such species as sulfhydryl, phenolic, imidazolium and quanidinium could be involved. Emphasis is placed on a significant role being played by the basic amino acids and cysteine in the collection process since the side groups of these molecules reflect a change in the charge in going from pH 5 (adsorption) and pH 8.5 (elution).

Recovery of virus from seawater using magnetic iron oxide indicated the salt content of seawater did not interfere greatly with the processes of adsorption and elution. No salt enhancing requirement for virus adsorption was shown in the study, and the similar recoveries of virus from each of four solutions of varying salinity did nothing to further the idea that effectiveness of adsorption might be increased in the

presence of salt.

The data obtained in the study strongly suggested that virus adsorption to a collecting surface was governed by electrostatic forces and elution could be accomplished by reversal of these forces. Adsorption was furthered by any influence increasing the electrostatic potential between virus and collecting surface, while elution occurred in the face of a decrease in this potential. The electrostatic potential developed with magnetic iron oxide layers was believed to be a function of the atomic structure of the hydrated iron oxide with four combing sites, and the ionization of amino acid structures. Adjustment of pH led to either furtherance or repression of ionization of amino acid structures, depending upon the pH value obtained with respect to the isoelectric point of the protein involved.

The success of virus recoveries obtained from water using magnetic iron oxide showed the feasibility of using iron oxide for quantitative enumeration of virus in surface waters. Of all the substances studied, magnetic iron oxide showed the greatest promise in laboratory trials. Some very practical problems require solutions before quantitative enumeration can be realized in the "real world". The two most important problems revealed in the study were the presence of organic substances interfering with virus adsorption, and a heavy load of particulate debris which clogged iron oxide layers and prevented maintenance of regular flow rates. Interference with virus adsorption by organic substances occurring in water

leads to loss of virus in effluents. Clogging of collecting surfaces by debris retarding or cutting off flow rates makes it impossible to sample volumes of water considered necessary in order to adequately concentrate virus occurring in small numbers. Failure to concentrate results in an inability to demonstrate small numbers of virus in cell cultures.

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