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Radioimmuno Detection of Virus

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RADIOIMMUNO DETECTION OF VIRUS

by

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Approved:

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TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF TABLES

ABSTRACT

Radioimmuno Detection of Virus

by

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The commonly used techniques for viral detection are tedious, time consuming and in many cases inadequate. As a consequence, a rapid, sensitive radioimmunoassay has been developed for detecting viruses. Reovirus is reacted with homologous ^{125}I labeled antibody after which the antigen-antibody complexes are separated from unreacted labeled antibody by density gradient ultracentrifugation. After centrifugation, the densty gradient is fractionated and the radioactivity counted in a liquid scintillation spectrometer.

The amount of activity in the lower fractions of the density gradient is directly proportional to the virus concentration. The radioimmunoassay developed has several advantages over other viral assay procedures; the method is rapid, viral samples can be assayed within six hours after receiving the sample, both viable and inactive viruses are detected, and the procedure is sensitive.

INTRODUCTION

The dissemination of many viral diseases is poorly understood because of the lack of good methods for concentrating and assaying viruses.

One of the major problems in regard to air and water quality is the detection and identification of viruses when they are present in low concentrations. A solution of this problem will require the development of new and improved techniques for concentrating and detecting viruses.

The antigen-antibody reaction is basically the same in all immunological tests. The only difference is in the manner in which the formation of the complexes are measured. Many of the existing procedures are inadequate because of the lack of sensitivity, rapidity and specificity. Viral detection procedures such as indirect hemagglutination and complement fixation have the specificity needed, but lack sensitivity. Other procedures such as fluorescent antibody staining of infected cells and plaque assay have sufficient sensitivity, but are time consuming because viral detection depends on multiplication of the virus in the host cells. At the present time the only available method for obtaining total viral particle counts requires the use of the electron microscope, and this method lacks sensitivity because high magnification is needed to observe viral particles.

The basic principle of the radioimmunoassay developed in this study, is that unreacted ^{125}I labeled antibody can be separated from the virus-^{125}I antibody complex by rate zonal density gradient

centrifugation. The rate of sedimentation of particles under centrifugal force is dependent on their size, shape and density, therefore, viruses with bound antibody will migrate at a faster rate than smaller labeled serum proteins that are not bound to virus. The radioactivity of the virus-^{125}I antibody complex, once separated from other components is an indication of the concentration of virus present.

The radioimmunoassay has several advantages. The assay is comparable with hemagglutination in rapidity. The virus sample can be assayed within a six hour period. Like many other immunological assays, the radioimmunoassay is specific and both viable and inactive viral particles are detected. The assay procedure is sensitive and therefore has application in detection of viral contamination in the environment.

LITERATURE REVIEW

General

Viruses as antigens react with their homologous antibody in the presence of an electrolyte to form a virus-antibody complex. Viruses, when neutralized, are modified in some unknown physical or chemical manner so the infectivity of the virus no longer exists.

Weiser, Myrvik and Pearsall, (1969) indicated that the forces involved in antigen-antibody complexes were low energy binding forces such as Coulombic forces, Van der Waal's forces and hydrogen bonding. They also stated that weak binding forces are effective only when the molecular surfaces have complementary structures. For this reason there must be a high degree of steric complementary, which accounts for the specificity of antigen-antibody reactions. If covalent linkages were involved in the antigen-antibody complexes, the dissociation of the complex would not take place as readily as it does in solutions of high salt concentration or high or low pH. Because of the disruption of the antigen-antibody complex by adverse ionic conditions, Kedar et al. (1971) was able to purify antibody by immunoadsorption techniques.

Fritz and Beard (1969) found that one virus binds many molecules of antibody, however, Fazekas de St. Goth (1962) found that one antibody would neutralize a single virus. The single antibody may change the isoelectric point of the virus and block attachment to a host cell receptor site. Recent studies by Brown and Smale (1970) revealed that foot-and-mouth disease virus contained within its protein coat three antigenically distinct sites of antibody reaction, thus further com-

plicating the explanation of an antibody molecule neutralizing one virus. When virus and antibody are mixed, various virus-antibody complexes form; these complexes vary with respect to the kinds and numbers of antibody molecules. Fritz and Beard (1969) showed that the nature of the complexes also varies with the ratio of antibody per virion and with the incubation time.

Labeling immunoglobulin

With the use of radioactive isotopes, the measurement of virus-antibody complexes can be achieved. Ideally it would be desirable to label immune serum by replacing one of the natural atoms with isotopes of carbon, sulphur or tritium similar to a method used by Collipp et al. (1965) to label insulin. This would involve feeding laboratory animals on radioactive amino acids, which would result in many proteins other than antibodies becoming labeled. For this reason immunoglobulins could only be very lightly labeled before causing injury to the animal. Kollman (1972) raised mice on ^{13}C yeast and estimated that about 50% of the ^{13}C was incorporated into living tissue. This would be an ideal system for internally labeling IgG molecules, since ^{13}C is a stable isotope and would not cause radiation damage. The ^{13}C associated with the antibody can be measured by a mass spectrometer or by a nuclear magnetic resonance spectrometer.

There are many relaible methods which can be used to iodinate proteins. Although iodination of immunoglobulin alters the physical properties of the molecule, such as molecular weight and isoelectric point, Rosa et al. (1964) has shown the immunological properties are

not altered if the protein molecules are not labeled too heavily.

Hunter (1967) stated the following advantages of using ^{125}I to label immunoglobulin. The labeling procedure can be carried out in vitro, and the specific activities needed for various experimental conditions can be obtained. Exchanges of iodine atoms with other atoms does not occur, because the iodine is covalently bonded to tyrosine groups. The primary reaction involves replacement of a H+ ion on a tyrosine ring with iodine. The labeled protein molecules lose very little antibody activity, and isotopes with short half lives (^{131}I) can be used to provide higher specific activities than possible with more slowly decaving isotopes.

There are three basic methods described in the literature for labeling immunoglobulins. Free iodine will react with tyrosine rings to form mono- or diiodotyrosine; in addition, the iodine may react with sulphydryl groups and with the histidine ring. The reaction forming moniodotyrosine is most commonly used. Farr(1958) showed that the other reactions produce adverse molecular alterations and loss of specificity.

McFarland (1958) and Helmkamp et al. (1961) describe the use of iodine monochloride in a technique which has wide application in labeling plasma proteins. Rosa et al. (1964) used an electrolytic method in which small amounts of proteins were labeled without denaturation. Hunter and Greenwood (1962) have developed the most widely used method for labeling hormones and protein molecules with very high specific activities.

In the Hunter and Greenwood method, the labeling procedure is

extremely rapid, therefore, denaturation of the proteins is minimized. The original procedure buffers the protein with phosphate buffer pH 7.6. Chloramine T is used as an oxidizing agent and when used in large amounts could cause damage to the protein. Sodium metabisulphite used as a reducing agent to stop the reaction can also cause damage to the protein when used in high concentrations.

There are many other causes of loss of antibody activity during iodine labeling. In many cases activity is lost in the purification procedures, however,this can be minimized. Over labeling of molecules with iodine can be a major cause of inactivation of antibody molecules. Freeman, Matthews and McFarlane (1959) and Bianchi et al. (1966) showed that albumin can be iodinated with five atoms of iodine per molecule of albumin, while human, rat and rabbit gamma globulin can be labeled with 1.5 atoms of iodine per molecule of globulin without showing loss of antibody activity. Radiation damage can cause inactivation of antibody molecules when high specific activities are required, however this can be overcome by adding carrier protein.

The rate of reaction

Carpenter (1965) described many conditions which effect the rate of the antigen-antibody reaction such as concentration of antibody, antigen, kinds of electrolytes present, pH, temperature and agitation of the reaction mixture. Mandel (1960) has shown that the antibody concentration influences the rate of the antigen-antibody reaction.

Heidelberger (1939) found that the precipitate of rabbit anti-

body formed in 0.9 M NaCl contained only one third as much protein nitrogen as that formed in 0.15 M NaCl. Electrolytes are essential for the antigen-antibody reaction, but high concentrations inhibit the reaction. The addition of electrolyte reduces the negative charge possessed by most proteins and viruses in neutral solutions. This effect is greater with multivalent ions than with univalent ions. Heidelberger (1939) showed that the antigen-antibody reactions take place better when the charge drops below about 15 millivolts.

Mandel (1960) stated that the optimun hydrogen ion concentration for most serological procedures is between pH 6.5 and 8.5; however, certain reactions proceed satisfactorily in more acid or more alkaline media. Northrop and DeKruif (1922) found that a typhoid bacterial suspension hemagglutinated within a narrow pH range.

Mandel (1960) also showed that serological reactions are usually accelerated as the temperatures are increased from 0 C to 30 C and in some cases higher temperatures. In most cases temperatures above 56 C reduce the rate of the reaction.

Mechanical shaking accelerated the antigen-antibody reaction, apparently by providing increased opportunity for contact between molecules and virus.

Reactivation of neutralized virus

There are two methods used to reactivate neutralized viruses; the first of which is dissociation of the virus-antibody complex without destroying the antibody, and the second is dissociation of

the virus-antibody complex with destruction of the antibody. The degree of dissociation has been evaluated by determining the degree of infectivity that was restored.

Low and high pH treatment

High and low pH dissociation have been used on viruses that are capable of withstanding the extreme pH necessary to bring about dissociation. Mandel (1961) presented evidence that pH 2.5 was required to achieve complete dissociation of neutralized poliovirus. It was also concluded that neither virus nor antibody suffered any functional impairment at pH 2.5.

Kjellen (1965a) showed that neutralized adenovirus can be reactivated by pH 2.5 treatment; however, his conclusion was that the virus-antibody complex did not dissociate, but rather reorganized in such a way that the infectious capacity of the virus was restored. Kjellen's conclusion was based upon the observation, that reactivated virus and neutralized virus in CsCl had a buoyant density of about 1.30 gm/ml, and the control virus had a buoyant density of about 1.35 gm/ml. Many other workers have also shown that reactivation of neutralized virus takes place at extreme pHs. These include the following: Pinheiro and Hsiung (1963) with coxsackie virus, Granoff (1965) using Newcastle disease virus, and Rappaport (1961) with tobacco mosaic virus.

Reactivation by proteolytic digestion of antibody

If viral proteins are insensitive to the enzyme it is sometimes possible to reactivate neutralized virus using proteases. Many viruses

have shown a high degree of insensitivity to proteolytic enzymes; however, Spendlove, McClain and Lennette (1970) showed that reovirus infectivity was enhanced by removing or altering the outer protein coat with proteolytic enzymes. If the reaction were allowed to go long enough the subcapsid layer was completely digested, which resulted in loss of infectivity. Kalmanson, Hershey and Brofenbrenner (1943) showed that complete reactivation of neutralized Escherichia coli phage could be achieved with papain if the ratio of antibody to virus was low; however, as the ratio was increased, the degree of reactivation decreased to zero. More recently Keller (1968) found neutralized poliovirus was 20-25% reactivated with papain or pepsin since neither of these enzymes have an effect on poliovirus. An additional observation was, that virus reactivated with papain could be reneutralized by a serum prepared against rabbit gamma globulin. These results indicated that reactivated virus still retained a fragment of the antibody molecule of the original rabbit antiviral antibody.

Reactivation by fluorocarbon treatment

With the use of fluorocarbons such as Freon 113 and Genetron 226, 65% of neutralized poliovirus can be recovered as infectious particles. Hummeler and Ketler (1958) found that the reactivation rate depended on the blending time of the neutralized virus with the fluorocarbon. Similar results were obtained by Brown and Cartwright (1960) with the foot-and-mouth disease virus.

Reactivation by high salt concentration

One would expect high salt concentrations to dissociate virus-antibody complexes because of the weak forces involved in the antigen-antibody complex. Svehag (1965) carried out a series of experiments with poliovirus which indicates that a 5 M NaCl concentration could reactivate poliovirus when neutralized with IgM; however, when IgG was used to neutralize poliovirus, reactivation did not take place. He also found that if NaCl was reacted with the poliovirus and antibody before the reaction took place the formation of the complex was prevented.

Reactivation by ultrasonic treatment

Ultrasonic waves have been used to reactivate neutralized virus. The effect of sonication results from the cavitation that occurs when dissolved gases respond to the progressive condensations and rarefactions of the impinging high frequency sound waves. Lafferty (1963a) observed 50% recovery of influenza virus infectivity after three minutes sonication and 100% recovery after five minutes at a frequency of 16 kc/sec. and 50 watt output. The specimen was assayed immediately after sonication because the titer would decline, suggesting that at least some active antibody survived sonication. Influenza with no bound antibody showed complete resistance to fifteen minutes of sonication.

Keller (1965) showed that poliovirus was reactivated by sonication and neither prolonged incubation of the antibody complex or a high ratio of antibody to poliovirus affected the degree of react-

vation by sonication. Kinetically, the reactivation process showed first order characteristics.

Reactivation by freezing and thawing

Successive freeze-thaw cycles have been used to reactivate neutralized poliovirus. Aliquots of the specimens were frozen in a Revco freezer at -70 C, then thawed at room temperature for five successive cycles and assayed. The degree of reactivation was influenced by the number of antibody molecules per virus. Keller (1965) showed that reactivation was more complete if thawing occurred at room temperature rather than 37 C. Mandel (1961) showed that reactivation was independent of the virus type and degree of neutralization if precautions were taken to stop reassociation, therefore, they were able to get complete reactivation of the neutralized viruses by dilution.

Characterizations of virus-antibody complexes

Many workers have shown that viral characteristics, such as size, mass, surface charge, and configuration were altered by neutralizing the virus with antibody. By measuring the change in physical characteristics of neutralized virus, the antibody activity of a serum sample can be determined.

Two phase polymer separation

A two phase polymer system which is the distribution of virus and antibody between two aqueous polymer phases can be used to separate virus-antibody complexes. Philipson et al. (1966) used dextran

sulfate and polyethylene glycol to detect and characterize virus-antibody complexes. The distribution coefficient of the virus-antibody complex was different from the virus and the antibody distribution coefficients. (The distribution coefficient is the ratio of virus-antibody complex in the upper phase to the concentration in the lower phase.) Poliovirus type 1 (in dextran sulfate and polyethylene glycol system) has a distribution coefficient (K) of 4.5, IgG antibody has a (K) value of 0.55, while the virus-antibody complex has a (K) value of 0.05. Because of the difference in the distribution coefficient, virus-antibody complexes can be characterized by a two phase polymer system.

A procedure described by Clark and Lister (1971) which has application in the separation of the virus-antibody complex from unreacted components involves centrifugation of virus through a reverse concentration gradient of polyethylene glycol (PEG). The reverse PEG gradient is stabilized with a positive sucrose gradient. The concentration of PEG at which the virus particles resolubilize is termed the solubility concentration, and the solubility concentration of virus particles is dependent upon their surface/volume ratio and their surface charge characteristics. Because the surface characteristics of viruses are altered by the addition of antibody, separation of neutralized virus can be achieved.

Ultracentrifugation

Particles with different physical characteristics can be separated by equilibrium density gradient ultracentrifugation or by zonal

rate density gradient ultracentrifugation. In both cases density gradients are used as an anti-convection medium. In the zonal rate method, particles are separated in accordance with their sedimentation coeffecients, which depend on size, shape and density. Whitcomb and Spendlove (1966) demonstrated the presence of virus-antibody complexes by rate zonal ultracentrifugation. The method developed by Whitcomb and Spendlove (1966) revealed a sensitivity tenfold greater than the precipitation test, and it should be borne in mind, that very high concentrations of virus were required before the band was seen. The smaller the virus, the higher the concentration of virus needed to visualize the band. A similar study of tobacco mosaic virus (TMV) and Southern bean mosaic virus (SBMV) was carried out by Ball and Brakke (1969), and the only difference between the two procedures was the method of observing the virus-antibody complex which was with a scanning (253.6 mu) fractionator.

In isopycnic ultracentrifugation particles are separated by their buoyant densities. Salt gradients with average densities approximating those of the particles to be separated are used. When isopycnic ultracentrifugation is used to separate the virus-antibody complex, the time of ultracentrifugation should be long enough to insure that the density gradient forms. Fritz and Beard (1969) illustrated the use of isopycnic centrifugation for the study of virus-antibody complexes. Avian leukosis virus was purified by sodium sulfate precipitation and then iodinated with ^{125}I. The density of avian leukosis virus was found to be 1.176 gm/ml which was quite low due to its lipid content. The density of IgG antibody was around 1.381 gm/ml, a value calculated from the assumed partial specific

volume of 0.725 ml/gm. The densities of the virus-antibody complexes varied from 1.192-1.218 gm/ml, and the ratio of antibody molecules per virion varied from 970-3458. When the ratio of antibody molecules per particle was plotted against density, a good linear relationship was observed.

The separation by isopycnic centrifugation depends upon the difference between the densities of reactants; however, in the absence of such a difference the virus-antibody complexes can still be separated by rate zonal ultracentrifugation. McCombs (1967) reported that viruses with buoyant densities in CsCl, less than 1.27 gm/ml (eg. influenza and Rauscher murine leukemia viruses) increased in buoyant density, and viruses with densities more than 1.27 gm/ml (adenovirus, reovirus) decreased in density after reacting with antibody. Viruses with densities of 1.27 gm/ml (herpes simplex) showed no measurable change in buoyant density.

Steensjaard and Hill (1970) used a zonal rotor to separate soluble immune complexes. The reaction between an antigen and the homologous antibody resulted in the formation of both soluble and insoluble complexes. By using a B-XIV rotor they were able to carry out preparative and analytical studies on the soluble complexes at the same time. The reaction mixture consisted of human serum albumin and rabbit anti-human serum albumin.

Electrophoresis

A great majority of polymers are electrically charged, and macromolecules can be classified as strong or weak electrolytes depending on the ionization constants of the acidic or basic groups.

Electrophoresis has been used to separate mixture macromolecules according to their charge; the rate of migration of a molecule varies according to the net charge, mass and geometry of the protein. Since antibodies and viruses have been separated from each other by electrophoresis, the technique can be used to separate virus-antibody complexes from free antibody or virus. Mandel (1971) used a sucrose gradient for electrophoresis of poliovirus. The electrophoretic analysis alone showed a complex behavior suggesting that the virus has two conformational states with respect to isoelectric points, one at pH 7.0 and the other at pH 4.5, but neutralized virus had a single isoelectric point at pH 5.0.

Brakke, Allington and Langille (1968) measured electrophoretic mobilities of tobacco mosaic virus (TMV) and brome mosaic virus (BMV) in a density gradient zonal electrophoresis apparatus. The virus zone was located by pumping the gradient column through an ultraviolet flow cell; the pumping of the gradient did not disturb the virus zone.

Isoelectric focusing, an extension of density gradient zone electrophoresis, was a technique developed by Svensson (1961) and Vesterberg and Svensson (1966). It involves establishing a pH gradient by applying a voltage to a mixture of ampholytes, posessing a range of isoelectric points. Macromolecules migrate to their isoelectric point and stop. The technique of isoelectric focusing offers the advantage that protein zones become sharpened, since the forces producing the separation act in such a way as to minimize spreading due to diffusion. Isoelectric focusing is superior to electrophoresis for separation of virus from neutralized virus,

since diffusion is counteracted.

Electron microscopy

Negative staining techniques have extended the sensitivity of the electron microscope and made it a convenient method for observing virus-antibody complexes. Several papers have been published on virus-antibody complexes, in the monomeric and multimeric form.

Hummeler, Anderson and Brown (1962) showed the applicability of negative staining by differentiating two different antigenic forms of type 1 poliovirus. The virus and antisera were purified before reacting and the complexes were observed directly after staining with 2.0% solution containing neutralized phosphotungstic acid and 0.2% sucrose. Lafferty and Oertelis (1963) used similar methods to show the demensions of antibody molecules of the IgG type. By careful preparation of the samples the authors were able to show evidence for bivalent attachment of antibody molecules to the virus. Svehag and Bloth (1967) carried out studies to show the attachment and configuration of antibodies to poliovirus. Brown and Smale (1970) studies virus-antibody complexes of foot-and-mouth disease virus, and because of exceedingly fine definitions they concluded that three distinct antigenic sites were present in the viral capsid.

Filtration

Selective filtration has often been used as a rapid method for separation of virus-antibody complexes from free virus. Frazekas de St. Groth and Webster (1963) used a Millipore (cellulose acetate)

filter to separate uncombined antibody from virus-antibody complex. The pore size was small enough to allow rapid passage of antibody. They first treated the filters with antibody which prevented adsorption of molecules onto the filter. Wallis and Melnick (1967) reported the successful separation of mondispersed virus particles from aggregated virus particles by using membranes or appropriate pore diameters.

Chromatography has been used for many years for the separation of two or more solutes in the same solution. These methods have been refined and have been adapted to the separation of virus-antibody complexes from other components. There are basically two types of chromatography; exculsion chromatography which utilizes particle size for separation, and adsorption chromatography which utilizes surface charge properties as the differentiating characteristic. A chromatography procedure used by Thomssen (1963) for the fractionation of free virus from neutralized viruses makes use of adsorption chromatography in which the adsorbent used was an aluminum hydroxide gel.

METHODS AND MATERIALS

Chemicals and reagents

The following chemicals and reagents were used in this study: Iodine 131 and 125 and Aquasol (New England Nuclear, Boston, Massachusetts), chloramine T and potassium tartrate (Matheson Coleman and Bell, Norwood, Ohio), sodium metabisulfite (J.T. Baker Chemical Company, Phillysburg, New Jersey), Biuret reagent (American Monitor Corps., Indianapolis, Indiana), bovine serum albumin (BSA)(Sigma Chemical Company, St. Louis, Missouri). Immune goat serum was a gift from R.S. Spendlove (Utah State University, Logan, Utah.)

Cells and viruses

The cells used in this research were Madin-Darby canine kidney (MDCK) cells. The cell line was purchased from the American Type Culture Collection Cell Respository, Rockville, Maryland, and has been in our laboratory since March 1968. The experiments were conducted while the cells were between 1 and 128 passages in this laboratory. The MDCK cells were maintained on Eagle's minimum essential medium (Difco Laboratories, Detroit, Michigan) enriched with 10% fetal calf serum, 1% glutamine and 1% antibiotic solution, containing the following: 25,000 units of penicillin, 25,000 ug each of streptomycin and neomycin and 250 units of bacitracin per ml.

The reovirus used in this study was type 1, Lang 17-1 strain, a plaque isolated from the California State Health Laboratory, Berkeley, California.

General

Goat antireovirus antibody was fractionated on DEAE-cellulose by a method first used by Levy and Sober (1960). After fractionation the serum was dialized overnight at 4 C in borate buffered saline pH 8.6. The immune globulin was then diluted to 4 mg/ml and dispensed into 0.5 ml quantities and stored at -90 C. The antibody was labeled with ^{125}I or ^{131}I by a modification of a procedure developed by Hunter and Greenwood (1962). The modification was in the amounts of reactants used. The immune serum and ^{125}I concentration were varied to give the desired specific activity, and the stable ^{127}I concentration was decreased to insure that each antibody molecule would be labeled with ^{127}I or ^{125}I. The procedure is outlined in detail below.

Labeling procedure

The following reactants were added to a small glass vial with mild agitation:

(1) Immune globulin (1 mg/ml) . . . . . 1.0 ml

(2) Potassium iodide (8.3 ug/ml) in borate buffered saline (BBS) pH 8.6 . . . . 0.1 ml

(3) One millicurie of ^{125}I (Na ^{125}I)

(4) Chloramine T (0.5 mg/ml) fresh in BBS, pH 8.6 . . 0.1 ml

The above mixture was reacted for 15 minutes at room temperature after which the reaction was stopped by adding 0.1 ml of sodium metabisulfite (0.5 mg/ml fresh in BBS pH 8.6) and 0.3 ml of carrier iodide (potassium iodide 10 mg/ml in BBS, pH 8.6). One

milliliter of bovine serum albumin (BSA) (10 mg/ml in BBS pH 8.6) was then added as a carrier protein to protect the labeled globulin.

Two ul of the reaction mixture plus 0.1 ml of BSA (10 gm/ml) were added to 3.0 ml of 5% cold trichloracetic acid. The preparation was agitated in a vortex mixer and centrifuged for five minutes at 1000 rpm to yield a pelleted precipitate.

The supernatant fluid was separated from the precipitate and the precipitate was dissolved in 3 ml of BBS pH 8.6. One ul each of the precipitate and supernatant was counted separately in the Isocap 300 liquid scintillation spectrometer (Nuclear Chicago Corporation, Des Plaines, Illinois.) The efficiency was determined by the following equation.

$$\frac{\text{precipitate (CPM)}}{\text{precipitate (CPM)} + \text{supernatant (CPM)}} \times 100 = \text{efficiency}$$

The remaining labeled mixture was desalted by passing the reaction mixture through a G-25 Sephadex column and twelve different fractions were collected. The fractionation procedure was monitored with a survey meter (CDV-700 model, Anton Electronic Laboratories, Inc. Brooklyn, New York.)

One ul of each of the twelve samples was counted in the liquid scintillation counter to determine which fractions contained the labeled antibody, Each of the twelve samples was assayed for protein using a Biuret assay. A hemagglutination-inhibition test was carried out to determine which fractions contained antibody activity.

The labeled immune serum was then centrifuged at 40,000 rpm,

to remove sedimentable labeled serum proteins and stored at a -90 C until used.

Liquid scintillation cocktails

All liquid scintillation cocktails contained the following ingredients:

(1) 1 ml of sample.

(2) 1 ml of H_2O.

(3) 10 ml of Aquasol.

Protein determination procedure

The protein content of the 12 fractions obtained from the G-25 Sephadex column was determined by a method described By Gornall, Baradawill and David (1949) which makes use of the Biuret reaction. One milliliter from each of the 12 fractions was mixed respectively with 4 ml of Biuret reagent. These mixtures were incubated at room temperature for 30 minutes, and the optical density was measured in a Spectronic 20 spectrophotometer (Baush and Lomb Optical Company, Rochester, New York) at 540 mu. A standard protein curve, using BSA, was used to determine the mg of protein per ml of sample.

Micro-hemagglutination-inhibition procedure

The hemagglutination-inhibition-procedure described by Spendlove and Schaffer (1965) was adapted to a micro-hemagglutination-inhibition test and used in this study. Microdilutors and plastic plates containing wells for holding reaction mixtures were obtained

from Cooke Engineering Company in Alexandria, Virginia. Figure 1 is a representation of the hemagglutination-inhibition micro-titer plate. Twenty five ul of 0.85% physiological saline were added to each well. In the first row, 25 ul of the fractions containing protein from the G-25 Sephadex columns were added and two-fold serial dilutions were made with microdilutors, except in the last two rows (rows 11 and 12) which were controls. Row 11 was a serum control containing 25 ul of physiological saline, 25 ul of the different fractions containing protein from the G-25 Sephadex column, and 50 ul of a 0.3% RBC suspension. The twelfth row was a saline control containing 25 ul of saline and 50 ul of 0.3% suspension of erythrocytes (RBC). Four hemagglutination units of reovirus were added to all wells except the controls. The microtiter plate was incubated at room temperature for two hours before 50 ul of a 0.3% suspension were added to each well. After another two hours of incubation at room temperature the results were read.

Preparation of the potassium tartrate density gradient

Figure 2 represents the potassium tartrate gradient prepared on 1.5 ml of 70% sucrose in the bottom of cellulose nitrate tubes (9/16" x 3 3/4"). The 5-20% potassium tartrate was prepared by adding 8.8 ml of 20% potassium tartrate to one chamber of the gradient mixer containing the vibrating rod and 8.2 ml of 5% potassium tartrate to the other chamber. Using the gradient mixer and a polystaltic pump (Buchler Instrument, Inc., Fort Lee, New Jersey), 8.5 ml of 5-20% potassium tartrate were layered on the 70% sucrose layer. Two cell-

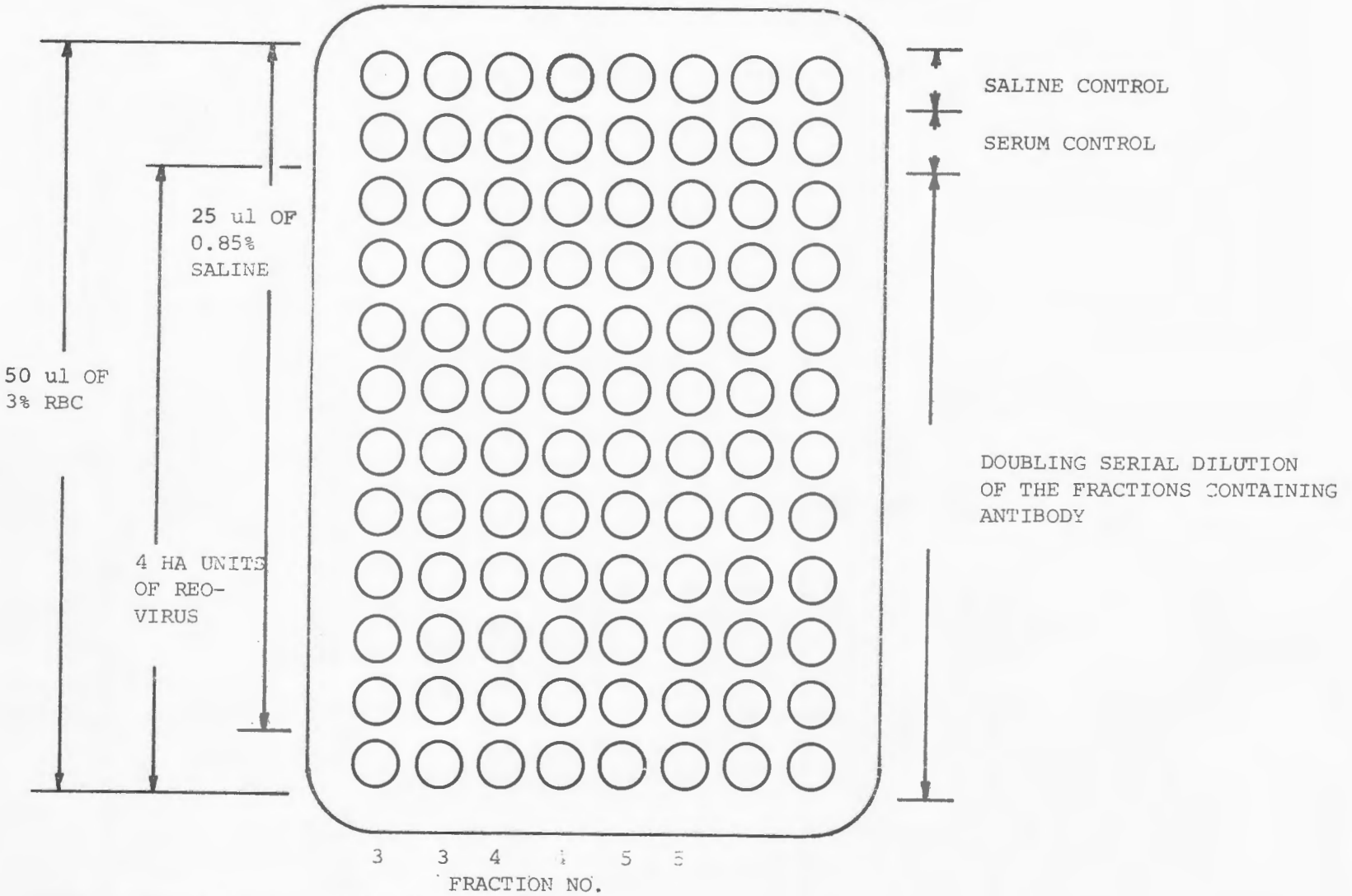


Figure 1. Hemagglutination-inhibition micro-titer plate.

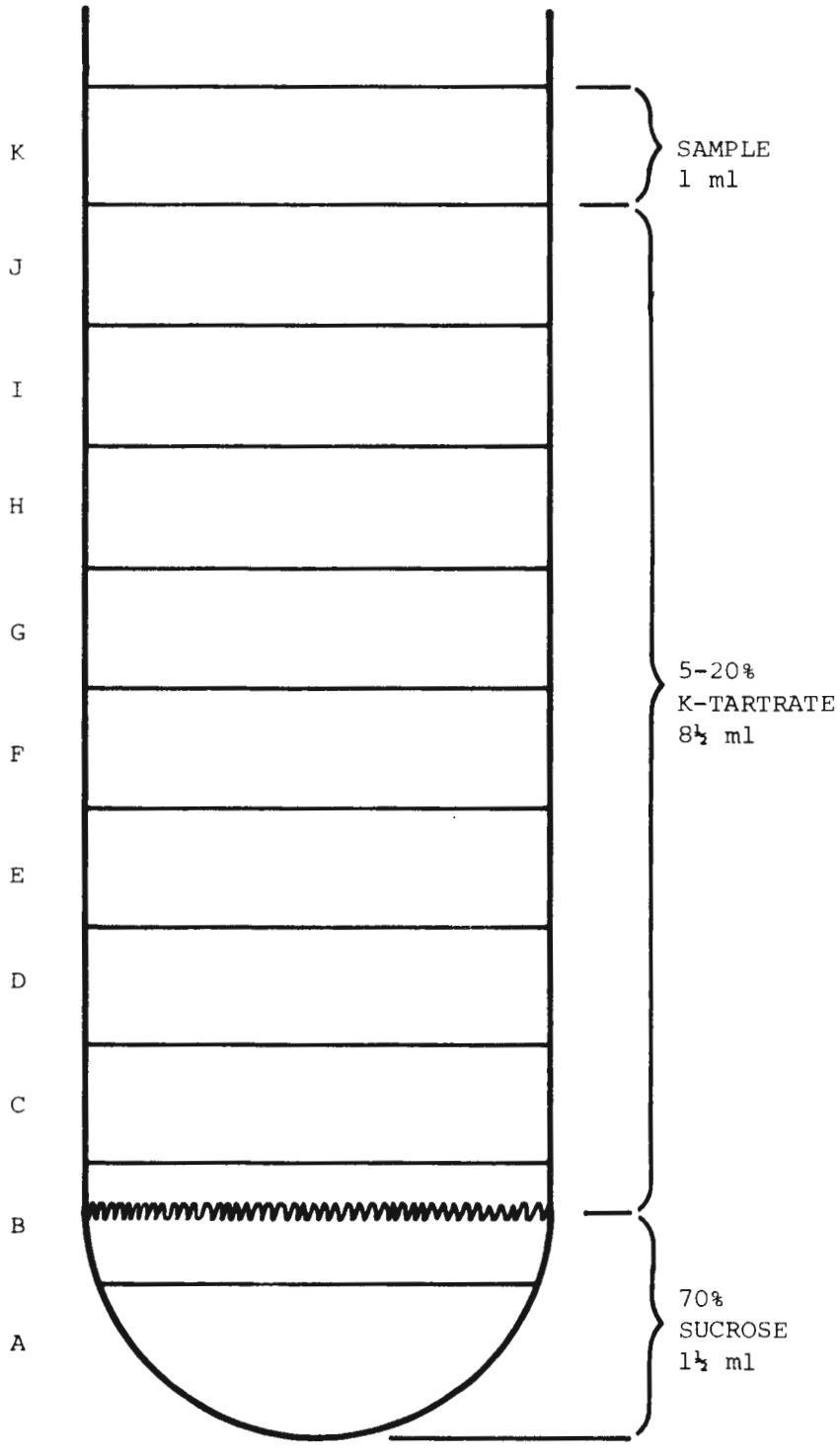


Figure 2. Preparation of the potassium tartrate gradient.

ulose nitrate tubes were filled at the same time and the flow rate on the Buchler polystaltic pump was set to give a combined flow rate of 1.7 ml/min.

The general procedure for the radioimmunoassay will be outlined here and the various conditions will be described later. Figure 3 is a representation of the radioimmunoassay procedure.

One tenth milliliter of the labeled immune globulin from fraction four of the G-25 Sephadex column was reacted with 1.0 ml of reovirus. The reaction mixture was incubated for six hours at 23 C in a shaker water bath with mild agitation, after which the mixture was layered on a 5-20% potassium tartrate gradient and centrifuged in a B-60 preparative ultracentrifuge with a 283 rotor (International Equipment Company, Needham Heights, Massachusetts) at 4 C for one hour at 40,000 rpm (130,000 X g). The gradients were fractionated into 1 ml samples; the first five fractions being collected in liquid scintillation vials with 1 ml of water and 10 ml of Aquasol. The controls, which consisted of Eagle's medium obtained from uninfected cell cultures and labeled antibody, were handled in the same manner. Figure 4 depicts the fractionation procedure used.

Optimal antibody concentration

Seven different volumes of labeled antibody ranging from 0.02 ml to 0.5 ml were reacted respectively with 10^6 infectious reoviruses contained in 1.0 ml to determine the amount of antibody needed to saturate this number of viruses. The antibody activity of the labeled immune globulin was standardized by the hemagglutination-inhibition test, and in all cases, the labeled immune globulin contained

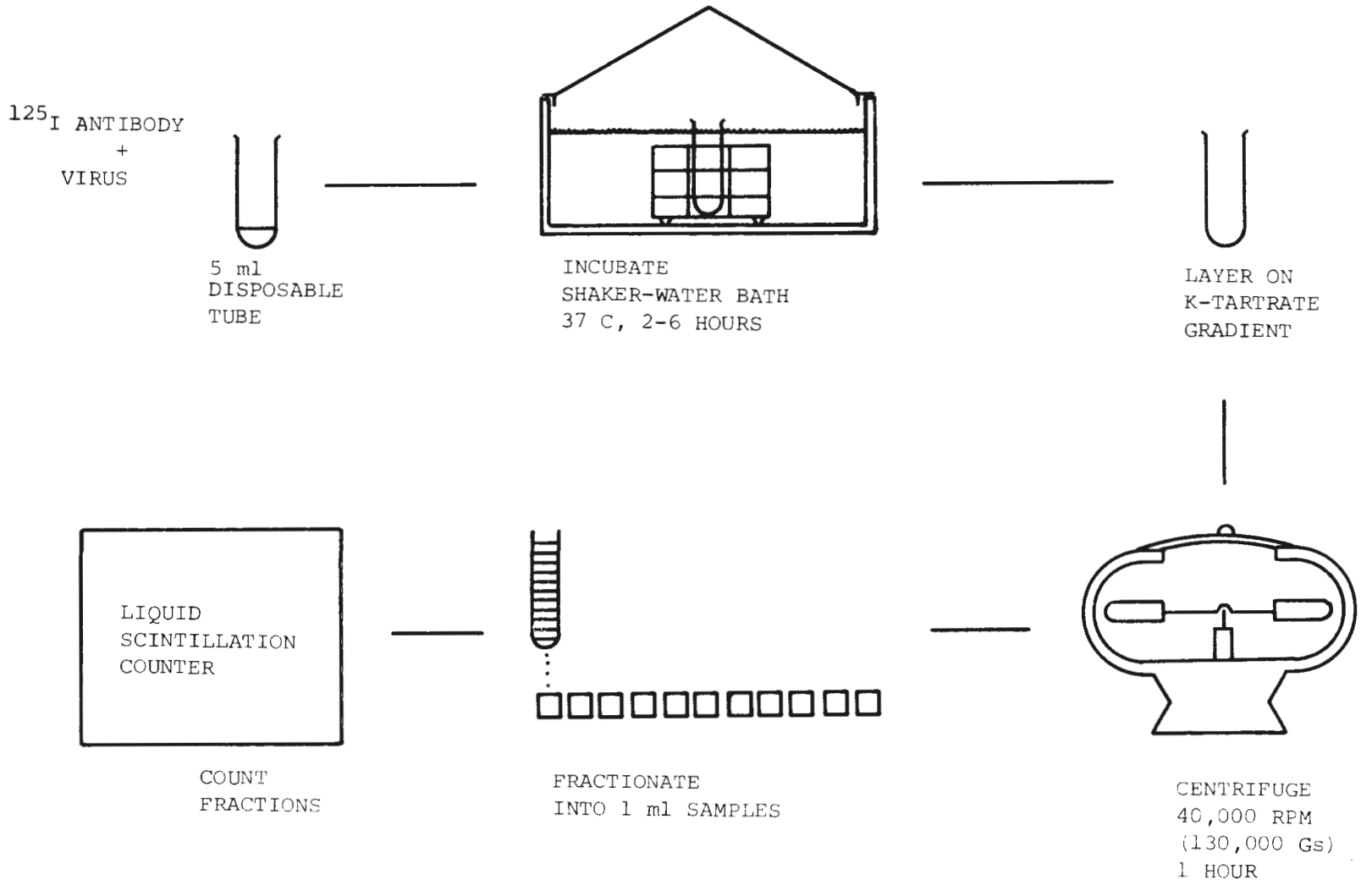


Figure 3. Flow sheet illustrating the radioimmunoassay procedure.

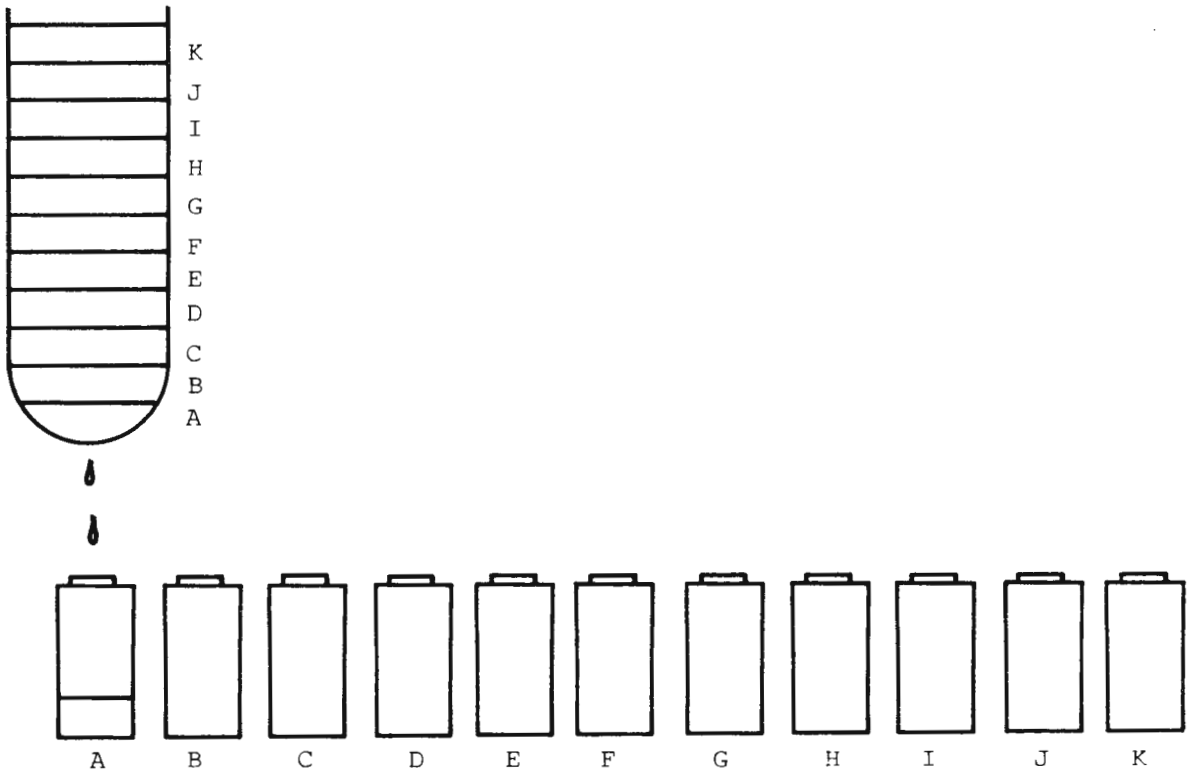


Figure 4. Fractionation of the potassium tartrate gradient after centrifugation.

two hemagglutination-inhibition units of antibody per 50 ul.

The virus samples plus various concentrations of labeled antibody were incubated in a shaker water bath at 37 C for six hours. The reactants were centrifuged on a 5-20% potassium tartrate gradient layered on 70% sucrose, fractions were collected and the fraction containing the tartrate-sucrose interface (fraction B) was counted in the liquid scintillation spectrometer. The controls in this experiment consisted of 1 ml aliquots of Eagle's medium obtained from uninfected cell cultures, to which were added respectively volumes of labeled immune globulin ranging from 0.02 ml to 0.5 ml.

Optimal time of incubation

To determine the optimal time of incubation for the virus-antibody reaction, a time sequence was carried out. A virus preparation containing 10^6 infectious particles was reacted with 0.1 ml of labeled antibody and the mixture was incubated in a shaker water bath at 37 C for various time periods ranging from one hour to eight hours. The reactants were then centrifuged and counted in the liquid scintillation spectrometer as previously described. The controls for this experiment consisted of 1 ml of Eagle's medium (previously collected from a cell culture) which was reacted with 0.1 ml labeled immune globulin and incubated at the same temperature and time period as the virus sample.

Incubation of virus-antibody mixtures

A set of tubes each containing 10^6 infectious virus particles and 0.1 ml of labeled globulin was incubated respectively at 4 C,

23 C (room temperature) and 37 C for six hours in a water bath. The controls were treated in a similar manner and consisted of 1 ml of Eagle's medium obtained from a cell culture and 0.1 ml of labeled immune globulin. The samples were centrifuged, fractionated and counted as previously described.

The effect of pH

Stock virus (10^8 infectious viruses/ml) was diluted 100-fold in McIlvaines citric acid phosphate buffer, (Weast and Tuve, 1968) at various hydrogen ion concentrations ranging from pH 2.5 to pH 8.0, and then reacted with 0.1 ml of labeled immune globulin. The reactants were incubated in a shaker water bath at 23 C (rotating at 130 rpm) for six hours; the samples were then layered on a potassium tartrate gradient, centrifuged and counted in a liquid scintillation spectrometer as mentioned previously. The controls of this experiment (Eagle's medium obtained from cell cultures plus 0.1 ml of labeled immune globulin) were handled in the same manner as the virus sample. Aliquots of Eagle's medium were also diluted 100-fold in McIlvaines buffer ranging from pH 2.5 to pH 8.0.

The effect of NaCl

Sodium chloride concentrations ranging from 0.1 M to 0.5 M were prepared in McIlvaines buffer pH 7.5. A stock virus suspension (10^8 infectious viruses/ml) was diluted 100-fold with the various salt solutions. One milliliter of diluted virus sample (10^6 viruses) was then reacted with 0.1 ml of labeled immune globulin and incubated in a shaker water bath at 23 C for six hours. The samples were then

treated as in previous experiments. The controls in this experiment consisted of a 1:100 dilution of Eagle's medium obtained from cell cultures plus labeled antibody. Eagle's medium was diluted in various concentrations of NaCl prepared in McIlvaines buffer pH 7.5.

The effect of sonication

To determine the effects of sonication, 10^6 infectious virus particles (diluted in McIlvaines buffer pH 7.5) were sonicated for various time periods ranging from 10 seconds to 100 seconds. Ten milliliter virus samples and controls were sonicated in a Biosonik BP III sonicator (Bronwill Scientific, New York) at an intensity setting of 35% maximum. The probe was 136 mm long and 4 mm in diameter. The samples were never sonicated more than 20 seconds without a one minute cooling period. After sonication 1.0 ml of the virus sample was reacted with 0.1 ml of labeled immune globulin. The samples were then treated as described in the General section of the Methods and Materials. The controls consisted of a virus control with no sonication in which 1.0 ml of 10^6 infectious viral particles/ml was mixed with 0.1 ml of labeled immune globulin. The buffer control was tissue culture fluid diluted 100-fold in McIlvaines buffer pH 7.5. This solution was sonicated after which 0.1 ml labeled immune globulin was added. The controls were handled in the same manner as the virus samples.

Specificity of the radioimmunoassay

To determine the specificity of the radioimmunoassay, 1.0 ml of 10^8 infectious canine hepatitis virus (ICHV) particles was reacted

with 0.5 ml of labeled antireovirus antibody. The mixture was centrifuged, fractionated and counted in an Isocap 300 liquid scintillation spectrometer as described above. A small aliquot (0.01 ml) from each centrifuge gradient fraction was added to 2 ml of Eagle's medium and assayed for ICHV by an immunofluorescent cell counting technique, used by Spendlove and Lennette (1962) for assaying reovirus.

To determine the sedimentation pattern of reovirus that had not been exposed to antibody, 1.0 ml of reovirus was centrifuged and fractionated by the methods described above. Each fraction was diluted 1:200 and then all ten fractions were assayed for infectious virus by the immunofluorescent cell counting procedure.

The sedimentation pattern of neutralized reovirus was determined by reacting 10^8 infectious viruses contained in 1.0 ml, with 0.5 ml of immune globulin and then incubating in a shaker water bath at 37 C for six hours. After incubation the sample was centrifuged and fractionated as described above. Fractions A, B, C and D were then transfered to a dialysis bag and dialyzed against 0.85% saline overnight at 4 C to remove the potassium tartrate and sucrose. The dialyzed fractions were examined by electron microscopy for viral particles. Viral samples were prepared for electron microscope examination by a technique developed by Horne and Wilde (1963) and modified by Hayes (Personal Communication). Phosphotungstic acid (2%) was adjusted with 1 N NH_4OH to a pH of 7.0 and then 0.05 ml of Kadak Photoflo was added per 20 ml of stain. The virus was added to a grid and allowed to adsorb for 30 minutes. The excess fluids were blotted free and the above stain was applied in excess before

the grid dried. After 10 seconds the grids were blotted with filter paper to remove the excess stain.

Sensitivity of the radioimmunoassay

Two different experiments were carried out to ascertain the sensitivity of the radioimmunoassay. In the first experiment, serum globulin was labeled with 1 mc of ^{125}I which would be enough iodine to label one in every 12 antibody molecules if 100% labeling efficiency were achieved. Preparations containing 10^5, 10^6 and 10^7 infectious viral particles were reacted with 0.25 ml of ^{125}I labeled antibody, while 10^8 and 10^9 infectious viral particles were reacted with 0.5 ml of ^{125}I labeled antibody. The above samples were incubated at 4 C for 24 hours and then centrifuged, fractionated and assayed for radioactivity as previously described. The controls consisted of Eagle's medium from tissue cultures treated in the same manner as the virus samples.

In the second experiment, globulins were labeled with 10 mc of ^{131}I, which was enough ^{131}I to label one in nine antibody molecules if a 100% labeling efficiency were achieved. Virus particles containing 10^3 and 10^4 infectious viral particles were reacted with 0.5 ml of ^{131}I labeled antibody, 10^5 and 10^6 infectious viral particles were reacted with 0.25 ml of ^{131}I labeled antibody and 10^7 and 10^8 infectious viral particles were reacted with 0.1 ml of ^{131}I labeled antibody. The above samples were incubated at 23 C for six hours and then handled as previously described. The controls consisted of Eagle's medium obtained from cell cultures.

Stock virus production

Stock virus was produced by a procedure described by Spendlove et al. (1966). Cylindrical bottles were seeded with MDCK cells and incubated at 37 C on a roller device for 24 hours or until a complete monolayer was achieved. The cells were then infected with 20 ml of culture fluids containing 10^7 infectious reoviruses per ml (type 1, Lang 17 - 1 strain.) The virus was enzyme treated by a method described by Spendlove et al. (1970) to enhance the infectivity. After a two hour viral adsorption period, 180 ml of Eagle's medium without fetal calf serum was added to each roller bottle. Reovirus was harvested every 24 hours and the medium was replaced until the cells were no longer confluent. Virus concentrations ranged from 10^6 to 10^8 infectious viruses/ml when assayed by a fluorescent antibody technique developed by Spendlove and Lennette (1962). The stock virus was centrifuged at 10,000 rpm for ten minutes in a GSA rotor in a RC 2 B centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut), to remove debris. Four milliliter quantities of the virus were stored in a -90 C freezer. Tissue culture fluids were processed in a similar manner and stored in a -90 C freezer until used.

RESULTS

Labeling procedure

When immune golbulin was iodinated and desalted, the labeling efficiency ranged from 65-71%. Figure 5 shows that the protein was found in fraction four and very little protein, if any, was detected in other fractions. The void volume of the Sephadex column was 12.9 ml. The first three fractions contained 4.3 ml of eluant and each fraction, after fraction three, contained 5 ml. Pretreatment of the G-25 Sephadex column with BSA prevented loss of labeled immune globulins due to adsorption in the column. The percent of protein recovered was 98%, however, there was no way to determine if part of the protein recovered was BSA used to pretreat the Sephadex column.

Figure 6 shows that the antibody activity as measured by hemagglutination inhibition (HI) was also found in fraction four. In all cases the antibody activity was five HI units before labeling and two HI units after labeling. This was the result of a six fold dilution that occurred when the labeled immune globulin was passed through the Sephadex column. No antibody activity was found in any of the other fractions.

Two peaks of radioactivity were observed (Figure 7) when the labeled immune globulins were desalted on a G-25 Sephadex column. One peak (fraction four) was the labeled antibody, and the other (fractions six through ten) was unbound ^{125}I. The efficiency of labeling the globulins in Figure 7 was 71%.

The effect of antibody concentration

Various volumes of a radioactive antibody preparation were reacted respectively with 10^6 infectious viruses to determine the amount of antibody required to saturate this number of viruses. Figure 8 shows that antibody contained in 0.3 ml of labeled immune globulin was sufficient to saturate the viruses and that higher concentrations of radioactive antibody did not appreciably increase the activity in fractions A and B of the virus sample. The radioactivity of the control also increased when larger volumes of labeled immune serum were used; however, the radioactivity in all cases was less than the radioactivity in the virus sample. The optimal amount of antibody was found to be 0.3 ml when reacted with 10^6 infectious viruses; however, in later experiments 0.1 ml of labeled antibody was used since the antibody in this volume would not saturate the virus and mask the effects of pH, salt concentration, sonication and time of incubation.

The effect of incubation time

To determine the optimal time for incubation, aliquots of infectious virus (10^6 virus) were reacted respectively with 0.1 ml amounts of labeled immune globulin and incubated for various time intervals. Figure 9 shows that any time of incubation between two and six hours is satisfactory. Although the radioactivity of the control increased with time of incubation it always contained about 3/4 of the radioactivity of that found in the test samples during the two to six hour incubation time. Therefore, a two hour incubation period can be used successfully when a rapid viral assay is

needed. In the remaining experiments a six hour incubation with mild agitation was selected. This provided a period of time for preparation of the density gradient.

The effect of temperature

Mixtures containing 10^6 infectious viruses and 0.1 ml of labeled immune globulin were incubated respectively in a shaker water bath at 37 C, room and 4 C temperatures to determine which temperature was best for the antigen-antibody reaction. The rate of the antigen-antibody reaction increased as the temperature increased (figure 10). Correspondingly the same relationship was observed in the controls, viz., the level of radioactivity increased as the temperature increased. The temperature of incubation in the remaining experiments was carried out at 23 C because of convenience.

The effect of pH

Aliquots of stock virus were buffered at various pHs and reacted with labeled immune globulins to determine the optimal pH of the antigen-antibody reaction. Figure 11 shows that the radioactivity in fractions A and B of the virus sample was greatly increased at pH 5.0; however, the activity in fractions A and B of the control was also increased at pH 5.0. Figure 11 also shows that the pH for the antigen-antibody reaction is not critical, however, if results from different experiments are to be compared the pH must be the same in all of the experiments. It is evident from these results that the pH of the virus sample and the pH of the control must also be the same.

The effect of NaCl

Figure 12 shows that the optimal salt concentration under the conditions of the experiment was 0.3 M NaCl, however, the activity of the control was also maximum at 0.3 M NaCl. The data also show that lowest activity in the control was with 0.5 M NaCl. The concentration of NaCl is not critical in the antigen-antibody reaction as long as it is held constant in every experiment.

The effect of sonication

To determine the effect of sonication on the capacity of reovirus preparations to react with antibody, reovirus samples were sonicated for various time periods, and then reacted with a labeled antibody. The results in figure 13 show that sonication enhanced the reactiveness of the reovirus preparation probably by disrupting viral aggregates. The optimum effect occurred after 50 seconds of interupted sonication. In subsequent experiments the virus samples were sonicated for 50 seconds before they were reacted with labeled antibody.

Specificity of the radioimmunoassay

The iodination procedure could alter the structure or charge of antireovirus antibody so that labeled antibody could combine non-specifically with unrelated viruses. To determine the specificity of the radioimmunoassay, ICHV reacted with antireovirus ^{125}I antibody was centrifuged and assayed for radioactivity. The results from table 1 show that very little radioactivity was found in fraction A,

B or C, however, when ICHV was assayed by an immunofluorescent cell counting technique most of the virus was found in these fractions. The results in table 1 indicate that very little of the labeled antibody reacted with ICHV, so the test was considered to be immunospecific.

Table 1 also shows that when 1 ml of reovirus was layered on the potassium tartrate density gradient, centrifuged and assayed for infectious virus, most of the virus was found in fractions A, B and C.

Electron microscope studies confirmed that the radioactivity found in fractions A and B was associated with the virus-antibody ^{125}I complex. Figures 14 and 15 are electron micrographs showing the virus-antibody complex, very little scanning was needed to find viral aggregates. Figure 16 shows an individual virus particle which was found only after a thorough examination of the grid. In fraction D no virus-antibody complexes were observed; figure 17 shows a representative view of fraction D.

Sensitivity of the radioimmunoassay

To determine the sensitivity of the radioimmunoassay, two different experiments were carried out. In the first experiment, immune globulin labeled with 1 mc of ^{125}I was reacted with reovirus; the results in figure 18 indicate that the lowest concentration of virus that could be detected was 10^5 infectious viral particles. These results also indicate that the activity in fraction A and B are proportional to the virus concentration. The control consisted of Eagle's medium and 0.25 ml of labeled immune globulin. The radioactivity found in fractions A and B of the control was 864 CPM. In the second

experiment 10 mc of ^{131}I were used to label the antibody preparation. Three different labeled antibody concentrations were reacted with various concentrations of reovirus ranging from 10^3 - 10^8 infectious viral particles; however the sensitivity for the radioimmunoassay was no greater when using 10 mc of ^{131}I. In the second experiment controls were used that had the same labeled antibody concentration (0.1 ml, 0.25 ml and 0.5 ml) as used in the virus samples. Figure 19 indicates that the increased radioactivity in the control makes the increased radioactivity in the virus sample. The results in figure 19 suggest that highly purified antibody can be used to increase the sensitivity of the radioimmunoassay.

Figure 5. Fraction containing protein when labeled globulins were desalted on G-25 Sephadex.

 Bovine serum albumin and antireovirus antibody protein.

Figure 6. Fraction containing antibody activity (HI) when labeled globulins were desalted on G-25 Sephadex.

 Antibody activity.

Figure 7. Fractions containing radioactivity when labeled globulins were filtered on G-25 Sephadex.

 Radioactivity.

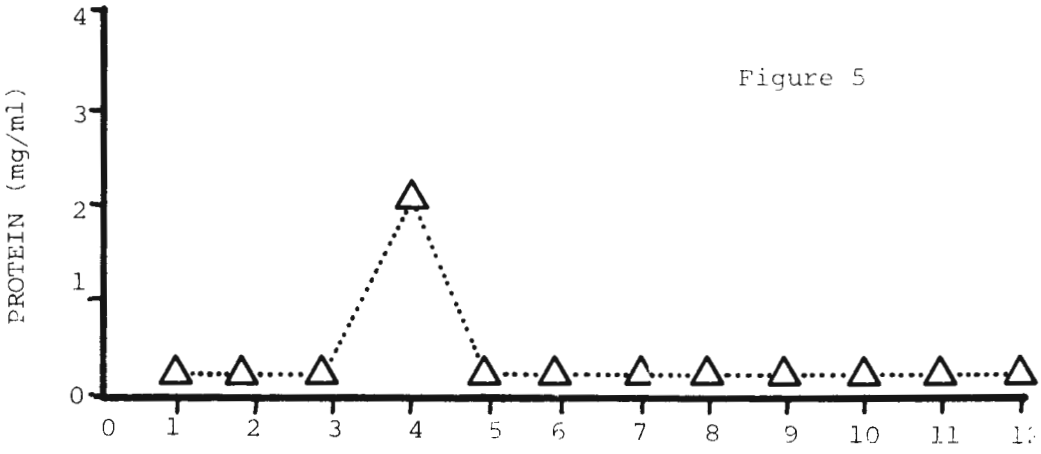


Figure 5

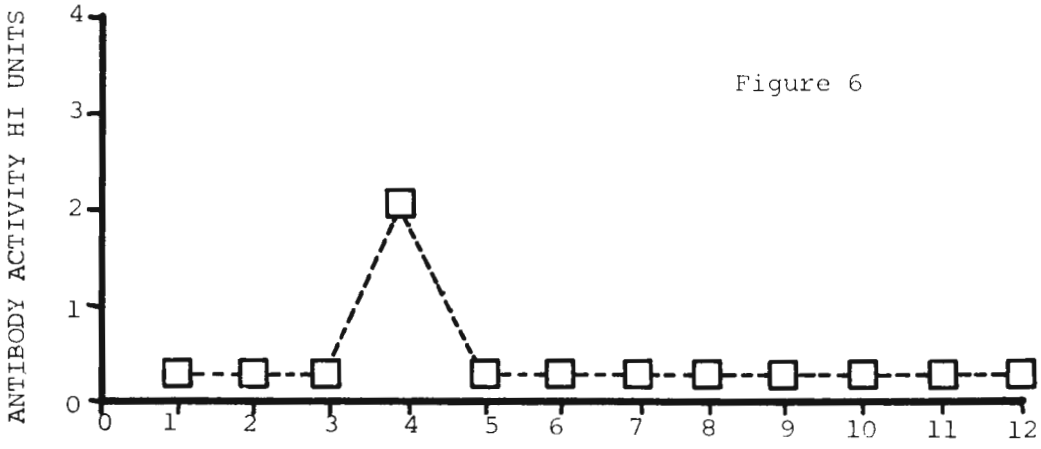


Figure 6

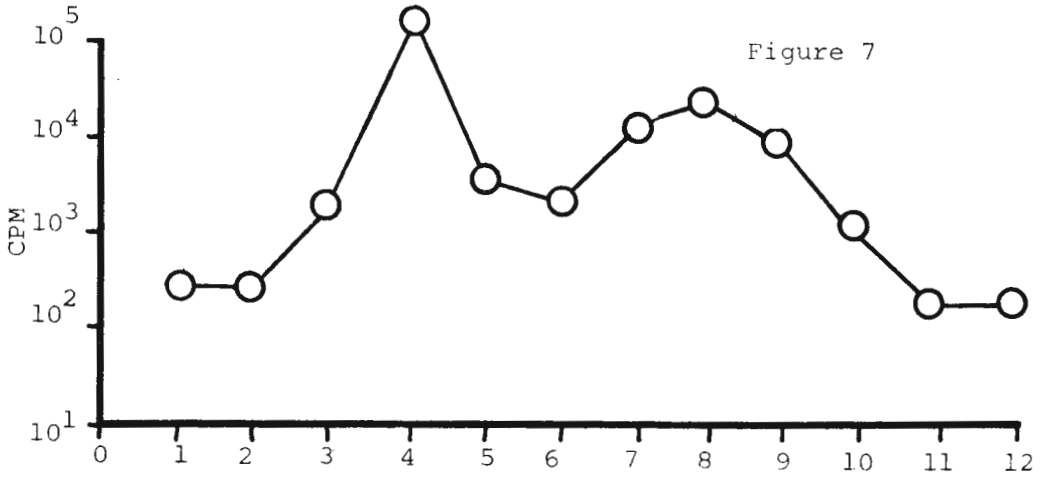


Figure 7

Figure 8. Effect of Antibody Concentration on the Virus-Antibody Reaction.

Various volumes of a radioactive antibody preparation ranging from 0.05 ml to 0.5 ml were reacted with 10^6 infectious viruses contained in 1.0 ml. Fractions A and B from the density gradient were counted in an Isocap 300 liquid scintillation counter.

Radioactivity (CPM) in the virus-^{125}I antibody complex.

Radioactivity (CPM) in the control.

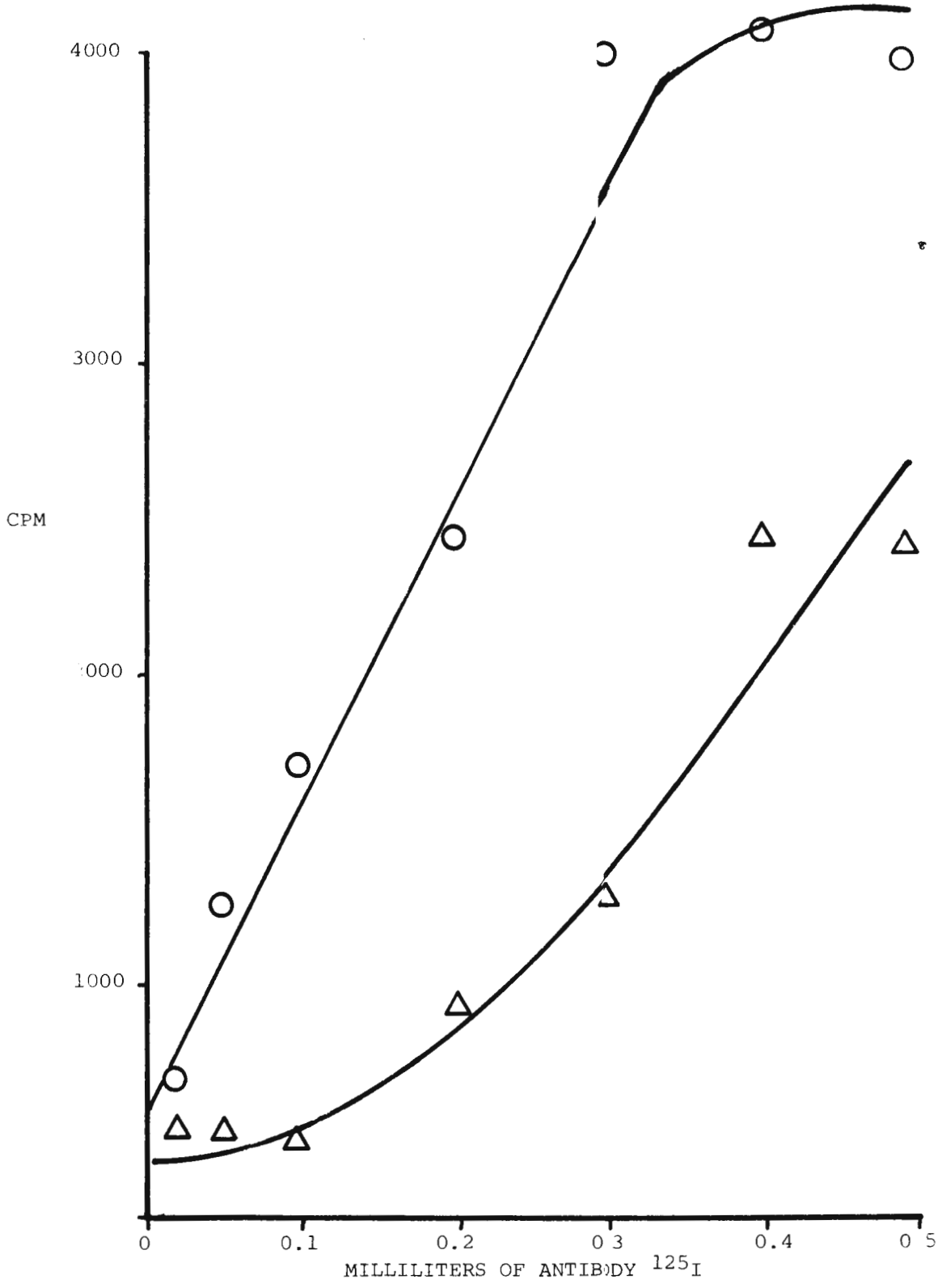

4000
3000
CPM
000
1000
0
0.1
0.2
0 3
0.4
0 5
MILLILITERS OF ANTIBODY 125I

Figure 8

Figure 9. Effect of Incubation Time on the Virus-^{125}I Antibody Reaction.

Infectious reovirus (10^6) was reacted with 0.1 ml of labeled antibody and incubated in a shaker water bath at 37 C for various time periods. The CPM are the combined counts found in fractions A and B of the density gradient.


Radioactivity (CPM) in the virus-^{125}I antibody complex.


Radioactivity (CPM) in the control.

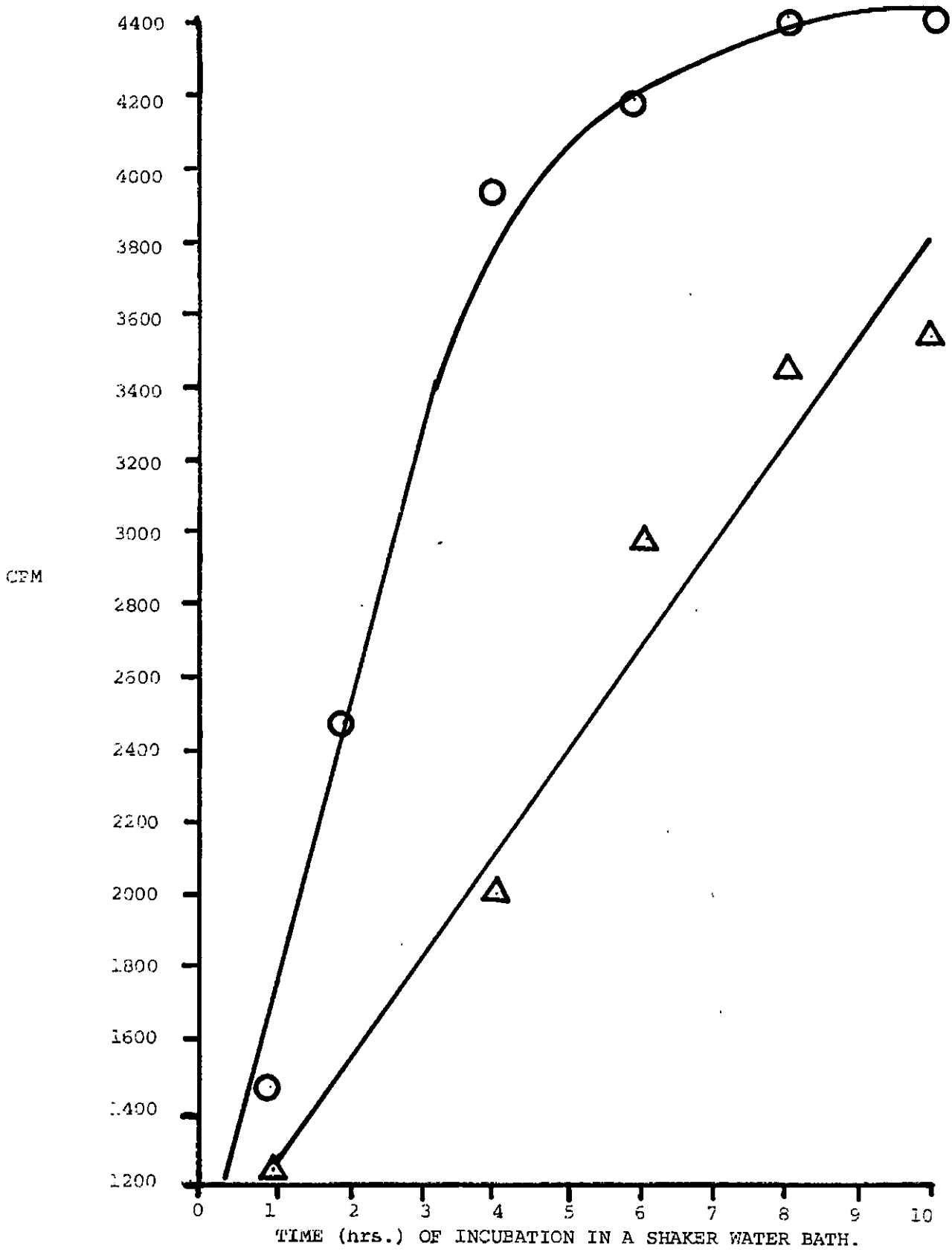

4400
4200
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
CPM
0
1
2
3
4
5
6
7
8
9
10
TIME (hrs.) OF INCUBATION IN A SHAKER WATER BATH.

Figure 9

Figure 10. Effect of Temperature on the Virus-^{125}Antibody Reaction.

Aliquots of infectious reovirus (10^6 virus) plus 0.1 ml of labeled immune globulin were incubated at three different temperatures (4 C, 23 C and 37 C) for six hours in a shaker water bath. The CPM are the combined counts found in fractions A and B of the density gradient.

Radioactivity (CPM) in the virus-^{125}I antibody complex.

Radioactivity (CPM) in the control.

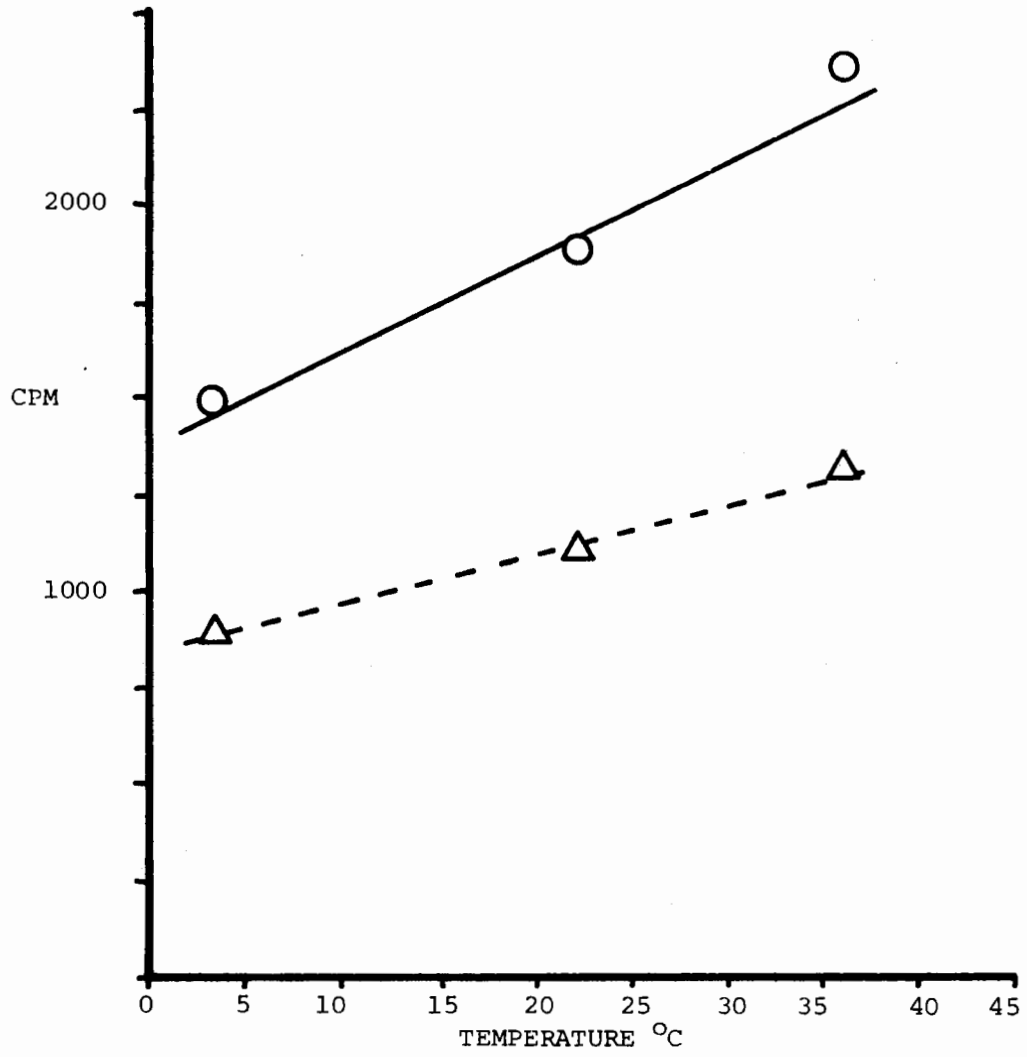


Figure 10.

Figure 11. Effect of pH on the Virus-^{125}I Antibody Reaction.

Stock virus was diluted in McIlvaines buffer at various pHs ranging from pH 2.5 to pH 8.0. The virus samples (10^6 infectious viruses), buffered at different pHs, were then reacted with 0.1 ml of labeled antibody. The CPM are the combined counts found in fractions A and B of the density gradient.

Radioactivity (CPM) in the virus-^{125}I antibody complex.

Radioactivity (CPM) in the control.

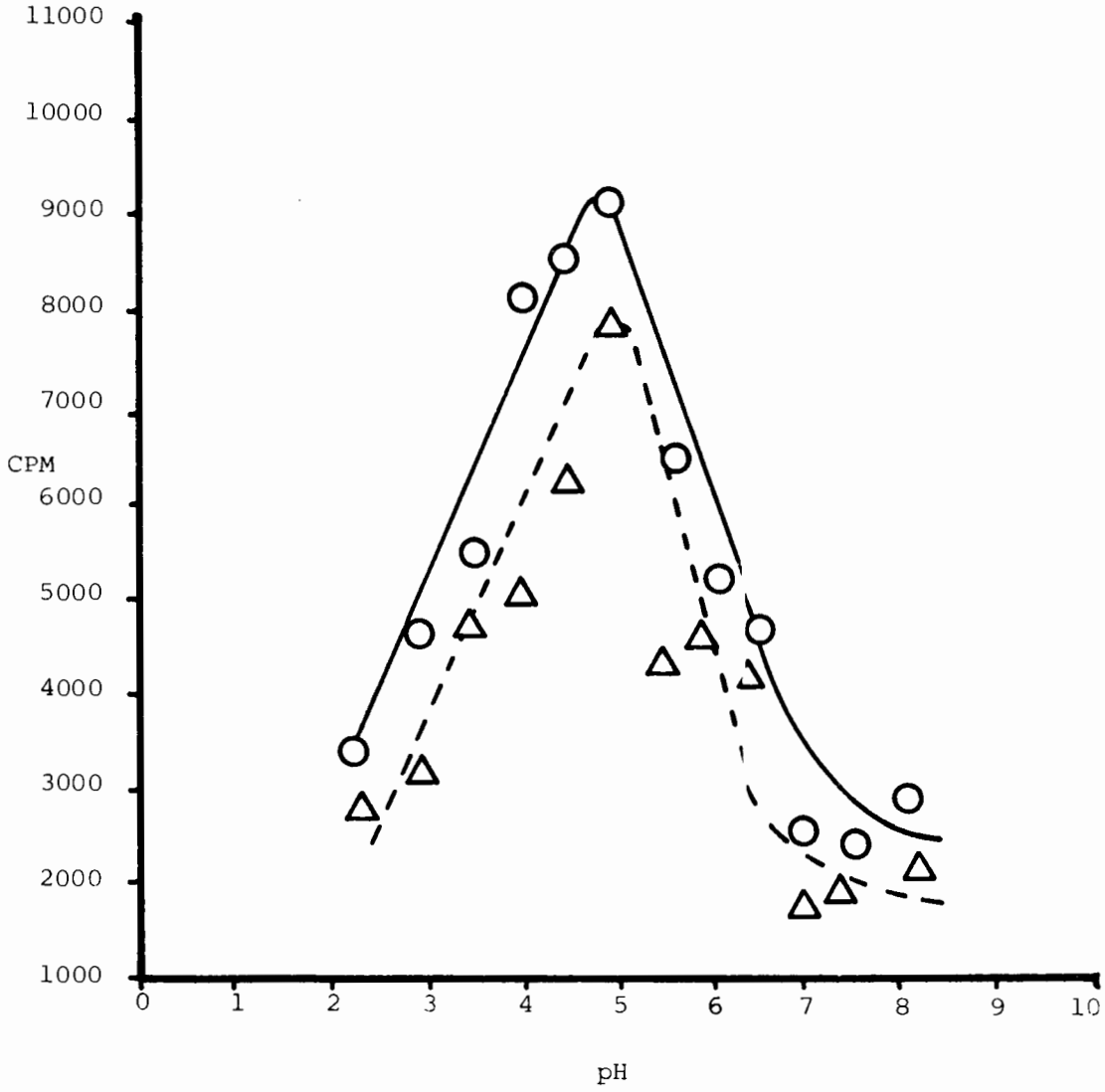

11000
10000
9000
8000
7000
CPM
6000
5000
4000
3000
2000
1000
0
1
2
3
4
5
6
7
8
9
10
pH

Figure 11.

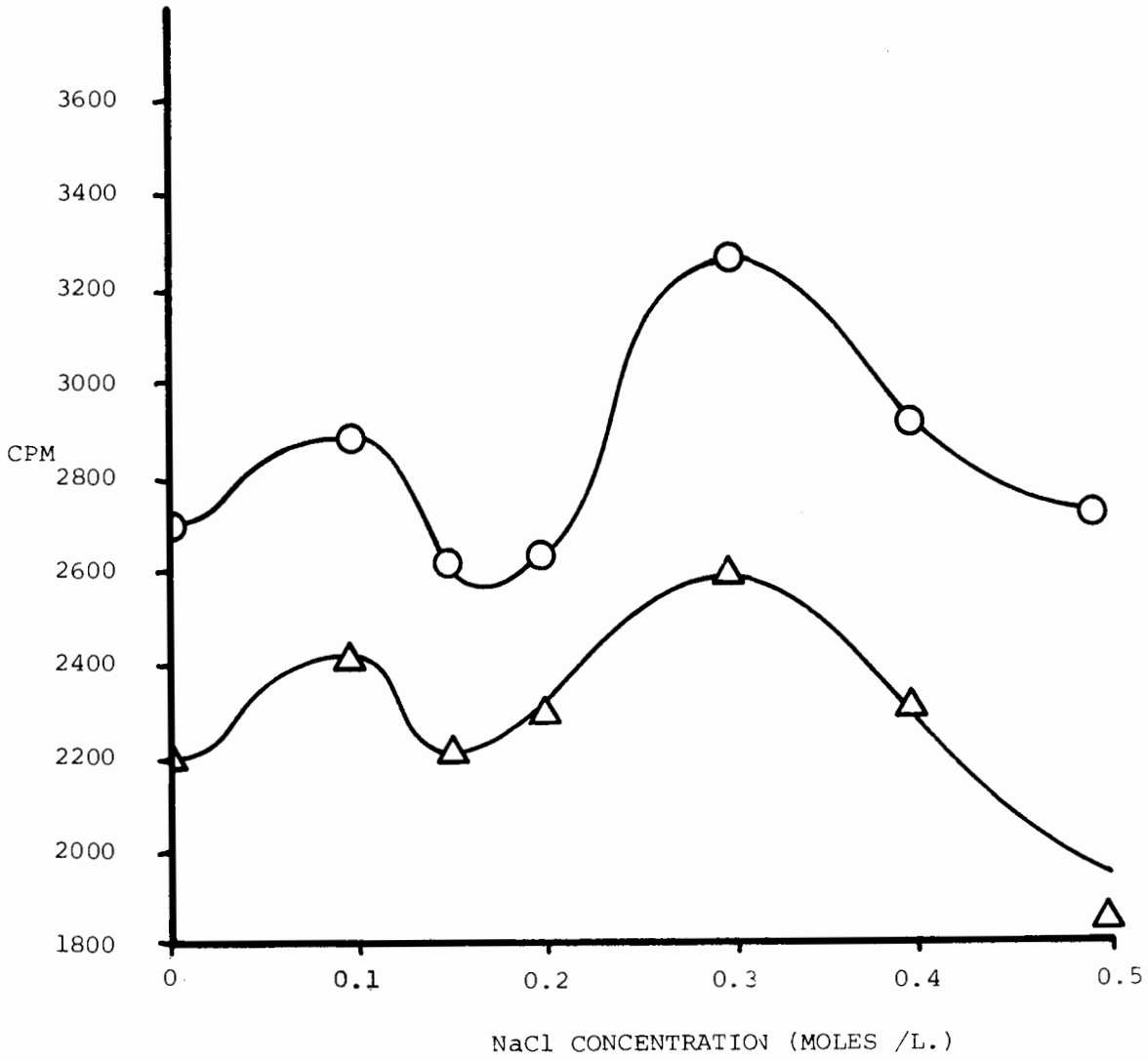

3600
3400
3200
3000
CPM
2800
2600
2400
2200
2000
1800
0
0.1
0.2
0.3
0.4
0.5
NaCl CONCENTRATION (MOLES /L.)

Figure 12.

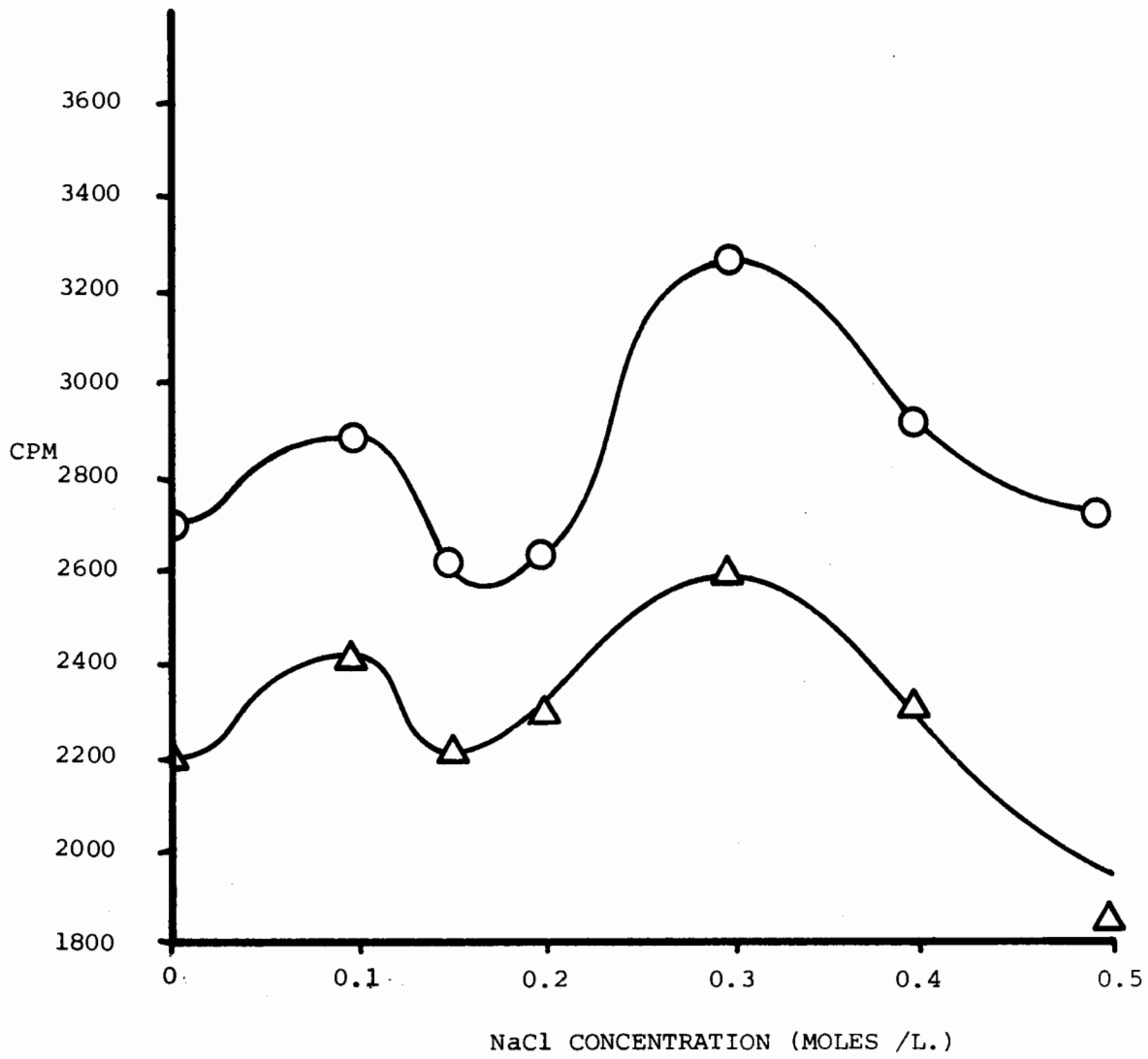

3600
3400
3200
3000
CPM
2800
2600
2400
2200
2000
1800
0
0.1
0.2
0.3
0.4
0.5
NaCl CONCENTRATION (MOLES /L.)

Figure 12.

Figure 13. Effect of Sonication on the Virus-^{125}I Antibody Reaction.

Reovirus diluted in McIlvaines buffer pH 7.5 was sonicated for various time periods ranging from 10 seconds to 100 seconds. After sonication, 1 ml of the virus sample was reacted with 0.1 ml of labeled antibody. The controls consisted of reovirus with no sonication, plus 0.1 ml of labeled antibody. The second control was Eagle's medium, diluted in Mcilvaines buffer and sonicated for 100 minutes, after which 0.1 ml of labeled antibody was added. The count rates for the controls were respectively 2567 CPM and 2201 CPM. The CPM are the combined counts found in fractions A and B of the density gradient.

Radioactivity (CPM) in the virus-^{125}I antibody complex when sonicated virus was used.

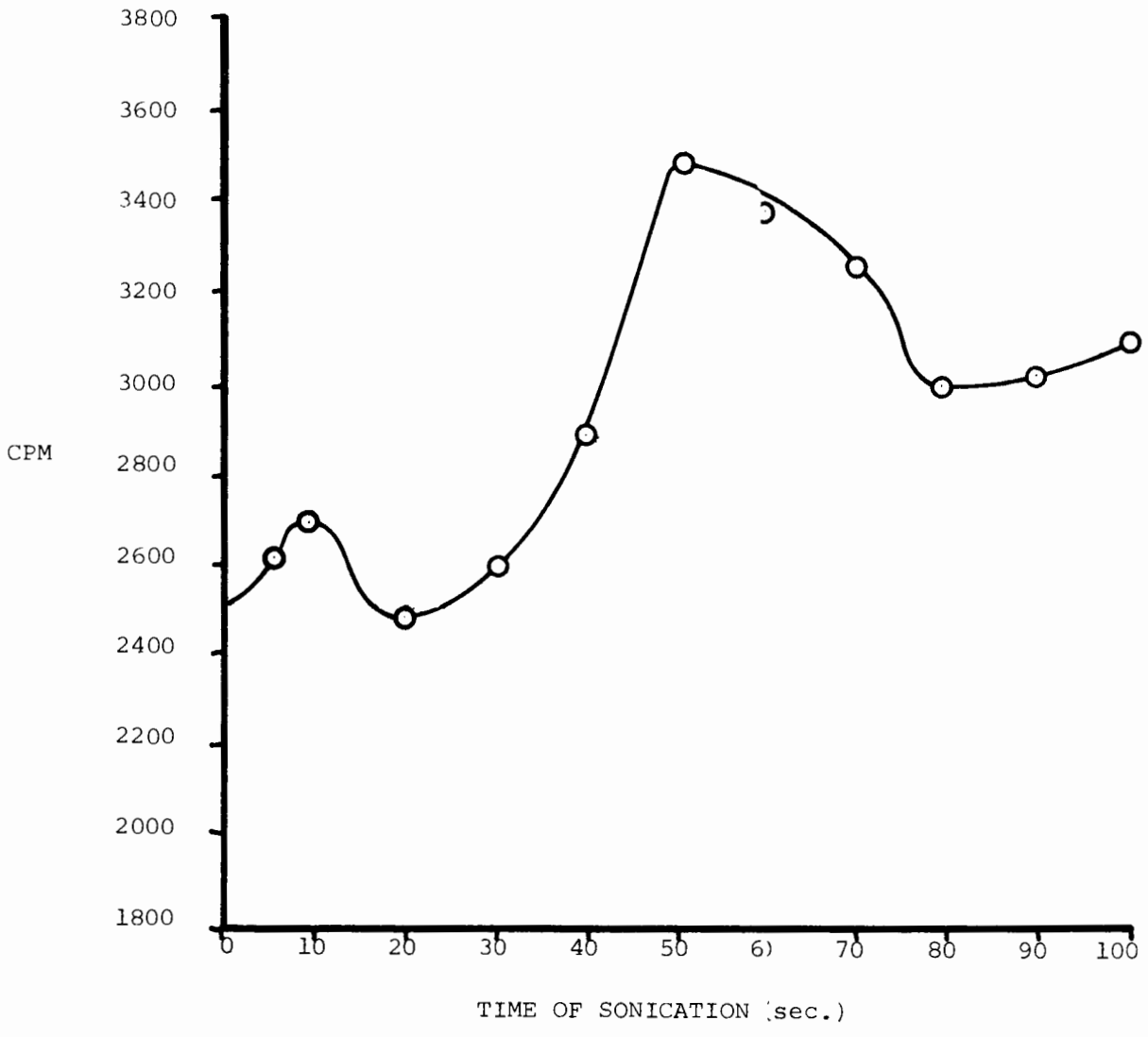

3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
CPM
0
10
20
30
40
50
60
70
80
90
100
TIME OF SONICATION (sec.)

Figure 13.

Table 1. Specificity of the radioimmunoassay

Fract. No.	Number of infected cells/cover slip	CPM	Fract. No.	Number of infected cells/cover slip	CPM
a/			a/		
1a	0	17411	2a	0	16260
1b	0	28080	2b	0	28908
1c	0	5534	2c	0	3870
1d	0	2116	2d	0	5106
1e	0	18412	2e	0	12107
b/			b/		
3a	362	2601	4a	285	2187
3b	931	3203	4b	751	2251
3c	464	1142	4c	520	1079
3d	10	1585	4d	51	1807
3e	0	19812	4e	0	19976
c/			d/		
5a	0	1894	6a	321	0
5b	0	2463	6b	891	0
5c	0	1308	6c	492	0
5d	0	2041	6d	82	0
5e	0	16431	6e	6	0

a/ Reovirus sample containing 10^8 infectious viral particles reacted with 0.5 ml of labeled antireovirus antibody. The samples were centrifuged and fractionated into 1 ml fractions; fractions A through E represent the lower five fractions. Results of duplicate experiments are shown.

b/ Infectious canine hepatitis virus (ICHV) sample containing 10^8 infectious ICHV particles reacted with 0.5 ml of labeled antireovirus antibody. The samples were centrifuged and fractionated into 1 ml fractions; fractions A through E represent the lower five fractions. Results of duplicate experiments are shown.

c/ Eagle's medium control reacted with 0.5 ml of labeled antireovirus antibody. The control was centrifuged and fractionated into 1 ml fractions; fractions A through E represent the lower five fractions.

d/ Infectious reovirus particles (10^8) reacted with 0.5 ml of Eagle's medium. The sample was centrifuged and fractionated into 1 ml fractions; fraction A through E represent the lower five fractions.

Figure 14. Electron micrograph of virus-antibody complex showing presences of antibody on virion in fraction A. (183,000 X)

Figure 15. Electron micrograph of aggregates of virus, both coreless and complete with associated antibody in fraction B. (167,000 X)

Figure 16. Electron micrograph of isolated virus particles in fraction C. (134,000 X)

Figure 17. Electron micrograph of a random sample of fraction D. (134,000 X)

Figure 18. Sensitivity of the Radioimmunoassay Using 1 mc ^{125}I.

One half milliliter of labeled antibody was reacted respectively with 10^9 and 10^8 infectious viruses, while 0.25 ml of labeled antibody was reacted respectively with 10^7, 10^6 and 10^5 infectious viruses at 4 C for 24 hours. The control consisted of Eagle's medium with 0.25 ml of labeled antibody. The combined count rate of fractions A and B of the control was 864 CPM and the control is not shown in figure 18. The CPM are the combined counts found in fractions A and B of the density gradient.

Radioactivity (CPM) in the virus-^{125}I antibody complex.

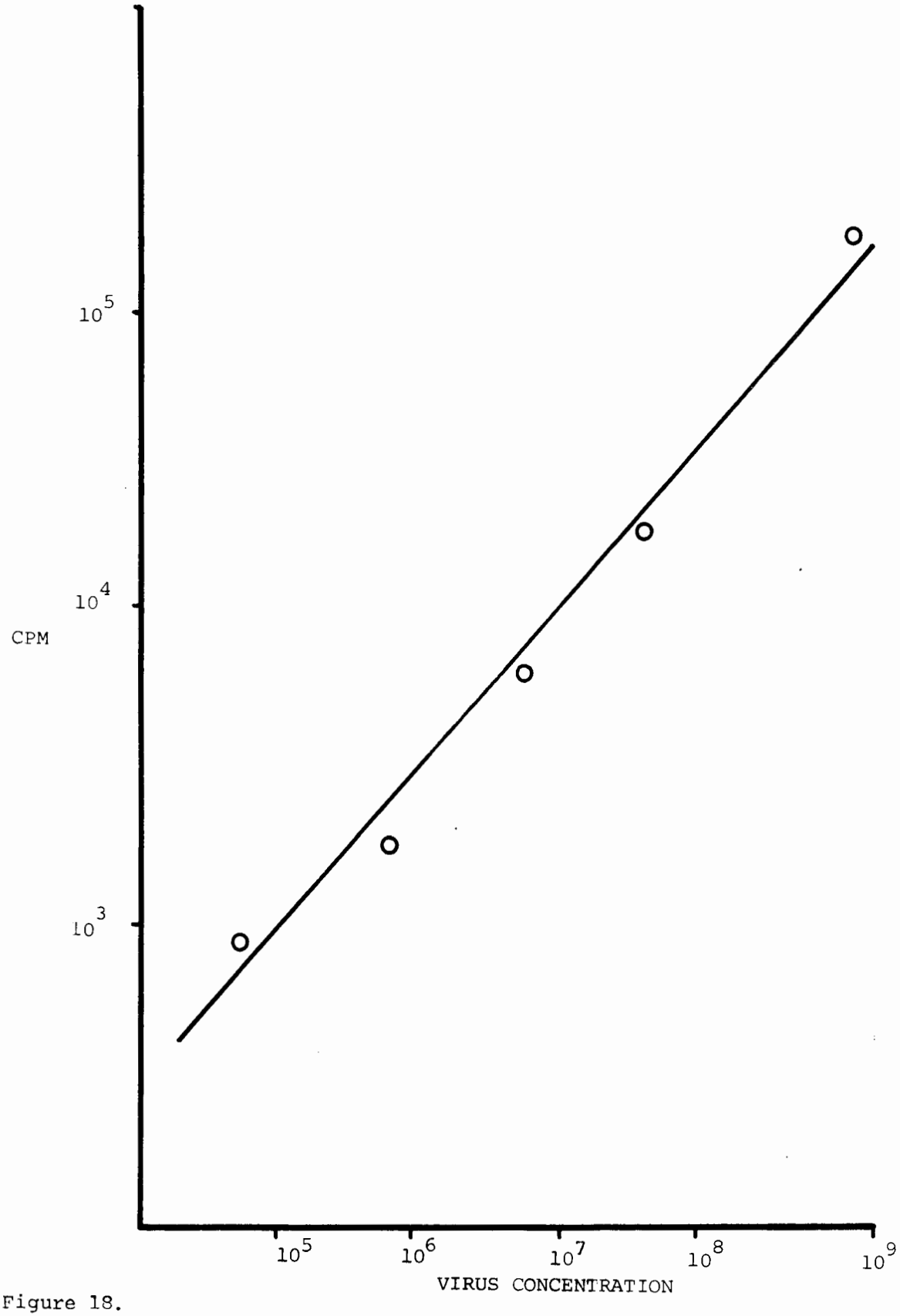
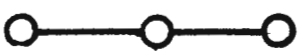


Figure 18.

Figure 19. Sensitivity of the Radioimmunoassay Using 10 mc of ^{131}I.

One half milliliter of labeled antibody was reacted respectively with 10^8 and 10^7 infectious viruses, 0.25 ml of labeled antibody was reacted respectively with 10^6 and 10^5 infectious viruses, while 0.1 ml of labeled antibody was reacted respectively with 10^4 and 10^3 infectious viruses at 23 C for six hours. The controls consisted of Eagle's medium reacted respectively with 0.5, 0.25 and 0.1 ml of labeled antibody. The CPM are the combined count rates of fractions A and B.



Radioactivity (CPM) in the virus-^{125}I antibody complex.

Radioactivity (CPM) in the control.

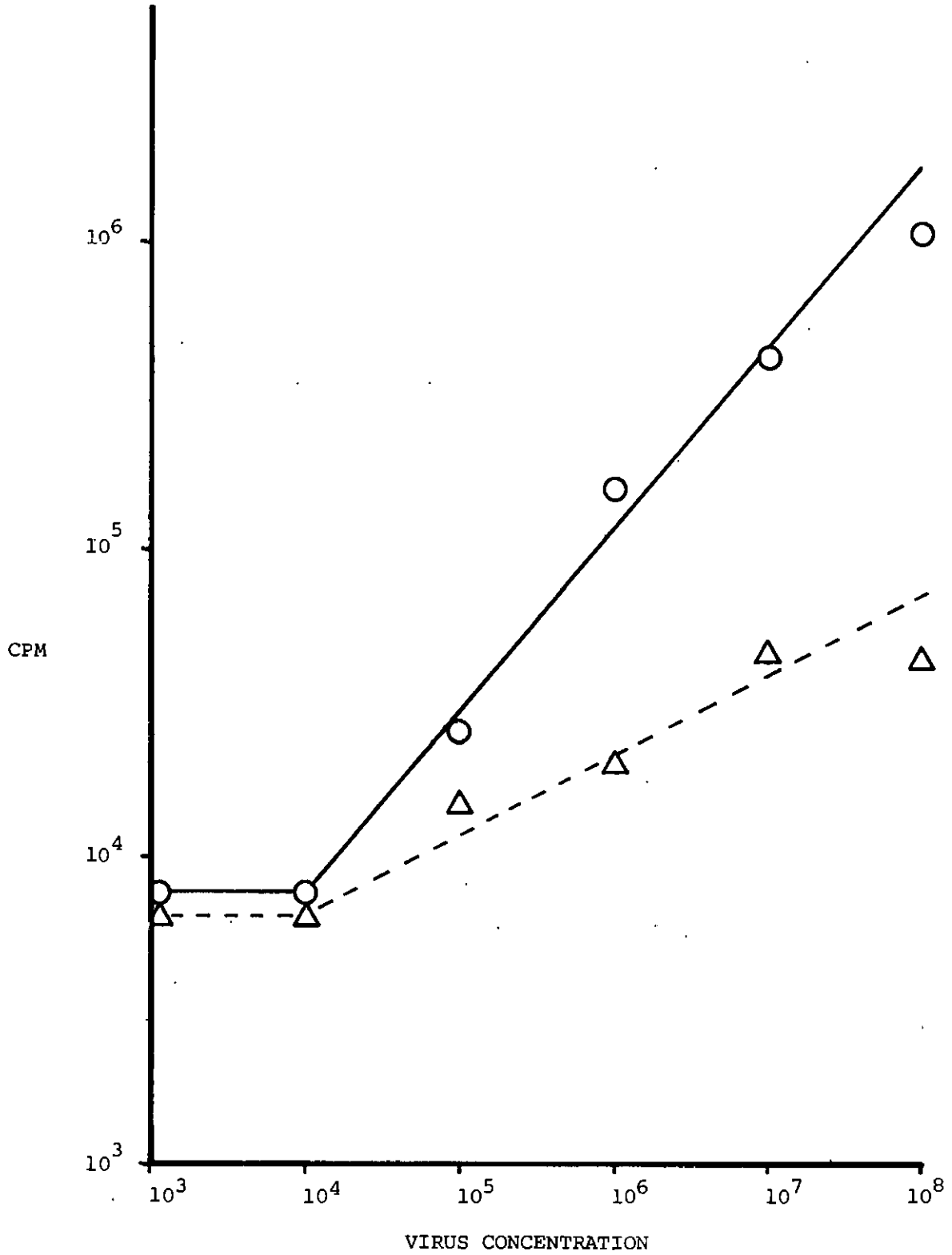

10^6
10^5
10^4
10^3
CPM
10^3
10^4
10^5
10^6
10^7
10^8
VIRUS CONCENTRATION

Figure 10.

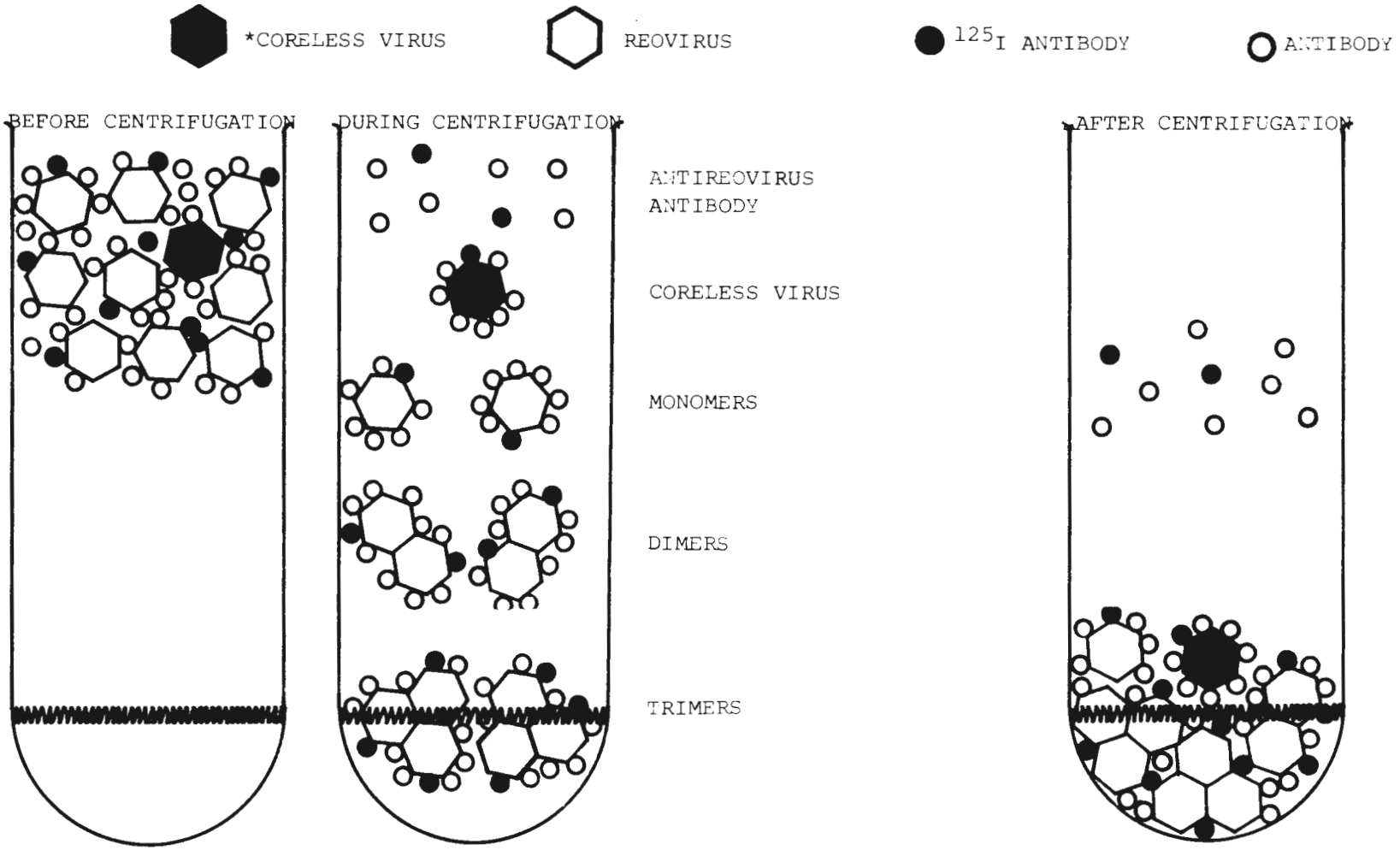
DISCUSSION

The detection of viral antigen with ^{125}I labeled immune serum in virus infected cells has been demonstrated by Hayashi, Rosenthal and Notkins (1972) and by Evans and Yohn (1970); however, the purpose of this study was to develope a sensitive and rapid radioimmunoassay for detecting virus, without prior cultivation in cells.

The iodination procedure developed by Hunter and Greenwood (1962) was used for labeling antibody molecules. A minor variation in the labeling procedure used in this study was adding enough cold iodine to insure that all antibody molecules were labeled with cold or hot iodine. Unlabeled antibody may have a greater affinity for antigen and this insured that all antibody molecules had the same affinity for reovirus.

By rate zonal ultracentrifugation, molecules separate according to size, shape and density, consequently larger antibody-viral aggregates migrate at a faster rate than smaller aggregates. Separation of polymers, trimers, dimers and monomers can be achieved by rate zonal ultracentrifugation. Figure 20 demonstrates the sedimentation of viral aggregates in a potassium tartrate density gradient with a 70% sucrose pad. Whitcomb and Spendlove (1966) developed a serological test in which reovirus-antibody reaction mixtures were centrifuged on a sucrose gradient. When very little antibody was present in the test, a large monomer band and one or more bands containing small complexes was observed. This method was ten times more sensitive than the precipitation test in detecting antibody.

The radioimmunoassay developed in this study was an extension



*Coreless virus is depicted as it might appear in an electron micrograph of a negative stained virus preparation.

Figure 20. The separation of viral aggregates by rate zonal ultracentrifugation.

of the earlier work of Whitcomb and Spendlove (1966) in which the sensitivity was increased by labeling the antibody with ^{131}I or ^{125}I. The separation of viral aggregates by rate zonal centrifugation is useful when studying the antigen-antibody reactions, however, when determining the number of viruses in a sample it is desirable to collect all viral aggregates into the same fraction. For this reason viral samples were centrifuged at a 130,000 Xg to insure that both virus and viral aggregates collect at the 70% sucrose-tartrate interface. The viral aggregates collect at the interface because of the high viscosity and density of the 70% sucrose.

The optimal conditions for the radioimmunoassay procedure were determined as were the physical conditions which would bring the antibody molecules in contact with the virus without denaturing the labeled antibody. Generally the physical conditions which increased the reaction of virus with labeled antibody also increased the radioactivity in the controls, which indicates that a certain fraction of the labeled antibody always precipitates. Radioactivity in the controls was always significantly lower under all conditions tested.

Increases in antibody concentration, time of incubation, and temperature were parameters which increased the rate of the antigen-antibody reaction, but also increased the radioactivity in the controls. It was important to determine the conditions for the virus-antibody reaction. By knowing the conditions that saturate the virus with labeled antibody; the radioimmunoassay can be quantitated by equating the radioactivity in fractions A and B to infectious viral particles in a sample. Also the radioactivity in the control can be decreased by using the smallest amount of labeled globulin that would saturate

the virus. It was found that 0.3 ml of labeled immune globulin would saturate 10^6 infectious viral particles within six hours, using antibody preparation that contained two HI units. In most experiments 0.1 ml of labeled antibody was used, thus enabling the effects of incubation, temperature, pH, NaCl concentration, and agitation to be better elucidates. If the antibody were used in high concentration so it rapidly saturated the virus, this would have masked the effect of the other parameters.

Drastic changes occurred in the amount of radioactivity found in fractions A and B of the control and virus sample, when the pH and/or NaCl concentrations were changed slightly. Consequently if the results from different experiments are to be compared the pH and salt concentration must be the same. Also the test and control samples in an experiment must have the same pH and salt concentration if the results are to be reliable. Because of the effect of pH and salt concentration on the solubility of protein, it is very desirable to use highly purified antibody. If a pH and salt concentration could be chosen in which the labeled antireovirus antibody would not sediment into the gradient, the radioactivity in the control would be eliminated. This would allow the concentration curve to be extrapolated to 80 CPM rather than 800 CPM.

Deland, Tilden and Baer (1972) developed a screening procedure for the detection of hepatitis associated antigen. Iodinated antigen plus antibody were reacted in an agar well and electrophoresis was carried out. One of the problems encountered was that radioactivity found in the control was very high which may have resulted from the wrong pH and salt concentration.

Sonication of the virus sample, before reacting with the labeled immune serum resulted in increased sensitivity of the test. Monodispersed virus can react with more labeled antibody molecules than equal numbers of viruses in the aggregated form. Sharp (Personal Communication) routinely sonicates poxvirus preparations for two minutes to break up aggregates so total particle counting can be carried out with monodispersed virus. Spendlove et al. (1970) showed that reovirus can be sonicated for two minutes without loss of infectivity. The radioimmunoassay measures total viral particles rather than infectious particles; however, prolonged sonication can reduce the sensitivity of the radioimmunoassay by causing the nucleic acid cores to fall out. When viruses lose their nucleic acid, the density becomes less and the sedimentation rate is altered, thereby affecting the amount of radioactivity in fractions A and B of the density gradient.

Table 1 indicates that the specificity of the globulin was not altered when labeled with iodine. Only small amounts of radioactivity was found in fractions A and B of samples 3 and 4, which indicates that the labeled antibody did not combine with ICHV. Samples 3 and 4 were a duplicate experiment in which labeled antireovirus antibody was reacted with ICHV virus. Table 1 also shows that ICHV was not neutralized with antireovirus antibody.

The electron micrographs substantiate that the radioactivity found in fractions A and B came from the virus antibody complexes since reoviruses were observed in fractions A, B and C.

The radioimmunoassay gives quantitative results and, in many cases, the sensitivity of the assay can be calculated by knowing the

physical properties of the virus. The fact that more labeled antibody can combine specifically to larger viruses results in a greater sensitivity when larger viruses are used. Equation 1 relates radioactivity to the number of atoms of an isotope by a decay constant, which is dependent upon the half life.

$$\text{Equation 1: } A = \lambda N = \frac{(N)\ln_2}{T_{\frac{1}{2}}} = (N)\ \frac{.693}{T_{\frac{1}{2}}}$$

A = activity in disintegrations per second (dps)

N = number of atoms isotopes

λ = decay constnat

$T_{\frac{1}{2}}$ = half life of isotope

Isotopes with shorter half lives have higher activities per atom and therefore, have greater sensitivity when used in the radioimmunoassay. The radioimmunoassay can be easily quantitated and the sensitivity of the assay can be predicted by knowing the physical properties of the virus. Equation 1 was used to quantitate the assay procedure and to predict the minimum number of reoviruses that could be detected. By knowing the number of radioactive iodine atoms per virion per sample, one can calculate the activity in fractions A and B with equation 1. The number of radioactive iodine atoms per virion per sample (N) can be calculated by equation 2 and this value is used to calculate the activity in equation 1.

$$\text{Equation 2: } N = \frac{(^{125}I)(ab\ \ )(virus)(ml)}{ab\ \ \ \ virus\ \ ml}$$

ab = antibody molecules

^{125}I = Iodine-125 atoms

ml = milliliters

Equations 3, 4 and 5 are derived from combining equation 1 and 2, and can be used to calculate the radioactivity, of virus/ml and antibodies/virus.

Equation 3: $A = \frac{(^{125}I)}{ab}\frac{(ab\quad)(virus)(ml)\,\lambda}{virus\quad ml}$

Equation 4: $\frac{(ab\quad)}{virus} = \frac{A(ab\quad)}{^{125}I}\frac{(ml\quad)(1\ )}{virus\quad ml\quad \lambda}$

Equation 5: $\frac{(virus)}{ml} = A\ \frac{(ab\quad)(virus)(1\ )}{^{125}I\quad ab\quad \lambda}$

All the variables in equation 5, except (virus/ab), are known, and this can be estimated. Fritz and Beard (1969) stated that the number of antibody molecules per avian leukosis virion ranged from 970-3458 antibody molecules per virion. In the latter case the avian leukosis virus was saturated with antibody. Another estimation would be to calculate the surface area of a reovirus and determine how many antibody molecules can fit around the virion. If IgG molecules have the following average dimensions, $2.5A^{o}$ x $41A^{o}$ x $250A^{o}$, Almeida (1963), and if a reovirus has a surface area of $1.86 \times 10^{6}A^{o2}$, Dales, Gomatos and Konrad (1965), there is physical room for 2000 antibody molecules per virion. This is the value used in equation 5.

Equation 5 shows that the activity of fractions A and B of the density gradient should be a linear function of virus concentration. This has been verified experimentally by plotting virus concentration against radioactivity in figure 19. The lowest calculated virus concentration detectable when using 10 mc of ^{131}I is 4.5×10^{3} reoviruses/ml., however, the actual sensitivity of the radioimmunoassay as used

in this study is only 10^5 infectious viral particles. The difference between the estimated and actual concentration is due to the radioactivity found in the controls. The sensitivity of the radioimmunoassay was not further increased by labeling with 10 mc of ^{131}I since the radioactivity of the unbound antibody in the control increased proportionally as the amount of ^{131}I used in labeling the serum protein was increased.

Greater sensitivity of the radioimmunoassay could be achieved by labeling highly purified antibody with 10 mc of radioactive iodine, (figure 22). By purifying the antibody 10 fold and labeling with 10 mc of ^{131}I; it would be possible to achieve the same specific activity as labeling crude antibody with 100 mc of ^{131}I. If one could then select conditions in which the purified labeled antibody would not sediment into the gradient then the background activity would be reduced. By using highly purified antibody and reducing the activity in the control, the radioimmunoassay has the potential of detecting 4.5×10^3 infectious reovirus/ml. The cost and safety hazard would also be minimal by labeling highly purified antibody.

Using the radioimmunoassay, virus can be assayed within six hours after receiving the sample. This by-passes growing the virus in tissue cultures and saves considerable time. The assay procedure is sensitive and nonspecific reactions are minimal.

One of the major problems in regard to air and water quality is the detection and identification of viruses when they are present in low concentrations. Because of the advantages of the radioimmunoassay it should have application in detecting virus not only at the clinical level, but also in water and aerosol samples.

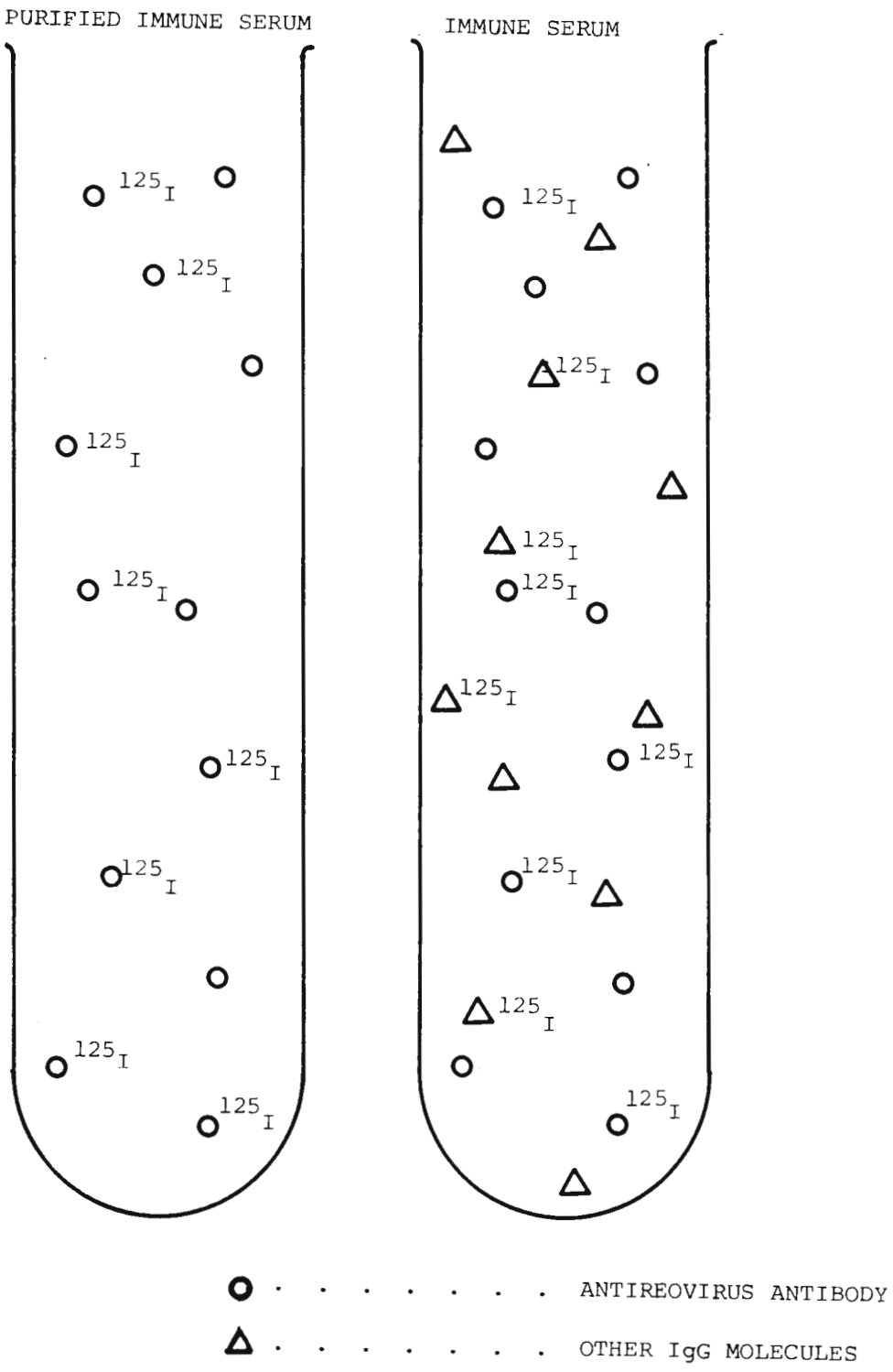


Figure 21. Labeling of immune globulin and highly purified immune globulin.

SUMMARY AND CONCLUSION

The purpose of this study was to develope a rapid and sensitive radioimmunoassay which could be used in the detection of virus in water, air, soil and clinical specimens.

Immune serum was purified by DEAE cellulose fractionation and the globulin was labeled with ^{131}I or ^{125}I without loss of antibody activity. The labeled immune globulin was then reacted with reovirus and unreacted antibody was separated from the virus-antibody complex by ultracentrifugation. After the radioimmunoassay was developed, optimal conditions for the assay were determined. It has been shown that changes in antibody concentration, time of incubation, pH, NaCl concentration and sonication all effect the sensitivity of the radioimmunoassay.

The radioimmunoassay has several advantages. The assay is rapid, virus can be assayed within six hours after receiving the sample; problems with nonspecific reactions are minimal; both viable and inactive viruses are detected; and the procedure is a sensitive assay technique which has practical application in the detection and quantitation of viruses.

BIBLIOGRAPHY

Almeida, J.D., B. Cinder and A. Howatson, 1963. The structure of antigen-antibody complexes. J. Exp. Med. 118: 327-339.

Ball, E.M. and M.K. Brakke, 1969. Analysis of antigen-antibody reactions of two plant viruses by density gradient centrifugation and electron microscopy. Virology 39: 746-758.

Bianchi, R., G. Federighi, L. Donato, U. Rosa, G. F. Pennisi and G.A. Scassellati. 1966. Iodinated albumin for metabolic studies. Symposium on labelled plasma proteins for metabolic studies. Pisa.

Brakke, M.K., R.W. Allington and F.A. Langille, 1968. Mobility measurements by photometric analysis of zone electrophoresis in a sucrose gradient column. Analyt. Biochem. 25: 30-39.

Brown, F. and B. Cartwright, 1960. Purification of the virus of foot-and-mouth disease by fluorocarbon treatment and its dissociation from neutralizing antibody. J. Immunol. 85: 309-313.

Brown, F. and C.J. Smale, 1970. Demonstration of three specific sites on the surface of foot-and-mouth disease virus by antibody complexing. J. Gen. Virol. 7: 115-127.

Carpenter, P.L., 1965. Immunology and Serology. W.B. Saunders Company, Philadelphia and London. p. 162-165.

Clark, M.F. and R.M. Lister, 1971. The application of polyethylene glycol solubility - concentration gradient in plant virus research. Virology 43: 338-351.

Collipp, P.J., S.A. Kaplan, D.C. Boyle and C.S. Shirmizu, 1965. ^{14}C-acetyl bovine growth hormone, physiological and antigenic properties. J. Biochem. 240: 143-147.

Dales, S., P.J. Gomatos and H.C. Konrad, 1965. The uptake and development of reovirus in strain L cells followed with labeled viral ribonucleic acid and ferritin - antibody conjugates. Virology 25: 193-211.

Deland, F.H., R.L. Tilden and H. Baer, 1972. Electrophoretic radioimmunoassay: application to Australian antigen. Abstracts of the 72nd annual meeting of the American Society for Microbiology. p. 214.

Evans, M.J. and D.S. Yohn, 1970. Application of the paired radioiodine labeled antibody technique (PRILAT) to the detection of adenovirus 12 tumor (T) antigen. J. Immunol. 104: 1132-1142.

Farr, R.S., 1958. A quantitative immunochemical measure of the primary interaction between I BSA and antibody. J. Infect. Dis. 103: 239-262.

Fazekas de St. Groth, 1962. "The neutralization of viruses", Advances in virus research. K.M. Smith and M.A. Lauffer, ed. Academic Press Inc., New York and London. p. 1-125.

Fazekas de St. Groth, S. and R.B. Webster, 1963. The neutralization of animal viruses. III Equilibrium conditions in the influenza virus-animal system. J. Immunol. 90: 140-150.

Freeman, T., C.M.E. Matthews and A.S. McFarlane, 1959. Albumin labelled with ^{131}I in an albuminaemic subject. Nature, Lond. 183: 606.

Fritz, R.B. and J.W. Beard, 1969. Density of BAI strain A avian leukosis virus-antibody complexes relative to antigen-antibody ratio. J. Immunol. 102: 1326-1329.

Gornall, A.G., C.J. Baradawill and M.M. David, 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.

Granoff, A., 1965. The interaction of Newcastle disease virus and neutralizing antibody. Virology 25: 38-47.

Greenwood, F.C. and W.M. Hunter, 1963. The preparation of ^{131}I labelled human growth hormone of high specific radioactivity. Biochem. J. 89: 114-123.

Hayashi, K., J. Rosenthal and A.L. Notkins, 1972. Iodine - 125-labeled antibody to viral antigen: binding to surface of virus-infected cells. Science 176: 516-518.

Heidelberger, M., 1939. Quantitative absolute methods in the study of antigen-antibody reaction. Bact. Rev. 3: 49-95.

Helmkamp, R.W., R.L. Goodland, W.F. Bale, I.L. Spar and L.E. Mutschler, 1961. High specific activity iodination of gamma-globulin with iodine - 131 monochloride, Cancer Res. 20: 1495-1500.

Horne, R.W. and P. Wilde, 1963. Virus structure revealed by negative staining. Advanc. Virus Res. 10: 101-170.

Hummeler, K., T.F. Anderson and R.A. Brown, 1962. Identification of poliovirus particles of different antigencity by specific agglutination as seen in the electron microscope. Virology 16: 84-90.

Hummeler, K. and A. Ketler, 1958. Dissociation of poliomyelitis virus from neutralizing antibody. Virology 6: 297-299.

Hunter, W.M., 1967. The Preparation of Radioiodinated Proteins of High Activity, Their Reaction with Antibodies in Vitro: The Radioimmunoassay. D.M. Weir, ed. F.A. David Company, Philadelphia. p. 608-654.

Hunter, W.M. and F.C. Greenwood, 1962. Preparation of iodine 131 labeled growth hormone of high specific acitivity. Nature Lond. 495: 195.

Kalmanson, G.M., A.D. Hershey and J. Bronfenbrenner, 1943. Factors influencing the rate of neutralization of bacteriophage by the antibody. J. Immunol. 45: 1-12.

Kedar, E., M. Wiener, N. Goldblum and D. Sulitzeanu, 1971. Antigenic relationships between cell membranes of normal and SV_{40} transformed hamster kidney cells. Israel J. Med. Sci. 7: 132-143.

Keller, R., 1965. Reactivation by physical means of antibody-neutralized poliovirus. J. Immunol. 94: 143-149.

Keller, R., 1968. Studies of the mechanism of the enzymatic reactivation of antibody-neutralized poliovirus. J. Immunol. 100: 1071-1079.

Kjellen, L., 1965 a. Stabilization in acid of mercaptolethanol reduced adenovirus antibody by combination with virus. Virology 27: 580-588.

Kjellen, L., 1965 b. Reactions between adenovirus antigens and papain dijested rabbit immune globulin. Arch. Ges. Virusforch 14: 189-200.

Lafferty, K.J., 1963 a. The interaction between virus and antibody. I. Kinetic studies. Virology 21: 61-75.

Lafferty, K.J., 1963 b. The interaction between virus and antibody. II Mechanism of the reaction. Virology 21: 76-90.

Lafferty, K.J. and S. Oertelis, 1963. The interaction between virus and antibody. III Examination of virus-antibody complexes with the electron microscope. Virology 21: 91-99.

Levy, H.B. and H.A. Sober, 1960. A simple chromatographic method for preparation of gamma globulin. Proc. Soc. Exptl. Biol. Med. 103: 250-251.

Mandel, B, 1960. Neutralization of viral infectivity; characterization of virus-antibody complex, including association, dissociation and host-cell interaction. Annals of the New York Academy of Sciences 83: 515-527.

Mandel, B., 1961. Reversibility of the reaction between poliovirus and neutralizing antibody of rabbit origin. Virology 14: 316-328.

Mandel, B., 1971. Characterization of type 1 poliovirus by electrophoretic analysis. Virology 44: 554-568.

McCombs, R.M., 1967. Density gradient centrifugation of virus-antibody complexes. Bacteriol. Proc. 67th annu. meeting. p. 138.

McFarlane, A.S., 1958. Efficient trace-labelling of proteins with iodine. Nature. Lond. 182: 53.

Northrop, J.H. and P.H. Dekruif, 1922. Stability of bacterial suspensions, hemagglutination of bacillus of rabbit septicemia and of bacillus typhosus by electrolytes. J. Gen. Physl. 4: 639-654.

Philipson, L., 1966. Interaction between poliovirus and immunoglobulins. II Basic aspects of virus-antibody interaction. Virology 28: 35-46.

Philipson, L., J. Killander, and P. Albertsson, 1966. Interaction between poliovirus and immunoglobulins. 1. Detection of virus antibody by partition in aqueous polymer phase system. Virology 28: 22-34.

Pinheiro, F. and G.D. Hsiung, 1963. A study of dissociation of coxsackie B4 virus-antibody complex. Virology 20: 457-463.

Rappaport, I., 1961. Acid dissociation of aggregates of antibody and tobacco mosaic virus, with recovery of infectious virus. Biochim. Biophy. Acta. 47: 206-208.

Rosa, U., G.A. Scassellati, F. Pennsis, N. Riccioni, P. Giagnoni and R. Giordani, 1964. Labelling of human fibrinogen with ^{131}I by electrolyte iodination. Biochem. Biophys. Acta. 86: 519-526.

Sharp, D.G., 1962. Sedimentation counting of virus particles by electron microscopy. RCA Scientific Instruments News. 7: 1-4.

Spendlove, R.S. and E.H. Lennette, 1962. A simplified immunofluorescent plaque method. J. Immunol. 89: 106-112.

Spendlove, R.S. and F.I. Schaffer, 1965. Enzymatic enhancement of infectivity of reovirus. J. Bact. 89: 598-602.

Spendlove, R.S., E.H. Lennette, C.D. Knight, and J.N. Chin, 1966. Production in FL cells of infectious and potentially infectious reovirus. J. Bac. 92: 1036-1040.

Steensgaard J. and R.J. Hill, 1970. Separation and analysis of soluble immune complexes by rate-zonal ultracentrifugation. Analyt. Biochem. 34: 485-493.

Svehag, S.E., 1965. Formation and properties of poliovirus neutralizing antibody .5 changes in quality of 19S and 7S rabbit antibodies following immunization. Acta. Pathol. Microbiol. Scand. 64: 103-119.

Svehag, S.E. and B. Bloth, 1967. Examination of virus YM antibody complexes by electron microscopy. I. Configuration of YM antibody and mode of attachment to antigen. Virology 31: 676-687.

Svensson, H., 1961. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. I. The differential equation of solute concentrations at a steady state and its solution for simple cases. Acta. Chem. Scand. 15: 325-341.

Thomssen, V.R., 1963. Ein chromatographisches verfahren zur bestimmung typensperzifischer poliovirus-antihorper mit ^{32}P-markiertem poliovirus. Z. naturforch. 18: 798-799.

Tu, K.C. and R.S. Spendlove, 1969. Enzyme sensitive reovirus. A thesis submitted for a Master of Science. Bacteriology Department. Utah State University. p. 36.

Vesterberg, O., and H. Svensson, 1966. Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. IV Further studies on the resolving power in connection with separation of myoglobin. Acta. Chem. Scand. 20: 820-834.

Wallis, C. and J.L. Melnick, 1967. Virus aggregation as the cause of the nonneutralizable persistent fraction. J. of Virol. 1: 478-488.

Weast, R.C. and G.L. Tuve, 1968. ed. Handbook of Biochemistry Selected Data for Molecular Biology. The Chemical Rubber Company, Cleveland, Ohio. p. j-195-j-196.

Weiser, W.S., Q.N. Myrvik and N.N. Pearsall, 1969. Fundamentals of Immunology. Lea and Febiger, Philadelphia. p. 105-110.

Whitcomb, R.F. and R.S. Spendlove, 1966. Density gradient centrifugation of virus-antibody complexes: a sensitive serological method. Virology 30: 752-754.

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