RAPID DETECTION OF ENTEROVIRUSES IN PUBLIC WATER SUPPLIES

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CHAPTER I

INTRODUCTION

Enteroviruses are present in natural waters in various amounts. The amount of virus present in the waters depends on population density, method of sewage treatment, and volume of the water body the sewage is disposed in.

The current standards for water quality and safety regarding microbial content are based on the bacterial content only. The number of bacteria is determined and a coliform test is done to check for fecal contamination. There are no standards for viral content and no tests are done to identify or quantitate viruses in water. Because viruses do not replicate outside the host cell, they do not numerically increase in water, and may even be reduced by environmental factors. Presence of viruses in low numbers and their small size render them difficult to retain after a sample is prepared for an assay.

The enteroviruses are a group of small, naked RNA viruses. They inhabit the gastrointestinal tract for a short period before they reach their target organs where they cause different diseases. Diseases caused by this group of viruses range from the common cold, idiopathic myocarditis, congenital heart disease to poliomyelitis. They are excreted with nasopharyngeal secretions and with the feces which may then contaminate bodies of water.

The main problem in assaying for enteroviruses in a water sample is the absence of a rapid, sensitive and inexpensive assay method for them. The method currently used is done by plaquing the virus in tissue cultures and then neutralizing the virus with specific antisera to determine the strain. The method is sensitive but takes from 48 hours to 7 days to show plaques for some strains of enteroviruses.

The goal of this study was to develop a radioimmunoassay method using tritium labelled antibody bound to a solid-phase for detecting enteroviruses. This would provide a simple method that does not involve the complexities of a plaque assay in tissue cultures. The assay could be done in a few hours instead of several days and with higher levels of precision.

CHAPTER II

LITERATURE REVIEW

2.1 General

The group of enteroviruses is composed of polioviruses, Coxsackie viruses, ECHO viruses, and reoviruses. They were grouped together in 1957 by the committee on enteroviruses because of their many similarities. The committee defined enteroviruses as transient inhabitants of the alimentary tract, although they may be found in the nasopharynx. They exist in multiple antigenic types; all those so far examined have the following properties: a diameter of 28 nm, ribonucleic acid core, ehter resistant, with cationic stabilization to thermal inactivation (2). The enteroviruses have a seasonal incidence, increasing in summer. They are harbored with greater frequency among those living under poorer socioeconomic and lower hygienic conditions (2). Some strains produce neural lesions in primates and rodents; others produce lesions in the muscle, pancreas and brown fat of newborn mice. Some have produced no illness in test animals (2). Most strains are cytopathogenic for primate cells, but several have not yet been cultivated in cell cultures. Although monkey kidney cell cultures are preferred for most enterovirus types, some strains are known which grow best or only in human embryo, amnion, or HeLa cell cultures. Others are more fastidious in regard to growth requirements than most and grow optimally at 33° to 34°C under slightly acidic conditions in rolled cultures. Some enteroviruses

possess specific haemagglutinins. They contain complement-fixing antigens which are specific when used with hyperimmune sera prepared in laboratory animals. Many of the enteroviruses cause diseases in man ranging from severe paralysis to myocarditis, skin rashes, and common colds, depending upon target organ attacked. Infection is usually at the subclinical level, however, with clinically manifest disease more common for some types than for others. Different enteroviruses, as well as viruses outside the group, may produce the same syndrome. On the other hand, the same enterovirus may cause more than a single syndrome. For these reasons clinical disease has not been considered as a satisfactory basis for classification (2).

The study of this group of viruses has been delayed due to the absence of an efficient and practical laboratory host for them. Landsteiner and Popper used the monkey as an experimental animal. They injected the monkey with bacteria free filtrate of the spinal cord from a human poliomyelitis patient. However, this method was expensive, cumbersome and limited in the amount of data that could be obtained from it (16). Adaptation of poliovirus to the cotton rat by Armstrong represented a substantial step forward. The turning point came when Enders, Weller and Robbins showed that polioviruses could be isolated and readily propagated in cultures of non-neural human or monkey tissue (18) (47). The tissue cultures used were mixed human embryonic skin and muscle, human embryonic brain and human embryonic intestine (18). They also described the degenerative changes that occurred in cell cultures after infecting them with poliovirus (40). Dulbecco et al. (1954) (17) produced plaques of poliovirus on monolayer cultures of monkey kidney tissues and showed that the plaques

could be used for titration purposes and the isolation of pure lines of the virus.

2.2 The Problem of Virus Transmission by Water

Viruses excreted in the feces and capable of producing infection when ingested should theoretically be transmissible by drinking water. Infectious hepatitis has been the only viral disease which is known to be a water-born disease (36). This does not exclude the possibility of transmission by the water route of other viral diseases.

It is difficult to determine the role of water as a vehicle in transmission of viral diseases due to several factors. Viruses are found in low numbers (as compared to bacteria) in the water because they normally multiply only within living cells. Also, the ingestion of small quantities of virus by relatively small numbers of people daily would result in disseminated illnesses that would produce an epidemiological picture consistent with person-to-person transmission (48).

The status of immunity of exposed population could markedly affect the likelihood of recognizing a water-born outbreak of virus disease. Obviously if a substantial proportion of the population were immunized, the number of water-born infections might be small and scattered and escape recognition. Furthermore, the magnitude of the ratio of unapparent infection to overt disease, characteristic for a given agent in a particular population, is significant. If only a small proportion of individuals exposed and infected by a given agent suffered overt disease, detecting an outbreak and tracing its source to potable water would be very difficult (21).

It has been observed that cases of poliomyelitis occurring in families most frequently cluster in a period shorter than the average incubation period, suggesting infection by the virus from a common source rather than transmission of the virus within the family itself (48).

The virus level in different water sources showed seasonal variation being greater in summer than in winter (29). The human enteric viruses have been recovered from 36 percent of the surface water samples examined. Underground water has also been shown to contain viruses (3).

Vaccine-like poliovirus is continuously shed by immunized persons. Through contamination of water supplies, it may cause infection in persons drinking the contaminated water (33).

2.3 Effects of Environmental Factors on Enteroviruses

The presence of particulate matter has its effect on the removal of poliovirus from natural waters. Enteroviruses adsorbed to clay particles and were then removed with them. However, in the presence of large amounts of organic matter in the water, inactivation of the viruses was inhibited by competition for adsorption sites (21). An adsorption of the virus to sand particles was not sufficient to produce a virus free filtrate (7). It has also been found that enteroviruses can be inactivated by certain bacteria. The protein coat of Coxsackie type A9 was degraded by proteolytic enzymes of animal and microbial origin. Poliovirus type I was found to be protease resistant but susceptible to Pseudomonas aeroginosa proteolytic action. These bacteria inactivate enteroviruses in natural water (24). Natural factors such as sunlight inactivated the virus in shallow waters (7). While in waters highly polluted by sewage, poliovirus had a longer survival time than in moderately polluted bodies

of water. Extremes of pH and temperature were also found to inactivate the enteroviruses (20) (7).

2.4 Basic Steps for Water Purification in a Municipal Purification Plant

Wastewater is sedimented in large tanks for a holding period where large particulate matter settles. Alum is added to flocculate precipitating material. The water is then passed through sand filter beds where 99 percent of the bacteria are removed. Chlorination of the water is done to a final residual dosage of 0.2 to 1.0 mg/liter of free chlorine. For natural waters penetrating through soil it undergoes filtration, there is no viral hazard as long as the soil strata are continuous and the usual public health practices of separation of wells and disposal systems were followed (20).

2.5 Sewage Treatment and Its Effect on Viral Content and Survival

The United States Environmental Protection Agency (EPA) has set the guidelines for the treatment of wastewaters. Authority for this approach was given in the 1970 Environmental Protection Act (45). This act requires that all wastewaters be treated to a technologically feasible level. Feasibility is construed to include economic considerations.

Treatment processes for wastewater can be divided into primary, secondary and tertiary stages. These stages are synonymous with sedimentation, biological treatment with sedimentation, and removal of residual or nonbiological materials by any means, respectively.

Primary-solids removal is used where the wastewater contains a sufficient concentration of large, dense, suspended or settleable solids to make removal economic. Biological treatment can be accomplished in a number of ways, but the basic characteristic of the system is the use of heterogeneous bacterial culture for conversion of pollutants. In most cases, organic materials are converted to oxidized end products, mostly CO_2 and new bacterial cells. Physical-chemical treatment is done to remove soluble or colloidal organic materials. The principle processes used are coagulation, filtration, and adsorption on activated carbon. In the final treatment the effluent is chlorinated and discarded in a body of water. The sludge is disposed of in water, on land or incinerated.

Chlorination of water inactivated 90 percent of the virus in sewage effluent. The time of contact between the chlorine and the water proved to be more important than its dosage (6). However, in the presence of organic matter, chloramines were produced and these were relatively slow disinfectants. When hypochlorous acid was added and maintained in the water, disinfection was achieved rapidly (4).

Removal of the viruses by ferric chloride and/or alum has been reported but viruses were not inactivated in this coagulation procedure. Care must be taken in sludge disposal if this method was used. Presence of organic material interfered with virus removal by coagulation (12).

It has been found that titres of reoviruses and enteroviruses were not significantly reduced by the purification process between influent and secondary humus tank effluent. Only occasional low-titre reovirus and enterovirus isolations were made from maturation pond effluents (34). During the 1959 poliomyelitis epidemic in Des Moines, Iowa, 94 sewage samples were obtained from eight sites in different parts of the city.

The frequency of recovery of poliovirus from the samples varied from 9.1 to 100 percent. The titres of poliovirus in the positive samples ranged from less than 200 to 560 $TCID_{50}$/100 ml of sewage (11). Foliquet et al. (20) reported that enteroviruses were found in 35.2 percent of all untreated wastewater samples tested in the Meurth-et-Moselle district of France. Experiments on the survival of poliovirus and Salmonella typhi in sludge digestion at various temperatures were done by Frandetti et al. (20). The results indicated that poliovirus survived digestion at 32°C for 12 days and for 5 days at 39°C while S. typhi disappeared by the seventh day at 32°C and by the third day at 39°C.

The per cent removal of enteroviruses from sewage after the different processes of treatment was studied and compared by Pound et al. (37). It was found to increase from fine screens, plain sedimentation, trickling filters, chemical sedimentation, and chlorination of effluent, respectively. The per cent removal in activated sludge varied from 0 to 88 percent. The per cent removal of bacteria in each process was always higher than that of virus. In a study made for a four-year period in London, the enteroviral content of samples from influent sewage and unchlorinated effluent were compared. The results showed an increase in the viral content of samples from 24 to 86 percent in influent samples, and from 8 to 41 percent in unchlorinated effluent samples during the period from 1971 to 1974. The amount of virus was 50 percent less in effluent samples as compared to the corresponding influent samples. The low recovery of virus in 1971 was attributed to deficiency in recovery methods (43).

2.6 Current Standards of Water Quality for Drinking

The present criteria used to determine the safety of drinking water microbiologically are all based on bacterial content: a plate count to determine the number of bacteria present, and a coliform test. These methods are inadequate, since they give no information on the viral content of water. A positive coliform test would certainly indicate possible virus contamination. However, a negative test does not necessarily indicate freedom from viruses; on the contrary, it may indicate the presence of high titres of bacterial viruses. The water may thus be contaminated by undetected sewage (3). Water can be treated always to be free of biological pollutants including viruses by using current treatment processes and disinfectant measures at all times. However, the result of the National Community Water Supply Study (3) showed that adequate treatment and disinfection has not been uniformly applied to all systems (3).

2.7 Methods of Sampling Water, Concentration and Recovery of the Virus From Water

One of the major problems of detecting enteroviruses is the recovery of small amounts of virus from large volumes of water. There are two methods for sampling water for viruses. A gauze pad was suspended in water for a certain period of time, then treated with an eluent to remove the virus (1N NaOH pH 8). This method was strictly qualitative but very useful in turbid or highly polluted (fecal matter) water. The other method was simply to collect a sample of water in a container. This latter method allowed for quantitative analysis (25).

Several methods of concentrating and recovering enteroviruses from water have been reported. Shuval et al. (1965) reported two methods: concentration by hydroextraction and separation by an aqueous polymer two-phase.

In concentration with hydroextraction, the water sample was dialyzed using an hydrolic agent like polyethylene glycol. One liter of water was concentrated to a few milliliters in 18 hours. After dialysis the dialyzing bag was flushed with phosphate buffer solution to remove any adsorbed virus. The concentrated sample was brought to 10 ml with phosphate buffer. The calculated virus concentration factor was 100, efficiency of recovery 40 to 50 percent and 0.57 PFU were detected. In the other method, the sample was divided into two liquid phases according to the differences in particle surface properties. The sample was added to a mixture of sodium dextran phosphate, polyethylene glycol and sodium chloride. The mixture was shaken and incubated over night at 4°C. It separated into two phases, a small bottom one that contained the virus, and a larger top phase. The bottom portion was precipitated by potassium chloride to remove the dextran. The precipitate was collected by centrifugation and the supernatant was assayed for virus. The method gave the following results: a concentration factor of 100, efficiency of recovery of virus, 40 to 50 percent 0.66 PFU were detected. The major disadvantage of this method was the inhibitory effect of dextran on some of the enteroviruses (44).

Concentration of enteroviruses from water on membrane filters has been described by Rao et al. (38). The water sample was passed through a membrane filter (type HA, with pore size 0.45 μ of either 25 mm or 45 mm diameter) and a prefilter pad, AP25, combined as a single unit. The

prefilter clarified the turbid water, therefore facilitating filtration of large volumes of raw water through the membrane filter which was directly under it. Some of the virus was adsorbed to the prefilter. This was eluted by beef extract pH 8 in one single step. The virus that was adsorbed to the prefilter was usually lost if prefiltration was carried out in a separate step. Addition of divalent cations like Ca^{++} increased virus recovery.

For turbid estuarine water, celite was used to prevent clogging of the prefilter. Celite was added to the water sample, after the pH was adjusted to 3.5 $\pm$0.1 and mixed. The sample was pressure filtered through an adsorbent filter. Virus was eluted from the celite-filter complex using 5x nutrient broth in 0.05 M carbonate buffer pH 9.0. This was followed by aqueous polymer two-phase separation (24). Combination of membrane filtration and aqueous polymer two-phase separation was reported to have provided a more sensitive, simple and reliable sequential method for quantitative recovery of polioviruses occurring at multiplicities as low as 1-2 PFU/five gallons of water.

Adsorption of virus to precipitable salts, iron oxide or polyelectrolytes was used to recover enteroviruses from water. Stevenson et al. (46) used alum flocculation precipitation. Aluminum sulfate, under specified conditions, was added to the water. A floc formed in situ. Virus was eluted from the floc by elevating the pH to 8.0. The virus concentration factor was 100 and as little as 0.00625 LD_{50}/ 0.02 ml were detected by assaying in mice (46).

Gärtner (19) used alginate filters which are soluble in sodium citrate to recover enteroviruses from water. It allowed retention and

adsorption of the virus. The alginate citrate solution did not inactivate the virus and became clogged by turbid water.

Anderson et al. (1) reported the adaptation of isopycnic-zonal centrifugation to the concentration of viruses from water. By using this procedure as a continuous flow ultracentrifugation, a continuous stream of water was passed over a density gradient in a special rotor. Sedimentation of the virus by rate zonal centrifugation has been reported by Schwerdt et al. (1).

Forced flow electrophoresis was employed for the concentration of virus from water. This method was reported by Bier et al. (5). Electrophoretic transport was used to bring about adsorption of virus on dialyzing membranes which are part of the electrophoretic cell. The membranes were directly applied to a prepared substrate for plaque development. McHale et al. (35) concentrated viruses from water by forced-flow electrophoresis and electro-osmosis. Enteroviruses act as anions at a pH above their isoelectric point and, therefore, can be separated according to the difference in their electric potential.

2.8 Methods of Assay for Enteroviruses

There is one method of assay for enteroviruses that is used for all purposes of titration. That is titration by plaque formation in tissue cultures. This method was first described by Dulbecco and Vogt in 1953 (17). They showed that poliovirus types 1, 2 and 3 could be isolated from these plaques. This method was later simplified by Hsiung and Melnick in 1955 (27), and its sensitivity increased for a larger number of enteroviruses. Dahling et al. (13) used a continuous cell line of Buffalo Green Monkey kidney cells which was more sensitive, less

expensive and produced plaques for several enteroviruses: three types of poliovirus, echoviruses 3, 6, 9, 11, and 27, Coxsackieviruses B1-3, and reovirus 1.

2.9 Method of Titration

Trypsinized Buffalo Green Monkey Kidney (BGMK) cells were prepared with the nutrient medium and put in tissue culture flasks or plates. The flasks were then incubated at 37°C until a monolayer was formed (3 to 7 days). The nutrient medium was poured off and the virus sample added to the cells which were incubated at 37°C for 30 to 60 minutes. This allowed the virus to attach to the cells. An agar overlay containing neutral red and the flasks were reincubated at 37°C after the agar solidified. Plaques started showing microscopically, after 24 hours of infection, as small clear areas in the middle of a lawn of red-stained living cells. The plates were observed to obtain a daily plaque count. The number of plaques doubled between the first and fifth days of incubation for poliovirus types 1 and 2. Poliovirus type 3 took 48 hours to start showing plaques. Echovirus plaques took 5 to 6 days to appear, and some strains needed 12 to 13 days. The morphology of the plaques of the different enteroviruses was found to be similar. Polioviruses and Coxsackie A9 had indistinguishable plaques macroscopically and microscopically (27).

Dulbecco showed that a plaque was formed by a single virus particle. He defined a single virus particle as the amount that could produce a plaque and could not be further divided by dilution and still form a plaque. There was a linear relationship between the number of plaques and the amount of virus in the sample. The titre was expressed as

PFU/ml: plaque forming units per ml of sample.

Another method commonly called the $TCID_{50}$ assay has been used to quantitate viruses. The tissue culture procedure consists of making serial logarithmic dilutions of the material to be assayed and inoculating a number of tubes containing cell monolayers. Following an incubation period, the presence or absence of cytopathic effect (CPE) in the cells of each tube is observed microscopically. The 50 percent end point was calculated by the Reed and Muench method (25). This method was used for viruses that could not form plaques in tissue cultures. However, it was not sensitive enough and lacked the precision of the plaque assay method.

A new method of identifying poliovirus isolates was described by Hatch et al. (23). One-tenth to two-tenths of a milliliter of prepared stool suspensions were inoculated into tubes of BGMK cells. Then a flourescent antibody was used. As soon as 1 to 2 + cytopathic effect (CPE) was observed, the maintenance fluid was poured off and the cells were rinsed with versene in buffered saline. One milliliter of fresh versene was added to the tubes which were incubated for about 15 minutes at 37°C. The cells were removed from the glass by gentle shaking and were collected by centrifugation. Smears of the sedimented cells were made on slides. The smear was air dried and fixed in acetone at room temperature for 10 minutes, then stained. The samples that showed no CPE on first passage were passed a second time. The negative tubes were reincubated for seven days, frozen, thawed, and 0.1 ml passed to fresh tubes. Second passage tubes remaining negative were harvested at the end of seven days for staining. The direct method of fluorescent antibody staining was employed and preparations were examined under ultraviolet

light microscopy. The brilliance of staining, as well as the number of stained cells, increased as CPE increased to about the 2+ stage. But smears showing 3+ or 4+ CPE did not stain well (possibly because of release and dispersion of viral antigen from cells). There was also occasional cross-staining between poliovirus types 1 and 3 but staining with homologous conjugate was brighter. This method was a relatively simple and rapid one, only if the samples showed CPE after the first passage. Otherwise, the cultures needed 7 to 15 days to show a positive CPE. This method gave no information on quantity of virus in the sample.

Currently the method of choice for titrating enteroviruses is the plaque assay method. It is accurate and gives quantitative data on the virus tested. Also, it is used to type the viruses after neutralization of the sample with the anti-sera pools for enteroviruses as described by Lim and Benyesh-Melnick (31). However, it takes a long time to perform a plaque assay, and it is expensive.

2.10 Solid-Phase Radioimmunoassay in Antibody-Coated Tubes

With the advance of surgery and surgical procedures, the use of plastics has increased. Plastics are used in heart-lung machines, kidney machines, artificial heart valves and patches. Several problems developed. One of which was that plastics had the tendency to cause coagulation of blood and this necessitated the use of anticoagulants. Research has been done to make plastic surfaces nonthrombogenic. The first substantial advance came when Gott (22) treated a graphited plastic surface with zephiran and then with heparin. These surfaces proved to be nonthrombogenic in dogs over a period of two years.

Leininger et al. (30) investigated another method that could be applied to flexible plastics. They bound heparin chemically to plastic surfaces. This created a surface similar to the natural vascular surface which contains heparin and other sulfated polysaccharides that have heparin-like activity. The initial step in chemical heparinization of the plastic has been the formation of quaternary ammonium sites on the plastic surface. The heparinization was done by simple contact with a heparin solution. It has been found that unmodified plastics strongly adsorbed blood proteins (30). Most forms of radioimmunoassay include a procedure to separate free and bound tracer antigen when equilibrium was reached in the incubation mixture. The separation procedure was done by several methods such as electrophoresis, ion exchange, solvent fractionation, etc. (9).

Based on Leininger's work, a radioimmunoassay method was developed by Catt et al. (19). This included the antigen-antibody reaction and the separation of bound and free tracer antigen in a single step. The antibody was coupled to an insoluble polymer, the antigen and radioactive tracer were added and when the antigen bound to the antibody, it simultaneously became attached to the solid supporting medium. By simple washing, the free tracer was removed and the system was counted to get the amount of bound antigen. The binding of the labeled antigen was reduced by the presence of unlabeled antigen (9). This method was used to detect and assay proteins in biological fluids.

Catt et al. (9) first used the isothiocyanate derivative of a graft copolymer of poly-tetrafluoroethylene (Fluou G4) and styrene. This copolymer allowed for a variety of reactive substituents to be attached to the aromatic rings on the surface of the dense and inert core of the

poly-tetrafluoroethylene. The material used for solid-phase radioimmunoassay was first available in the form of powder and later as discs. Various polymeric materials were examined for their adsorbency of proteins. It was found that certain unsubstituted polymers could adsorb antibody from serum which was able to bind antigen. Plastic tubes were coated with the antibody and used in the radioimmunoassay. The most commonly used disposable tubes were made of polypropylene or polystyrene. The tubes received no treatment before the antiserum was incubated in them. After the incubation period, the duration of which was not critical, the tubes were washed with 0.15 M NaCl, a protein solution and merthiolate. A volume of the serum to be assayed was incubated in the tubes for 16 hours. After aspiration of the contents, each tube was washed and counted for one minute in an automatic gamma counter (8). This method was inexpensive and simple. The antibody was strongly adsorbed to the tube surface by forces that were unaffected by several washings and physical stress like vigorous rubbing of the tube interior with the tip of the pipette during aspiration of the washings (8). The specific binding of the antigen to the solid-phase antibody was found to be irreversible (9).

2.11 Morphological Changes in Cell Cultures Infected With Poliovirus

The sequence of intracellular changes occurring in monolayer cultures of monkey kidney cells after infection with poliovirus has been related to the growth cycle of the virus. Reissig et al. (39) studied these changes with phase contrast microscopy. The preparations were examined after fixing, washing and staining with haematoxylin and eosin.

Samples were taken every 30 to 60 minutes for a period of 10 hours after infection. Portions of the samples were titrated to compare the amount of intracellular virus with the morphological changes. The results showed that the whole sequence of changes ending with death of the cell was completed in about seven hours after virus inoculation. The virus was detected in the cells three hours after infection. Intracellular virus was found in the cells after nuclear changes were seen which preceded cytoplasmic alterations.

Lowff et al. (32) studied the kinetics of release of virus from single cells and reported that the infected cells did not release any virus for the first five and one-half hours. Then, within one-half hour the bulk of the virus was released. During the next one-half hour virus particles continued to appear in the medium at an almost uniform rate. Thus, practically all the virus was released in less than one hour.

2.12 Tritiated Antibody Binding Studies

Sanborn and Durand (42) labelled antibodies with tritium by the borohydride exchange reaction. These antibody preparations were used to follow the infection process of Sendai virus in host cells. This method of labeling was simple, gentle and incorporated high ^{3}H specific activity into the antibody with little detectable loss of antiviral activity. The tritium labeled antibodies also retained their radioactivity longer than iodine labeled antibodies. This allowed for experimentation over extended periods of time with one antibody preparation. The host cells were infected with Sendai virus, fixed at hourly intervals with 70 percent alcohol, reacted with antibody, and counted in a scintillation counter. Two antibody preparations were used to monitor

intracellular viral antigens, anti-nucleocapsid, and the surface associated antigens of Sendai virus. Both types of antigens were traced over a period of 15 hours.

CHAPTER III

MATERIALS AND METHODS

3.1 Growth Medium

Dry powdered tissue culture medium, MEM with Earl's base and L-glutamine, without $NaHCO_3$, was the base for the growth medium (KC Biological, Inc., Lenexa, Kansas). To 9.49 gm dry base was added 10 percent foetal calf serum, 9.53 gm Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid) buffer and 50 μ gm/ml Gentamicin. The volume was brought to one liter with double distilled water. The pH was adjusted to 7.2 using 20 percent NaOH, and the medium was filter sterilized and frozen at -20°C until used.

3.2 Balanced Salt Solution Without Ca^{++} and Mg^{++} (BSS) 10X

Ten X-BSS was made by mixing 80 gm NaCl, 4.0 gm KCl, 3.5 gm $NaHCO_3$, 10.0 gm glucose, and 100 ml of 0.2 percent phenol red in double distilled water. The 10X solution was stored at room temperature with the addition of a few drops of chloroform. The stock solution was diluted to 1X and autoclaved before using as a wash solution for the BGMK and Vero cells.

3.3 Trypsin

Trypsin-EDTA, the enzyme solution used to dissociate the cells for monolayer preparation, was made by simple mixing 0.6 gm/100 ml of 1:250

trypsin, 0.02 percent EDTA and one liter of BSS together. The solution was filter sterilized and frozen at -20°C until used.

3.4 Phosphate Buffered Saline

Ten X PBS was prepared by combining 105.7 gm of $Na_2HPO_4 \cdot 7H_2O$, 27 gm KH_2PO_4, 41 gm NaCl, and 100 ml of distilled water. For use the stock soltuion was diluted to 1X PBS with double distilled water (0.037 M and pH 7.2).

3.5 Barbital Buffer (Veronal)

Barbital Buffer was used to dilute the antibody; it had the following composition: 0.331 gm diethyl barbituric acid, 1.848 gm sodium diethyl barbiturate, 0.030 gm thimerasol, and 120 ml distilled water. The pH of the buffer was 8.6 (Millipore Corp., Bedford, Mass.).

3.6 Red Blood Cells

Whole chicken blood in Alsever's solution was centrifuged at 1,700 rpm for five minutes in an International Clinical Centrifuge Model CL. The pelleted RBD's were resuspended in PBS. This washing procedure was repeated three times. A 0.6 percent RBC working suspension was prepared in PBS.

3.7 Melnick's Medium B

Melnick's medium B was used to dilute the anti-poliovirus pools after their rehydration. The medium has the following composition in grams/liter: NaCl 6.8, KCl 0.4, NaH_2PO_4 0.14, $CaCl_2$ anhydrous 0.20, $MgSO_4 \cdot 7H_2O$ 0.2, glucose 1.0, lactalbumin hydrolysate 5.0, phenol red 0.01 and $NaHCO_3$.

3.8 Propagation and Purification of Sendai Virus

Sendai virus (parainfluenza 1) provided by Dr. M. R. Sanborn, was propagated in 10 day old embryonated turkeys. A tenth of a milliliter of the virus suspension was injected asceptically in the allantoic cavity of 10 day old embryonated turkey eggs. The eggs were incubated at 31°C for 48 hours. The eggs were kept at 4°C for 2 hours before the allantoic fluid was harvested. The virus was isolated from the allantoic fluid by precipitating it with polyethylene glycol. A 6 percent solution of polyethylene glycol (PEG) in the allantoic fluid was made, 0.5 M with NaCl, and kept at 4°C for 24 hours whereupon a white precipitate formed. The solution was then centrifuged at 5000 rpm for 30 minutes in a Sorval-type SS 34 rotor. The pellet was resuspended in phosphate buffer saline to the original volume. The pellet and supernatant were checked for the presence of virus by the viral haemagglutination test. The resuspended pellet was ultrafiltered (XM 300, 62 mm, Diaflo ultrafiltration membranes, Amicon Corp., Lexington, Mass.) to remove the polyethylene glycol. The residue containing the virus was centrifuged through a discontinuous sucrose gradient, 15, 30 and 60 percent, at 20,000 rpm for 120 minutes in a Beckman SW 27 rotor. The gradient showed three bands: upper, middle, and lower. Each band was collected separately using a bent needle and syringe. Each band was mixed with phosphate buffer saline and was ultrafiltered to remove the sucrose. The residue containing the virus was put on top of a continuous sucrose gradient from 15 to 60 percent and centrifuged at 20,000 rpm for 120 minutes in a Beckman SW 27 rotor. A single homogeneous band was seen in every gradient. This band was collected with a bent needle and syringe. Phosphate buffer saline was added and it was

ultrafiltered to remove the sucrose. The residue was checked for the presence of virus by viral haemagglutination reaction. The virus was divided into three aliquots and frozen at -20°C until used.

3.9 Anti-Sendai IgG

The specific IgG was prepared by immunizing rabbits against Sendai virus. The virus was first inactivated by ultraviolet light. One tenth of a milliliter of the viral suspension and 1 ml Freund's complete adjuvant were emulsified through a needle and syringe. Weekly injections of 1 ml of the emulsion were given sub-scapularly for three weeks, and one month after the last injection a booster dose of 1 ml was given. One week after the booster injection blood was collected via a cardiac puncture. The IgG fraction was precipitated by ammonium sulphate. Then it was centrifuged at 1,700 rpm for 15 minutes in an International Clinical Centrifuge model CL and the pellet was resuspended in PBS to the original volume. The precipitating procedure was repeated two more times to get a more purified IgG precipitate. In the last centrifugation, the pellet was resuspended to half the original volume. The ammonium sulphate was removed by dialyzing the precipitate against borate buffered saline at 4°C for three days. The anti-Sendai IgG was titrated using viral haemagglutination inhibition test.

3.10 Rabbit Anti-Equine Antibody

Anti-equine serum was prepared in rabbits by immunizing them with intravenous injections against the equine anti-poliovirus pools in the same method previously described for the preparation of anti-Sendai virus serum. The IgG fraction was purified by ammonium sulphate precipitation.

The anti-equine immunoglobulin G was tritium labeled by the method described by Sanborn and Durand (42).

3.11 Propagation and Isolation of Poliovirus

Poliovirus type 1, strain Chat (American Type Culture, Rockville, Maryland) was propagated in roller bottles of Buffalo Green Monkey kidney cells (Microbiological Associates, Bethesda, Maryland). After a monolayer of BGMK cells was formed, they were infected with the virus. The nutrient medium was first poured off the cells, then 50 ml of BSS, 5 ml of the virus (titre about 2×10^6 PFU/ml), and 1 ml of a 50 percent solution of $MgCL_2$ were added to the cells. The bottles were incubated at 37°C for 5 to 10 minutes for the virus to attach to the cells. After the incubation, MEM medium was added to the cells and they were reincubated for 48 hours at 37°C. Following the incubation period, the medium with the infected cells was harvested and centrifuged at 2,400 rpm for 10 minutes in an International Clinical Centrifuge model CL. The pellets were resuspended in 2 ml phosphate buffer saline and passed through a 10 ml syringe and a 23 gauge needle to break down the cells and release any virus that might have been inside. This was repeated 10 to 15 times or until no whole cells were seen under the phase contrast microscope. They were then centrifuged at 3000 rpm for 15 minutes in a Sorval SS 34 rotor. The supernatant was taken together with the first supernatant and ultracentrifuged at 20,000 rpm for 3 hours in a Beckman SW 27 rotor. The pellets were resuspended in 3 ml phosphate buffered saline and stored at -20°C until used.

3.12 Preliminary Studies on Solid-Phase Antibody

Sendai virus, a paramyxovirus, has the characteristic of agglutinating chicken red blood cells. It was used as an antigen to test the coating of the scintillation tubes (Polyethylene mini vials, 7 ml, from Rochester Scientific Co., Rochester, New York) before working with the radiolabeled antigen or antibody. The red blood cells were used as an indicator of Sendai virus binding to the solid-phase antibody. The number of RBCs bound to the virus was then counted with a light microscope. Solid-phase antibody refers to the antibody bound to a polymer.

3.13 Effect of Temperature on Binding Antibody to Solid-Phase

Anti-Sendai IgG titre 128 HAIU/ml and protein concentration 20 mg/ml was diluted 1:10, 2 ml of which were put in each of four scintillation tubes. Two tubes were incubated at 37°C and the other two at room temperature. The incubation period was 30 minutes, after which the contents were poured out and the tubes washed three times with 0.15 M NaCl, and once with 0.01 percent merthiolate solution in normal saline. One to two ml of a 1:10 dilution of Sendai virus, titre 160 HAUI/ml were added to each tube and incubated at room temperature for 45 minutes. After the incubation period the virus suspension was poured out and the tubes washed once with normal saline. A 6 percent solution of chicken red blood cells was incubated in the tubes for 45 minutes at room temperature, each tube had 2 ml of the RBC suspension. The tubes were then washed twice with phosphate buffer saline, cut into three slips longitudinally, and examined under a light microscope using 10 x objective to count the red blood cells binding.

3.14 Minimum Titre of Antibody Binding to the Solid-Phase

The anti-Sendai antibody was diluted 1: 10, 1:50 and 1:100 in barbital buffer. These dilutions corresponded to 16 HAUI/ml 2 mg/ml protein, 8 HAUI/ml 1 mg/ml protein, and 4 HAUI/ml and 1/2 mg/ml protein concentration, respectively. Two ml of each dilution were incubated in two scintillation tubes which were washed, virus added, then red blood cell suspension added, and the number of RBCs counted under the microscope. Control tubes were incubated with non-specific IgG, and bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) instead of adding Sendai virus. Reb blood cells were then added to give the number of RBC non-specifically binding to the antibody.

3.15 Antigen Detection by Solid-Phase Antibody

To get the average number of red blood cells adsorbing to the solid-phase, twelve tubes were incubated with 2 ml of a 1:10 dilution of anti-Sendai antibody. Three tubes were incubated with 2 ml of non-specific IgG to serve as control. Test tubes were washed, virus added, washed again, and the red blood cells added. Control tubes were washed and bovine serum albumin and the red blood cells were added, respectively.

3.16 Sensitivity of Virus Detection by Solid-Phase Antibody

Sendai virus was diluted 1:10, 1:20 and 1:100. Two ml of each dilution were incubated in antibody coated tubes, three tubes for each viral dilution. Three control tubes were incubated with bovine serum albumin. All tubes were washed and red blood cells added.

3.17 Antigen Detection Using ^{14}C Sendai Virus

Anti-Sendai virus immunoglobulin G titre 80 HAIU/ml, diluted 1:10 was incubated in 14 tubes, 0.5 ml in each tube. After 30 minutes incubation, seven tubes were washed and 0.5 ml of 1 mg/ml solution of bovine serum albumin were added to each tube. The tubes were then reincubated at 37°C for 30 minutes. All tubes were washed and 1 ml of a serial twofold dilution to a final dilution of 2^{-7} was incubated in each tube at 37°C for one hour. All tubes were washed five times with phosphate buffer saline. Five ml of cocktail (Aquasol, New England; Nuclear, Boston, Massachusetts) were added to each tube before counting them for one minute in a liquid scintillation counter.

3.18 Antigen Detection Using Whole Serum

Anti-Sendai virus immunoglobulin G was mixed with normal rabbit serum in a ratio of 1:1 to coat all binding sites on the surface of the tube. One ml of this antiserum was incubated in seven tubes. Tubes were washed and 0.1 ml of ^{14}C Sendai virus serially diluted twofold to 2^{-7} was added to each tube and incubated at 37°C for one hour, then washed five times with phosphate buffer saline and counted for one minute.

3.19 Poliovirus Detection by Solid-Phase Antibody

Lyophilized equine source anti-poliovirus pools were provided by the National Institute of Health, Bethesda, Maryland. Each pool was rehydrated with 5 ml sterile distilled water. For use in the tests, the rehydrated pool was diluted tenfold in Melnick's B medium and was stored at -20°C until used. The working solution contained 50 antibody units

of each antiserum per 0.1 ml. One antibody unit was defined as the dilution which would neutralize 100 $TCID_{50}$ of virus.

Scintillation tubes were incubated at 37°C for one hour with 0.3 ml of anti-poliovirus serum. The tubes were then washed five times with normal saline, once with normal rabbit serum, and once with 0.01 percent merthiolate solution in normal saline. To six of the tubes 0.1 ml of poliovirus suspension was added while the other six tubes received 0.1 ml phosphate buffer saline and served as control. All tubes were incubated at 37°C for one hour, then washed twice with phosphate buffer saline. One-tenth ml of tritium labeled rabbit source anti-equine antibody was added to all tubes and incubated at 37°C for one hour. The tubes were then washed five times with phosphate buffer saline, filled with 5 ml cocktail, and counted for one minute in a liquid scintillation counter. To diminish the non-specific binding of the tritium labeled antibody, bovine serum albumin was used to wash the scintillation tubes. Twelve tubes were incubated with 0.3 ml anti-poliovirus serum, washed, filled with a 1 percent solution of bovine serum albumin, and incubated at room temperature for 15 minutes. This was done to bind the BSA to the naked binding sites on the surface of the tube after the antibody has bound. The tubes were washed and 0.1 ml poliovirus suspension was added to six of them. The other six control tubes were incubated with buffer. The tubes were incubated, washed, and 0.1 ml ^{3}H anti-equine IgG was added to each tube, incubated, washed, and counted.

3.20 Effect of Diluting the ^{3}H Anti-Equine Antibody

To fourteen scintillation tubes 0.1 ml equine anti-poliovirus serum was added, the tubes incubated, then washed, filled with bovine serum

albumin for 15 minutes at room temperature. A serial twofold dilution of poliovirus was made in phosphate buffer saline to a final dilution of 2^{-7}, two tubes per dilution. The tubes were washed and to one set of seven tubes 0.1 ml of undiluted tritium labeled anti-equine antibody was added. To the other seven tubes 0.1 ml ^{3}H anti-equine antibody diluted 1:2 by cold anti-equine antibody was added. All tubes were incubated, washed, and counted.

3.21 Sensitivity of Virus Detection by Solid-Phase Antibody

Scintillation tubes coated with anti-poliovirus antibody were washed and each received 0.1 ml poliovirus. Poliovirus was serially diluted in phosphate buffer saline to a final dilution of 2^{-7}; for each dilution three tubes were incubated. The tubes were incubated, washed, tritium labeled antibody added, and counted.

A serial twofold dilution of the ^{3}H anti-equine immunoglobulin G was made in PBS to a final dilution of 2^{-10} before incubating it with solid-phase antibody. The tubes were then washed and counted for one minute.

3.22 Growth Curves of Poliovirus in Tissue Cultures

Sterile plastic cover slips were asceptically put in the bottom of the wells of multidish tissue culture plates. Then 1 ml of a suspension of 10^{6} cells/ml of Vero cells (a gift from the Oklahoma Animal Disease Diagnostic Laboratory of the College of Veterinary Medicine, Oklahoma State University) of BGMK cells (Microbiological Associates, Bethesda,

Maryland) in MEM was added to each well. The plates were incubated at 37°C until monolayers formed. Each column in the plates had four wells and one column was used for each hour of infection. The growth medium was carefully removed with a pipette from the first column. Then each well received 0.1 ml poliovirus suspension and incubated at 37°C for 15 minutes to allow the virus to attach to the cells. Then 1 ml of MEM was added to each well and the cells were incubated at 37°C for 45 minutes. The remaining columns were infected at one hour intervals using the same procedure. The sixth column of each plate was not infected with virus and was kept as control. One hour after the last column was infected, the growth medium was removed carefully from the wells and the cells were washed with phosphate buffer saline to remove all traces of the medium. The cells were then fixed with 0.5 ml of 70 percent ethyl alcohol for 15 minutes at room temperature. The cells were washed five times with phosphate buffer saline. One-tenth ml of equine source anti-poliovirus pool C was added to each well and the cells were incubated at room temperature for 45 minutes. The antibody was washed off the cells with PBS and ^{3}H anti-equine immunoglobulin G were added and the plates were incubated at room temperature for 45 minutes after which they were washed five times with phosphate buffer saline. The cover slips were removed and put in scintillation tubes and counted.

3.23 Detection of Poliovirus by Tissue Cultures

A serial twofold dilution of poliovirus was made in MEM to a final dilution of 10^{-5}. One column was infected per dilution, each well received 0.1 ml of the virus suspension. The sixth column received no virus and was left as control. The virus was incubated with the Vero

cells for five hours to give maximum production of the virus inside the cells. The cells were fixed and reacted with both antibodies as previously described. The discs were put in the scintillation tubes and counted.

CHAPTER IV

RESULTS

4.1 Preliminary Studies on Solid-Phase Antibody

The experiments done to compare the binding ability of the antibody to the solid-phase at 25°C room temperature and 37°C gave an average of 6.9 chicken red blood cells/mm^2 at 25°C and 13.39 red blood cells/mm^2 at 37°C. The temperature of 37°C was chosen as the incubating temperature to bind the antibody to the solid-phase in all experiments.

The results of the solid-phase antibody's ability to detect the antigen showed that the anti-Sendai virus tubes bound an average of 75 red blood cells/microscopic field, while the control tubes which were incubated with normal rabbit serum and received no virus, contained only 6.9 red blood cells/field.

The antibody was diluted to find the highest dilution that binds to the solid-phase and remains active to detect the virus. The tubes coated with 2 mg/ml solution of antibody bound an average of 17.8 red blood cells/mm^2. The tubes coated with 1 mg/ml protein concentration had an average of 4.22 red blood cells/mm^2 and the 0.5 mg/ml protein concentration had 0.7 red blood cells/mm^2 binding to the solid-phase. Control tubes bound an average of 8 red blood cells/mm^2.

In the experiments done to find the lowest titre of Sendai virus that could be detected by the solid-phase anti-Sendai virus antibody, the tubes that were incubated with Sendai virus of a titre 16 HAU/ml

bound an average of 26.95 red blood cells/mm^2. The Sendai virus with a titre 8 HAU/ml bound an average of 0.42 red blood cells/mm^2 and the tubes incubated with a virus titre of 1.6 HAU/ml bound an average of 0.37 red blood cells/mm^2 while the control tubes had an average of 10 red blood cells binding/mm^2.

4.2 Solid-Phase Antibody and ^{14}C Sendai Virus

Figures 1 and 2 represent the results of detection of ^{14}C Sendai, virus titre 32 HAU/ml, by the solid-phase antibody. Figure 1 is the result of incubating the serial twofold dilution of the virus with solid-phase antibody without previously adding an extra protein to coat the rest of the active sites in the tubes. The curve shows a decrease in the number of counts with the decrease of viral titre. The difference between consecutive viral counts binding was not proportional to the decrease in the number of virus particles. The curve leveled off around the fifth dilution. In Figure 2, where the same virus dilutions were added to the solid-phase antibody after incubating it with nonspecific bovine immunoglobulin G, the curve also leveled off at about the fifth dilution. The difference in counts before the curve leveled off was more proportional to the difference between viral counts.

The results of mixing the anti-Sendai virus antibody with normal rabbit serum before adsorbing it to the solid-phase are shown in Figure 3. This solid-phase antibody was used to detect a serial twofold dilution of ^{14}C Sendai virus. The curve showed the same trend as the previous ones, without any points being off the curve. The counts were proportional to the virus dilution and the curve still leveled off at the fifth dilution.

Figure 1. Detection of ^{14}C Sendai Virus by Solid-Phase Antibody
A serial 2 fold dilution of ^{14}C Sendai virus was incubated with the solid-phase antibody for 60 minutes at 37°C. The tubes were then counted for 1 minute in a liquid scintillation counter.

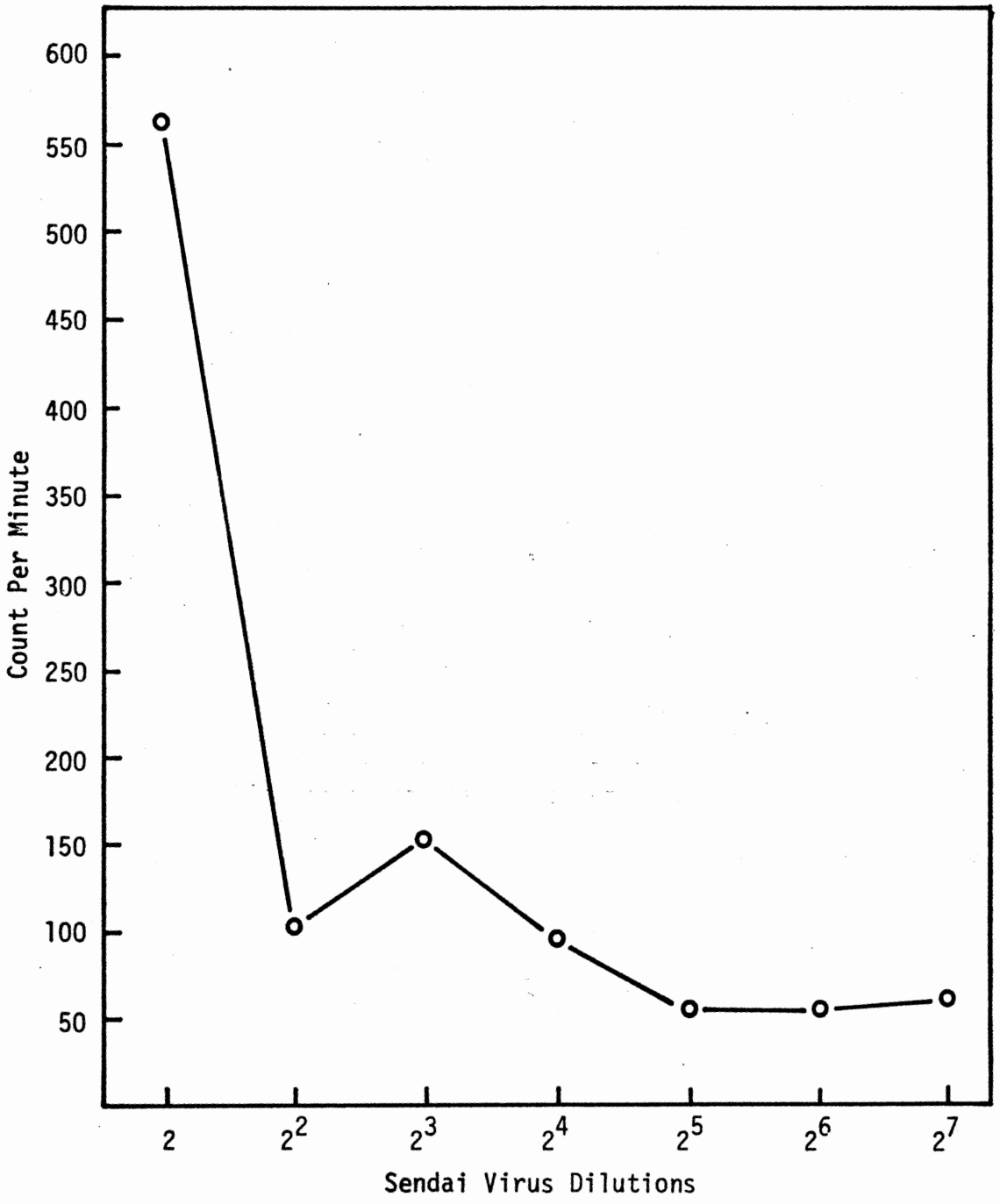

600
550
500
450
400
350
300
250
200
150
100
50
Count Per Minute
2
2^2
2^3
2^4
2^5
2^6
2^7
Sendai Virus Dilutions

Figure 2. Detection of ^{14}C Sendai Virus by Solid-Phase Antibody Incubated With Non-Specific Immunoglobulin G

Solid-phase antibody was incubated with non-specific IgG. A serial twofold dilution of ^{14}C Sendai virus was then added, the tubes were incubated at 37°C for 60 minutes and counted for one minute in a liquid scintillation counter.

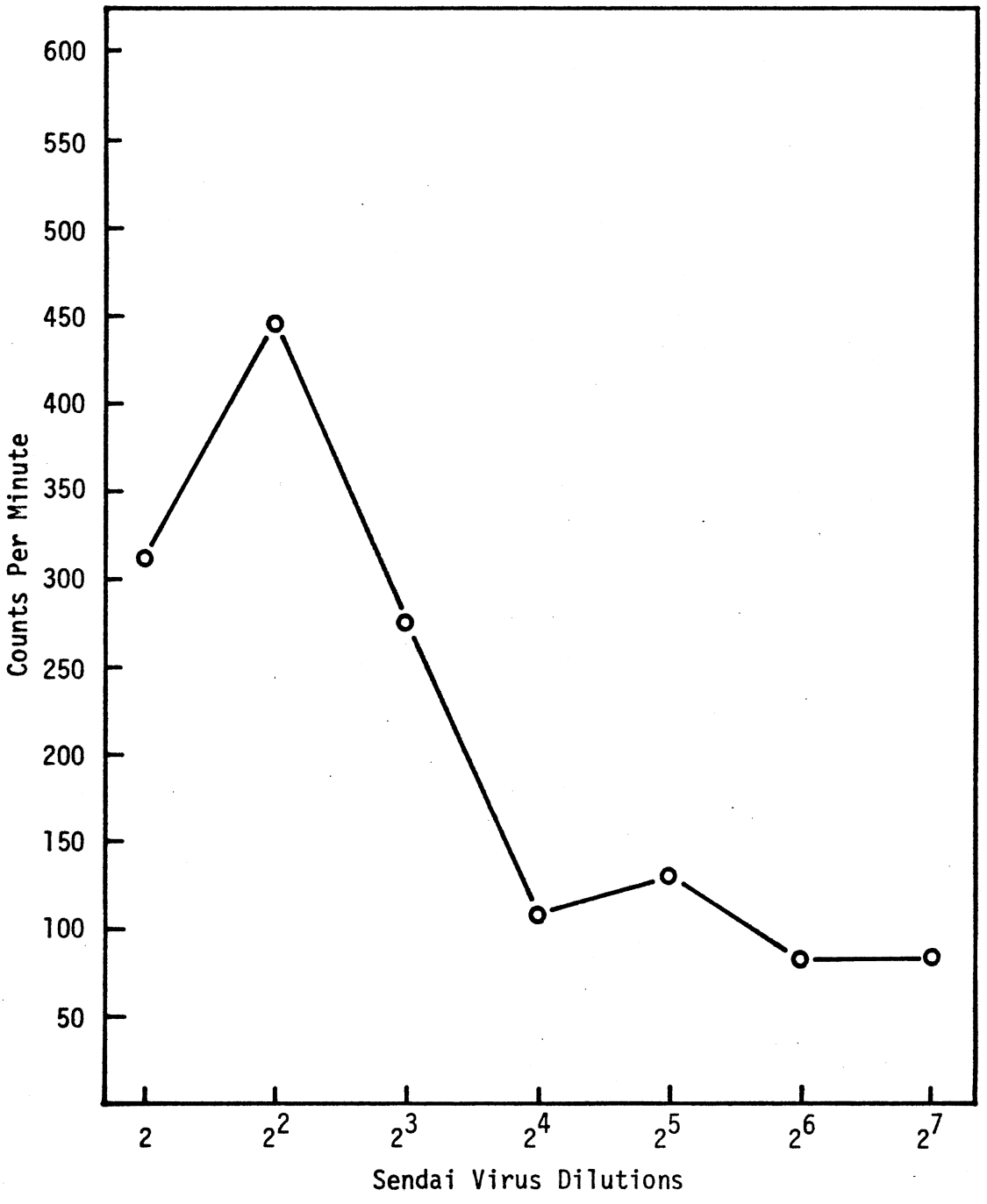
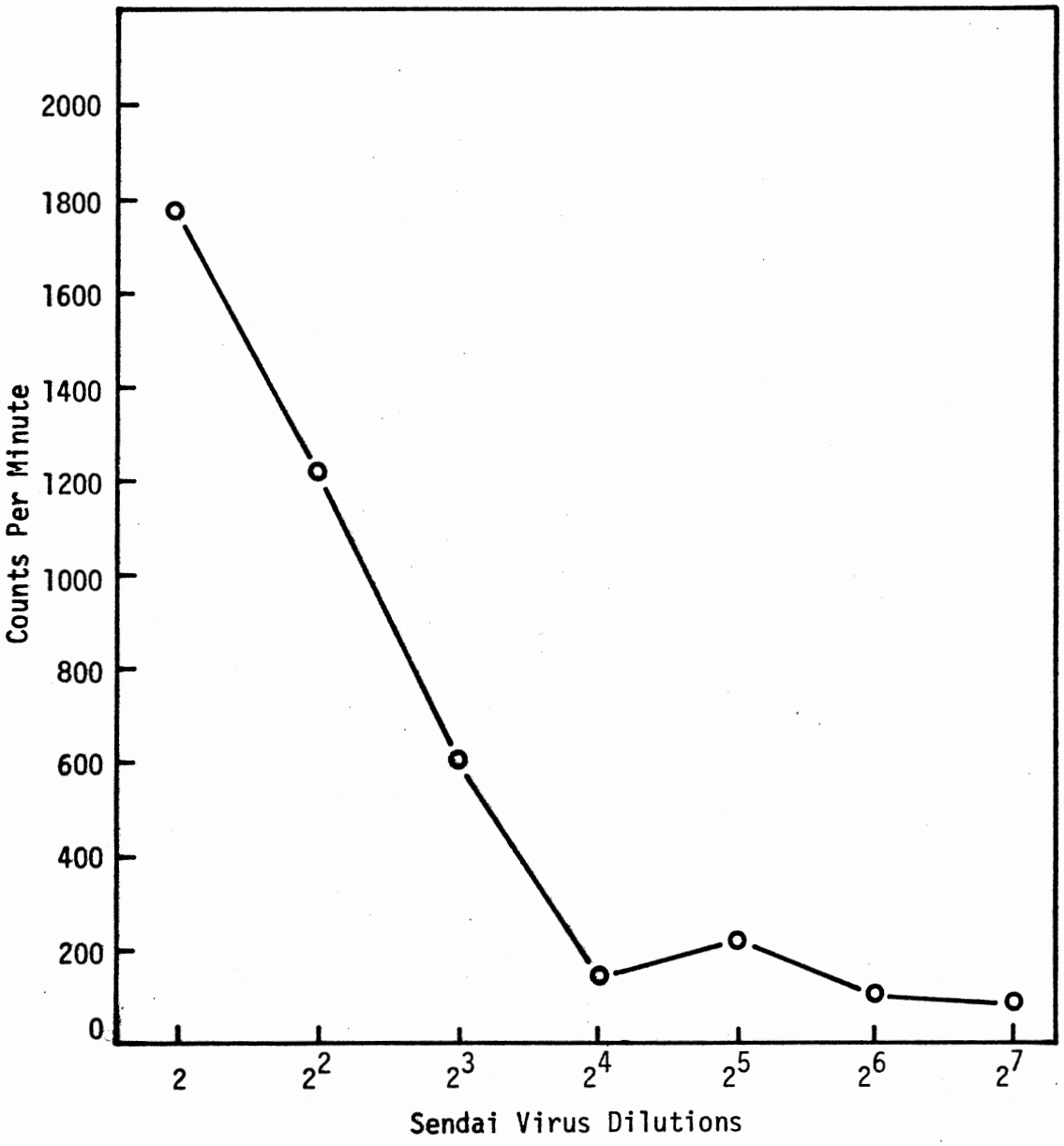

600
550
500
450
400
350
300
250
200
150
100
50
Counts Per Minute
2
2^2
2^3
2^4
2^5
2^6
2^7
Sendai Virus Dilutions

Figure 3. Detection of ^{14}C Sendai Virus by Solid-Phase Antibody Coated by a Mixture of Anti-Sendai Virus Antibody and Normal Rabbit Serum

Solid-phase was coated by a mixture of normal rabbit serum and anti-Sendai virus antibody. This solid-phase was then incubated with a serial twofold dilution of ^{14}C Sendai virus for 60 minutes at 37°C. The tubes were then counted in a liquid scintillation counter for one minute.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
Counts Per Minute
2
2^2
2^3
2^4
2^5
2^6
2^7
Sendai Virus Dilutions

4.3 Indirect Assay of Poliovirus

To prove that the solid-phase immunoglobulin G could detect poliovirus, antibody coated tubes were incubated with poliovirus and compared with control tubes incubated with normal rabbit serum. Tritium labeled anti-equine immunoglobulin was used to indicate viral binding. The average count of labeled antibody in the control tubes was 36,762 counts per minute, while the average count in the tubes incubated with the virus was 27,170 counts per minute. The solid-phase antibody was incubated with bovine serum albumin before adding the virus or the normal rabbit serum in the control tubes. The tubes were washed with bovine serum albumin for 15 minutes. The average count in the control tubes was 15,698 counts per minute and in those with virus, 12,934 counts per minute. Statistical analysis was done on all these results, where the average count was changed from a Poisson distribution to a normal one. There was a difference between the counts in the control tubes and the test tubes at a 90 percent confidence level. The same difference was found between the two sets of tubes when they were washed with bovine serum albumin for 15 minutes.

Figures 4 and 5 show the results of the experiment done to test the effect of competitively diluting the tritium labeled antibody. Figure 4 represents the undiluted labeled IgG; the number of counts increased in the beginning with the decrease in the virus but the last three points were scattered. Figure 5, which represents the diluted antibody, showed a scattering behavior for the first four points after which the counts increased and leveled after the sixth point. A statistical analysis was done to find the correlation between the counts and the virus dilutions for each curve. The correlation coefficient for the curve in

Figure 4. Indirect Detection of Poliovirus 1. by Solid-Phase Anti-Poliovirus Pool, Using Undiluted Anti-Equine IgG

Scintillation tubes were coated with equine antipoliovirus pool. A serial twofold dilution of poliovirus 1 was then incubated in these tubes at 37°C for one hour. Labeled anti-antipoliovirus pool IgG was then added and the tubes were reincubated at 37°C for one hour. The counts were taken for one minute in liquid scintillation counter.

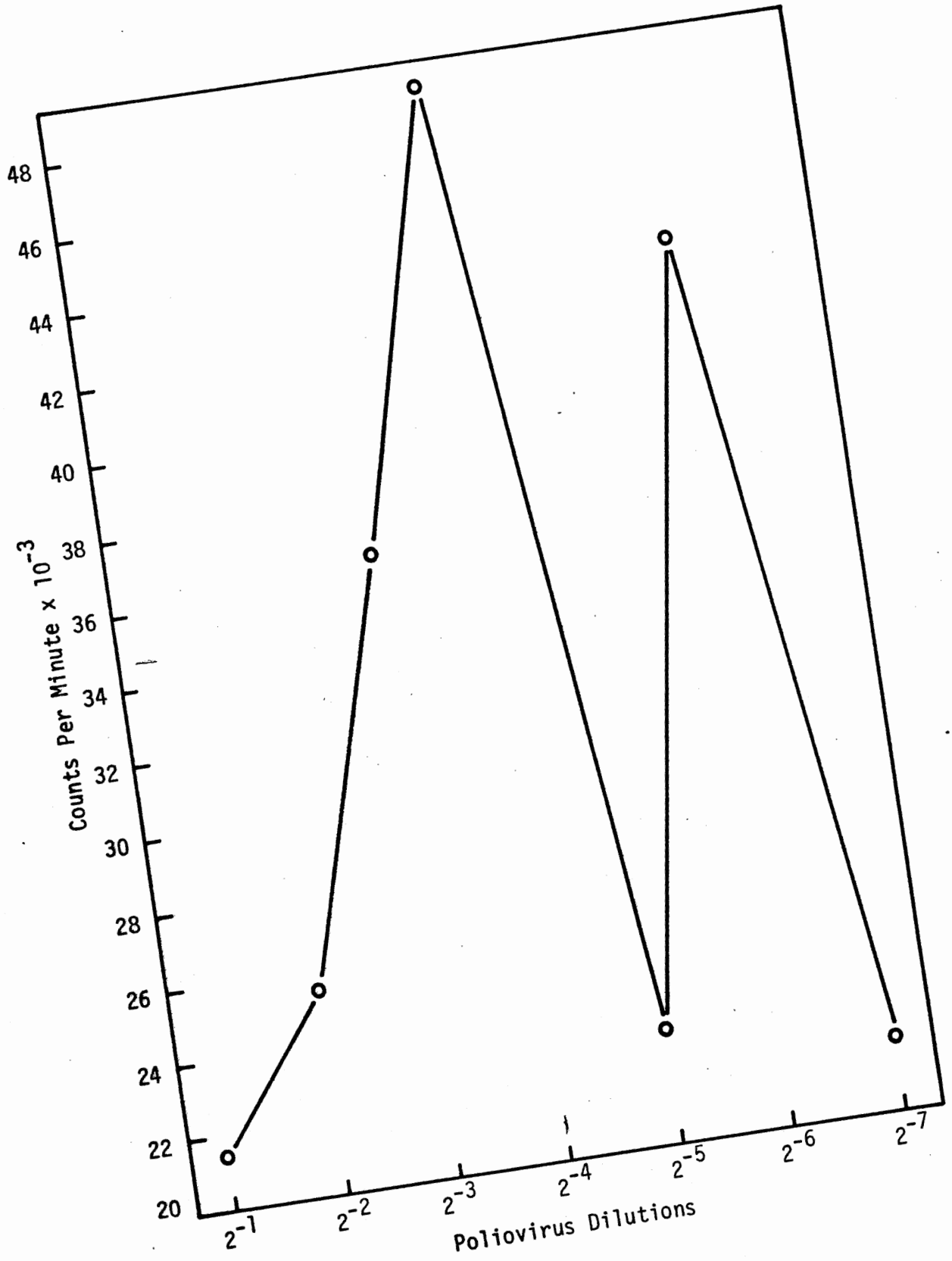

48
46
44
42
40
38
36
34
32
30
28
26
24
22
20
Counts Per Minute x 10^{-3}
2^{-1}
2^{-2}
2^{-3}
2^{-4}
2^{-5}
2^{-6}
2^{-7}
Poliovirus Dilutions

Figure 5. Indirect Detection of Poliovirus 1. by Solid-Phase Anti-Poliovirus Pool and Diluted Anti-Equine IgG

Solid-phase antibody was incubated with a serial twofold dilution of poliovirus 1. Tritium labeled anti-equine IgG was diluted 1 to 2 by cold anti-equine IgG and incubated in the tubes. The tubes were washed and counted for one minute in a liquid scintillation counted.

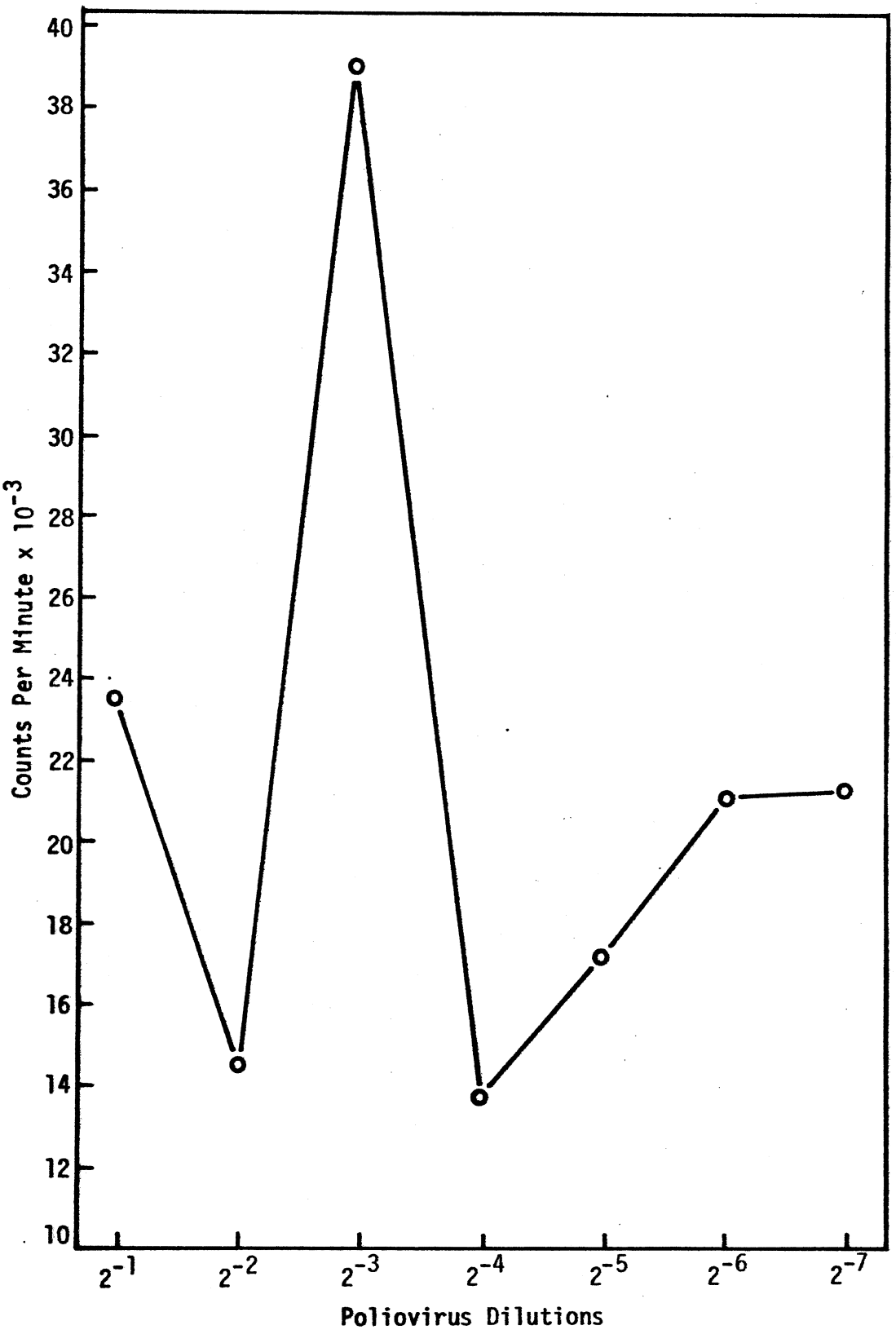

40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
Counts Per Minute x 10^{-3}
2^{-1}
2^{-2}
2^{-3}
2^{-4}
2^{-5}
2^{-6}
2^{-7}
Poliovirus Dilutions

Figure 4(a) was found to be -0.123 and the curve had a slope of -1.550, while the curve in Figure 5(b) had the values +0.243 and +3.460, respectively. There was a significant difference between the two curves at 95 percent confidence level. The counts on curve B were always lower than those on curve A.

To find the lowest dilution of the antibody needed to bind to all active sites on the surface of the solid-phase antibody, a serial dilution of the antibody was incubated with antibody coated tubes and the results are shown in Figure 6. The first three dilutions gave the same amount of ^{3}H immunoglobulin G bound to the solid-phase. The counts then started to decrease with lower concentrations of the anti-equine antibody.

Results of detecting different titres of poliovirus by the solid-phase are shown in Figure 7. The counts increased with decreasing virus concentrations as was expected. But the counts for the dilutions 2^{-2} and 2^{-7} are almost the same. The slope of the curve was found to be +0.8226 and the correlation coefficient of the counts and viral dilutions was 0.683.

4.4 Detection of Poliovirus in Tissue Cultures

The results of monitoring poliovirus in Vero cells are shown in Figure 8. The amount of poliovirus showed a slight decrease during the first hour after infection, then it gradually increased for the next three hours and reached a maximum five hours after infection after which it decreased steeply and reached the initial level in thirty minutes. By the seventh hour the amount of cell associated virus dropped significantly to a level less than the initial one; starting from the ninth

Figure 6. Optimum Dilution of ^{3}H Immunoglobulin G Needed to Bind to the Active Sites on the Surface of the Solid-Phase Antibody

Scintillation tubes coated with equine source anti-poliovirus pool were incubated with a serial dilution of tritium labeled anti-equine IgG for one hour at 37°C. The tubes were washed and counted for one minute in a liquid scintillation counter.

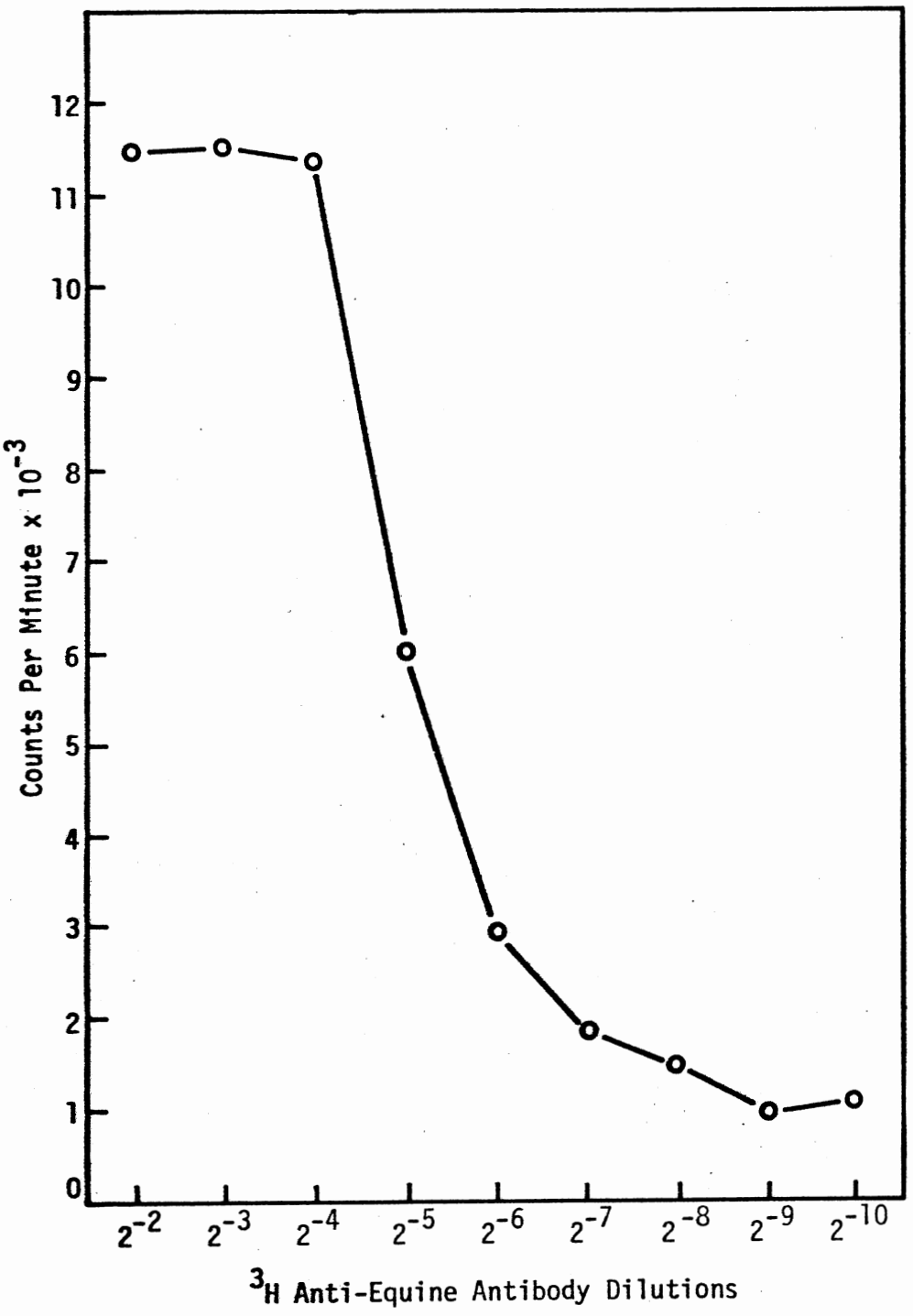

Counts Per Minute x 10^{-3}
12
11
10
9
8
7
6
5
4
3
2
1
0
2^{-2}
2^{-3}
2^{-4}
2^{-5}
2^{-6}
2^{-7}
2^{-8}
2^{-9}
2^{-10}
^{3}H Anti-Equine Antibody Dilutions

Figure 7. Titration of Poliovirus 1. Indirectly by the Solid-Phase Antibody

The solid-phase coated with equine source antipoliovirus pool was incubated with a serial twofold dilution of poliovirus 1. After the incubation period tritium labeled anti-equine IgG was added to all tubes. The tubes were incubated at 37°C for one hour. A one minute count was taken in a liquid scintillation counter.

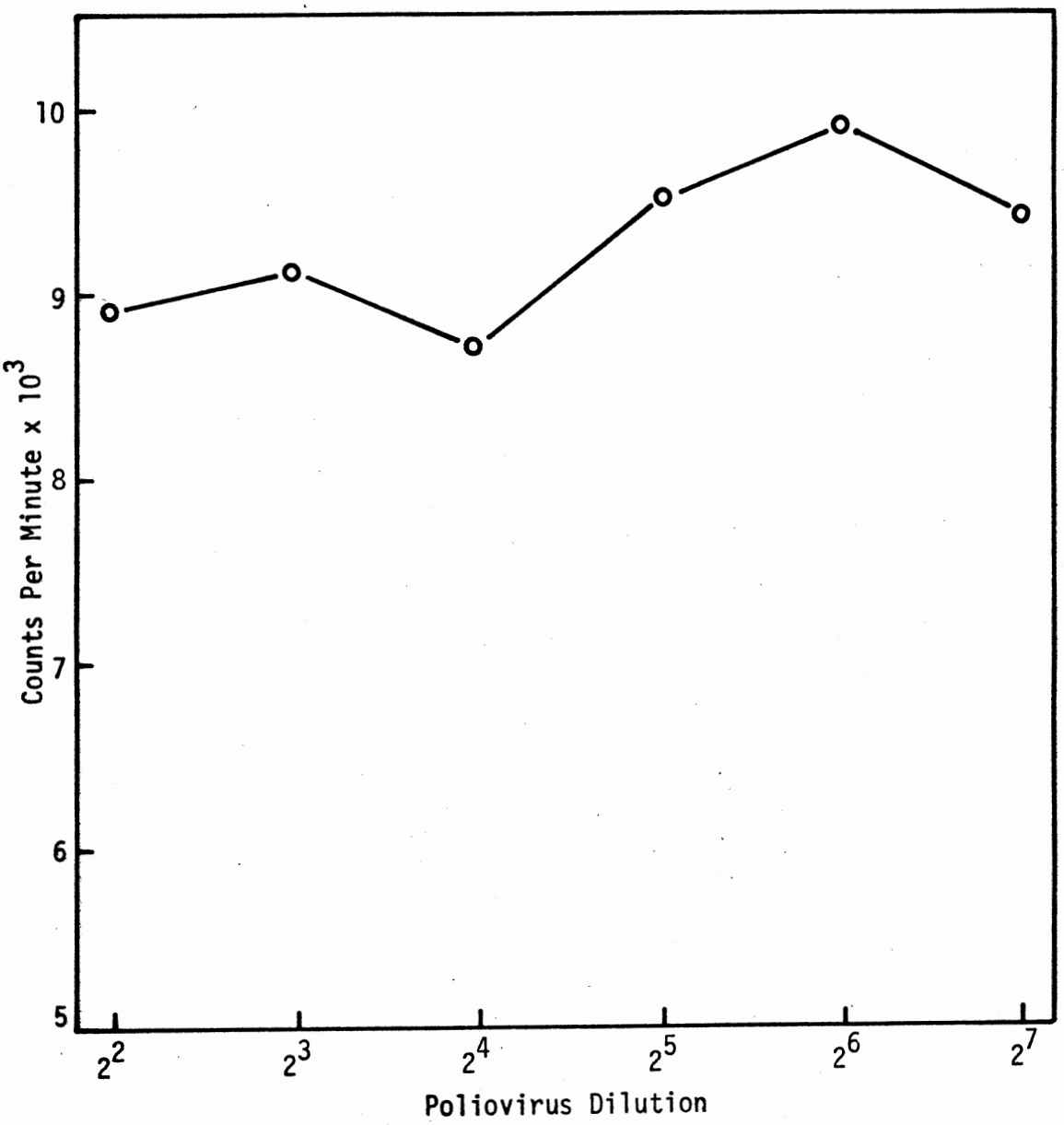
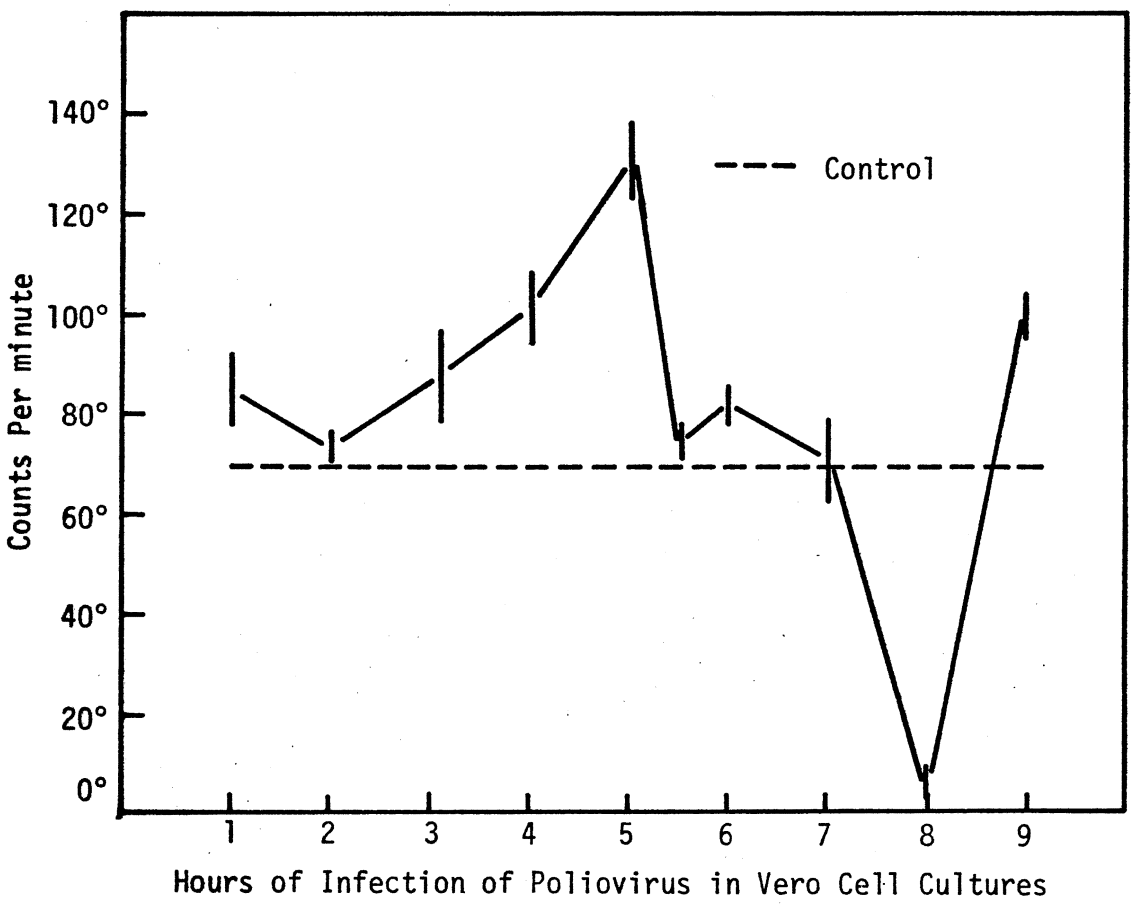

10
9
8
7
6
5
Counts Per Minute x 10^3
2^2
2^3
2^4
2^5
2^6
2^7
Poliovirus Dilution

Figure 8. Growth Curve of Poliovirus 1. in Vero Cell Cultures

Monolayers of Vero cells prepared on discs in tissue culture wells, were infected by poliovirus 1 at hourly intervals. The cells and virus were incubated at 37°C. After nine hours incubation, the cells were washed with PBS, fixed with 70% ethyl alcohol, and 0.1 ml equine source antipoliovirus pool was added to each well. The cells were incubated at room temperature for 45 minutes. The antibody was washed and ^{3}H labeled anti-equine IgG was then added. The cells were reincubated at room temperature for 45 minutes. A one minute count was then taken in a liquid scintillation counter.


140°
120°
100°
80°
60°
40°
20°
0°
Counts Per minute
Control
1
2
3
4
5
6
7
8
9
Hours of Infection of Poliovirus in Vero Cell Cultures

hour the curve shows an increase in the number of virus in the cells. Figure 9 shows the results of growing poliovirus in the BGMK cells. The virus shows almost immediate increase in amount to reach a maximum by the third hour after infection. Following this there is a gradual decrease in amount to reach the initial level by the fifth hour. During the period from six hours to nine hours post infection the amount of virus is lower than the initial level. In the tenth hour post infection the amount of virus shows an increase.

The results of detecting different titres of poliovirus using tissue culture and labeled antibody are shown in Figure 10. After five hours of incubating the virus with Vero cells, the curve shows that the number of counts decreases with the decreasing amount of poliovirus initially infecting the cells. All the values are greater than the value obtained from the control wells.

Figure 9. Growth Curve of Poliovirus 1. in BGMK Cell Cultures
Monolayers of BGMK cells were prepared in tissue culture wells. The cells were infected at hourly intervals with poliovirus 1. After 10 hours incubation at 37°C, the cells were washed and fixed. Equine source antipoliovirus 1 pool was added to all wells. The cells were incubated at room temperature for 45 minutes. After this period ^{3}H labeled anti-equine IgG was added and the cells were reincubated at room temperature for 45 minutes. The cells were then washed and counted for one minute.

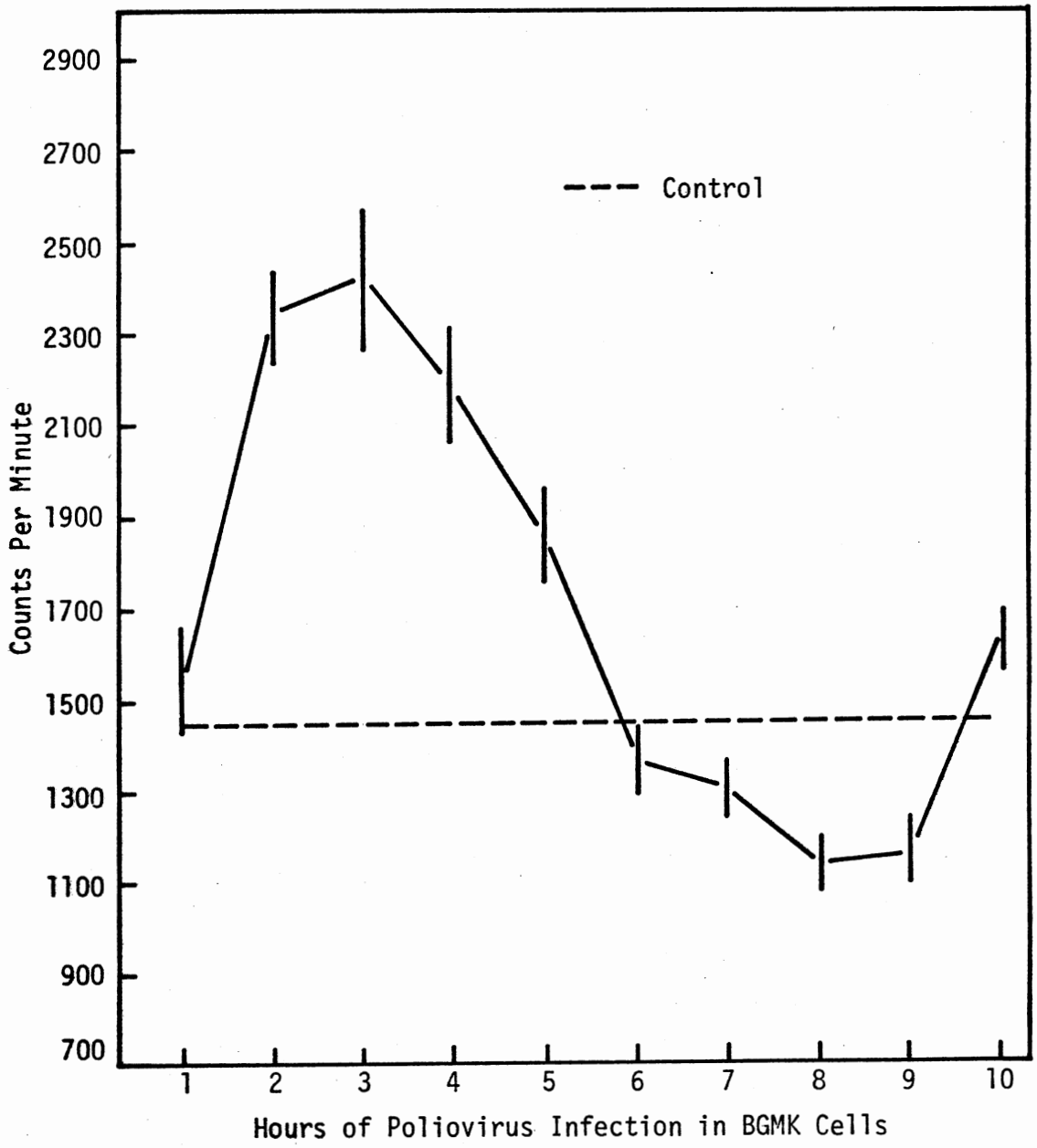
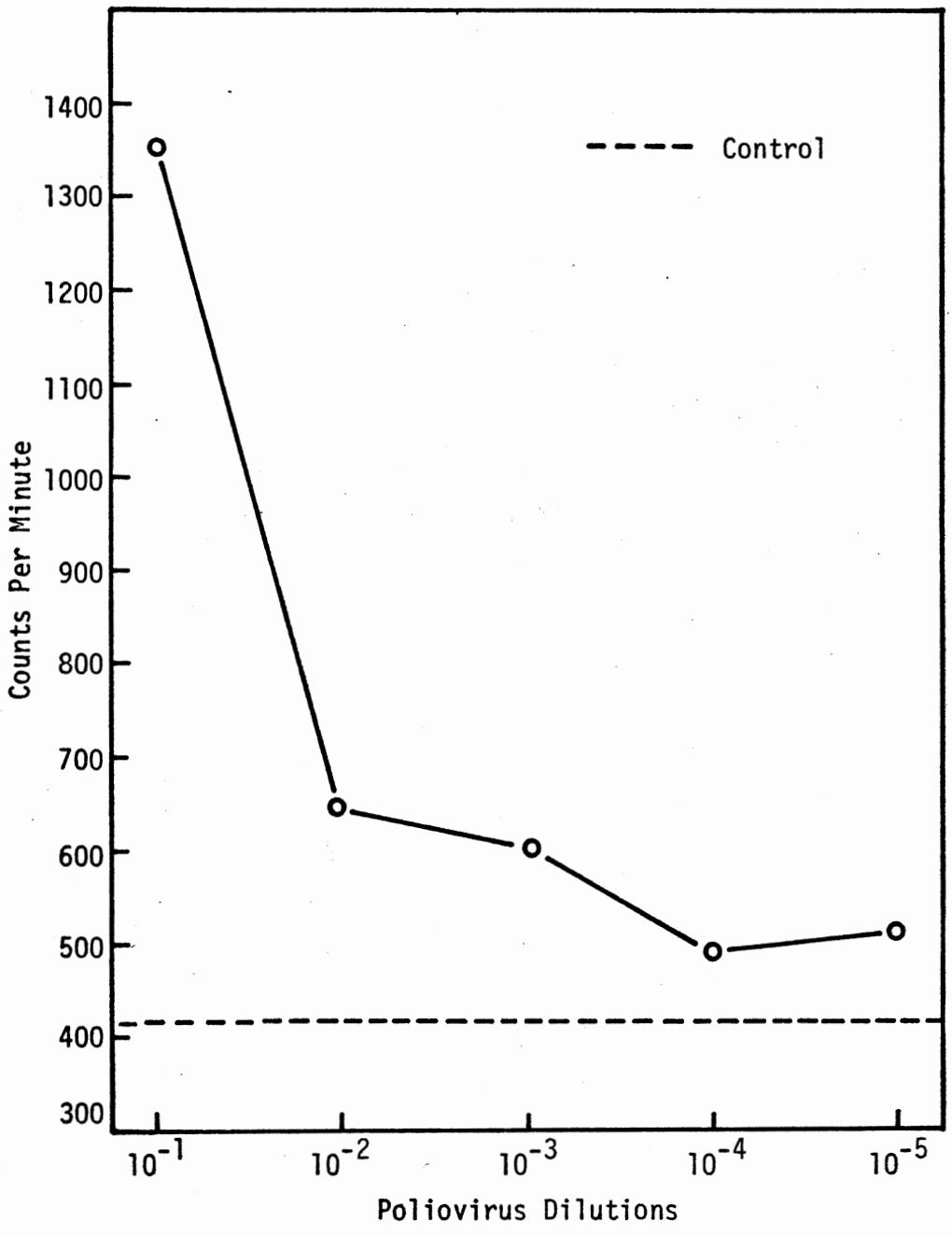

2900
2700
2500
2300
2100
1900
1700
1500
1300
1100
900
700
Counts Per Minute
Control
1
2
3
4
5
6
7
8
9
10
Hours of Poliovirus Infection in BGMK Cells

Figure 10. Titration of Poliovirus 1. in Vero Cell Cultures
Monolayers of Vero cells were infected with a serial tenfold dilution of poliovirus 1 and incubated at 37°C for five hours. The cells were washed and fixed. Antipoliovirus 1 pool was added and the cells were incubated at room temperature for 45 minutes. The cells were washed and ^{3}H labeled anti-equine IgG was added, the cell were incubated at room temperature for 45 minutes. The cells were washed and a one minute count was taken.


1400
1300
1200
1100
1000
900
800
700
600
500
400
300
Counts Per Minute
Control
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Poliovirus Dilutions

CHAPTER V

DISCUSSION

5.1 Preliminary Study on Solid-Phase Antibody

A solid-phase radioimmunoassay was developed and used to detect enteroviruses. Results of the preliminary study done with chicken red blood cells as indicators showed that antibody strongly bound to the scintillation tubes, a property shared by all blood proteins (30). It was observed that when the tubes were filled by red blood cell suspension, a large number of them bound to the tubes. When non-specific immunoglobulin G was incubated in the tubes to coat them prior to the addition of red blood cells, hardly any of the red cells adsorbed to the tubes. This indicated that IgG coated the surface of the tubes.

From the results on Sendai virus binding to the solid-phase antibody it is evident that the bound antibody was active and could bind the virus. It is also evident from the numbers of red blood cells binding to the solid-phase antibody incubated with Sendai virus which was much higher when compared to the number binding to the control tubes that were incubated with non-specific immunoglobulin G only. Binding of the antibody to polyethylene tubes was compared at room temperature and at 37°C, the physiological temperature. The results of binding at 37°C were much better than those at room temperature. The effect of temperature on the binding of antibody to any polymer was not discussed in the literature

and different researchers used a wide range of temperatures to coat the solid-phase with antibody.

It has been found that a wide range of antibody titres could be used to bind to the solid-phase. In order to detect specific virus a titre as low as 8 HAIU/ml may be used for Sendai virus. The sensitivity of the solid-phase antibody to detect virus as monitored by red blood cells was not expected to be very high; however, as little as 16 HAIU/ml of Sendai virus was detected.

Binding of red blood cells to the coated tubes was due to non-specific binding of the cells to the immunoglobulin G, and/or to adsorption of the cells to the naked sites on the wall of the tube that had no IgG binding to them.

The optimum time needed for the immune reaction between specific immunoglobulin G and Sendai virus to be completed was investigated. Incubation periods were varied from 30 minutes to 90 minutes with 15 minute intervals. The number of red blood cells binding to the solid-phase antibody-antigen increased and reached a maximum after 60 minutes incubation and then it started to decrease. These results did not give the expected trend. The amount of bound virus should have increased to reach a maximum and then level off. It should not decrease again because the virus does not elute from the antibody after binding to it.

The antibody preparations used in all experiments were purified immunoglobulin G. This decreased the amount of protein to that of the IgG present, which was 2 mg/ml. Non-specific binding observed in the results of the preliminary study was attributed to the absence of enough proteins to coat all the binding sites in the tube and prevent non-specific binding of the RBC.

5.2 Radioimmunoassay Studies on Solid-Phase Antibody

The results of the experiments done using ^{14}C Sendai virus showed the effect of increasing the protein content in the antibody preparation used to coat the scintillation tubes. Figures 1 and 2 both show that the counts of Sendai virus binding to the solid-phase antibody decreased with higher dilutions of the virus. After a certain point the solid-phase antibody could not detect lower virus concentrations. There is a difference between the two curves. Figure 2 showed better correlation between the counts and the virus dilutions and better proportionality between the increase in counts and the decrease in virus concentrations. It is evident that at the 2^{-5} dilution corresponding to 1 HAU/ml the curve levels off. Detection of the virus at this level does not show much difference between the consecutive dilutions. Several factors that decrease the sensitivity of the reaction are as follows: viral dilution, low precision of the pipette used, amount of antibody binding to the scintillation tube, and the non-specific binding. Improvement was made by incubating the solid-phase antibody with non-specific bovine immunoglobulin G. Even better results were obtained as shown in Figure 3 when the specific IgG was mixed with normal rabbit serum before binding to the solid-phase. This further reduced the amount of non-specific binding.

Experiments were done to detect Sendai virus by the solid-phase antibody. The results show that the solid-phase antibody was successfully applied to detect a wide range of viral titres. The decrease in counts per minute was a result not only of the increase in viral dilution but also of binding of the virus to the solid-phase.

The results of the experiments done using Sendai virus as a model suggested that one could use solid-phase antibody to detect the virus. The system was then applied to enteroviruses, specifically poliovirus. Detection of the virus was done indirectly. The scintillation tubes were coated with equine anti-poliovirus pools. The virus was then added and the binding detected indirectly by adding labeled anti-equine IgG prepared in rabbits. The decrease in counts would correspond to the amounts of binding virus as compared to the counts in the control tubes. The indirect method was used because it was simpler and less expensive to prepare one labeled anti-equine antibody rather than to prepare specific labeled IgG for every strain of enterovirus.

The counts of anti-equine antibody binding to the solid-phase antibody were compared with those binding with the solid-phase antibody after incubating it with poliovirus. The difference was significant in all tubes. The counts of tubes that had poliovirus incubated in them were less than the counts of the control tubes that had both antibodies but no virus. It was evident from the statistical analysis that there was a difference between the two sets of counts at a 90 percent confidence level. The solid-phase antibody was then incubated with bovine serum albumin before adding the labeled anti-equine IgG or the poliovirus, to decrease the non-specific binding. The difference between these two sets of tubes was the same at a confidence level of 90 percent. The difference was expected to be greater for the tubes incubated with bovine serum albumin. The bovine serum albumin had been added to coat the naked active sites on the wall of the tubes and thus to prevent the labeled IgG from binding to these sites and giving counts that do not represent binding of the labeled IgG to the equine antiserum.

To improve the results further a lower dilution of the labeled anti-equine antibody was used. The results were then compared to those of another experiment done at the same time using undiluted labeled anti-equine antibody. The ^{3}H anti-equine antibody was diluted by cold anti-equine antiserum. This process gave competitive binding of both antibodies to the solid-phase. The change in counts would be that of actual binding of antibody and not due just to a decrease in the amount of labeled antibody initially used. Neither gave satisfactory results and some of the points were off the curve for both experiments. But when compared statistically, there was a great difference and improvement when the antibody was diluted. The dilution of the labeled antibody eliminated the excess amount of immunoglobulin G. This gave a better correlation between the amounts of labeled antibody binding and the amounts of virus present. The results of these experiments yielded good and encouraging data indicating that poliovirus could be detected by the solid-phase antibody. Subsequently, other experiments were done to determine the sensitivity and accuracy of the system.

The equine anti-poliovirus pool was not a purified IgG fraction. Rather it was whole serum, so it was not mixed with normal rabbit serum to increase the protein content. The solid-phase antibody was still incubated with bovine serum albumin before adding the virus as a further precaution against non-specific binding.

The results improved in comparison to the previous tests. The sensitivity of the solid-phase antibody was higher and the counts correlated better with the virus dilutions. It was evident from the results of the statistical analysis. The slope of the curve was positive and it had a greater value than the previous curves, which indicated an improvement in

the trend of the counts. The solid-phase antibody could detect the specific virus as far as 2^{-6} dilution. Unfortunately, when this experiment was repeated several times, the results did not improve. Several minor changes were made each time, but still there was no improvement.

The binding of the antibody to the solid-phase has been described by Catt et al. (8) (9) (10), Leininger et al. (30), and Daugharty et al. (14) to be very strongly adsorbed and electrostatically bound. Once the antibody bound to the tubes, it was not easy to remove by the usual experimental manipulations as shaking, washing with 0.2 M HCl or 0.2 M NaOH, or even rubbing the walls of the tubes with the tip of the pipette while aspirating. Kalmakoff et al. (28) developed a theoretical model for solid-phase radioimmunoassays using labeled antibodies. The model was based on the mass action equation for antigen and antibody. It assumed that the reaction was reversible at equilibrium and that there was a single combining site for antibody and antigen. There was perfect separation of unreacted antibody from the antigen-antibody complex. The separation was most conveniently carried out by fixing the antigen to a solid matrix and washing away the unreacted antibody. In other forms of radioimmunoassays other steps have to be taken to separate unreacted antibody such as centrifugation, pH precipitation, immunoadsorption, electrophoresis immunoprecipitation, or several other methods. No attempts have been made to assess whether the reaction was at equilibrium. It has been observed that increasing the incubation period several times did not significantly improve the sensitivity of the assay.

Detection of the antigen by liquid scintillation has several advantages over the solid scintillation method used by Catt et al. (8) (10). Labeling the antibody with tritium by the method described by Sanborn

and Durand (42) was simple, quick, and resulted in minimal loss of antibody titre after labeling. The labeled preparations were stored for a period of 1.5 years with loss of 73 percent of its radioactivity. The loss was a result of the exchange of titrium with hydrogen in the environment rather than a result of radioactivity decay. However, iodine labeled preparations would lose 75 percent of their radioactivity due to decay in 12 days for ^{131}I and in 90 days for ^{125}I. This makes the tritium labeled preparations more desirable and practical to use. In the long run liquid scintillation is less expensive to use than iodine preparations and solid-scintillation. As for safety to the user tritium labeled preparations would be less hazardous to the health. For example, if the person were accidentally contaminated, tritium would be distributed throughout the body thus diluting its effects. Iodine would be exclusively concentrated in the thyroid gland.

5.3 Detection of Poliovirus in Tissue Cultures

The main stages through which a virus goes when it infects a host cell are adsorption to the host cell, penetration, an eclipse phase during which the virus breaks down into its constituent parts, virus replication, and finally release from the cell. Enteroviruses enter the host cell by melting in or by pinocytosis, and being non-enveloped viruses no part of the virus is left outside the cell. The new virus formed accumulates inside the cell until all replication ceases. Then, it is released with or without lysing the cell. The eclipse phase in the case of enveloped viruses is more evident when compared to that of a simple naked virus like the enteroviruses. Enveloped viruses would have more antigens to reveal after breaking down than naked viruses. The method used in

this research detects all cell-associated viral antigens whether or not they are infective. The only antigens that are not detected are the ones that have no specific antibody present in the pool used. These are revealed only after the virus was broken down in the host cell.

Poliovirus was successfully monitored in the host cell using labeled immunoglobulin G. The two cell lines used in this study to grow and monitor poliovirus gave similar results. Both curves showing almost the same trend. The BGMK cell line showed one early peak in the third hour that gradually decreased over a period of three hours. During the following three hours almost no virus was detected in the cells. However, by the tenth hour virus started to appear. These events represent the entry of the virus into the host cell and its immediate replication. This was evidenced from the immediate increase in virus number during the first three hours following infection. The decrease in cell associated virus during the following three hours represents the period of virus release from the cells. After the sixth hour there was little or no cell associated virus. The sharp decrease in counts during this period probably represents lysis of the cells where most of the non-specific binding to the cells is lost. The decrease in counts continues until the beginning of the tenth hour, at which time the virus reappears and this probably represents the beginning of a new cycle of infection and replication.

The Vero cells had some slight differences from the BGMK cells. Poliovirus replicates more slowly in them than in the BGMK cells. Use of Vero cells would be more beneficial in detecting the early events in the growth cycle of the virus in the host cells. The main differences are seen in the first hours following infection.

The slight decrease in the number of poliovirus during the second hour after infection probably represents the breakdown of the virus and the eclipse phase. During this phase some antigenic sites are revealed which had not been recognized by the anti-poliovirus serum. The virus then increased gradually in amount in contrast to the steep increase observed in the BGMK cells. It required five hours for the virus to reach a peak while it required only four hours in the case of the BGMK cells. Poliovirus is released from the cells in a short period; it took approximately 30 minutes for most of the virus to be released. By the eighth hour, there was virtually no cell associated virus. Furthermore, there was little non-specific binding of the antibody to the cells. This period may represent cell lysis. Subsequently, during the ninth hour the amount increased to about the initial level indicating the beginning of a new cycle.

Reissig et al. (39) and Lwoff et al. (32) reported the events that take place in the host cells after infection with poliovirus. The cells were examined under phase contrast microscope to detect the changes a cell goes through following infection. The amount of virus was calculated by taking samples from infected cells at different time intervals. The samples were incubated in monkey kidney cells. These were then examined for CPE. The results indicated that the synthesis of poliovirus in monkey kidney cells was rapid and required 8 hours. However, almost half of the cells in the culture failed to show cytopathic changes for up to 10 hours after infection. The number of cells showing cytopathic effect is proportional to the multiplicity of infection (39).

The cells started to release virus after 5½ hours post infection. Within 30 minutes the bulk of the virus was released, appearing in the

medium for about 30 minutes, during which time the rate of release was almost uniform (32).

It is evident from the above studies that the only virus detected was the whole infective virus particles released from the cells. Low titres of the virus would give delayed results because of the low multiplicity of infection, and appearance of microscopic changes later than 10 hours and consequently later formation of new virus.

With the use of radiolabeled antibody, infective and non-infective virus particles are detected and the degree of sensitivity would be expected to be much higher. The time required for detection of an unknown amount of virus in a sample can be minimized. The growth curves would be used to determine the amount of time required for the virus to reach its maximum amount.

The results of detecting various viral titres after five hours incubation in the Vero cells proved that this method could be applied to detect virus in a water sample. The counts decreased with decrease in the initial amount of virus incubated in the cells. The different titres of the virus used were detected by this method and all had counts higher than the control wells that had no virus and gave the background reading. The different strains of enteroviruses could be detected using the corresponding specific antibodies. The amount can be quantitated by plotting a standard curve every time. The curve showed the expected trend.

CHAPTER VI

SUMMARY AND CONCLUSION

Specific immunoglobulin G was adsorbed to the surface of scintillation tubes by simple incubation at 37°C. This provided a solid-phase antibody that was used to detect specific virus. The immune reaction took place on a solid matrix where, after incubation and completion of the immune reaction, the unreacted portions were poured out and simple washing by a buffer ensured removal of unreacted elements. The presence of binding to the solid-phase antibody was detected by adding a third layer of labeled antibody specific for the first antibody to bind to the active sites that are not occupied by the virus. The specific antibody was mixed with normal serum before binding to the solid-phase to cover all active sites on the surface of the tube to prevent binding of the virus or labeled antibody to them.

Results of this preliminary study have been encouraging and showed that the virus can be detected by the solid-phase antibody, but more investigation is needed to increase the sensitivity of the assay.

Two cell lines, Vero and BGMK cells, grown on sterile cover slips were infected with poliovirus at hourly intervals for ten hours, washed and fixed with 70 percent ethyl alcohol. Equine anti-poliovirus antiserum was added, then labeled anti-equine IgG was added. The cells were incubated for 45 minutes and the unreacted antibody was washed off. The results were read for one minute in a liquid scintillation counter. This

method was used to assay poliovirus. The cells were infected by a serial dilution of poliovirus and incubated for five hours which allowed for maximum production of the virus in the host cells which was determined from the previous experiments. The results showed that this method could be used to detect and quantitate enteroviruses.

A radioimmunoassay method using solid-phase antibody and labeled antibody was developed to detect viruses in a water sample. The antibody was successfully bound to the solid-phase. The antibody strongly bound to the solid-phase by electrostatic forces which were unaffected by experimental manipulations. The process of binding the antibody to the solid-phase was simple, direct, and rapid. After binding, the active sites of the antibody were still exposed and these were used to detect various titres of the virus both directly and indirectly. In this study better results were obtained by the direct method where the virus was labeled. In the indirect method a second labeled antibody specific for the bound antibody was used to detect how many sites on the solid-phase antibody have not been occupied by the virus. The indirect method would be the one recommended to detect virus in a sample. It provides a simple and inexpensive method for viral detection.

The antibody solution used to coat the solid-phase can be used again because only 1 percent of the antibody in the solution binds to the polymer which minimizes and saves on the amount of antibody solution needed to coat the solid-phase. Small volumes of the reactants ranging from 1.0 ml to 0.1 ml have been used and all reactants can be reused.

Separation of the unreacted antibody was a simple procedure, done by simple washing of the solid-phase with a buffer. Binding of the antibody-antigen-antibody to a solid-phase provided this simplicity. The total

time needed for the immune reactions to take place and the results obtained was in the range of four hours, which provides a rapid method for viral detection.

The sensitivity of the system was affected by the non-specific binding of the labeled antibody to the virus and its binding to exposed active sites on the surface of the solid-phase. To obtain higher levels of sensitivity the problem of non-specific binding should be investigated. Due to the great sensitivity of any radioimmunoassay method, high precision is necessary in performing the assay.

The solid-phase antibody assay system provides a simple, rapid, and inexpensive method to detect enteroviruses. To improve the sensitivity of the system, more research and investigation of the problems are needed.

Another simple method for virus detection and quantitation has been investigated. Tissue cultures were successfully used to grow, magnify, and detect poliovirus. This method can detect low titres of virus because it allows for viral replication and thus a better chance for the virus to be detected. It is also a sensitive radioimmunoassay method. The virus can be quantitated by comparing a standard curve to test results. The results of the assay can be obtained within five to seven hours depending on the cell line used.

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