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THE USE OF A LARGE VOLUME AIR SAMPLER FOR THE DETECTION OF AIRBORNE RESPIRATORY VIRUSES: A FEASIBILITY STUDY

by

Glenn W. Park

A thesis submitted to the School of Medicine
in partial fulfilment of the requirements
for the degree of Master of Science.

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ABSTRACT

The results of this study have demonstrated the feasibility of the recovery of Poliovirus Type I (Sabin) (Polio), Rhinovirus Type 14 (RV-14) and Human Coronavirus Strain 229E (HCV/229E) with the Large Volume Air Sampler (LVAS) from aerosol.

The addition of bovine serum albumin (BSA) to a final concentration of 0.5% was found to be the most effective and practical additive of those tested, for the recovery of each of the three viruses. The effect of BSA on the recovery of Polio and RV-14 was manifest equally during both the collection and assay phases of the procedure whereas with HCV/229E most of the benefit appeared to be during the collection phase.

Amicon centriflo ultrafiltration cones were found to be effective for concentrating the viruses within the collection fluid of the LVAS, thus increasing the detection capability.

Efficiencies of recovery of 60% and 40% were recorded for RV-14 and HCV/229E respectively.

On the basis of preliminary data, in order for the LVAS to recover one infectious particle within 10 ml of collection fluid, the air must contain at least three HCV/229E particles per 10^4 litres of air sampled or two RV-14 particles per 10^4 litres of air sampled.

Further work pertaining to the aerosol stability of RV-14 and HCV/229E in different spray fluids and at different humidities and temperatures is indicated as are field studies with the LVAS to test its effectiveness in actual environmental situations.

INTRODUCTION

The airborne route of infection has usually been considered the major mode of transmission of agents causing respiratory tract infections because the respiratory tract lends itself well to the collection and dissemination of airborne particles. The normal process of breathing brings particles into the respiratory tract where many are impacted onto the epithelial cell lining. Should infection of the cells occur, this site will become a source of the infectious agent. With the normal movement of air past this site some of the infectious particles are suspended in the airstream and are carried out to the environment. The numbers of these released particles are increased through sneezing and coughing which usually accompany an upper respiratory tract infection.

The aerosols released from the respiratory tract consist of a variety of sizes of particles ranging from visible droplets, which rapidly settle from the air, to extremely small particles (diameters of less than 10 micrometers (μm) that have a very slow settling rate of less than one foot per minute (Bacterial Samplers, 1968). When these small particles are aerosolized, the water within them is evaporated almost immediately leaving a residual particle containing salts, proteins and biological agent(s). These residual particles are called "Droplet Nuclei" (Wells, 1934) and are very important in disease transmission and agent survival studies because as well as being capable of remaining in the air for long periods of time these particles are easily retained within the respiratory tract.

According to Hatch (1961) the upper respiratory tract, from the nares down to and including the terminal bronchioles, retains (by impaction) more than 80% of the particles with diameters of 5 µm or larger. All others pass to the lower respiratory tract (pulmonary lobules) where 100% of those 1µm or larger are trapped. The retention of particles less than 1µm in diameter still remains high but is less than 100%.

These studies by Hatch (1961) were performed using non-hygroscopic particles consequently they did not absorb water and expand during their passage through the respiratory tract. Biological aerosols on the other hand do absorb water and consequently expand. Knight (1973) refers to studies which show that a 1.5 µm particle passing through the upper respiratory tract can absorb enough water to increase in size to a diameter of 4 µm. He also reported that the increase in diameter due to water absorption was only significant for particles with diameters of less than 6 µm and that the diameters of particles above this size were not appreciably increased due to the water they absorbed. This increase in particle diameter enhances the possibility of the particle being retained in a different location in the respiratory tract with altered consequences in regard to the establishment of infection.

Thus these small infectious particles of less than 10μm in diameter, referred to as "droplet nuclei", are extremely important in studies concerned with transmission of disease by the aerosol route.

Most transmission studies used aerosols produced from the respiratory tract of infected individuals (cough and sneeze aerosols) or from artificially generated aerosols. Aerosols generated by coughing and sneezing, as indicated previously, have a wide variation in particle size however the artificially produced aerosols are much more uniform and consequently the importance of particle size on the type of infection produced and survival of the infectious agent can be studied. Some of the aerosol generators used are the DeVilbiss #40 and Vaponefrin nebulizers (Larson et al 1976), the Wells type atomizer (DeOme et al 1944) and, the most popular model, the Collison nebulizer (May 1973). Each of these aerosol generators requires that compressed air be mixed with the virus suspension to be aerosolized. As the air expands the suspension is broken into airborne particles of a wide size variation. The majority of these particles are too large to be carried in the outgoing airstream consequently they drop back into to the virus suspension. The very small particles (droplet nuclei), on the other hand, are easily carried by the air consequently it is these particles which make up the aerosol.

Recent studies using these natural (cough and sneeze) or artificially produced (nebulized) virus aerosols on human volunteers have been concerned with the transmission characteristics of three respiratory viruses namely Coxsackievirus A type 21, Adenovirus type 4

and 7 and several Rhinovirus types. Both the Coxsackievirus and the two Adenovirus types are of interest to the military because each has been implicated in the Acute Respiratory Disease syndrome (ARD) which affects large numbers of recruits when they first arrive on base (Fenner et al 1974). While both of these viruses have been implicated in upper respiratory tract infections the viruses primarily associated with upper respiratory infections, within the general population, are Rhinoviruses (Jackson and Muldoon, 1973) and to a slightly lesser degree, the more recently classified human Coronaviruses (Monto, 1974).

In studies concerned with virus transmission it is important to be able to analyse the aerosol, thus various pieces of equipment have been designed for the collection of the aerosolized virus (droplet nuclei) in order that information pertaining to the particle size, concentration and survival of the airborne agent can be obtained.

Buckland et al (1965) using an impinger type sampler for the collection of airborne Coxsackievirus A type 21 were able to determine that this virus was easily inactivated in either small particle aerosol or when applied to surfaces, yet was fairly stable when aerosolized in large particles. Consequently, in order to achieve successful transmission of the agent, close contact between infected and non-infected (susceptible) individuals would be required.

Couch et al (1966) used a Litton large volume air sampler in studies with natural (cough and sneeze) Coxsackievirus aerosols. They found that the cough produced the greatest quantity of recoverable aerosolized virus and that the best recovery was obtained when the oral secretions contained the highest concentration of virus. In the same study with Coxsackievirus A type 21, Adenovirus 4 and a Rhinovirus species, each virus was tested for its ability to produce a natural type of infection when introduced into a susceptible individual as small particle aerosols produced by a cough or sneeze or by the Collison nebulizer. In all cases an infection was produced in which the individual exhibited symptoms similar to a naturally acquired infection.

Aside from these earlier findings there is still some controversy over the role(s) of the aerosol route in transmission of certain infections. Studies performed by Gwaltney et al (1978), using volunteers, have been interpreted to indicate that the majority of infections caused by Rhinoviruses are a result of hand to hand rather than airborne transmission. Air samplers were not used in these studies, however, even though their use would have been effective in determining the concentration of the virus in the air surrounding the susceptible individuals. Thus their results do not show whether or not virus was actually being shed by the infected hosts, a requirement crucial to airborne transmission. The samplers could be used in these and other studies to determine the

quantity of airborne infectious virus that is required before an individual becomes infected.

In addition to the various aerosol generators and air samplers used to study airborne viruses and disease transmission other pieces of equipment have been designed to transport and maintain these aerosols to provide additional information. Any aerosol of an infectious nature must be generated into containment equipment in order to be safely transported to susceptible hosts or maintained for extended periods of time for survival studies. The equipment most often used for such transportation is the Henderson apparatus (Henderson, 1958). This unit was designed to supply a continuous source of the infectious agent at a constant cloud density under controlled conditions of relative humidity and temperature. By placing appropriate test animals in the aerosol cloud they could then be exposed to the infectious agent for susceptibility and transmission studies.

Survival characteristics of the infectious agents can be studied in another type of maintenance chamber known as a rotating drum (Goldberg et al, 1958). This device was designed to maintain the infectious agent in the airborne state over long periods of time such

that the survival of the agent could be studied for several days with only a minimal loss due to "fallout" onto the walls of the drum.

Assessment of the aerosol within the Henderson apparatus, a survival study chamber, or within the environment with respect to the determination of the quantity of viable particles present, requires the use of an aerosol sampler. There are many types of samplers all operating on one of two principles; (a) impingement of the aerosol into a liquid recovery fluid or (b) impaction of the aerosol onto a solid or semi-solid surface. Quite often aerosols contain particles which are aggregates of more than one infectious unit and when these are collected into a liquid (as in the case of impingers) they disaggregate. When these aggregated particles are collected onto a solid or semi-solid surface (as with impaction) they do not disaggregate therefore the count from an impaction device represents the number of infectious aerosol particles (droplet nuclei) whereas the count from an impinging device represents the number of infectious units. Dividing the infectious unit count by the infectious aerosol particle (droplet nuclei) count gives the number of infectious agents per infectious aerosol particle (droplet nuclei).

The most commonly used samplers operating on the impingement principle, called impingers, include; (a) the All Glass Impinger (AGI) (Tyler and Shipe, 1959) in which the air particles are accelerated through an orifice and impinge into the liquid at an angle

perpendicular to the liquid surface, (b) the Shipe impinger (Shipe et al, 1959) which operates on the same principle as the AGI except the air impinges into the liquid at a slight angle to the liquid surface, and (c) the multistage liquid impinger (May, 1966) which consists of a series of three connected stages designed to separate the airborne particles into three size ranges based on their diameter so that the distribution of the infectious agent in the aerosol could be determined.

Impaction devices on the other hand are named for the type of opening through which the aerosol is accelerated. These samplers include (a) the slit sampler (Bacterial Sampler, 1958) in which the airborne particles accelerate as they are drawn through a narrow slit and impact onto a slowly revolving dish containing solid or semi-solid collection medium (b) the seive sampler (Bacterial Samplers, 1958) where the air particles accelerate through small holes and impact on the surface of the collection medium, and (c) the stacked seive or Anderson sampler (Anderson, 1958) which consists of six seives, each with different diameter holes, stacked one on top of the other. The Anderson sampler is unique because it separates the particles into six groups depending on their diameters so that one can assess the mean particle diameter in the aerosol being studied (Anderson, 1958, Guerin and Mitchell, 1964 and Couch et al, 1965).

As effective as the above samplers are at assessing infectious aerosols, none of them is very effective for recovering the small amounts of virus produced by, for example, a sneeze or cough from the air in a large room. Dimmick and Akers (1966) refer to work by Tyrrell in which he has found that for one sneeze about 1 to 10 infectious doses of virus are aerosolized into the environment as droplet nuclei. Thus the recovery of these nuclei from a large room would require the screening of hundreds of liters of air, volumes which are not practical for the above mentioned samplers. For this reason large volume air samplers have been designed so that airborne particles from large volumes of air can be concentrated into very small volumes of liquid and from there assayed with a minimum amount of time and expense. Many of these samplers have been described by Decker et al (1968). The most popular one used to date is the Litton large volume sampler which has seen practical application in both environmental (Winkler 1968) and laboratory (Artenstein et al 1967 and Couch et al 1966) studies. Because of the large size and complexity of operation of the Litton sampler a much smaller and simpler large volume air sampler (LVAS) was designed. It was originally described by Decker et al (1968) and was later tested by Buchanan et al (1972) who found it to be highly effective for the collection of bacterial spores. This model was further modified at the Defence Research Establishment Suffield (DRES), Alberta. White et al (1975) have described the modifications and have shown the LVAS to be

very efficient for the collection of a dye, bacterial spores (Bacillus subtilis var niger) and three species of bacteria in vegetative form (Serratia marcescens, Escherichia coli and Aerobacter aerogenes.

With the increasing importance placed on the study of airborne viruses it was appropriate to initiate a study concerned with the feasibility of using the LVAS for the recovery of viruses from aerosol. The viruses used for this study included Poliovirus type I (Sabin) and two respiratory viruses, Rhinovirus type 14 and Human Coronavirus (HCV) 229E. Poliovirus type I was used as a reference virus because it was safe to work with, had a well established plaque assay and because many of its characteristics in aerosol have been documented (de Jong and Winkler 1968 and Akers and Hatch 1968).

The study was composed of essentially three areas. The first was designed to determine whether or not each of the viruses could be collected by the LVAS. The second was concerned with increasing this recovery through the use of certain additives, determining where, during the recovery and assay phases of the virus recovery, the additive is most effective and was also concerned with the development of a concentration procedure, using Amicon Centriflo membrane cones to supplement the recovery capabilities of the LVAS. The third was concerned with the determination of the efficiency of the LVAS relative to the AGI for the recovery of the three viruses. Once the efficiencies were established, the values were used in an attempt to determine the

limits of virus detectability of the LVAS. This lower limit is the minimum number of plaque forming units/litre (pfu/l) of virus necessary before the LVAS will recover 1 pfu in 10 millilitres (ml) of collection fluid over a 10 minute (min) sampling period, as stipulated in the project protocol.

AIM OF RESEARCH

AIM OF RESEARCH

1. To determine whether or not the three viruses would survive aerosolization by the Collison and be recovered by the LVAS.

2. To enhance virus recovery through use of additives in the collection fluid, to determine where during recovery and assay enhancement is most effective and to develop a concentration procedure to improve the recovery capabilities of the LVAS.

3. To determine the efficiency of the LVAS, relative to a reference sampler, for each virus and using these values to attempt to estimate the limit of virus detectability of the LVAS for RV-14 and HCV/229E.

MATERIALS AND METHODS

Host Cells

L-132 cells

These were originally derived from human embryonic lung tissue by Davis and Brolin (1970) and were obtained from Mr. D.A. McLeod (Bureau of Virology, Laboratory Centre for Disease Control, Ottawa) who in turn had obtained them from the American Type Culture Collection (ATCC). The cells were maintained between passages 20 and 40 (from the time of establishment in our laboratory) at a split ratio of 1 to 3. They were grown in Eagle's Minimal Essential Media (MEM "Auto-Pow" Flow Laboratories Inc.) supplemented with 2 mM L-Glutamine (GIBCO), 20 mM sodium bicarbonate (Fisher Certified reagent), the antibiotics Penicillin (100 units/ml), Streptomycin (100µg/ml) and Neomycin (50µg/ml) from Nutritional Biochemical Co. and 10% fetal calf serum (FCS) from Flow Laboratories Inc. Further details have been included in Appendix II.

Unless otherwise noted, all cells were grown in 75 cm^2 disposable plastic flasks (Lux Scientific Inc.) and were used within 24 hours of reaching confluence.

A-5-HeLa cells

These were obtained from Dr. K. Lonberg-Holm (E.I. duPont de Nemours and Co., Central Research and Development Department, Wilmington, Delaware).

Both the cell growth medium and the split ratio were the same for the A-5-HeLa cells as they were for the L-132 cells. The monolayers used in all experiments were restricted to those below our passage level of 25.

Viruses

Poliovirus

The Sabin vaccine strain of Poliovirus type 1 (Polio) was used for these experiments. The virus was cultivated in L-132 cells supplied with growth medium (see Appendix II for details) plus 2% FCS. After incubation at 37°C for 48 hours virus was released from cells by freezing and thawing the flask and contents three times. The suspension was transferred to centrifuge tubes and clarified by centrifugation at 2000 rpm for 15 minutes at 4°C. The "poliovirus-containing" supernatant fluid (SNF) was then dispensed into 1 ml aliquots and stored at -80°C.

Coronavirus

Human Coronavirus strain 229E (HCV/229E) was originally obtained from Dr. A.Z. Kapikian at the U.S. National Institute of Health , Bethesda, Maryland. The virus was cultivated in L-132 cells supplied with cell growth medium (Appendix II) plus 2% FCS. After incubation at 33°C for 36 hours the virus was harvested as above, separated into aliquots and stored at -80°C.

Rhinovirus

Rhinovirus type 14 (RV-14) as classified by Kapikian et al (1961) was obtained from Dr. B.D. Korant, E.I. duPont de Nemours and Co. The virus was cultivated in A-5-HeLa cells supplied with growth medium

plus 2% heat inactivated FCS (56°C for 30 minutes). The infected monolayers were incubated for 2 days at 33°C. Following incubation the virus was harvested as before and the RV-14/SNF was aliquoted then stored at -80°C.

Plaque Assay Procedure

The following plaque assay procedure has been adapted from the original method used by Kennedy and Johnson-Lussenburg (1976).

Cell monolayers usually less than 24 hours old were used. The growth medium was poured off and the monolayers were washed twice with Phosphate Buffered Saline (PBS). The liquid from the second wash remained on the monolayers until they were to be infected. Dilutions of the virus were made at 0°C and kept on ice until used for inoculation. The PBS was drained from the monolayers which were then infected with 0.33 ml of the virus sample. Virus was adsorbed for one hour at room temperature during which time the flasks were gently shaken at 20 minute intervals to redistribute the inoculum over the monolayer. At the end of one hour, 25 ml of the appropriate overlay medium (medium and agar) at 45°C was poured onto the upper cell-free surface of each flask. When the first signs of hardening appeared the flasks were inverted allowing the overlay to harden in contact with the monolayer. The flasks were then placed in an incubator with the monolayer inverted to the upper position. When assaying Polio I the flasks were incubated for 2 days at 37°C, HCV/229E required a 6 day incubation at 33°C and RV-14 required 4 days at 33°C.

Following incubation 5.0 ml of 4% formaldehyde in saline was added to each of the flasks which were then placed in a 37°C incubator for one hour. The overlay was then carefully removed and the remaining monolayer was stained with crystal violet to enhance the visibility of plaques which were then counted.

Stock Solutions Used in Overlay Media

The final concentration of the stock solutions in each overlay are tabulated in Appendix II.

Medium-199 (M-199) with Earle's salts, Flow Laboratories, was prepared in stocks of a five-fold concentration using deionized distilled water. The solution was pressure filter sterilized and stored at 4°C. Also sterilized and stored at 4°C were 5.6% sodium bicarbonate, 1.0% 5-bromodeoxyuridine (BudR, Calbiochem.), 4.0% DEAE Dextran (SIGMA) and 3.0 M $MgCl_2$ (Fisher Certified reagent). Penicillin, Streptomycin and Neomycin were used at final concentrations of 100 units/ml, 100µg/ml and 50µg/ml respectively. The stock solution (1000-fold concentration) was prepared from the powdered form of each antibiotic dissolved in deionized distilled water. The solution was filter sterilized, dispensed into 1 ml aliquots and stored at -20°C.

A stock Mycostatin suspension obtained from GIBCO (10,000µg/ml) was used in all overlays at a final concentration of 100µg/ml.

Fetal calf serum, (FCS) stored at -20°C, was used without further treatment or was heat inactivated at 56°C for 30 minutes.

Stocks of Oxoid No. 1 agar (Oxoid) at 1.8% and 2.7% were prepared in distilled water, autoclaved and stored at 4°C.

The agar and nutrient portions (see Appendix II) were kept separate at 45°C until required at which time two parts medium and one part agar were combined, then 25 ml was poured onto each cell monolayer.

Equipment

Collison Nebulizer

The small particle aerosols used in this study were produced by the Druett modified Collison nebulizer (Druett 1969). A schematic representation of this nebulizer showing the design and operation of one of the three nozzles is shown in Figure 1.

There are two major parts to the Collison, the head and the reservoir vessel. The head consists of the air inlet tube (compressed air), the three nozzle (or jet) head piece and the aerosol outlet. The reservoir vessel is a thick walled glass

FIGURE 1

LEGEND

Figure 1: A schematic view of the Collison nebulizer.

(1) Air Inlet Tube

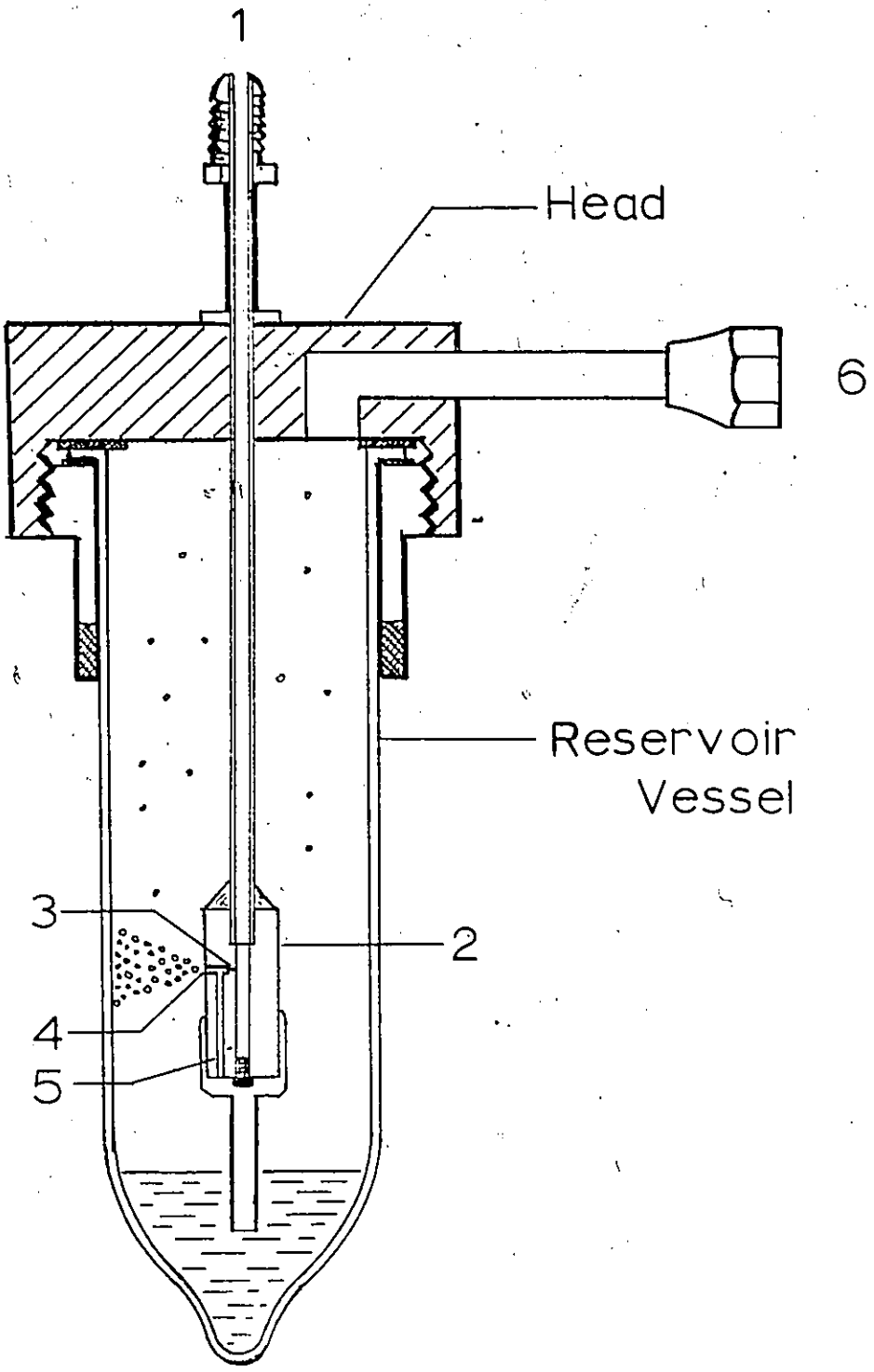
(2) Spray Head

(3) Air Jet

(4) Spray Nozzle

(5) Capillary Tube

(6) Aerosol Outlet


1
Head
6
Reservoir
Vessel
3
2
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5

container which, as well as containing the virus suspension, acts as a refluxing chamber into which the nozzles aerosolize the virus. The operation of the nozzle is easily observed through the glass vessel and should one or more of the nozzles not be functioning properly the experiment can be stopped and the nozzles cleaned. In this way inconsistent results from experiment to experiment caused by faulty aerosolization can be virtually eliminated.

The principle of operation is as follows; filtered, compressed air (26 psi*)is introduced into the Collison through the air inlet tube (1) where it flows to the spray head (2) which consists of three jets (one of which is shown (3)). Air passes through each jet into its associated nozzle (4) where the air expands reducing static pressure in the nozzle thereby drawing the liquid from the reservoir through a capillary tube (5) up into the nozzle. The liquid is then broken down in the expanding air into droplets of various sizes which are immediately expelled from the nozzle into the reservoir chamber. According to May (1972) the smaller droplets, those less than 43µm in diameter, are carried out of the Collison, through the aerosol outlet (6), in the departing air stream while the rest of the droplets (99.98%) impact on the glass surface and return to the liquid reservoir to be sprayed again.

* This is guage pressure - actual air pressure equals guage pressure plus atmospheric pressure (14.7 psi).

In all the experiments described in this work, the spray fluid consisted of 30 ml of cell growth medium (without fetal calf serum) plus 0.1 ml of 10% Antifoam "C" (Sigma).

Henderson Apparatus

The Henderson apparatus, as developed by Henderson in 1952, was designed to permit the continuous aerosolization of an infectious agent into a sterile, recycled air stream with the humidity and temperature controlled. For this reason the modified version of the Henderson apparatus, as shown in Figure 2, was used in this study.

The basic operation of the Henderson is as follows: sterile air enters the cloud chamber (2) from the circulating pump (8) through a port (3) adjacent to the point of attachment of the Collison nebulizer (1) (source of aerosol). The air flows through the cloud chamber past several ports for infection (animals), and/or sampling (mechanical sampling devices) and for measuring relative humidity and temperature (4) through a filter (5) and then through five critical orifices (7) which limit the air flow through the cloud chamber to approximately 30 L/min. The air is further filtered (5a) before it passes through the circulating pump (8) and on through the dessicant tanks (9 and 10) which are used to help control the humidity. The now filtered air passes through control valves (11 and 12) in the panel (13) and is returned to the cloud chamber. Thus, the aerosolized test agent is continuously introduced into the

FIGURE 2

LEGEND

Figure 2: A diagramatic representation of the Henderson Apparatus used in this study with the Collison and All Glass Impinger (AGI) in position.

(1) Collison Nebulizer

(2) Cloud Chamber

(3) Air Inlet Port

(4) Temperature and RH Measurements

(5) Filters

(6) All Glass Impinger

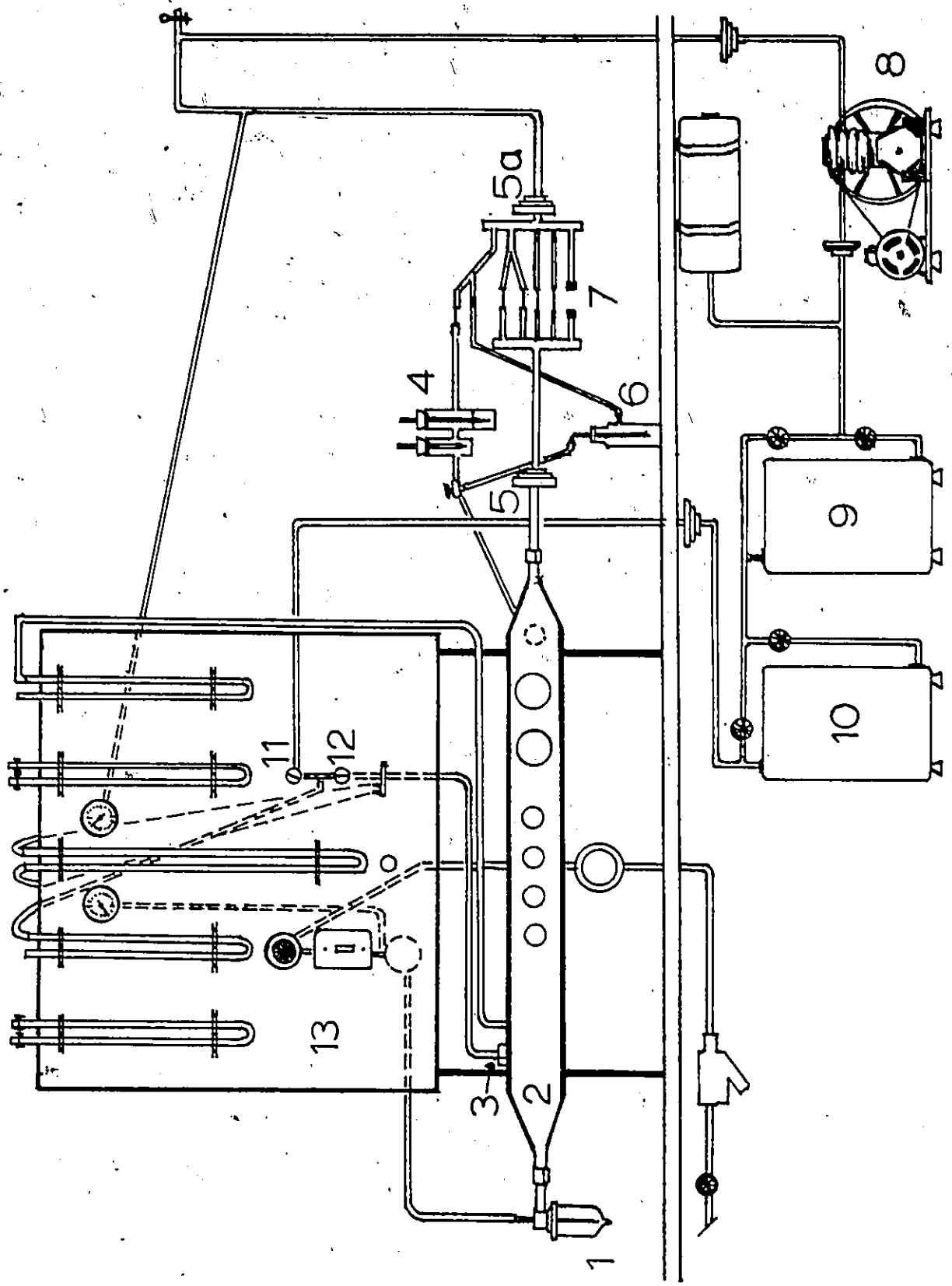
(7) Critical Orifices

(8) Circulating Pump

(9), (10) Dessicant Tanks

(11), (12) Control Valves

(13) Control Panel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sterile air stream at the source (1) and then removed by filtration after passing the sampling ports.

The circulating pump creates a vacuum of 40 cm of mercury between itself and the critical orifices so that the orifices will maintain the proper flow rate. This vacuum can be tapped when impingers are being used to sample an aerosol in the cloud chamber (6). When sampling from the chamber the pressure within the chamber is maintained at or near atmospheric so as not to subject the virus to any excess pressure and more importantly for safety because excess pressure may damage seals which could result in the leakage of aerosol into the work area. Usually the recommended condition within the cloud chamber is a slight vacuum of 2-3 mm of mercury. This vacuum keeps the aerosol from leaking from the chamber and is believed to be low enough that it has little or no effect on the virus or on its recovery from the chamber.

Impinger

The All Glass Impinger (AGI), as the name indicates, is made of glass and, as seen in Figure 3, consists of an aerosol inlet tube (1) which has a constriction (critical orifice) at its lower end (3), and

FIGURE 3

LEGEND

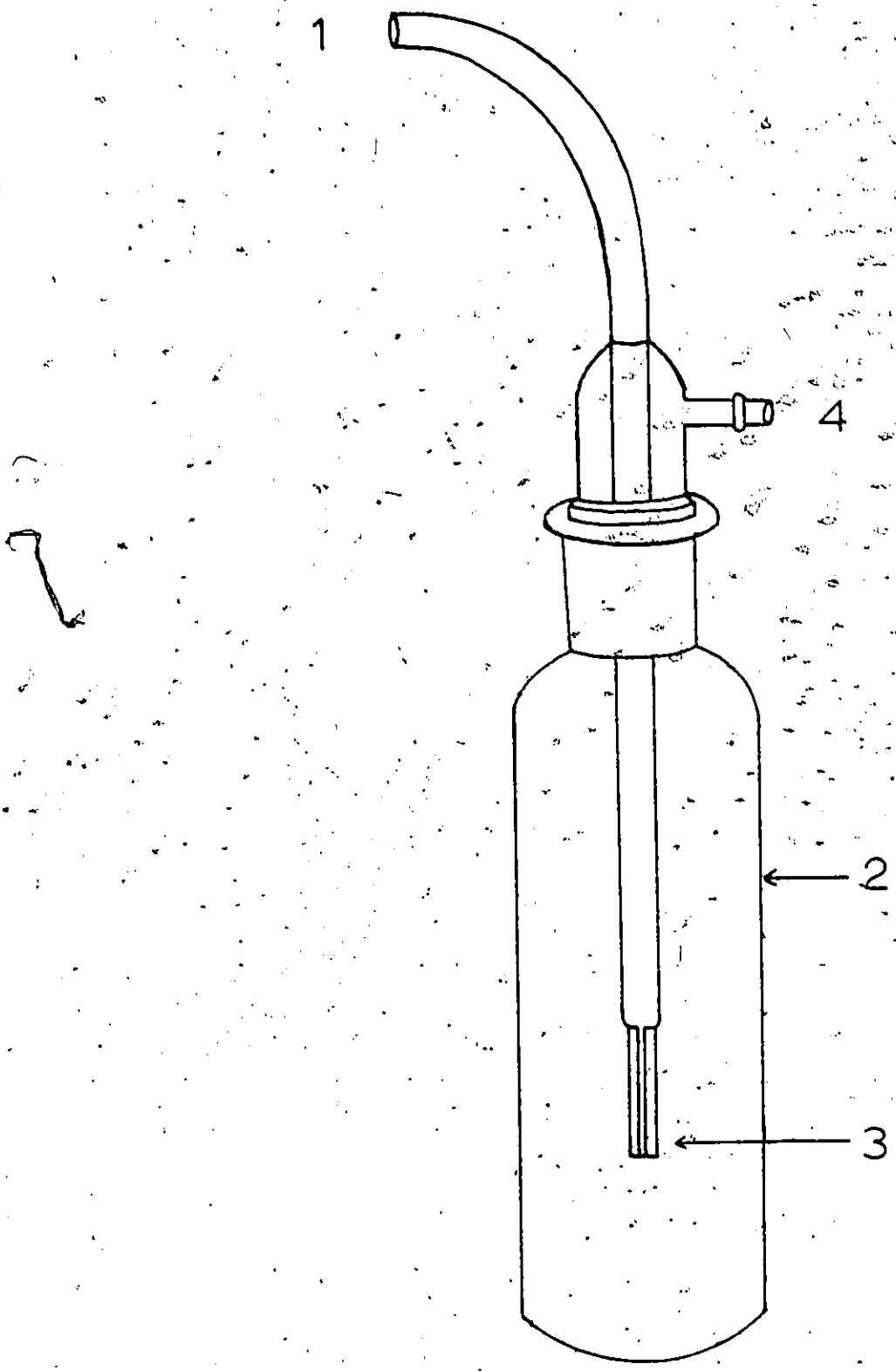
Figure 3: The All Glass Impinger (AGI)

(1) Aerosol Inlet

(2) Fluid Reservoir Vessel

(3) Critical Orifice

(4) Vacuum Port (Air Outlet)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3

a fluid reservoir vessel (2). This vessel is made of thick walled glass enabling it to withstand the sampling vacuum supplied through the vacuum port (4).

Unless otherwise stated the collection fluid used in the impinger consisted of MEM, approximately 0.05 ml of 10% Antifoam "C" and appropriate additive all to a total volume of 12 ml. During a typical sampling run the aerosol inlet tube of the impinger is connected to the aerosol source (Henderson cloud chamber) which is always at or slightly below atmospheric pressure. A vacuum is supplied to the vacuum port (4) and the particles of the aerosol are accelerated as they are drawn through the orifice and impinge into the collection fluid. The maximum velocity for these particles is approximately the speed of sound (332 m/sec, or 1088 ft/sec) which is reached with a vacuum of at least one half an atmosphere. Also under these conditions the maximum characteristic flow rate as determined by a rotameter, for the impingers used here was 5.6 L/min. An intermediate flow rate of 1.1 L/min. was also used for the experiments in which optimal conditions could not be achieved.

Large Volume Air Sampler

The Large Volume Air Sampler (LVAS) used in these experiments was designed and built under the supervision of the Preventive Medicine Section, Defence Research Establishment Suffield, Alberta and was on loan to this department for the purpose of these studies. A schematic representation of the LVAS is shown in Figure 4.

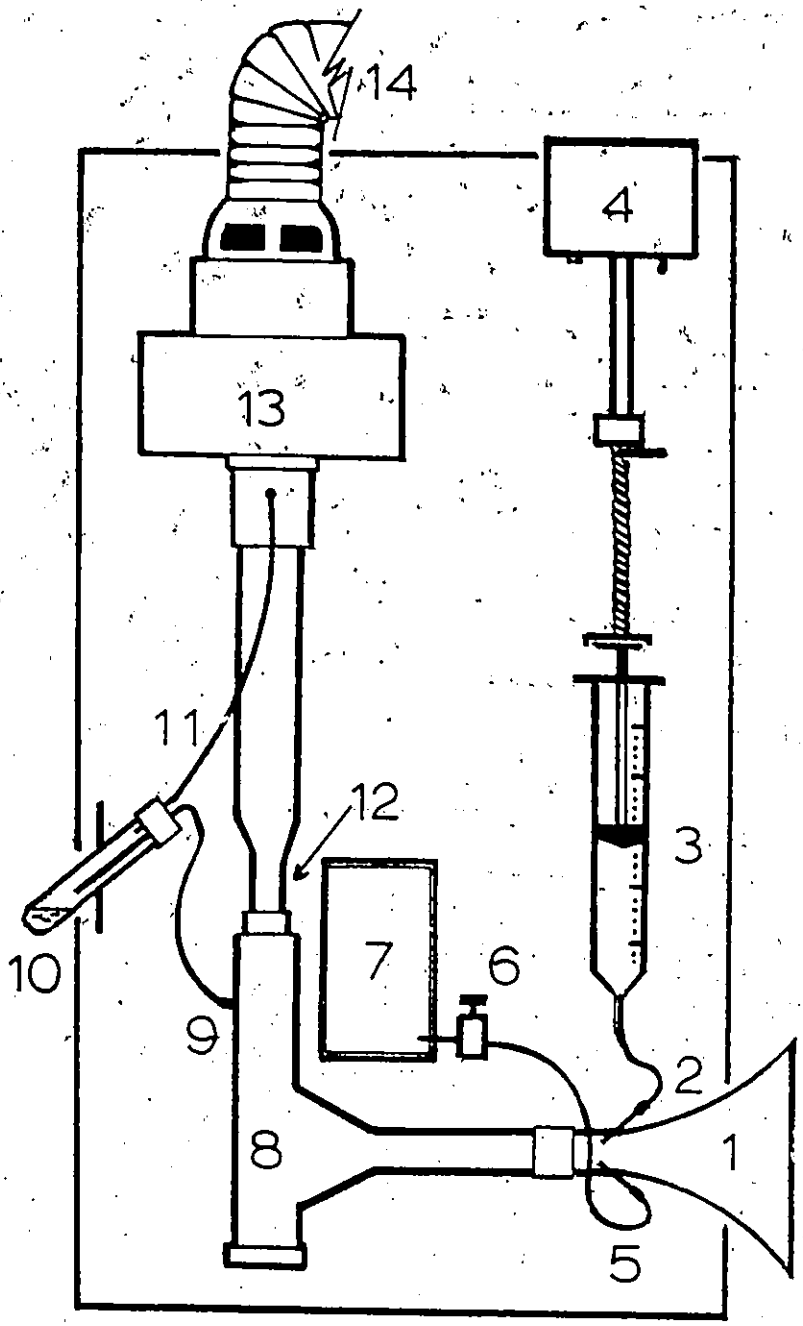
The air flow rate through the sampler is about 950 L/min. The flow of air is produced by the vacuum motor (13) which draws air into the sampler through the horn (1). Collection fluid from the syringe (3) is introduced into the air stream through a tube in the horn (2) by the syringe drive (4). The collection fluid travels within the air stream to the collector (8) where it impacts on the walls forming a film of liquid into which much of the particulate matter of the air stream impinges. The liquid moves in a helical path through the collector towards a constriction in the tube (12). The constriction serves two functions: (a) it stops the flow of fluid and associated particulates at the collection port so it can be removed from the collector and (b) it creates a pressure differential between the collection port and the vacuum motor. This pressure differential is used, with the aid of a vacuum tap (11), to draw the collection fluid from the collector through the collection port (9)

FIGURE 4

LEGEND

Figure 4: Schematic of the Large Volume Air Sampler (LVAS) showing the essential features.

(1) Horn
(2) Collection Fluid Inlet
(3) Collection Fluid Syringe
(4) Syringe Drive
(5) Make-up Water Inlet
(6) Control Valve
(7) Reservoir
(8) Collector
(9) Collection Port
(10) Collection Vessel
(11) Vacuum Tap
(12) Constriction
(13) Vacuum Motor
(14) Air Exhaust (HEPA Filter)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into the collection vessel (10). To compensate for water evaporation, make-up water is supplied to the system from the reservoir (7) and is controlled by the flow valve (6). The flow rate of the water entering the airstream (5) is adjusted so that the total collection fluid flow rate into the collection vessel is approximately 1.0 ml/min. The basic collection fluid in which the additives were evaluated was essentially the same as the spray fluid (Appendix II). As has been shown by White et al (1975) not all of the infectious particles entering the sampler under test conditions were removed from the air stream therefore to help insure that these particles (slippage) are not released into the environment all the air passing through the vacuum motor was exhausted through a HEPA filter (14).

Concentration Procedure

Amicon Ultrafiltration membrane cones (type CF 504A) (Amicon Corp.) were tested for their effectiveness in concentrating each of the three viruses. The cones were presoaked in distilled water for at least one hour after which they were drained, placed in the centrifuge tube supports and 8.0 ml PBS + 0.1% BSA was used to precondition the cones by centrifuging them at 2000 rpm for 30 minutes. Following this 8.0 ml of the sample was added to the cones which were then centrifuged at 2000 rpm for 20 minutes or until there was approximately 1.0 ml of liquid left in the filter cone. Part or all of this sample was then assayed for virus.

EXPERIMENTAL RESULTS

i) Sampling Virus from an Internal and External Source with the LVAS

ii) Enhancement of Virus Recovery

iii) Role(s) of BSA in Improving Virus Recovery

iv) Development of a Concentration Procedure

v) Concentration of LVAS Samples

vi) A Determination of the Efficiency and Limits of Virus Detectability of the LVAS

Sampling Virus From an Internal and External Source with the LVAS

This first part of the study was designed to obtain some basic information concerning the physical limitations of the LVAS and the survival of the three test viruses during recovery (by the LVAS) from both an "internal" and an "external" source.

As with virtually all samplers, a certain quantity of the aerosolized agent will pass directly through the sampler and will not become trapped in the collection medium. This portion of the aerosol represents the amount lost due to "physical slippage" and is most often determined through the use of a chemical dye or tracer. The amount of the tracer recovered by the sampler usually represents the maximum quantity of an agent that can be recovered. When biological agents are studied they are, as well as being lost through physical slippage, also subject to inactivation a process more commonly known as "biological decay".

Thus in this study, using the dye sodium fluorescein as a tracer, and each of the three viruses, an attempt was made to determine the amount of slippage and biological decay associated with the LVAS when the dye or virus were introduced into it from either the internal or external source.

Virus or dye for the "internal source" experiments was supplied to the LVAS air stream from the collection fluid syringe (Figure 5). In these experiments the virus was suspended in collection fluid (CF) (Appendix II) plus 0.1% Bovine Serum Albumin fraction V (BSA) whereas the dye was dissolved in CF. Under these conditions the virus was considered to be subjected only to the sampling process (entrapment and collection) and the ambient environmental conditions of temperature and humidity.

Following these "internal source" experiments, the "external source" experiments were concerned with the recovery of both the dye and test viruses as droplet nuclei. The Collison nebulizer (Figure 5) was used to aerosolize the dye or virus directly into the air stream of the LVAS. In these experiments the collection fluid syringe contained CF plus 0.1% BSA for virus recovery and CF without BSA for dye recovery. Under these conditions the physical slippage of droplet nuclei could be determined as could information pertaining to the survival of the viruses in aerosol and during subsequent recovery by the LVAS.

In the following two sub-sections the results of these sampling experiments are presented.

FIGURE 5

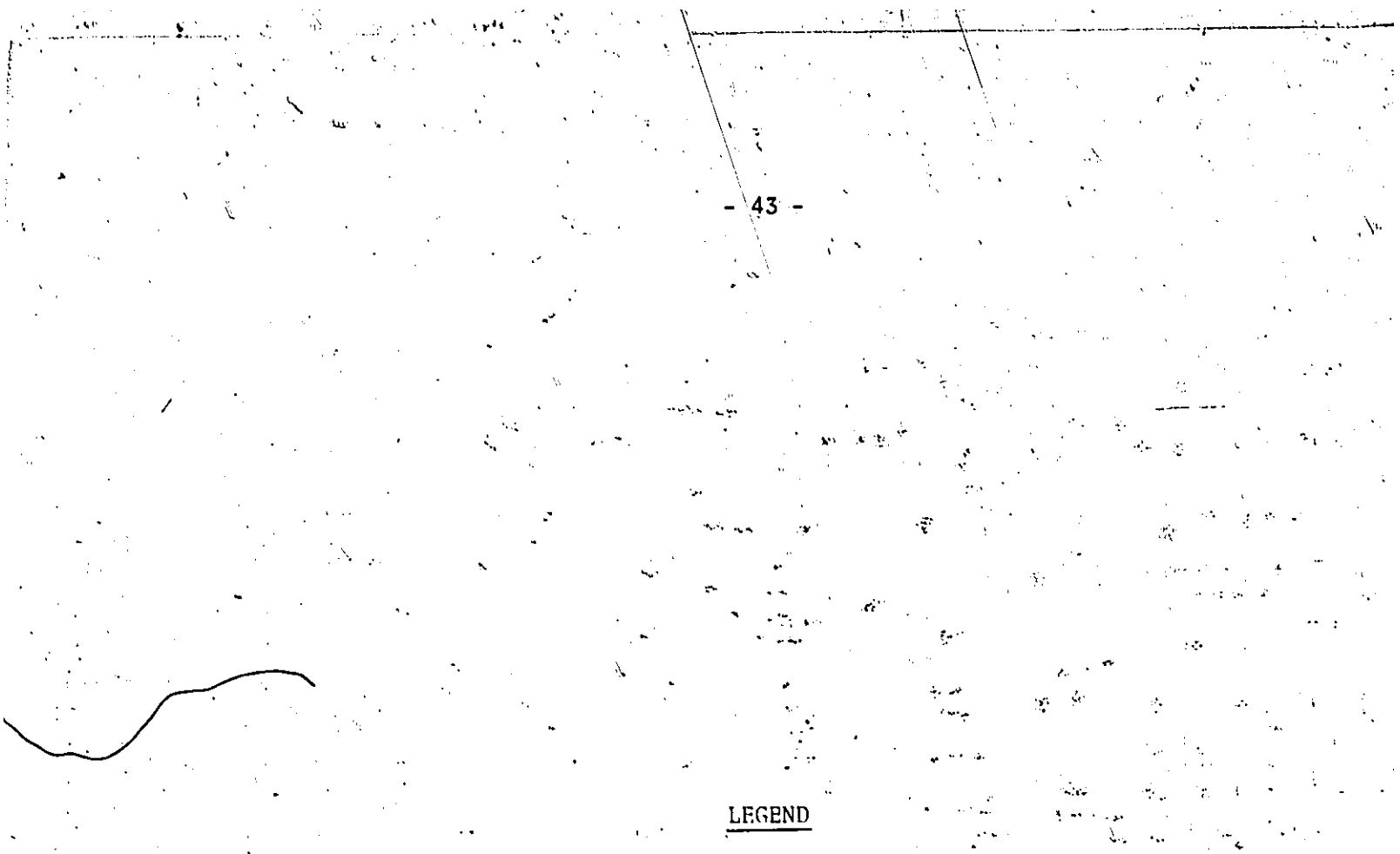
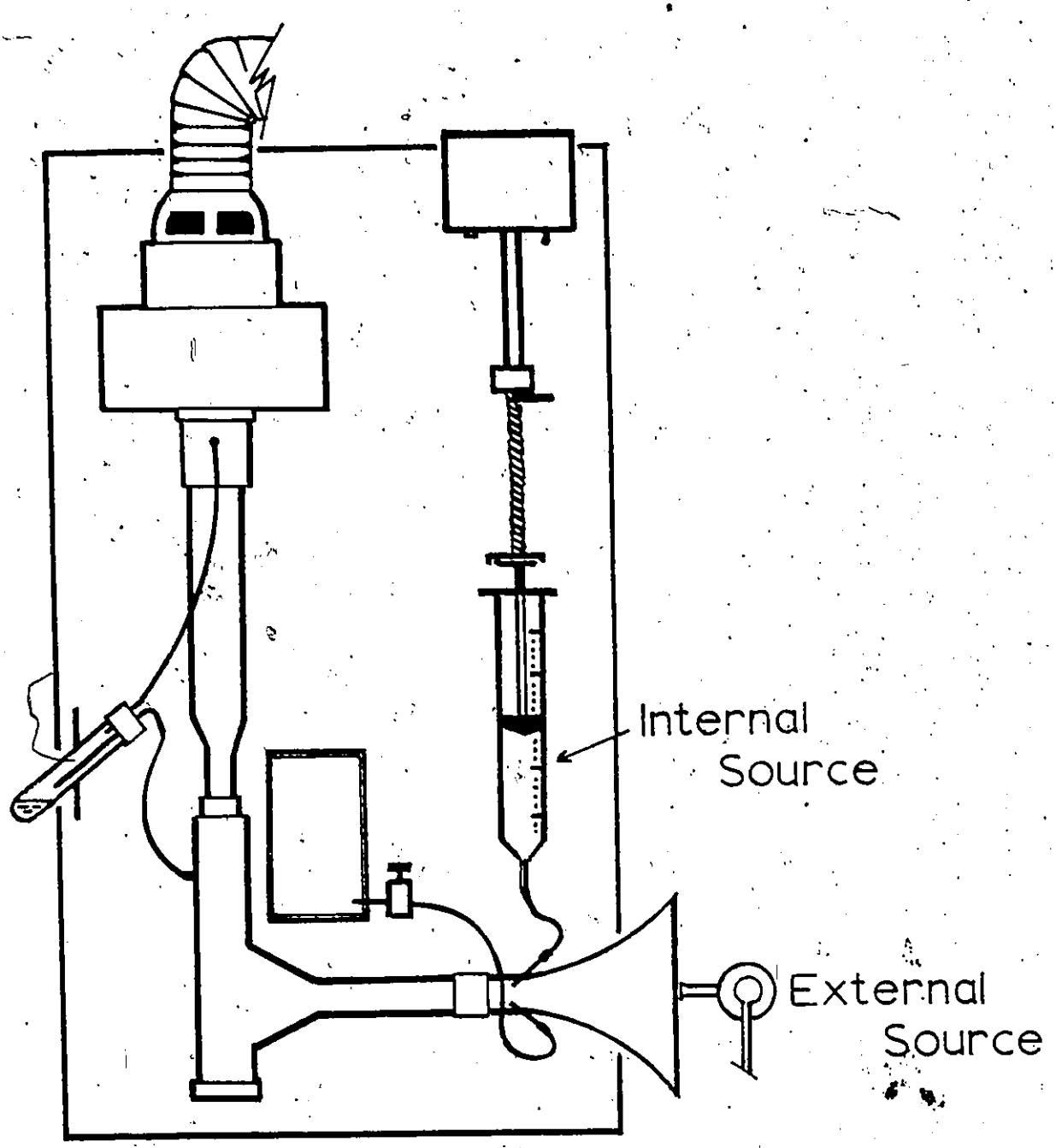


Figure 5:- LVAS indicating the positioning of the "external" and "internal" virus sources.


Internal
Source
External
Source

Recovery of Internal Source Virus

Three experiments were performed using the dye and each of the viruses, and the resulting averages are shown in Table 1, (syringe). The dye was assayed by measuring the optical density at 493.5 nm using a Beckman DB spectrophotometer. Sterile distilled water was used for dilution as required. The appropriate plaque assay techniques were used to measure virus recovery. (See Materials and Methods Section).

The LVAS sampling period for this and all subsequent experiments, unless otherwise stated, was 10 minutes. The results obtained in these experiments are expressed as percent recovery which is a comparison between the actual and theoretical recoveries based on the concentration of dye or virus at source and rate of delivery into the system.

From Table 1 it can be seen that recovery of the dye was 105% indicating negligible physical slippage. Recovery for each of the viruses was below theoretical which, since slippage was negligible, was probably a result of biological inactivation or aggregation.

From these results it appeared that Poliovirus type I was least affected by the sampling action of the LVAS, while Rhinovirus type 14 and Coronavirus 229E were considerably less stable. However, this instability was not as great as expected consequently the study was continued to the next phase.

TABLE 1

TABLE 1

DYE AND VIRUS RECOVERY BY LVAS FROM INTERNAL AND EXTERNAL SOURCES

Test component	Source	Concentration at source	Liquid volume	LVAS recovery		
				Estimated	Experimental	
		mg/ml	ml	mg	mg	%
Dye	syringe	0.15	0.9	0.14	0.15	107
	Collison	0.17	2.7	0.46	0.14	31
		$pfux10^3/ml$	ml	$pfux10^3$	$pfux10^3$	
Poliovirus type I	syringe	0.18	0.9	0.16	0.12	77
	syringe	0.27	0.9	0.24	0.17	71
	Collison	1.05	2.7	2.84	0.25	9
	Collison	1.23	2.9	3.57	0.39	11
Rhinovirus type 14	syringe	0.60	0.9	0.54	0.23	42
	syringe	0.63	0.9	0.57	0.22	38
	Collison	1.19	2.7	3.21	0.95	30
	Collison	1.18	2.7	3.19	0.87	27
Human Coronavirus 229E	syringe	24.60	0.9	22.14	8.87	40
	syringe	24.00	0.9	21.60	8.81	41
	Collison	149.00	2.7	402.30	59.04	14
	Collison	131.00	2.7	353.70	53.64	15

Recovery of External Source Virus (Collison Nebulizer)

The virus or dye was aerosolized by the Collison and introduced into the LVAS through the horn (Figure 5) instead of from the collection fluid syringe as before. The syringe now supplied only the collection fluid consisting of CF plus 0.1% BSA.

The resulting average of three experiments using the dye and two performed using each of the viruses are shown in Table I (Collison). As can be seen from the table there was a decrease in the amount of dye and virus recovered. The recovery of dye from the aerosol was approximately 30% indicating a loss of about 70%.

The amount of Polio recovered from aerosol was only 11%, considerably less than the 77% which had been recovered from the internal source. HCV/229E recovery decreased from 40% to 14% while Rhinovirus recovery, which appeared to be the least affected, was reduced by only 10% (from 40% to 30%). Thus for both the dye and viruses there was a decrease in recovery from aerosol for which there are several possible explanations.

The results of dye recovery in these experiments were different from those of White et al (1975) who, using the same type of sampler,

but collecting monodispersed aerosols of Rhodamine B, reported recoveries of approximately 100% for particles down to 2.3µm and recoveries of about 70% for 0.5µm particles. Their results incorporated an adjustment for the loss of 20% of the dye due to the entrapment, within the aerosol generator, of satellite particles. These particles were below 0.5µm in diameter and therefore less than 70% would have been recovered by the LVAS. Therefore had these particles been present in their aerosol, most of them would have slipped through the sampler and their loss would probably have decreased the efficiency results by approximately 20%. It has been reported that the Collison nebulizer produces these satellite particles but we were not able to collect and study them. However, Couch et al (1965), using sodium fluorescein, have performed some studies concerning the particle sizes of the aerosols produced by the Collison nebulizer. They have found that about 54% of the aerosol volume consists of particles with diameters of 1-2µm and that approximately 85% of all the particles produced by the Collison are below 1µm therefore it would seem likely that at least 20% of the remaining 46% of the volume of aerosol produced would be in the form of particles of less than 0.5µm in diameter, the size White et al (1975) classed as satellite particles, which are recovered by the LVAS at an efficiency of less than 70%. Of the particles of less than 0.5µm that are collected all will contain some quantity of dye however only a few will contain virus. Couch et al (1965) have shown that using

virus suspensions of less than 10^9 pfu/ml the average concentration of virus per resulting aerosol particle is less than one and by using the Anderson sampler they have found that over 90% of the virus is collected within the 3-0.5μm size range. They therefore concluded that the quantity of virus contained in particles of less than 0.5μm in diameter would be negligible. The results of Couch et al (1965) are similar to those obtained in this laboratory (Appendix I). It follows then that although approximately 70% of the dye was lost through physical slippage, a very small amount of virus would have been contained in and therefore lost with those particles. Thus the amount of dye lost will be relatively high compared to the amount of virus lost as is the case in the set of experiments presented here. For example, referring back to the results of RV-14 recovery (Table 1), biological decay of the virus recovered from the internal source was 60% when physical slippage (by recovery) was negligible. When physical slippage was 70% for collection of the dye aerosol, the recovery of RV-14 from the aerosol decreased slightly. Therefore it appears that although 70% of the aerosol was lost through physical slippage, only 10% of the virus was lost through a combination of physical slippage and biological decay.

From the same Table , recovery of HCV/229E from a Collison aerosol decreased considerably, relative to internal source recovery.

Assuming the same amount of physical slippage for both viruses it appears that HCV/229E is less stable than RV-14 under aerosol conditions.

The internal source experiments for Polio appeared to have little effect on the survival of the virus however when recovering it from aerosol there was a dramatic decrease in the amount of virus recovered. On initial observation, since the percentage loss of Polio was almost as large as the percentage loss of dye through physical slippage, it was felt that most of the loss of the virus was due to slippage. However, with the recovery of the two respiratory viruses (RV-14 and HCV/229E), irregardless of the virus, since the percent loss of both viruses was much less than Polio, it was assumed that much of the virus had been lost through biological decay. The relative humidity of the incoming air mixing with the virus aerosol was approximately 50%, a condition reported to cause rapid inactivation of Poliovirus (de Jong and Winkler, 1968 and Akers and Hatch, 1968) which is much more stable at high humidities. The components of the spray fluid, which are concentrated in the droplet nuclei, might also be contributing to the decrease in Polio. To understand this difference in recoveries between Polio and the two respiratory viruses, further work should be performed concerning the survival of each of the viruses at different relative humidities and temperatures in different spray and recovery fluids.

From these results one can see that though each of the viruses is recoverable the stability of each under the conditons employed is different. The following experiments were devoted to testing certain additives to the recovery fluid in an attempt to increase virus recovery.

Enhancement of Virus Recovery

The survival of Poliovirus type I, Coronavirus strain 229E and Rhinovirus type 14 during aerosolization by the Collison and recovery by the LVAS has been demonstrated in the previous section. In the next group of experiments it was necessary to devise and provide a more stable physical system in which humidity and temperature could be controlled in order that the necessary improvements in recovery fluids could be evaluated. A viral aerosol generation and recovery system (Figure 6) consisting of the Collison nebulizer, Henderson Apparatus, LVAS and AGI was used. This system was established to (a) provide a continuous source of aerosolized virus, (b) sufficiently mix the virus aerosol to obtain consistent recovery, (c) maintain a constant environment for the virus aerosol and (d) provide a virus aerosol that could be sampled by both the LVAS and AGI so that a comparison of their virus recoveries could be made.

The LVAS and AGI were connected to the Henderson as indicated in Figure 6. A 5.6 L/min. AGI orifice (5) was positioned 11.0 cm inside the centre of the LVAS horn (6) where a vacuum of 3.0 cm of Mercury was obtained when the LVAS was operating. Under these conditions the

FIGURE 6

LEGEND

Figure 6: Arrangement of the cloud chamber, LVAS and AGI for the evaluation of the additives.

(1) Collison Nebulizer

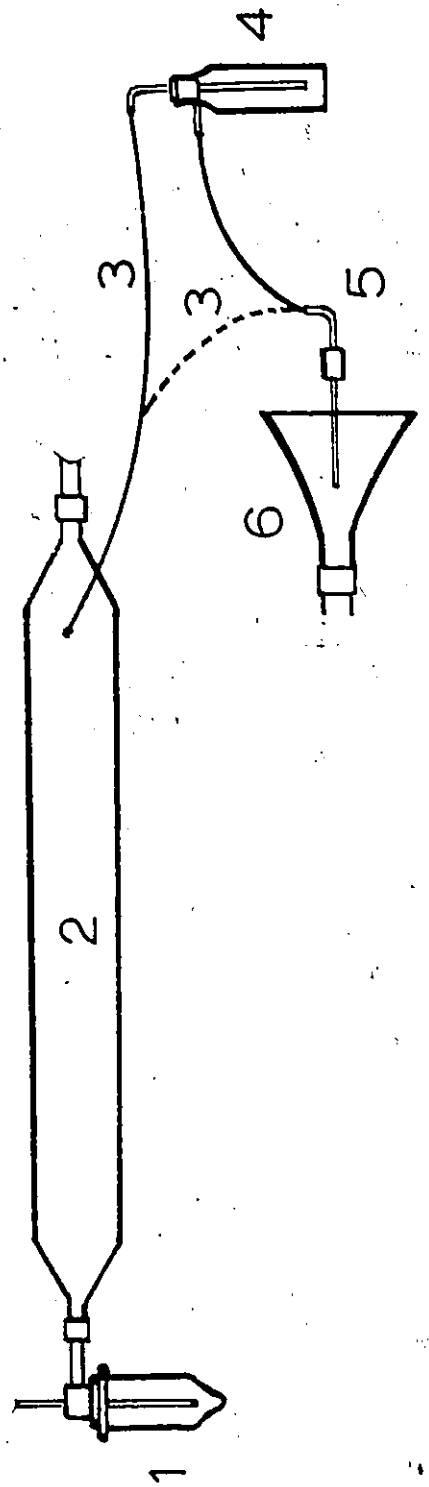
(2) Henderson Cloud Chamber

(3) Sampling Tube

(4) All Glass Impinger (AGI)

(5) AGI Orifice

(6) LVAS Horn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flow rate of virus aerosol from the Henderson to the LVAS, (through a 1 meter section of 1/2 inch diameter Tygon tube) as measured by a rotameter, was 1.6 L/min. When the impinger was used as the sampler it was connected between the LVAS and Henderson (3) using the LVAS as a vacuum source. The flow rate of the virus aerosol from the Henderson into the impinger was 1.1 L/min, slightly less than into the LVAS because the impinger was a restriction in the line between the LVAS (vacuum source) and the Henderson and impeded the aerosol flow.

In these experiments the impingers were used essentially to monitor the virus concentration in the aerosol of the cloud chamber during the sampling period. Thus for each additive to be tested one impinger sample was taken before the LVAS samples and one after. The two recoveries were averaged to better represent virus recovery by the impinger during the period when the LVAS was sampling the virus. In this way a more accurate comparison of virus recovery by the two samplers could be made. The additives used were tested for their ability to improve conditions in the recovery fluid in an attempt to optimize virus recovery.

Dimmick and Akers (1966) refer to a number of papers which have dealt with the concept of suspending fluids and their effect on virus survival in aerosol. The effect of the components varied with the virus used and the relative humidity. Serum from one source or another

was often used hence the reason for testing FCS. Because BSA is a concentrate of the albumin fraction of serum it was also tested to observe its effect on virus recovery. Glycerol protects cells during the freezing process by affecting the size of crystal formation, thus it interacts with the water during freezing. It was felt that glycerol could interact with water during rehydration by slowing the process down and thereby protect the viruses (especially HCV/229E) during collection.

Since four impingers were available for these experiments only two additives could be tested at one time thus each sample was a test sample.

Poliovirus (Type 1 Sabin) (Polio)

Using the previously outlined sampling technique the additives BSA (0.1%, 0.5% and 1.0%) and glycerol (10%) were tested for their ability to enhance the recovery of Polio by the LVAS and AGI. The results of this study are shown in Table 2A to 2D. The data in Table 2A represents the study comparing virus recovery by both the impinger and LVAS (pfu/L) using collection fluid with and without 0.1% BSA. It can be seen that the difference in impinger virus recovery with and without BSA (+13% and +92%) was not as pronounced as for the LVAS (+140%). This indicated that the requirement for BSA in the collection fluid was greater when using the LVAS suggesting that the sampling action of the LVAS was more detrimental to Polio than that of the impinger. The

TABLE 2

TABLE 2

POLIOVIRUS TYPE I (SABIN)

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

	Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
A.		No BSA	0.1% BSA	% Change+	No BSA	0.1% BSA	No BSA	0.1% BSA
	Impinger*	262	295	13				
	LVAS	59	142	140				
	Impinger**	425	818	92				
	Average				344	557	17	25
	Impinger*	851	556	-35				
	LVAS	196	268	37				
	Impinger**	753	753	0				
	Average				802	655	24	41
	Impinger*	229	295	29				
	LVAS	139	176	27				
	Impinger**	491	360	-27				
	Average				360	328	39	54
B.		0.1% BSA	0.5% BSA	% Change	0.1% BSA	0.5% BSA	0.1% BSA	0.5% BSA
	Impinger*	360	515	43				
	LVAS	99	260	163				
	Impinger**	395	295	-25				
	Average				377	405	26	64
	Impinger*	524	589	14				
	LVAS	220	330	50				
	Impinger**	785	425	-46				
	Average				655	507	34	65
	Impinger*	622	425	-32				
	LVAS	206	368	79				
	Impinger**	753	687	-9				
	Average				688	556	30	66

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample.

\+ All values are increases unless a decrease is indicated (-).

TABLE 2

POLIOVIRUS TYPE I (SABIN)

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
C.	0.5% BSA	1.0% BSA	% Change†	0.5% BSA	1.0% BSA	0.5% BSA	1.0% BSA
Impinger*	327	491	50				
LVAS	166	265	60				
Impinger**	687	425	-38				
Average				507	458	33	58
Impinger*	229	360	57				
LVAS	172	228	33				
Impinger**	458	360	-21				
Average				344	360	50	63
D.	0.1% BSA	10% Glycerol	% Change†	0.1% BSA	10% Glycerol	0.1% BSA	10% Glycerol
Impinger*	295	393	+33				
LVAS	93	125	+34				
Impinger**	458	196	-57				
Average				377	295	25	42
Impinger*	327	362	+11				
LVAS	196	108	-45				
Impinger**	295	327	+11				
Average				311	295	63	37
Impinger*	164	458	+179				
LVAS	138	224	+62				
Impinger**	393	982	+150				
Average				279	720	49	31

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample.

† All values are increases unless a decrease is indicated (-).

results of the other two experiments confirmed these results though not quite as dramatically. Analysis of the percent recovery confirmed that the impinger was a more effective sampler than the LVAS because virus recovery with the LVAS varied between 17 and 54% of impinger recovery. Furthermore, in related experiments, this recovery was much better in the presence of BSA further indicating the importance of BSA in improving virus recovery by the LVAS.

The results in Table 2B represent a comparative study of the effects of 0.1% and 0.5% BSA on Polio recovery. Virus recovery by the impinger in the presence of 0.5% BSA was either less than or equal to that for 0.1% indicating no apparent requirement for the additional BSA. All LVAS samples showed a definite increase in virus recovery with the additional BSA that were comparable to the increases found in the previous experiments. The percent recoveries are much better due to the additional BSA with the average recovery being 65%.

The results in Table 2C represent two experiments comparing virus recoveries in the presence of 0.5% and 1.0% BSA. As can be seen the results from the impinger samples were rather inconclusive, one half indicated an increase in recovery with additional BSA, the other a decrease. Thus it appeared that BSA was no more effective at 1.0% than it was at 0.1%. The results from the LVAS samples indicate that additional BSA resulted in an increase in the amount of virus recovered by the LVAS. However when comparing the two samplers the virus recovery with the

LVAS is still about 60% of that recovered by the impinger indicating the additional BSA did little to improve the overall efficiency of the LVAS.

These results suggested that increasing the concentration of BSA above 1.0% would not result in much of an increase in virus recovery. This became an important consideration because 1.0% or more BSA presented technical difficulties in performing the tests especially regarding foaming and delays between tests. Therefore 1.0% BSA was regarded throughout this work as being the upper limit for testing.

Finally 10% glycerol was compared with 0.1% BSA to determine its effectiveness on virus recovery. As can be seen from the results in Table 2D, virus recovery by the impingers was better in the presence of glycerol than BSA. In two of three experiments using the LVAS there was an increase in recovery while in the third a decrease.

These results were encouraging for the further testing of glycerol on Polio recovery however, work was stopped at this point pending similar findings with the two test viruses, RV-14 and HCV/229E.

Rhinovirus Type 14 (RV-14)

The results shown in Table 3A are from three experiments comparing the recoveries of RV-14 in collection fluid with and without BSA. Here, as with Polio, recovery by the impingers with BSA incorporated in the fluid resulted in an increase in virus for two experiments and a decrease for the third. Whereas all three experiments indicated an increase in virus when BSA had been added to the LVAS recovery fluid. The percent recovery (LVAS/AGI) was higher in the presence of BSA suggesting that, as was the case with Polio, the presence of BSA improves virus recovery by the LVAS more than by the impinger.

When comparing the virus recoveries at 0.1% and 0.5% BSA (Table 3B) one can see a small decrease in virus recovery when using the impingers whereas there is a slight increase using the LVAS. The effect of the additional BSA was minimal compared to the previous experiments thus it appears that there is little need for more than 0.1% BSA. When comparing the samplers however it becomes evident that there is a considerable increase in the recovery for the LVAS to the point where it is virtually equal to (82%) if not better than (148%) the impinger. Thus the additional BSA appears to have been important in increasing the effectiveness of the LVAS in comparison to the AGI.

Further increasing the BSA concentration to 1.0% provided the results shown in Table 3C. The results for the impinger indicate that virus recovery, although fluctuating, was better in the presence of the additional

TABLE 3

TABLE 5

RHINOVIRUS TYPE 14

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

A. Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
	No BSA	0.1% BSA	% Change+	No BSA	0.1% BSA	No BSA	0.1% BSA
Impinger*	1244	1669	34				
LVAS	410	586	43				
Impinger**	1604	2029	26				
Average				1424	1849	29	32
Impinger*	3305	2945	-11				
LVAS	1114	1619	45				
Impinger**	4844	3338	-31				
Average				4075	3142	27	52
Impinger*	5695	6709	18				
LVAS	2772	3965	43				
Impinger**	5236	6218	19				
Average				5466	6464	51	61

B.	0.1% BSA	0.5% BSA	% Change	0.1% BSA	0.5% BSA	0.1% BSA	0.5% BSA
Impinger*	458	398	-13				
LVAS	253	271	7				
Impinger**	294	262	-11				
Average				376	330	67	82
Impinger*	327	164	-49				
LVAS	197	244	24				
Impinger**	164	164	0				
Average				246	164	80	148

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample.

\+ All values are increases unless a decrease is indicated (-).

TABLE 3

RHINOVIRUS TYPE 14

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

Sampler	Particle Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
C.	0.5% BSA	1.0% BSA	% Change+	0.5% BSA	1.0% BSA	0.5% BSA	1.0% BSA
Impinger*	164	262	60				
LVAS	195	92	-53				
Impinger**	98	55	-66				
Average				131	148	145	62
Impinger*	1898	2389	26				
LVAS	2628	2686	2				
Impinger**	2880	4680	63				
Average				2389	3535	110	76
D.	10% Glycerol	0.1% BSA	% Change+	10% Glycerol	0.1% BSA	10% Glycerol	0.1% BSA
Impinger*	556	131	-76				
LVAS	244	39	-84				
Impinger**	196	327	67				
Average				376	229	65	17
Impinger*	785	524	-33				
LVAS	421	405	-4				
Impinger**	491	425	-13				
Average				638	478	66	85
Impinger*	327	196	-40				
LVAS	351	363	3				
Impinger**	33	327	890				
Average				180	262	195	139

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample.

+ All values are increases unless a decrease is indicated (-).

BSA whereas recovery by the LVAS appeared to be less. Therefore the addition of 1.0% BSA did not sufficiently increase virus recovery to warrent its use or the use of higher concentrations in further experiments.

The results of three experiments comparing virus recoveries in the presence of 10% glycerol or 0.1% BSA can be seen in Table 3D. As with Polio, RV-14 recovery by the impingers and LVAS was generally better with glycerol than BSA. Comparing the recoveries of the two samplers the results in the presence of glycerol are more consistent than those for BSA. Virus recovery by either sampler in the presence of glycerol is not as good as in the presence of 0.5% BSA. Further testing of higher concentrations of glycerol was indicated however these were postponed until the similar tests using HCV/229E had been performed.

Coronavirus 229E (HCV/229E)

Initial studies with HCV/229E under the conditions established for this portion of the study showed that it was inactivated in aerosol to a greater degree than previous experiments indicated (first section). In the following experiments the concentration of HCV/229E in the Collison nebulizer had to be ten times (10X) higher than Polio or RV-14 in order that all three viruses be recovered at similar concentrations. Because of this greater sensitivity a more intensive study of the additives was performed (Table 4A to 4F).

The results of experiments comparing virus recovery by the LVAS with and without 0.1% BSA are shown in Table 4A. The use of 0.1% BSA in both the LVAS and the impingers resulted in an increase in virus recovery of over 200% in all but one experiment. This indicated that there was a definite requirement for BSA for the recovery of HCV/229E, one that was much more pronounced than for Polio or RV-14. The percent recoveries were similar to those obtained for the other viruses indicating a proportionate increase in recovery for the AGI and the LVAS in the presence of BSA.

The comparison of virus recovery in the presence of 0.1% BSA or 0.5% BSA is shown in Table 4B. There was a slight increase in virus recovery in the LVAS samples but this was considerably smaller than that found in the previous experiments. There was virtually no increase in recovery using the impinger suggesting that the presence of 0.1% BSA in this sampler was sufficient for optimum virus recovery. The additional BSA permitted an increase in the percent recovered by the LVAS which indicates, as with the other viruses, that the increase in effect on virus recovery by the additional BSA was more pronounced in the LVAS samples than in the impinger samples.

These experiments demonstrated that the maximum advantage gained by using BSA may have been reached at a concentration of 0.5% however further test of 1.0% BSA was compared to 0.5% BSA the results of which

TABLE 4

TABLE 4

HUMAN CORONAVIRUS 229E

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		LVAS/AGI (%)	
A.	No BSA	0.1% BSA	Change	No BSA	0.1% BSA	No BSA	0.1% BSA
Impinger*	138	785	468				
LVAS	-	562	-				
Impinger**	65	458	604				
Average				102	622		90
Impinger*	327	1211	271				
LVAS		-	-				
Impinger**	65	785	1107				
Average				196	998		-
Impinger*	-	518	-				
LVAS	31	207	568				
Impinger**	-	229	-				
Average				-	524	-	40
Impinger*	-	1244	-				
LVAS	234	601	157				
Impinger**		360	-				
Average				-	802	-	73
Impinger*	-	5474	-				
LVAS	968	-	-				
Impinger***	-	7069	-				
LVAS	-	2936	203				
Impinger**	-	5302	-				
Average				-	5948	-	49
Impinger*	-	5531	-				
LVAS	961	-	-				
Impinger***	-	5413	-				
LVAS	-	2786	294				
Impinger**	-	4745	-				
Average				-	5230	-	53
Impinger*	-	2095	-				
LVAS	221	-	-				
Impinger***	-	2195	-				
LVAS	-	993	349				
Impinger**	-	2487	-				
Average				-	2258	-	44

* All glass impinger sample before LVAS sample.

** All glass impinger sample after LVAS sample.

*** All glass impinger sample between LVAS samples.

\- All values are increases unless a decrease is indicated (-).

TABLE 4

HUMAN CORONAVIRUS 229E

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
B.	0.1% BSA	0.5% BSA	% Change+	0.1% BSA	0.5% BSA	0.1% BSA	0.5% BSA
Impinger*	1047	1211	16				
LVAS	196	-	-				
Impinger**	720	753	5				
Average				884	982	22	
Impinger*	818	360	-56				
LVAS	209	-	-				
Impinger**	360	327	-9				
Average				589	344	25	
Impinger*	949	1211	28				
LVAS	586	791	35				
Impinger**	1509	1015	-22				
Average				1129	1115	52	11
Impinger*	1276	1113	-13				
LVAS	638	956	50				
Impinger**	982	1047	7				
Average				1129	1080	57	89
C.	0.5% BSA	1.0% BSA	% Change+	0.5% BSA	1.0% BSA	0.5% BSA	1.0% BSA
Impinger*	1177	1375	17				
LVAS	901	968					
Impinger**	1145	720	-37				
Average				1161	1048	78	92

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample.

\+ All values are increases unless a decrease is indicated (-).

TABLE 4

HUMAN CORONAVIRUS 229E

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

	Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
D.		5% Glycerol	10% Glycerol	% Change†				
	Impinger*	-	720	-				
	LVAS	334	-	-				
	Impinger**	-	556	-				
	LVAS	-	321	-4				
	Impinger*	-	327	-				
	Impinger**	-	622	-				
	LVAS	79	-	-				
	Impinger*	-	622	-				
	LVAS	-	89	13				
	Impinger**	-	491	-				

		10% Glycerol	0.1% BSA	% Change†	10% Glycerol	0.1% BSA	10% Glycerol	0.1% BSA
E.	Impinger*	589	2553	333				
	LVAS	405	1603	296				
	Impinger**	622	4320	595				
	Average				605	3437	67	47
	Impinger*	809	2487	207				
	LVAS	531	2563	345				
	Impinger**	527	2591	692				
	Average				568	2539	93	93
	Impinger*	720	2356	227				
	LVAS	681	3807	459				
	Impinger**	551	2618	208				
	Average				786	2487	87	153

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample.

† All values are increases unless a decrease is indicated (-).

TABLE 4

HUMAN CORONAVIRUS 229E

VIRUS RECOVERY USING THE AGI AND LVAS: A COMPARISON OF ADDITIVES

Sampler	Particles Recovered pfu/L of aerosol			Average AGI Particle Recovery Before and After LVAS (pfu/L)		Recovery LVAS/AGI (%)	
F.	0.1% FCS	0.1% BSA	% Change†	0.1% FCS	0.1% BSA	0.1% FCS	0.1% BSA
Impinger*	911	845	-7				
LVAS	255	880	245				
Impinger**	916	Alkaline	-				
Average				914	-	28	-
Impinger*	851	1276	50				
LVAS	489	843	73				
Impinger**	662	1996	201				
Average				757	1636	65	52
Impinger*	687	1575	100				
LVAS	137	754	450				
Impinger**	1244	1375	11				
Average				966	1375	14	55
G.	0.1% FCS	0.5% FCS	% Change†	0.1% FCS	0.5% FCS	0.1% FCS	0.5% FCS
Impinger*	818	851	4				
LVAS	376	383	2				
Impinger**	752	1636	118				
Average				735	1244	48	51
Impinger*	556	785	41				
LVAS	315	868	175				
Impinger**	916	1555	100				
Average				736	1309	43	66

* All glass impinger sample taken before LVAS sample.

** All glass impinger sample taken after LVAS sample

† All values are increases unless a decrease is indicated (-).

are shown in Table 4C. Coronavirus recovery, as had been the case with the other viruses, was only slightly increased due to the additional BSA. Again the problems encountered with the additional BSA offset the slightly increased recovery consequently further studies with BSA at concentrations of 1.0% or higher were not performed.

Because of the encouraging results using 10% glycerol in the collection fluid for the recovery of Polio and RV-14 glycerol was tested for its effect on the recovery of HCV/229E. Because 10% may have been too high a concentration both 5% and 10% were tested. The results are shown in Table 4D. It can be seen that the recovery of virus with the LVAS was similar at both concentrations and since 10% was so effective for the other two viruses it was the concentration used in the following comparison with 0.1% BSA.

The results in Table 4E show that virus recovery in the presence of 0.1% BSA was much greater (at least 200%) than 10% glycerol. This difference in recovery was similar to that found when recoveries with and without 0.1% BSA were compared. This was contradictory to the results found with the other viruses where 10% glycerol had been at least as equally effective as 0.1% BSA at enhancing virus recovery. The reason(s) for this finding are not known at present, however, possibilities for this difference will be discussed later.

The percent recovery in the presence of glycerol was quite high indicating that although the total quantity of virus recovered was not as high as it was in the presence of BSA, glycerol was quite effective at protecting the virus in the LVAS sample when compared to a similar impinger sample. However, since one of the objects of this study was to find a single collection fluid formula that would be effective for the recovery of all three viruses and because the recovery of HCV/229E was so low in the presence of 10% glycerol, no further tests were performed using this additive.

The effect of serum (FCS) at enhancing virus recovery was also tested. A comparison of virus recovery in the presence of 0.1% BSA and 0.1% FCS is shown in Table 4F. As can be seen, 0.1% BSA is a more effective additive (up to 450%) than 0.1% FCS when used in the LVAS recovery fluid although, as before, the increase in virus recovery with the impinger was not as substantial.

The results in Table 4G show an increase in recovery although the extent of it varies markedly. The increase in recovery due to the presence of 0.5% FCS is not as large as it was for 0.1% BSA suggesting that 0.1% BSA would be as equally effective as 0.5% FCS or even better. Thus it would seem that the concentration of FCS required to enhance recovery to the same extent as 0.5% BSA could be very high (possibly 5 times that of BSA).

No further experiments were performed using FCS in the recovery fluid because: a) The lower concentrations of BSA were equally, if not more, effective, b) Since the quality of serum varies from batch to batch it could affect the reproducability of the results and c) Finally there was the possibility of antibody and viral contaminants in the serum which could be detrimental to the results of field samples. In the latter case, the naturally occuring viruses may be inactivated by the antibody or the contaminating virus may interfer with the adsorption and infection of the virus.

Therefore, on the basis of the preceding results bovine serum albumin (BSA), at a final concentration of 0.5%, was selected on the basis of most reliable recoveries.

Role(s) of BSA in Improving Virus Recovery

Once the addition of 0.5% BSA to the collection fluid had been shown to increase recovery of each of the three viruses, the following attempt was made to determine where the effect of BSA was manifest.

The enhancement by BSA was considered to be provided in any one or a combination of the following ways during three phases of the recovery and assay procedure. First, BSA may "protect" the virus during the collection period from the mechanical action of the sampler as well as from the effects on rehydration when the dried aerosol

particles are immediately rehydrated, and possibly disrupted (inactivated), upon entering the collection fluid. Second, the increase in virus recovery may be due to disaggregation of viruses within the large aerosol particles providing more numerous infectious agents. Additionally the BSA may help maintain the disaggregated state of these agents when they enter the liquid. The BSA may also be required during the adsorption stage of the assay procedure where it may aid adsorption and subsequent infection of both damaged and undamaged virions into cells.

In this study the Collison, Henderson, LVAS and impingers were used as described previously however the collection and assay procedure was slightly different. Paired impinger samples were taken before and after LVAS samples using collection fluids either with or without 0.5% BSA. The samples without BSA were used for two evaluations. In the first, a 0.33 ml inoculum (for plaque assay) was taken immediately after sampling to determine the quantity of virus recoverable by the LVAS without BSA. In the second, BSA was added to the remainder of the sample to a ~~final~~ concentration of 0.5%. The sample was shaken for a few seconds then left on ice for approximately one minute before 0.33 ml was assayed. The results from these two tests were used to determine the effect of BSA on disaggregation or aggregation of the aerosol particles while in the collection fluid after sampling and/or its effect on virus recovery during the period

of adsorption. Finally the samples with 0.5% BSA present during the entire collection procedure were assayed to determine the overall effect of BSA on virus recovery.

Two experiments were performed for each of the three viruses (Polio, HCV/229E and RV-14) the results of which are shown in Table 5. In the first column are the results of virus recovery by the impinger-in using collection fluid without BSA, while in the second are those for virus recovery from the same sample after BSA had been added. In the third column the percent change between these is recorded and is interpreted to evaluate the effect of BSA on these latter stages of collection and assay. Virus recovery in the presence of 0.5% BSA during sampling is recorded in the fourth column. Also the difference between the remaining recoveries with and without 0.5% BSA was calculated and is recorded as percent change in the last two columns.

A comparison of the results of Polio recovery by the impinger, represented by the percent change columns, showed small and inconsistent decreases in virus recovery. The decrease occurred in samples where BSA had been added after sampling. This indicated that the effect of BSA could be due to a number of things. It could be causing an increase in the amount of aggregation occurring among the virus particles, it could be affecting virus adsorption through the action of non-specific inhibition or quite simply it could be due to a dilution of the virus by addition of BSA. In any event these results were low enough to be within the sampling errors of the procedure.

TABLE 5

TABLE 5
THE ROLE OF BSA IN VIRUS RECOVERY

Sampler	Particles Recovered: pfu/L of aerosol — No BSA	0.5% After	% Change	pfu/L of aerosol — 0.5% During	% Change 0.5% After VS 0.5% During	% Change No BSA VS 0.5% During
POLIOVIRUS TYPE 1 (SABIN)						
Impinger*	262	262	0	196	-25	-25
LVAS	184	201	9	309	54	68
Impinger**	327	396	21	327	-17	0
Impinger*	262	131	-50	229	75	-13
LVAS	177	208	21	423	103	146
Impinger**	295	393	33	164	-58	-44
RHINOVIRUS TYPE 14						
Impinger*	393	285	-27	425	49	8
LVAS	374	479	28	450	-6	20
Impinger**	196	98	-50	393	75	101
Impinger*	360	327	-9	327	0	-9
LVAS	356	321	-10	375	17	5
Impinger**	262	229	-13	327	43	25
HUMAN CORONAVIRUS 229E						
Impinger*	262	131	-50	491	275	87
LVAS	103	86	-17	716	733	595
Impinger**	131	33	-75	1047	3072	699
Impinger*	327	262	-20	876	234	168
LVAS	156	133	-15	1143	759	633
Impinger**	327	327	0	982	200	200

* All glass impinger sample taken before LVAS.

** All glass impinger sample taken after LVAS.

- All values are increases unless a decrease is indicated (-).

The results obtained with the LVAS however showed a consistant increase in virus recovery when BSA was added after sampling and a further increase when BSA was present during collection. Thus the previously proposed mechanism of non-specific inhibitors would appear to be invalid since no evidence for these was obtained in the LVAS work. Thus from these experiments the effect of BSA during sampling with the LVAS appeared to be manifest throughout all three phases of sampling.

As can be seen in Table 5 similar results were obtained for RV-14. Here the differences in virus recovery were smaller than those obtained for Polio indicating an even lesser requirement for BSA. Because of these small changes in recovery the specificity of the BSA was difficult to assess. Although some of the requirement appeared to be the result of disaggregation or aggregation and/or during subsequent adsorption to the cells, there also appeared to be a small amount of "protection" afforded during collection.

The results for HCV/229E (Table 5) were considerably more dramatic. The presence of 0.5% BSA during sampling, resulted in a 200% increase when compared to virus recovery in samples collected without BSA. Thus for HCV/229E virtually all of the requirement for BSA appeared to be during the collection phase of the experiments while its effect on disaggregation or aggregation, and during adsorption was negligible.

On the basis of this study it was apparent that the enhancement in recovery of Polio and RV-14 afforded by the addition of BSA was small and manifest during each of the three phases of recovery. On the other hand, for HCV/229E most of the effectiveness of BSA was provided during the recovery of the aerosol in the collection fluid with only a minimal amount contributed by aggregation or dissaggregation of aerosol particles and/or during subsequent virus adsorption.

Development of a Concentration Procedure

The recovery of one or two pfu of virus from within 10 ml of LVAS recovery fluid would require a minimum of 10 cell monolayers, each infected by 1.0 ml of inoculum, to be assured of a reasonable chance of their recovery. Assuming that more than one sample will be collected the number of flasks as well as the time and expense involved in their assay becomes of great concern. For these reasons a method to reduce the quantity of fluid for assay and yet still maintain the number and integrity of the infectious virus was required. The technique would have to be cheap, rapid, simple and furthermore should be adaptable to field operations. Several concentration procedures were investigated before selecting the one that best fitted our requirements.

Ultracentrifugation

Concentrating the virus from a 10 ml sample onto a 2-3 ml sucrose cushion (60% w/w) by ultracentrifugation would require immediate access to an ultracentrifuge which could not generally be available in the field. The volume to assay (3 ml) would be much less than before however a concentrate of less than or equal to one ml was still the ideal.

Two Phase Separation

This system would involve adding the LVAS sample to a mixture of dextran sulfate and polyethelene glycol (PEG). After the required separation time the virus would be collected from the dextran sulfate phase from which it could be assayed. Again the volume of liquid into which the virus is collected (3-5 ml) is still not appropriate and as well, since the time factor for phase separation is quite long, (24 hours) the virus may become inactivated before being added to a monolayer.

Adsorption-Elution

In this procedure, as used by Sattar and Westwood (1976), a filter medium composed of 75% talc and 25% Celite is used to which virus, from a large liquid sample, is adsorbed. The virus is then

eluted from the layer using a small volume of medium containing 10% FCS. Using small talc-Celite layers the virus can be eluted effectively with as little as 1.0 ml of medium. Sattar and Westwood have found that concentrating samples of poliovirus containing FCS was not as effective as concentrating the virus in samples without FCS indicating the FCS was interfering with the binding of virus to the filter medium consequently it was apparent that the presence of 0.5% BSA in the LVAS might reduce the amount of virus recovered. Due to the good results obtained from preliminary studies using the following procedure the adsorption-elution method was not explored at this time.

Ultrafiltration

In this procedure the pores of the filter membrane are large enough to permit the passage of liquids but small enough to retain the virus. Amicon Centriflo membrane filter cones used in a low speed portable centrifuge appeared to be the most practical ultrafiltration method for the solution to the problem. These membrane cones hold up to 8.5 ml of virus suspension which depending on the duration of centrifugation can be concentrated to any amount desired.

Preliminary experiments using these Amicon filtration cones were conducted to optimize the process for the smaller poliovirus. A preconditioning step in which the cones, containing 0.1% BSA in PBS (no virus), were spun at 2000 rpm for 30 min was found to increase

virus recovery. It was also found that when the virus suspension, containing BSA to a final concentration of 0.1%, was added to the preconditioned cones virus recovery was almost twice as much as from unconditioned cones without BSA in the virus suspension.

The following set of experiments were performed to determine which concentration of BSA (0.5% or 0.1%) in the virus suspension was more effective when concentrating with the Amicon Centriflo membrane filter cones.

The virus was diluted into two preconditioned cones containing collection fluid, one with 0.1% BSA the other with 0.5% BSA. The samples were centrifuged at 2000 rpm for 20 min at room temperature. Following this the contents of each cone (approximately 1 ml) were assayed for infectious virus. The results of the comparison of the three viruses (Polio, RV-14 and HCV/229E) are shown in Table 6. The estimated recovery (column 4) was calculated by multiplying the initial concentration (column 3) by the number obtained when the initial volume was divided by the volume remaining (column 2). From the results it can be seen that the quantity of virus recovered from the concentrate was not dependent on the concentration of BSA. The actual recovery in both the direct and diluted samples was almost always less than estimated. This loss of virus could have been due to inactivation during the concentration procedure, adsorption of virus onto the walls of the filter membrane and/or through

TABLE 6

TABLE 6

CONCENTRATION OF VIRUS SAMPLES CONTAINING 0.1% BSA OR 0.5% BSA BY AMICON ULTRAFILTRATION

BSA Concentration	Volume Remaining ml	Initial Concentration pfu/ml	Estimated Recovery pfu/ml	Actual Recovery	
				Direct pfu/ml	10^{-1} Dilution pfu/ml
POLIOVIRUS					
0.1%	0.60	45	600	336	390
0.5%	0.85	45	423	360	270
0.1%	0.65	36	443	354	330
0.5%	0.75	36	384	375	420
RHINOVIRUS					
0.1%	0.55	12	175	99	30
0.5%	0.85	12	113	108	30
0.1%	0.60	12	160	105	120
0.5%	0.60	12	160	240	210
0.1%	0.73	12	132	114	60
0.5%	0.85	12	113	96	150
CORONAVIRUS 229E					
0.1%	0.50	33	528	276	720
0.5%	0.90	33	293	129	420
0.1%	0.55	48	698	120	420
0.5%	0.85	48	452	318	180
0.1%	0.53	18	272	120	60
0.5%	1.00	18	144	108	210
0.1%	0.55	54	785	222	270
0.5%	1.00	54	432	135	240

the formation of virus aggregates. The presence of these aggregates is suggested by the results of the last two columns where the quantity of coronavirus recovered from the sample after ten-fold dilution was usually higher than that from the concentrated sample (6 out of 8 tests). This difference in recovery was probably due to the disruption of putative viral aggregates upon dilution. However, the results with RV-14 and Polio were equivocal and no specific changes could be associated with increased (or decreased) recovery.

Specifically, the results with RV-14 were similar to those for Polio in that virus yield was virtually unaffected by the concentration of BSA used. Again there were differences in detectable virus following dilution of the concentrate however these were not as large as those found for Polio which suggested that possibly the extent of viral aggregation was not as great with RV-14.

Finally the recovery of HCV/229E (Table 6) appeared to be better in the presence of 0.1% BSA than 0.5% BSA. The reason for this is not known however the additional BSA could have increased viral aggregation and thereby reduce the quantity of detectable virus. As with the other viruses there was generally an increase in the amount of virus assayed from the diluted sample, here again virus recovery in the presence of 0.1% BSA was better than 0.5%.

From these results it is apparent that both concentrations of BSA (0.1% and 0.5%) are equally effective in the concentrating of Polio or RV-14 using the Amicon Centriflo membrane filter cones. However concentrating HCV/229E appears to be slightly better in the

presence of 0.1% BSA than 0.5% BSA. Thus for concentrating, 0.1% BSA would be the best concentration to have in the sample. However the Amicon technique was to be used in conjunction with the LVAS and since collection fluid with 0.5% BSA was so effective for the recovery of HCV/229E by the LVAS it was felt that 0.5% BSA would result in the best overall virus recovery.

Thus the samples collected by the LVAS could be transferred directly to the Amicon cones for concentrating. To confirm this concentration procedure the following experiments were performed using actual LVAS samples and concentrating them using the Amicon cones.

Concentration of LVAS Samples

The virus aerosol was generated by the Collison into the Henderson apparatus and a ten minute LVAS sample was taken using collection fluid plus 0.5% BSA, following the routine procedure. Two 0.5 ml aliquots were assayed for virus before the sample was concentrated. The remaining sample was divided equally between two Amicon filter cones and concentrated as outlined. After concentrating, the fluid remaining in each cone was assayed for virus.

The results of these experiments are shown in Table 7. The quantity of virus in the LVAS sample before concentration was based on the number of pfu obtained from the two 0.5 ml samples. The results of virus recovery after concentration were adjusted to include the number of pfu removed by the previous 0.5 ml samples in order to better assess the actual quantity of virus present.

In most cases the quantity of infectious virus in a sample before concentration was higher than the amount after concentration. However when the quantity of virus in the collection fluid was low the difference between virus recovery before and after concentration was also low and, as in the case of one RV-14 sample, the amount of virus recovered after concentration was actually greater than before. It would appear that as the concentration of virus in aerosol decreases, the relevance effectiveness of the concentration procedure increases. Thus for low concentrations of airborne virus the Amicon centriflo method of concentration appears to be not only a feasible application but an essential requirement for use with the LVAS.

TABLE 7

TABLE 7

CONCENTRATION OF LVAS SAMPLE BY AMICON ULTRAFILTRATION

	Collison Conc. pfu/ml	LVAS Recovery	
		Before concentration pfu/sample	After concentration pfu/sample
POLIOVIRUS			
	960	84	36
	1050	97	81
RHINOVIRUS	510	19	23
	630	48	32
CORONAVIRUS	5100	176	128
	3600	52	44

A Determination of the Efficiency and Limits of Virus Detectability of the LVAS

The results of this study had so far established that: a) the test viruses were recoverable from aerosol with the LVAS, b) of the additives tested, BSA, to a final concentration of 0.5% in the LVAS collection fluid, was the additive best suited for recovery of all three viruses, and c) concentration by means of the Amicon Centriflo membrane cones could provide a further increase in sensitivity.

The next step was to determine the efficiency of the LVAS with respect to the AGI and, using these values, calculate the limits of virus detectability of the LVAS for each of the two test viruses (HCV/229E and RV-14).

In the previous experiments the vacuum for the impingers was supplied by the LVAS (Figure 6) and resulted in a flow rate of only 1.1 L/min. The design of the present experiment changed this by connecting the impinger to the vacuum side of the Henderson apparatus (vacuum of 40 cm Hg) to allow it to operate at the recommended optimum efficiency of 5.6 L/min (Figure 2). At these conditions the concentration of virus in the cloud was determined from the quantity of virus collected by two one-minute AGI samples.

The results of these experiments are presented in Table 8. The concentration of virus in the cloud chamber (pfu/L) was calculated from the average amount of virus recovered by impinger samples taken before and after the LVAS samples. For example, the virus aerosol concentration calculated for the first Polio experiment was based on an average recovery (2 impinger samples) of 115 pfu from a 0.33 ml inoculum of a 12.0 ml impinger sample. Thus the concentration of virus within the impinger was 348 pfu/ml (116 pfu/0.33 ml) and the total amount of virus collected by the impinger was 4176 pfu (348 pfu/ml x 12.0 ml). Since this quantity of virus was collected from 5.6 liters of cloud chamber aerosol, the virus aerosol concentration was therefore 746 pfu/L (4176 pfu ÷ 5.6L). The "estimated" value for LVAS recovery was based on the assumption that the LVAS recovers 100% of the virus detected by the impinger and was determined by multiplying the virus aerosol concentration (746 pfu/L) by the LVAS sampling rate (1.6 L/min). Thus the "estimated" LVAS recovery for this first experiment was 1194 pfu/min. The experimental recovery is the amount of virus actually recovered by the LVAS which in this case was 708 pfu/min or 59% of the virus detected by the impinger.

The concentration of virus in the Collison was reduced ten-fold for each subsequent experiment in an attempt to determine the lower limit of virus detectable by the LVAS within this system as well as determine the changes, if any, in the efficiency of recovery as a

TABLE 8

TABLE 8

DETERMINATION OF LVAS EFFICIENCY

	Collison conc. pfu/mlx10^{-4}	Virus aerosol* conc. pfu/l	LVAS Recovery pfu/min		LVAS efficiency %
			Estimated	Experimental	
Poliovirus type I	16.60	746	1194	708	59
	1.94	90	144	59	41
	0.09	8	13	2	15
Rhinovirus type 14	11.70	573	917	558	61
	1.62	95	152	72	46
	0.10	2	3	2	67
Human Coronavirus 229E	158.00	1494	2390	1013	42
	4.50	36	58	19	33
	0.50	9	14	6	43

* As determined by AGI operating at maximum efficiency.

result of the decreasing virus aerosol concentration. The results of Table 8 show that the virus aerosol concentration, as determined by the impinger, is directly dependent on the virus concentration in the Collison. This result was also found by Couch et al (1965) in a more extensive study using Collison nebulized particles. Both studies show that the concentration of virus to be aerosolized does not affect its stability during aerosol generation or during the airborne state. Since decreasing the concentration of virus in the Collison does not appear to affect the survival of the virus in aerosol, any change in the efficiency of the sampler under these conditions would be representative of the effect of the sampler, not the aerosol, on the virus. The results of Polio indicate a decrease in efficiency with decreasing virus aerosol concentration whereas the results for RV-14 and HCV/229E show consistent efficiencies over the range of concentrations tested. The reasons for the apparent decrease in efficiency in Polio recovery is not known therefore more experiments at these lower concentrations should be performed to determine whether this was characteristic of Polio or was within the limits of experimental error associated with the small amount of virus used.

While the average efficiency for the recovery of HCV/229E was almost 20% less than for RV-14 the efficiencies of recovery for both Polio and RV-14, at least for the first two experiments, were very similar. These results for Polio and RV-14 were contrary to those obtained in the very first part of this study concerning the recovery of "Internal" and "External" source viruses.

As suggested at the time, because the relative humidity for those experiments was low, a condition known to be detrimental to the survival of poliovirus the recovery was poor. In the experiments performed here the relative humidity was about 80%, the level at which poliovirus appears to be most stable (de Jong and Winkler, 1968). This higher humidity therefore could explain the apparent increase in the recovery of Polio to a level equal to RV-14. Also the recovery of RV-14 may be less than optimal under these conditions therefore there could have been a decrease in its stability which along with the possible increase in survival of Polio could have resulted in the similar efficiencies. Further studies concerned with the stability of Rhinovirus in aerosol would provide more information ~~which~~ might help explain the above results.

Initially the detectability limits were to be determined by decreasing the virus aerosol concentration until no more virus could be recovered by the LVAS. However because the concentration became too low for recovery with the impinger an accurate determination of the concentration of virus in the aerosol could not be made. Since the efficiencies of recovery for RV-14 and HCV/229E were similar over their respective ranges of aerosol concentration, the average efficiency for the three experiments was used to calculate the lower limits of virus detectability of the LVAS under the conditions prevailing.

The calculations are based on a ten minute LVAS sampling period during which time most of the particles in 9500 liters (950 liter/min) of environmental air are collected into approximately 10 ml of collection fluid.

Assuming an efficiency of about 40% for HCV/229E, the air being tested would have to contain about 3 infectious units of virus (pfu) per 9500 liters of air to allow recovery of at least one infectious unit from the 10 ml of collection fluid. Thus the detection capability of the LVAS, for this virus is 3.2, in that 3.2 infectious units of virus are required within a 10^4 liter volume of air. In order to attempt to provide a definition of this capability in relation to the efficiency of the LVAS relative to the standard AGI we propose that this be expressed as the ratio of percent efficiency to detection capability and termed the Detectability Factor. This value can now be used for comparison of other agents as well as a value which would be appropriate to studies on the effects of defined conditions (e.g. temperature and humidity). Thus HCV/229E has a Detectability Factor of 12.5 (40/3.2).

Similarly for RV-14 collected with an efficiency of 60%, at least two infectious units of virus are required per 9500 liters of air resulting in a detection capability of 2.2. Here the Detectability Factor is 27 (60/2.2). Thus the higher the Detectability Factor the better the chance of recovering the virus. In this case RV-14 would be more readily recoverable than HCV/229E at similar concentrations in the air.

Although the efficiencies for virus recovery are lower with the LVAS than the impinger the large quantities of air sampled during a relatively short sampling period make it an extremely attractive sampler for the collection of aerosols from enclosed environments which normally have low concentrations of infectious virus. For example, as reported earlier the amount of infectious virus present in droplet nuclei particles produced in a sneeze is in the range of 1-10 infectious doses (Dimmick and Akers 1968). The LVAS could conceivably recover these particles even when they have been dispersed in as much as 5×10^3 liters of air.

Thus, assuming these particles were dispersed into a small room 9'x10'x8 (2.7mx3.1mx2.4m) containing 870 ft^3 (20×10^3 L) of air, the LVAS with a sensitivity of one infectious unit per 5×10^3 L, might be expected to recover approximately 25% of the virus present during one-10 minute sampling period.

The importance of this sampler for use in areas requiring the sampling of large volumes of air becomes apparent when these results are compared to those estimated for other more commonly used samplers. For example two popular and efficient samplers, the impinger (12.5 L/min) and the Anderson (28 L/min) would be quite impractical for use in sampling the same sized room. Assuming both samplers have efficiencies of 100% and are compared to the LVAS with an efficiency of 50% (actually 60% for RV-14) the results are rather interesting.

Had the Anderson sampler been used instead of the LVAS, ten samples would have been required to have recovered a similar number of particles (sampling only 50% of the air). Each plate in the Anderson contains 6 ml of collection fluid (Appendix I) therefore each sample requires the assay of 36 ml of fluid (6 ml x 6 plates), that times 10 samples means 360 ml of collection fluid must be assayed in order to recover the same amount of virus found in 10 ml of LVAS collection fluid.

Using an impinger (12.5 L/min), also sampling only 50% of the air, 200 one-minute samples would be required to recover the same quantity of virus as the LVAS. The virus would be suspended in 2.4 litres of collection fluid (12 ml x 200) which would all have to be assayed. Concentrating would be useful but would still require a considerable amount of time that could result in the inactivation of the virus.

DISCUSSIONS AND CONCLUSIONS

This project was designed as a study concerned primarily with the feasibility of using the DRES modified LVAS for recovery of virus from the environment rather than as a statistical evaluation of a small group of parameters. Considerable interesting information was gained concerning the survival of the three viruses in aerosol and their recovery from it.

The first studies performed demonstrated that the virus could be successfully recovered by the LVAS. From those results it appeared that Rhinovirus type 14 was the most stable of the viruses, followed by Coronavirus 229E and finally Poliovirus type I. If the inactivation of Poliovirus was due to the low and mid-range relative humidities (RH) that have been shown to rapidly inactivate it (de Jong and Winkler 1968) then it appeared that the two respiratory viruses (RV-14 especially) are only slightly effected by aerosolization into low humidity air. Further studies concerned with virus survival at these and other RH's are indicated and would provide a better understanding of virus behaviour in aerosol. From this knowledge possible ways of controlling enclosed environments to limit the spread of infection may become apparent.

Following these experiments were those designed to improve the recovery of the viruses by the LVAS. An analysis of the results shows that at the higher RH's (approximately 80%) within the Henderson there

was a considerable increase in the inactivation of Coronavirus 229E. Whereas initially HCV/229E appeared to be less sensitive then Polio, now it was considerably more sensitive than either Polio or RV-14 which, for these experiments, exhibited similar recoveries. This increased sensitivity of HCV/229E was manifest by a required ten-fold increase in the virus concentration (with respect to RV-14 and Polio) in the Collison in order that all three viruses be recovered in similar quantities. Since inactivation of HCV/229E in the Collison generator was found to be low (unreported data) most of it probably occurred during aerosolization and recovery. Because of the short time span between aerosolization and collection (less than 5 seconds) the inactivation was most probably due to the "immediate" effects of aerosolization rather than any "long term" effects. Further survival studies should verify this point and help determine the "long term" effects (in aerosol) on virus survival. The additives used during this portion of the study generally increased virus recovery though the extent varied with each additive. The most effective single additive for recovery of all three viruses was BSA at a final concentration of 0.5%. Thus in the event that one of these three viruses is suspected of being a contaminant within the air to be sampled 0.5% BSA should provide the most effective recovery conditions.

With both Poliovirus type I and Rhinovirus type 14 the difference in recovery using collection fluid with and without BSA was small however with Coronavirus 229E there was an increase in recovery of over 200% in

the presence of BSA. This difference in recovery was studied in an attempt to determine if the increase, was due to the presence of BSA during the collection (recovery) phase, aided in the disaggregation or aggregation of virus containing aerosol particles and/or was important in the adsorption of the infectious particles and their subsequent infection of the cells.

The results showed that for HCV/229E virtually all of the requirement for BSA was during the collection phase with very little increase attributed to disaggregation (aggregation) and/or adsorption and infection. The BSA therefore appeared to be "protecting" the virus, to a considerable degree, from the detrimental effects of each method of sampling. This is not surprising when one considers the architectural complexity of the coronavirus virion which would make it more subject to inactivation by the LVAS than would a simpler and more stable non-enveloped structure such as that of the picornaviruses (Polio and RV-14) used here. The manner in which this protection is afforded is not known however there are several possibilities. Protein molecules of the BSA could be adsorbed onto the aerosol droplet immediately upon its entry into the collection fluid. The combination of this protein and salts from the spray fluid surrounding the virion could stabilize the particle during collection. Also, the protein, in surrounding the virus particle, may be slowing the rate of rehydration of the particle when it enters the collection fluid thereby enhancing its survival. Benbough (1971) has suggested that salts and other

solutes (proteins) from the fluid of aerosolization may limit the rate of rehydration and thereby increase the recovery of non-lipid containing (non-enveloped) such as Polio and RV-14 thus there is a very good possibility that the presence of protein in the collection fluid enhances their recovery due to a reduction in the rehydration rate. The process of rehydration could be affected through the use of a prehumidification step just prior to collection. This technique subjects the aerosol to a relative humidity (RH) of 95-100% just prior to collection at which time the particles absorb water and essentially begin their rehydration process. This procedure has been shown to enhance the recovery of coliphage (Hatch and Warren, 1969) but according to Warren et al (1969), was not successful in enhancing the recovery of two animal viruses, vesicular stomatitis virus (enveloped) and mengovirus 37A (non-enveloped). Benbough (1971), however, has shown a 10-fold increase in Poliovirus recovery when the virus in a 40% RH was subjected to a prehumidification at 95% RH prior to collection. Thus through the use of prehumidification one could possibly ascertain to what extent rehydration is responsible for inactivation of the virus. In our experiments the humidity was approximately 80% thus there is a good possibility that prehumidification may not affect the recovery however this does not mean that the experiments could not be performed. Whatever the effect of the BSA its presence appears to be much more crucial to the recovery of the enveloped, lipid containing Coronavirus 229E than to either of the non-enveloped, non-lipid containing viruses (Poliovirus type 1 or Rhinovirus type 14).

Fetal calf serum (FCS) was tested to see how effective it was, in comparison with BSA, for the recovery of Coronavirus 229E. The results indicated that, although virus recovery improved as the concentration of FCS increased, FCS was not as effective as BSA at the same concentration. Since BSA contains mostly albumin and very little in the way of other serum components it appears that the albumin portion of serum plays an important role in the protection of Coronavirus 229E. On the other hand however, FCS, containing approximately 3% albumin was almost as effective as 0.1% BSA even when it showed an overall effective albumin concentration of 85% less than BSA. Therefore it is suggested that there are other components in FCS which are involved in the protection of HCV/229E and these should be studied further.

The final additive tested with the three viruses was glycerol. When used in the recovery of Rhinovirus type 14 and Poliovirus type I the results were very similar to those found for BSA. However when it was used in the recovery of Coronavirus 229E the results were considerably (200%) less than those for BSA. Thus it appears that for recovery of the two non-enveloped, non-lipid containing viruses the effects of glycerol and BSA are similar. This does not mean that they serve the same purpose, as can be seen from the results with Coronavirus 229E (lipid containing enveloped) where BSA becomes extremely important to virus recovery whereas glycerol is essentially non-effective. The sites and/or mode of action of each of these additives during collection would be considerable interest in further aerosol studies with these viruses.

Also from these additive experiments information concerning the differences in virus recovery with respect to the type of sampler (impinger (AGI) or LVAS) and sampling procedures was gained.

The recovery of virus by the LVAS was usually lower than by the AGI indicating that even when operating at less than optimum the AGI is a more efficient sampler than the LVAS. The viruses also exhibited a greater requirement for the additives when being collected by the LVAS than by the AGI suggesting that the mechanics of sampling by the LVAS are more detrimental to the viruses than are the mechanics of the AGI. From this it appears that although the additives may be sufficient for virus recovery with one type of sampler it may not be as effective for another, hence the additive(s) should be tested on both samplers and the concentration most appropriate for each sampler should be used when performing further comparative studies.

Using 0.5% BSA in the recovery fluid the efficiencies of recovery of the two respiratory viruses, Coronavirus 229E and Rhinovirus type 14, were determined to be 40% and 60% respectively. Earlier work by White et al (1975), using a similarly designed LVAS determined its efficiency for the recovery of BG spores and three vegetative bacterial species (Serratia marcescens, Escherichia coli, and Aerobacter aerogenes) to be 82, 90, 80, and 90% respectively. Thus it appears that the viruses used in this study are quite readily recoverable when compared to the recoveries of four relatively hardy

bacterial species. Further evaluation of virus recovery by the LVAS in field experiments would provide information pertinent to any future field sampling assignments.

Although the efficiency of virus recovery by the LVAS is lower than the AGI, the LVAS is nevertheless superior to the more conventional low volume air samplers (AGI, Anderson, Shipe, slit) for the recovery of airborne virus because not only is the volume of air sampled much greater but, as has been shown previously, the volume into which the particles are collected is smaller.

The values for the efficiency of recovery of the two respiratory viruses were used to determine virus detectability of the LVAS for each virus. These values were expressed in terms of a Detectability Factor which as was said before represents the ratio of the efficiency of recovery to the detection capability. The detection capability of the LVAS being the minimum quantity of virus required in 10^4 liters of air before it will be detected. Therefore from the amount of virus recovered, the original quantity of virus present in the air sample could be determined and combined with information pertaining to the infectious dose of the virus one could determine the possibility of an airborne infection within that particular environment.

The quantity of airborne virus present within the sampling environment depends upon the virus concentration at the source of infection and the stability of the virus through all stages of aerosol generation, maintenance and recovery (infection). The extent of virus stability depends on composition of the fluid the virus was aerosolized from as well as other environmental conditions such as temperature and RH.

Experiments concerned with the effect of different spray fluid components, although not performed here, would provide a more complete indication of the effectiveness of the sampler especially if fluids similar to those present in a natural infection were used. The results from these experiments concerning virus survival and recovery would also supply good background information of importance to airborne transmission studies.

The LVAS, because of its ease of handling and operation can be used virtually anywhere that requires sampling of an aerosol with a low virus concentration. Many studies concerned with airborne transmission of respiratory diseases, such as the common cold, could incorporate the LVAS into their volunteer studies. For example Gwaltney et al (1978a and 1978b) have shown that transmission of Rhinovirus infection is most often caused by hand to hand transmission. They have had limited success with airborne transmission and have not been able to isolate virus from the air (Gwaltney et al 1978b). The

LVAS could be used to sample the room air during an experiment to determine whether or not the virus was being aerosolized by the infected individual and if so how much.

Other studies that could benefit from the use of the LVAS are those concerned with the aerosol transmission of disease caused by viruses not normally associated with the respiratory tract. One example of such a study is the one performed by Petersen et al (1976). This was an attempt to recover hepatitis B surface antigen from the air within a hemodialysis unit. Using a low volume air sampler (25 litres/minute) in conjunction with the radioimmune-assay technique they are able to recover hepatitis B surface antigen from controlled aerosols generated within the laboratory. However when samples were taken in the hemodialysis unit no antigen was recovered. The use of the LVAS (950 litres/min) in this situation, for a 10 minute sampling period would have increased the chances of antigen recovery and would have helped make it a more conclusive study.

Another potentially hazardous source of airborne viral infections are the aerosols produced within sewage treatment plants and during spray irrigation of fields with untreated sewage. Teltsch and Katzenelson (1976) using an AGI-30 sampling at the rate of 12.5 L/min were able to recover coliforms at a distance of 70 meters down-wind of a spray irrigation system. Using the Anderson

sampler at a flow rate of 28 litres per minute, coliforms were collected at a distance of 350 meters from the source. Isolation of these agents does not necessarily mean there will be an infection. Thus in another study Katzenelson et al in 1976 compared the incidence of disease in Kibbutzim populations practicing waste water spray irrigation with the incidence for populations not spraying waste water. They found waste water irrigation increases the occurrence of certain viral diseases such as Infectious Hepatitis, Influenza (symptom cases but not clinically confirmed ones) and a considerable number of bacterial diseases (Typhoid, Salmonellosis, and Shigellosis), and that these diseases were most probably disseminated by the airborne route. The LVAS could be used to increase the detection capabilities of such a study and/or be used to sample the air in a community on a routine basis. The quantity of airborne virus could be correlated with the quantity of virus found in the sewage, after which the results could be related to the incidence of disease caused by that virus within the population. These are just a few of the possible uses of the LVAS in field studies concerning airborne virus recovery.

All of the information gained in this study indicates that the LVAS would be of considerable importance in environmental studies pertaining to recovery of airborne viruses. The addition of BSA to the collection fluid to a final concentration of 0.5% results in an effective medium for the collection of both enveloped

(Coronavirus 229E) and non-enveloped (Rhinovirus type 14 and Poliovirus type I) viruses. Furthermore the use of the Amicon concentration procedure in conjunction with the normal LVAS recovery procedure substantially increases the capabilities of the sampler for recovering very low concentrations of airborne viruses.

APPENDIX I

Evaluation of Collison Produced Aerosols Using the Anderson Sampler

The following series of experiments was performed to verify the size distribution of the virus containing particles within an aerosol produced by the Collison nebulizer. In this study both the aerosol produced directly from the Collison (Collison Aerosol) and the aerosol from the cloud chamber of the Henderson Apparatus were analysed. The aerosols of Polio and HCV/229E were compared using the Anderson sampler (Anderson, 1958) to collect and size the aerosol particles. The Anderson is divided into six stages each consisting of a plate containing holes of a different size from the preceding stage. Each stage contains a glass petri dish with collection medium into which the aerosol is collected. The stages are stacked on top of each other, the stage with the largest holes at the top and the one with the smallest at the bottom. At a flow rate of 28 L/min the aerosol particles collected will separate in the following way: (Dimmick and Akers, 1969):

Stage	Particle diameter µm
1	>9.2
2	5.5-9.2
3	3.5-5.5
4	2.0-3.3
5	1.0-2.0
6	<1

The following experiments were performed and the results were compared to those obtained by Couch et al (1965) who used aerosols of Coxsackievirus type A_{21} produced by the Collison nebulizer.

The first two experiments were designed to assay the virus aerosol directly as it is produced by the Collison (prior to entering the cloud chamber). In these tests a funnel was fitted into the top of the Anderson and to it was attached a piece of 6 inch diameter stove pipe as shown in Figure 7. The Collison was positioned as illustrated so that the aerosol from it would enter the air stream as it was drawn through the pipe into the sampler. Within the sampler one of six (6) special glass petri plates containing 21 ml of 2% Oxoid #1 agar with 6 ml of 12% gelatin layered on top was placed in each of the six stages. Two minute samples were taken during which time the aerosol particles were collected into the 6 ml of gelatin. Upon completion of the sampling the plates were removed from the sampler and warmed at 37°C. At this temperature the gelatin was easily poured into vials from which, samples for plaque assay were taken.

The results of these "Collison Aerosol" experiments are shown in Figure 9A. From the data obtained, one can see that 89 and 93% of the virus collected were recovered from stages 4, 5 and 6. These results are similar to those found by Couch et al (1965) and indicated that the Collison nebulizer used in this study produced an aerosol of which approximately 90% of the particles were 3μm or less in diameter, the

FIGURE 7

LEGEND

Figure 7: Experimental design for sampling a virus aerosol directly from the Collison using the Anderson sampler.

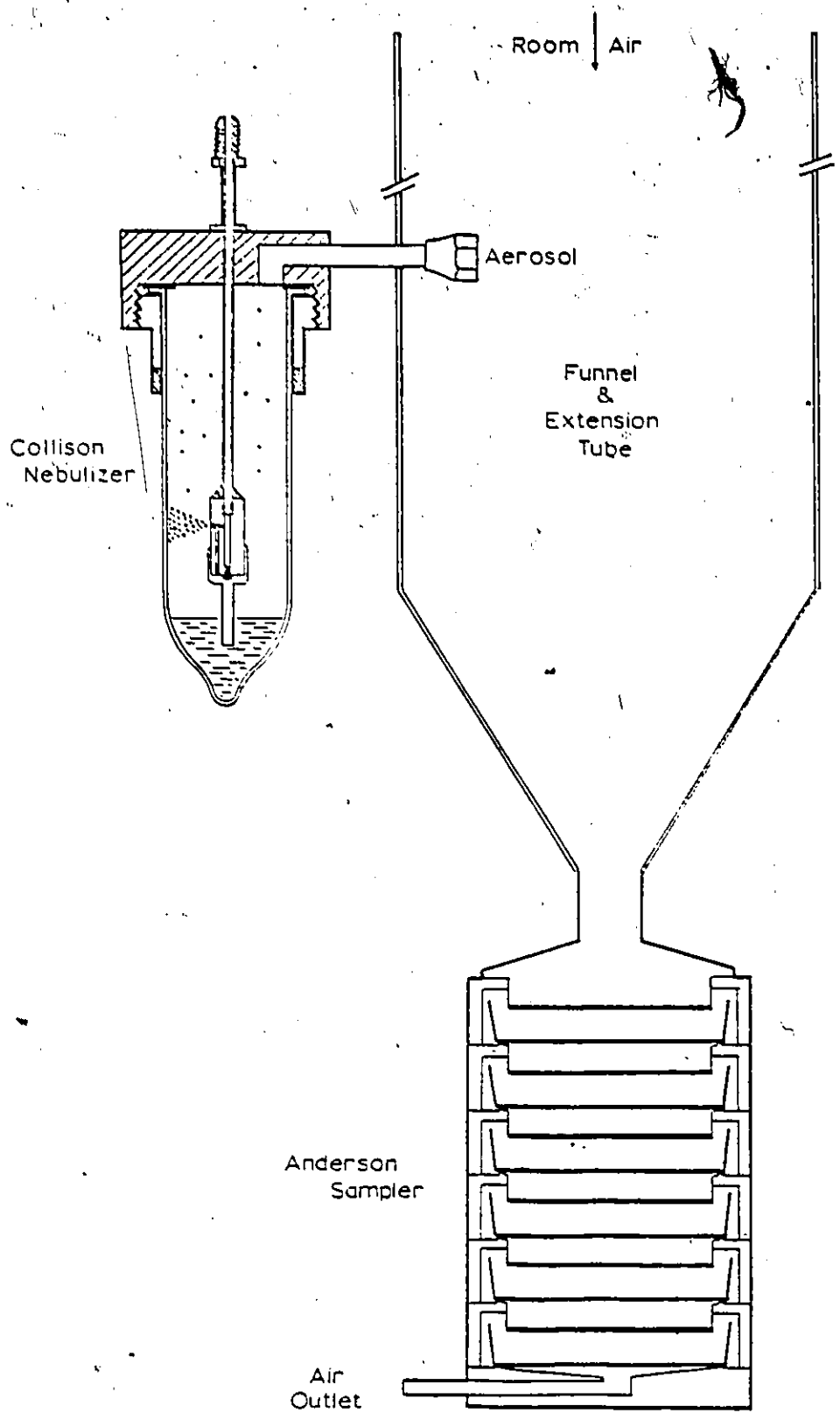

Room Air
Aerosol
Funnel
&
Extension
Tube
Collison
Nebulizer
Anderson
Sampler
Air
Outlet

FIGURE 8

LEGEND

Figure 8: Sampling a virus aerosol from the Henderson Cloud Chamber using the Anderson sampler.

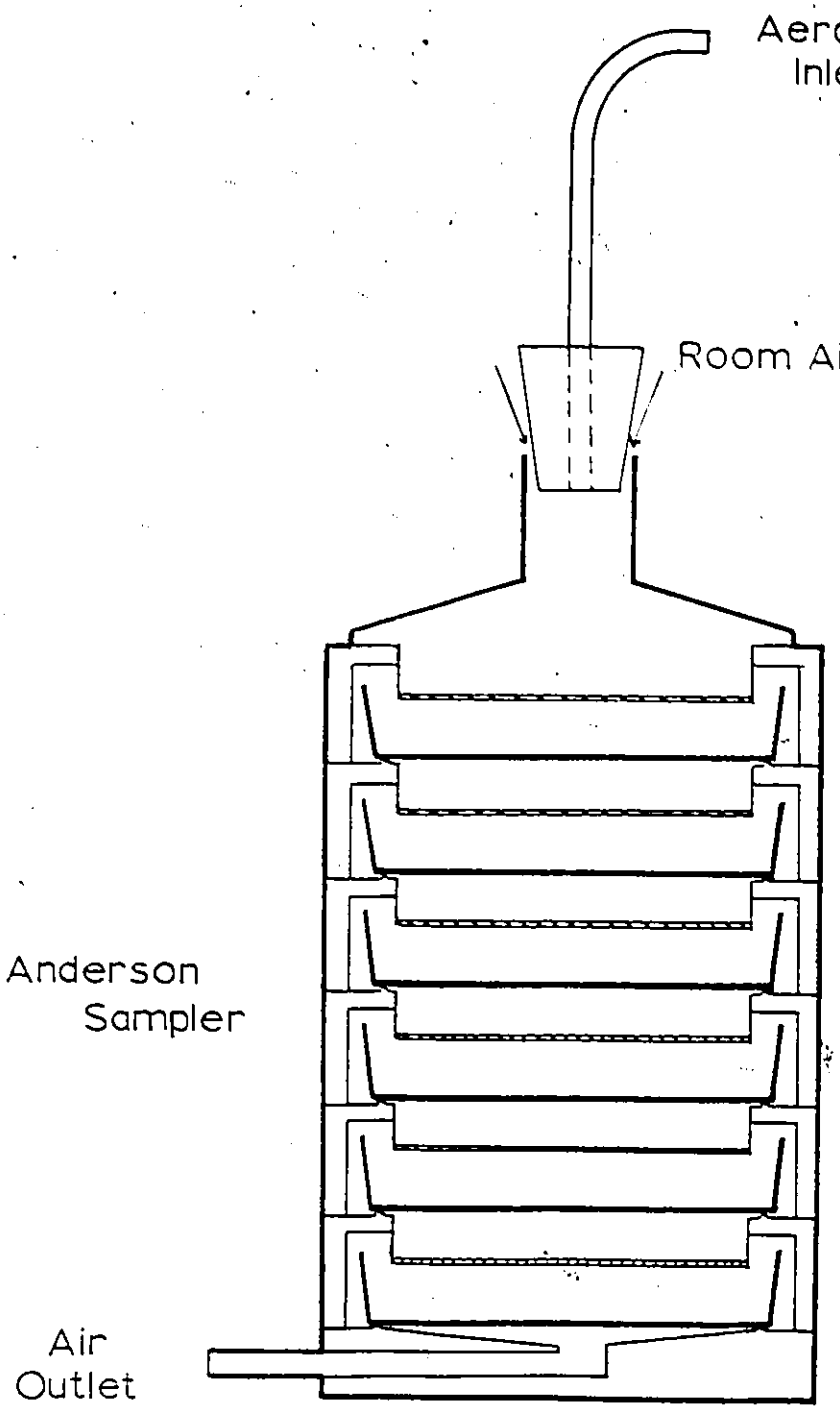

Aerosol
Inlet
Room Air
Anderson
Sampler
Air
Outlet

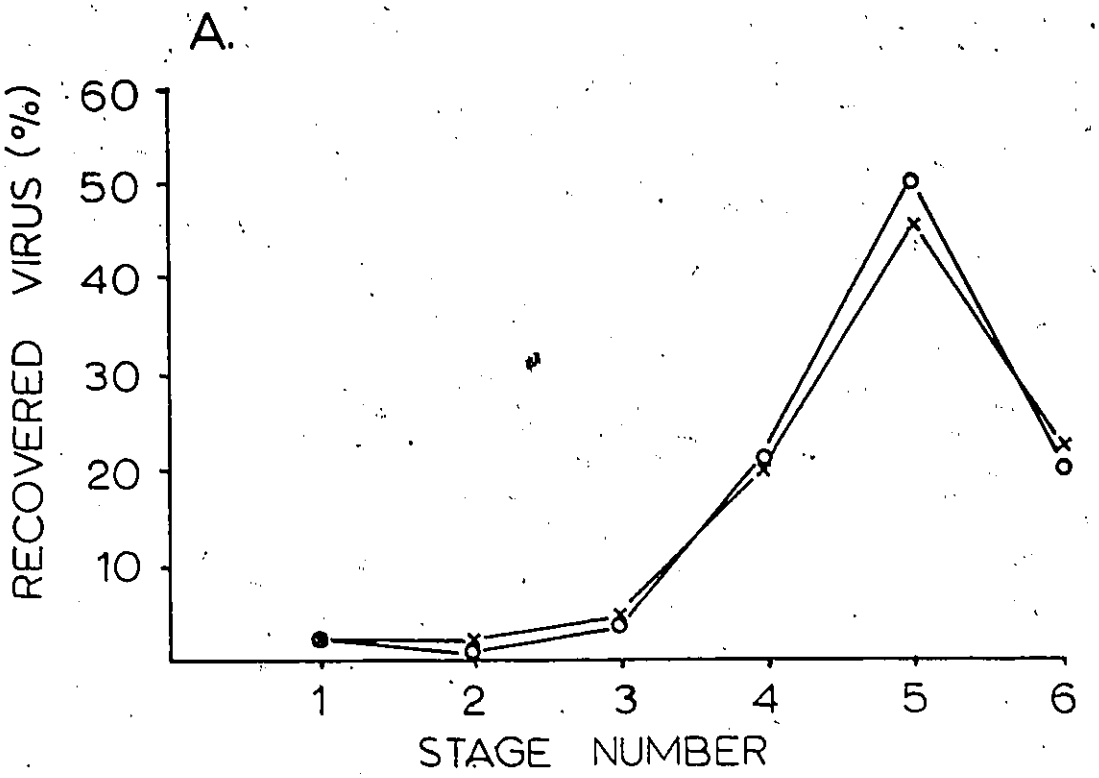
FIGURE 9

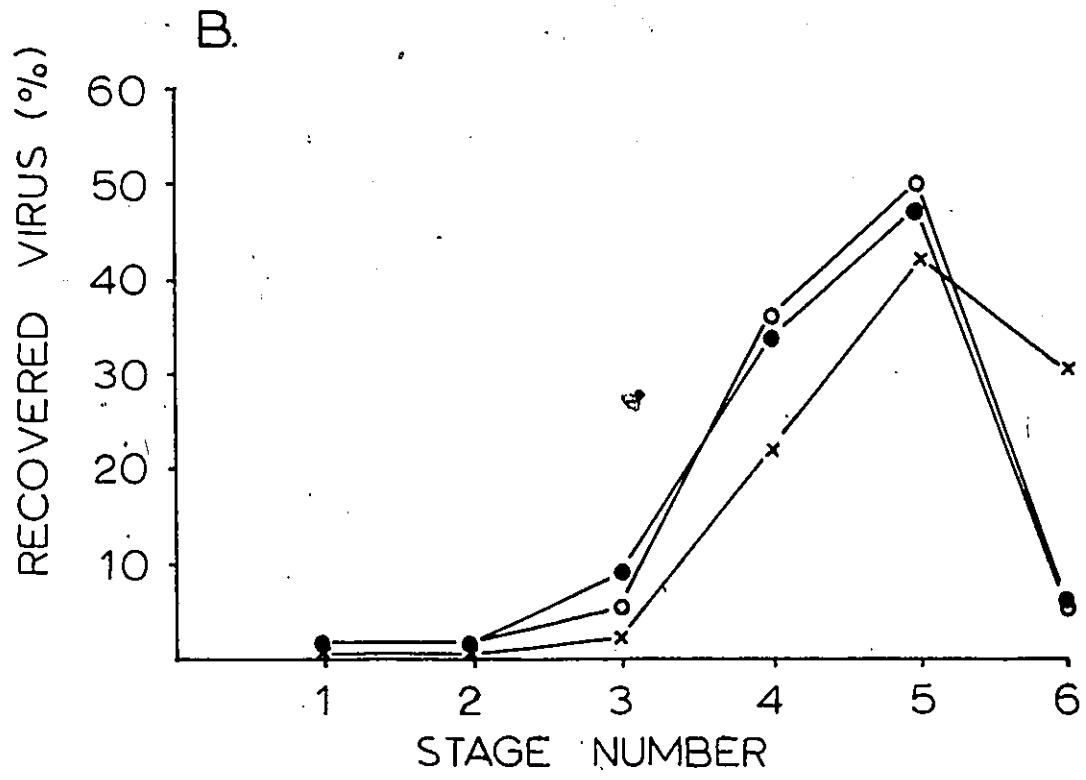
LEGEND

Figure 9: A. The results of duplicate experiments showing the particle distribution of Polio within an aerosol produced by the Collison nebulizer as recovered by the Anderson sampler. (See Figure 7).

B. The results of three experiments showing the particle distribution of HCV/229E and Polio within the Henderson cloud chamber aerosol as recovered by the Anderson sampler. (See Figure 8).

o —— o) HCV/229E
● —— ●)
x —— x Polio


A.
RECOVERED VIRUS (%)
60
50
40
30
20
10
1
2
3
4
5
6
STAGE NUMBER


B.
RECOVERED VIRUS (%)
60
50
40
30
20
10
1
2
3
4
5
6
STAGE NUMBER

usual size range for most Collison produced aerosols (Guerin and Mitchell, 1964; Couch et al, 1965 and May 1973).

Throughout the project aerosols generated into the cloud chamber of the Henderson were used extensivly therefore the size distribution of airborne particles (containing Polio and HCV/229E) within cloud chamber aerosols was studied. In these experiments the modification shown in Figure 8 was used. A rubber stopper fitted with a glass tube was inserted into the air inlet of the Anderson such that air could get into the sampler (at 28 L/min) through both the glass tube and between the stopper and the walls of the air inlet. Under these conditions the aerosol from the Henderson passed through the glass tube into the Anderson at the rate of 1.6 L/min while the total flow rate of air (and aerosol) through the sampler could be maintained at 28 L/min.

From the results of the one experiment using Polio in Figure 9B (Cloud Chamber Aerosol); the aerosol collected, as was expected, appeared to have a similar particle distribution to that of the Collison aerosol, i.e. the majority of the virus (about 90%) is found within stages 4, 5 and 6. There appears to have been a slight increase in virus recovery in stage 6 whereas virus recovery at stages 4 and 5 was similar to what it had been in the previous study. This suggests that conditions (biological and physical) within the cloud chamber are beneficial to the survival of Polio within aerosol particles of less than 1μ. These results imply that the environmental conditions can be selectively

detrimental or beneficial to virus survival depending upon the size of the particle within which the virus is contained.

The results of a similar study involving HCV/229E are also presented in Figure 9B. Virus recovery in stages 4 and 5 (36.3 and 49.5% for the first experiment) are similar in both experiments and considerably higher than recoveries from stages 3 and 6 (less than 10%). As with Polio, approximately 90% of the virus collected was in particles with diameters of 3µm or less (stages 4, 5 and 6). However, unlike Polio, very little of the virus was recovered in stage 6 which recovers most particles with diameters of 1µ or less.

Although the reasons for this low recovery are not known it appears that, in contrast to Polio, the virus is more susceptible to inactivation in the smaller particles. Thus it appears that the size of particle in which HCV/229E is aerosolized is important in considering its aerosol stability than say the stability of Polio.

The results of these experiments indicate that at least 90% of the aerosolized virus produced by the Collison nebulizer for the purpose of this project were within particles of 3µm or less in diameter.

APPENDIX II

Cell Growth Medium

Stock Solutions	Volume (ml)
MEM (Auto-Pow)	431.0
$NaHCO_3$ (5.6%)	13.4
L-Glutamine (200 mM)	5.0
Pen-Strep-Neo (1000X)	0.5
FCS	50.0
	500.0

This medium was used for the growth of both A-5-HeLa and L-132 cells. The cells were incubated in 30 ml of media at 37°C until they reached confluence at which time they were either used for plaque assay or split into more flasks for further experiments.

Spray and Collection Fluid

Stock Solutions	Volume (ml)
MEM (Auto-Pow)	481.0
$NaHCO_3$ (5.6%)	13.4
L-Glutamine (200 mM)	5.0
Pen-Strep-Neo (1000X)	0.5
	500.0

The above was the basic medium used for the spraying and collection of the virus. Antifoam (0.1 ml of 10% Antifoam "C") is added to this medium for spraying with the Collison and collection with the AGI. The various enhancement components were also added to the medium for collection with the LVAS and AGI.

Overlay Media

Stock Solutions	Polio† (ml)	HCV/229E† (ml)	RV-14* (ml)
Medium-199 (5X)	120.0	120.0	120.0
Sterile Distilled Water	228.0	234.0	230.0
$NaHCO_3$ (5.6%)	21.4	21.4	24.0
BudR (1%)	3.0	3.0	6.0
DEAE-Dextran (4%)	3.0	3.0	1.0
$MgCl_2$ (3.0M)	6.0	-	6.0
Pen-Strep-Neo (1000X)	0.6	0.6	0.6
Mycostatin (100X)	6.0	6.0	6.0
FCS	12.0	12.0	-
Heat Inactivated FCS	-	-	6.0
	400.0	400.0	400.0

† Mixed with 200 ml of 1.8% Oxoid #1 Agar or in the ratio of 2:1 medium to agar.

* Mixed with 200 ml of 2.7% Oxoid #1 Agar or in the ratio of 2:1 medium to agar.

The overlay media for the plaque assay of Polio and HCV/229E had been previously established within this laboratory. The assay for RV-14 was developed for this project through the following comparisons:

$NaHCO_3$ (5.6%) - Concentration of 0.06, 0.11, 0.17 and 0.22% were compared. From the results, 0.22% $NaHCO_2$ in the medium appeared to be most effective.

BudR (1.0%) - Concentration of 50, 100, 200 and 400µg/ml were compared. Plaques formed in the presence of 100µg/ml were the most numerous and well defined.

DEAE-Dextran (4.0%) - Concentrations of 50, 100, 200 and 400µg/ml were compared. The number of plaques formed and plaque definition were better when 50µg/ml was used.

M-199 or MEM - Plaque numbers and cell integrity were similar for both however the outer edges of the plaques formed in the presence of M-199 were slightly more defined.

Heat Inactivated FCS, FCS and BSA - These were compared at a concentration of 1.0% with an additional test for BSA at a concentration of 2.0%. Both the

quantity and size of plaques were better in the presence of 1.0% Heat Inactivated FCS than FCS or BSA.

Agar - Oxoid #1 (Oxoid), Ionagar #2 (Oxoid), and Noble agar (Difco) were tested at concentration of 0.6, 0.8 and 1.0%. Plaque morphology and number were better when the agar concentration was 0.8% or 1.0% for each agar type however the best results were obtained when 1.0% Oxoid #1 agar was used. A final concentration of 0.9% agar was chosen because 1.0% was difficult to remove from the cell monolayer and 0.8% agar was not quite as effective as 1.0% for the development of plaques.

APPENDIX III

Reliability of the Plaque Assay

The reproducibility of any assay is dependent on the accuracy of the equipment and the consistency with which the operator uses it. In the case of a biological assay, such as the plaque assay, factors affecting the interaction of cells and virus will also alter the outcome even between duplicate tests.

Virus stocks for use in all the aerosol experiments were aliquoted and stored frozen at -80°C. Each aliquote contained 1.0 ml of the virus suspension. Because of virus inactivation due to repeated freezing and subsequent thawing, a fresh aliquot from storage was used for each experiment.

Variations in batches of media, serum and cell passage levels all affect quantitation of the virus. Therefore in order to minimize the effects of such variables, the results of all plaque assays were grouped according to the time period during which the assays were performed when there were essentially no differences in the media, serum or cell passage level.

The variations from the mean titre for Polio, RV-14 and HCV/229E (Table 9) ranged between ±45%, ±44% and ±29% respectively. The ranges of both Polio and RV-14 titres are considerably higher than for HCV/229E. This indicated that monthly changes in media serum and cell passage levels were enough to cause substantial fluctuations in the results of assays for Polio and RV-14. This did not appear to be the case for

TABLE 9

SUMMARY AND ANALYSIS OF THE RESULTS OF PLAQUE ASSAYS

Number of Tests	Mean pfux10^7	Standard Deviation "σ"	Coefficient** of Variation %	Maximum pfux10^7	Minimum pfux10^7	Range pfux10^7	Maximum Range From Mean %
Poliovirus Type I †							
5	36	8.0	22	49	28	21	±36
8	42	11.5	27	59	29	30	±40
6	53	15.8	30	77	32	45	±45
4	33	3.4	10	39	31	8	±18
Rhinovirus Type 14							
5	31	8.4	27	44	21	23	±42
5	59	5.3	9	69	55	14	±17
5	41	14.2	35	59	24	35	±44
Human Coronavirus Strain 229E							
11	24.5	3.9	15	30.2	19.8	10.4	±23
4	21.8	4.6	21	28.3	16.9	11.4	±29
6	18.7	2.7	14	21.4	13.4	8.0	±27
6	17.0	2.6	15	21.1	12.5	8.6	±27

* Derived using the formula: $\sigma = \sqrt{\frac{\sum_{j=1}^{N} (X_j - \overline{X})^2}{N}}$

** Derived using the formula: $V = \frac{\sigma}{\overline{X}}$

† Polio and HCV/229E were grown in L-132 cells, RV-14 was grown in A-5-HeLa cells.

HCV/229E because variations in these conditions did not result in such wide fluctuations in virus titre during similar periods.

However, with respect to the HCV/229E assay, the results showed a small steady decrease in virus titre which was proportional to the length of storage. This was not seen with the other two viruses.

The reduced amount of HCV/229E virus due to this inactivation over the eight month storage period did not apparently contribute any significant problems to the experiments as judged by the overall virus recovery. It is however recommended that fresh stocks of virus be prepared at least once a year to guarantee that the virus available would always be above the critical level of detectability.

In view of these inherent variations it was essential to set limits to permit interpretation of the effects of the additives on virus recovery. On the basis of our analysis differences of up to ±50% were considered to be within the expected range of experimental variation. However differences in recovery of more than ±50% were indicative of a change that could be considered as being directly due to the affect of the additive.

Thus when comparing two additives, virus recovery had to be at least 50% better for one than the other otherwise they were considered to be of equal benefit.

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