High resolution immunautoradiography and tritium-labeled antibody binding studies detecting cell associated Sendai virus antigens during early phases of infection

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High resolution immunoautoradiography and tritium-labeled antibody binding studies detecting cell associated Sendai virus antigens during early phases of infection

by

Mark Robert Sanborn

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Bacteriology Major: Bacteriology (Virology)

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For the Graduate College

Iowa State University
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INTRODUCTION

The replication cycle of viruses can be divided into several stages: attachment, penetration, uncoating, production of progeny virus components, assembly, and release. The eclipse phase of this cycle is defined as that period during the infection process when little or no infectious virus can be detected in the host cell. It is the period during which the least amount of viral material is associated with the host cell and the period when the parental virion, devoid of its protective coverings, may enter a latent state "loosing" itself in the myriad of host-cell organelles. As its name implies many of the events of the eclipse phase are "hidden" from even the most sophisticated scientific investigations.

The use of the electron microscope and radioactive tracers has made many important contributions into our understanding of the eclipse phase of viral replication. However, important questions are still unanswered in many viral systems: In which cellular compartments are the invading viral antigens sequestered? Once progeny antigens are produced, which cellular organelles are responsible for their transport to the assembly site? Can antiviral agents be found which will interfere with these processes while not harming the host?

Paramyxoviruses are large, enveloped, RNA viruses which impart many changes upon their host during the infection process. The disease causing potential of this group includes: Newcastle disease of chickens, canine distemper, mumps, measles, and newborn pneumonitis in man. Sendai virus
is a paramyxovirus originally isolated from cases of fatal newborn pneumonitis. This virus is easily propagated and purified and its replication cycle is longer than other paramyxoviruses such as Newcastle disease. This makes the Sendai virus system a good model for studying early events in enveloped virus replication.

One of the problems encountered by many investigators, when probing the eclipse phase of viral infection, has been the inability to sufficiently label parental viral antigens with radioactive isotopes in order to limit, to a reasonable extent, the input multiplicity of infection.

With this in mind it was my goal to develop a specific label which would allow visualization and quantitative monitoring of the fate of Sendai virus antigens during the early phases of Sendai virus infection in both productive and non-productive host systems. If such a technique could be developed it would contribute to our overall knowledge of the early events of virus-host interaction.
REVIEW OF THE LITERATURE

In 1952, a previously undescribed infectious agent was isolated during an epidemic of fatal pneumonitis in children of Sendai Japan (Kuroya et al., 1953). At that time they were unable to cultivate the virus in vitro, but were finally able to passage the agent in mice by intranasal innoculation with autopsy material. The mice developed influenza-like lesions of the lung. When the agent was transferred to eggs, allantoic fluid was collected which could agglutinate chicken red blood cells. When the agent could not be identified as a known virus it was designated as newborn pneumonitis virus, type Sendai (Kuroya et al., 1953).

Evidence supporting the validity of the human source of the original isolation was supplied when a volunteer was fed mouse passaged virus and then developed pneumonitis (Kuroya et al., 1953). However, the human source of the infection was questioned when other investigators found an identical virus in normal strains of laboratory mice (Fukumi et al., 1954).

The host range of Sendai virus was extended to include hamsters by Matsuzawa et al. (1953). Swine were also indicated as a host for the virus since the animals developed pulmonary edema, hemorrhage, and respiratory symptoms upon innoculation with the virus. Additional isolations from human populations soon followed. Serologic surveys of human populations in 1953 showed that the virus was present throughout Japan (Matsuzawa et al., 1953). Russian investigators found Sendai virus
circulating with influenza A, during a moderately severe epidemic of influenza (Gemgросс, 1957).

Kuroya et al. (1953) showed that Sendai virus replicated in embryonated chicken eggs, hemagglutinated red blood cells and was approximately 150 to 180 nm in diameter. Hemolytic activity for Sendai virus was shown by Fukai and Suzuki (1955). Jensen et al. (1955) determined that the erythrocyte receptors for Sendai virus hemagglutinin were destroyed by *Vibrio cholerae* RDE (receptor destroying enzyme). They also agreed with the findings that Sendai virus was antigenically distinct from other myxoviruses; influenza viruses, mumps and Newcastle disease virus (NDV). Jensen et al. (1955) felt that this information was enough to suggest that the virus be classified as influenza D.

Sendai virus is referred to by many names in the literature. Among the more popular names are: Sendai virus, parainfluenza virus I, and HVJ (hemagglutinating virus of Japan). This situation reflects the state of present day viral nomenclature; with no one system being used by all virologists. The following is a historical summary of the naming of Sendai virus.

In 1955, Andrewes et al. published a non-Linnaean description of the myxovirus group. This group included: Myxovirus influenzae A, Myxovirus influenzae B, Myxovirus influenzae C, Myxovirus multiforme, Myxovirus pestisgalli, Myxovirus parotitidis. The names implied an affinity of all the members for certain mucins. Myxovirus multiforme was originally named Newcastle disease virus (NDV). Sendai virus was not included in this group, even though Jensen et al. (1955) had previously suggested
influenza D as a proper name for the virus. By 1959 the list of myxoviruses was extended to include Sendai virus, croup-associated virus, hemadsorption virus 1 (HA 1), and hemadsorption virus 2 (HA 2) (Andrewes et al. 1959). The four new viruses were closely related. They were somewhat larger than influenza A and had the ability to hemolyse as well as agglutinate certain red blood cells. These viruses were more closely associated with mumps and NDV than influenza. Antigenic interrelationships were shared among the group but no single antigen was common to the whole group. The HA 2 virus was considered to be a subtype of Sendai virus. For these reasons Andrewes et al. (1959) renamed the four viruses to conform to the nomenclature of the 1955 myxovirus descriptions. The following names were given the four viruses: Myxovirus parainfluenza 1 (Sendai and HA 2 viruses), Myxovirus parainfluenza 2 (croup-associated virus), Myxovirus parainfluenza 3 (HA 1 virus). These names were approved by the Virus Subcommittee of the International Nomenclature Committee.

As the members of the myxovirus group increased it was apparent that at least two subgroups existed. In 1962 Waterson divided the myxoviruses into subgroups 1 and 2. Influenza was the model for subgroup 1 and NDV represented subgroup 2 which included the parainfluenza viruses.

Lwoff et al. (1962) based viral classification on the type of nucleic acid, capsid symmetry, presence or absence of an envelope, and the diameter of the nucleocapsid or number of capsomers. According to this system Sendai virus was placed in subgroup 2 of the myxoviruses as a separate entity from the parainfluenza viruses.
In the classification system reported by Melnick (1973), which was approved by the International Committee on Viral Nomenclature, Sendai virus is placed in the paramyxovirus group and is referred to as a parainfluenza virus.

Physical Characteristics of Sendai Virus

The morphology of Sendai virus ranges from spherical to pleomorphic when examined by negative staining with the electron microscope (Horne et al., 1960; Choppin and Stoeckenius, 1964). The diameter of the virion ranges from about 120 nm to 300 nm (Hosaka et al., 1966). The fine structure of the virus conforms to that of the paramyxovirus group. The outer surface of the virion is a modified host-cell membrane containing projections of viral glycoproteins. The projections are approximately 8 nm in length, and are responsible for the hemagglutinin and neuraminidase activity of the virus (Horne et al., 1960). The viral envelope also contains an innermost layer which is not found in the host-cell membrane. This innermost layer encloses the nucleocapsid which is about 1 um in length (Hosaka et al., 1966). The nucleocapsid is a single-stranded left-hand helix containing 11 to 13 subunits in each turn of the helix (Finch and Gibbs, 1970). The protein subunits of the nucleocapsid cover the single stranded RNA of the virion. Depending upon the host system, Sendai virus has either 8 or 9 structural proteins ranging in molecular weight from 3.47 X 10^4 to 1.45 X 10^5 (Lamb and Mahy, 1975). The highest molecular weight protein has been consistently found in polyacrylamide gel preparations of Sendai virus. Lamb and Mahy (1975)
suggest that this protein could be: (1) A structural polypeptide equivalent to the P polypeptide found in influenza or the L polypeptide found in VSV. (2) An uncleaved precursor virion polypeptide. (3) An artifact of the gel system. Viral protein 1 (VP1), found in association with the nucleocapsid, has a molecular weight of $8.13 \times 10^4$ and seemed to be associated with RNA-dependent-RNA polymerase activity (Stone et al., 1972). The hemagglutinin and neuraminidase activities reside in the glycoproteins VP2, VP3, and VP6 of molecular weights $7.4 \times 10^4$, $6.2 \times 10^4$, and $4.6 \times 10^4$, respectively (Chen et al., 1971). VP3 is an extra protein found in virus grown in chick embryo lung cells (Lamb and Mahy, 1975). VP4 is the nucleocapsid subunit and has a molecular weight of $5.75 \times 10^4$. VP8, the matrix or M protein, has a molecular weight of $3.4 \times 10^4$ and moves the farthest on polyacrylamide gels (Lamb and Mahy, 1975). As of yet VP5 and VP7 have not been associated with a particular structural unit or function of the virus.

A single stranded RNA, of at least $5.33 \times 10^6$ daltons, is needed to code for the above listed proteins. Lamb and Mahy (1975) have determined that the molecular weight of untreated Sendai RNA is $5.5 \times 10^6$ daltons. After formaldehyde treatment, which eliminates the secondary structure of the molecule, the molecular weight was estimated to be $10 \times 10^6$ daltons. Thus the actual molecular weight of the virion RNA still remains in doubt.

Replication of Sendai Virus

The following sequence of events is required for a successful infection of a host cell by Sendai virus: attachment to the host cell,
penetration into the cytoplasm of the host, uncoating of the virion, release of the nucleocapsid, production of viral components, insertion of viral envelope proteins into the host membrane, alignment of progeny nucleocapsid under the modified host membrane, and finally the outward budding and release of the new viral particle.

There has been some debate as to the actual mode of entry of paramyxoviruses into the host system. Morgan and Howe (1968), provided evidence that the early penetration of Sendai virus was by fusion of the viral membrane and the host cell membrane with release of the nucleocapsid into the cytoplasm. Another route of entry for the particle might be by phagocytosis (Hosaka and Koshi, 1968; Durand et al., 1970; Dales, 1973). It may be that the type of entry is host dependent and/or dependent upon such culture conditions as temperature and serum concentration (Durand et al., 1970; Dales, 1973).

The mechanism for release of the nucleocapsid has not been determined. Some insight has been provided by Durand et al. (1970) showing that isolated lysosomes can completely uncoat NDV. Howe and Morgan (1969) have shown that Sendai nucleocapsids seem to be injected into the cytoplasm of human erythrocytes after dissolution of the fused viral-cell membrane.

Once the nucleocapsids are free in the cytoplasm they seem to unwind, deproteinize to some extent, and increase in buoyant density (Bukrinskaya et al., 1969a). After association with viral specific polysomes the viral nucleoprotein remains within the host cell for at least 6 hours and
may take part in the formation of polysomes synthesizing early viral proteins (Bukrinskaya et al., 1969a, and Bukrinskaya et al., 1969b).

Marx et al. (1974) found that after the negative (non-messenger) stranded RNA was released that a replicative intermediate was formed using the virion associated transcriptase. This heterogeneous, ribonuclease resistant, RNA was found to have a sedimentation coefficient ranging from 20s to 30s (Zhdanov and Bukrinskaya, 1970).

Kingsbury (1974) proposed that the nucleocapsid need not uncoat to be transcribed, but local stretching occurs to allow the polymerase to interact with recognizable bases in the stretched region. He based this hypothesis on the fact that it has been the nucleocapsid, and not naked RNA, which was the active template for RNA transcription in cell-free systems (Stone et al., 1972, and Marx et al., 1974). Kingsbury (1974) has also found that the complementary strand is encapsidated. Thus a mechanism similar to transcription could have existed for the replication of the viral genome. He considered these reactions to be cyclic, in that first the material must be transcribed and translated and then it is replicated.

Viral proteins are not produced in the host in equal molar amounts but in proportion to their amount in the virion (Kingsbury, 1974). The envelope proteins are inserted into the host membrane with the matrix protein forming the layer directly beneath. These patches of modified host cell membrane provide a focus point for the nucleocapsid to become enveloped. Electron microscopic studies have shown that Sendai virus follows the pattern of other paramyxoviruses and buds through the host
cell membrane (Comans and Dimmock, 1969). The actual mechanism of release from the cell has not been determined. However, Robinson and Duesberg (1968) suggested that the neuraminidase may play a role in particle release.

Localization of Cell Associated Antigens

In recent years several techniques have been developed to extend the potential of electron microscopy in the area of virus research. These techniques involved the use of a specific label which distinguishes viral antigens from host-cell material.

Singer (1959) pioneered in the field by first conjugating ferritin to an antibody molecule and using this antigen-specific marker in electron microscopy. Ferritin is a protein isolated from mammalian tissue, with a molecular weight of about $4.6 \times 10^5$. The ferritin granule consists of a protein shell about 9.5 nm in diameter which encloses an iron micelle which ranges in diameter up to 5.5 nm (Morgan et al., 1961). Only the iron micelle is visible in the electron microscope. Singer's method of conjugating the antibody and ferritin was a two-step reaction. First, ferritin was mixed with an aromatic diisocyanate to obtain a ferritin-xylene isocyanate complex. This material was then mixed with rabbit IgG. Approximately one third of the IgG molecules were labeled. Since then others have improved upon this method. Williams and Gregory (1967) have increased the amount of ferritin incorporated into the antibody while limiting titer loss by using bis-diazotized benzidine as the coupling agent.
One disadvantage that ferritin conjugated-antibodies have is that the complex has a greater size than the parent antibody molecule. This fact makes labeling of intracellular antigens difficult because the complex doesn't penetrate cell membranes. In spite of these difficulties ferritin conjugated-antibodies have been used in many studies involving virus-cell surface interactions (Morgan et al., 1961; Duc-Nguyen et al., 1966; and Coward et al., 1972).

Sternberger et al. (1963) introduced uranium as another heavy metal labeling technique. Specific antibody was attached to the antigen, thus protecting the reactive site from labeling. The antigen-antibody complex was then labeled with uranium. After the reaction was complete the labeled antibody was removed from the antigen. In this way a labeled antibody was produced which contained label in only the non-specific areas of the molecule. It was found that uranium-labeled antibody as well as other labeled antibodies would not consistently adhere to antigens, when added as a stain to plastic thin sections.

Enzyme-conjugated antibodies have also been used to detect antigens within cell systems. Four enzymes have been used to label antibodies: peroxidase, alkaline and acid phosphatases, and glucose oxidase (Kurstak, 1971). Of these enzymes only peroxidase has been extensively used in detection of viral antigens (Kurstak, 1971).

Peroxidase is a colorless protein combined with a dark brown iron-porphyrin and has a molecular weight of about $4 \times 10^4$. It is resistant to heat and most solvents used in histological techniques. The very stable peroxidase labeled antibodies are able to penetrate cells better
than ferritin tagged antibodies due to their lower molecular weight (Kurstak, 1971). Smaller tracer molecules would be even more desirable than peroxidase labeled antibodies for use in electron microscopy by increasing penetration and resolution.

Both the antibody molecule and the label have been reduced in size. The IgG (antibody) molecules were digested with papain by the method of Porter (1959). This reaction produces fragments (Fab fragments) containing the reactive site of the antibody molecule. The enzyme cytochrome C was selected as a smaller conjugate. The enzyme was reduced to a chain of eight amino acids and a porphyrin group. When Fab fragments were coupled to this micro-peroxidase, an active label was produced with a molecular weight of only $4.5 \times 10^4$ (Tijssen and Kurstak, 1974).

Fab fragments have also been labeled with radioactive iodine and used as an intracellular marker with autoradiography (Gonatas et al., 1974). In this case developed silver grains served to locate the antigen with the resolution obtained being comparable to that of the peroxidase labeled antibody.

One of the major problems in using labeled antibodies in locating intracellular antigens has been the inability of the labeled antibody to penetrate the cell membrane. Glutaraldehyde has been one of the most satisfactory fixatives for electron microscopy. However, it does not allow for penetration of antibody. This problem has partially been avoided by using paraformaldehyde in various combinations with other fixatives (Gonatas et al., 1974, and McLean and Nakane, 1974). Even
though antibody penetration was achieved, preservation of the cell ultrastructure suffered, especially when viewed at high magnification.

Labeled antibodies have also been incorporated into three dimensional electron microscopic work. Phillips and Perdue (1974) have successfully used hemocyanin conjugated-antibodies to map the distribution of cell-surface antigens in avian tumor virus-infected cells. In this system the hemocyanin complex produced an identifiable lump when carbon-platinum replicas of the cell surface were made.

Molday et al. (1974) have conjugated latex spheres to antibody and used these as markers in scanning electron microscopy. Antibody was covalently bonded to the latex spheres with either carbodiimide or glutaraldehyde. The latex tagged antibody served to distinguish a positive, antigen carrying, cell from a negative cell by producing a beaded coat on the positive cells.

Radioactive Labeling of Compounds

In recent years radioactive labeling of compounds for tracer studies has gained wide acceptance. Radioactive tracers have become of particular value in virus research because of the sensitivity achieved with the labeled compounds in tracing viral antigens within cells.

Probably the best known method of tritium labeling of organic compounds was that of Wilzbach (1957). In this method the compound of choice was sealed in a container with tritiated hydrogen gas of high specific activity. The tritium, being at a higher energy state, exchanged with normal hydrogen in the target compound. The Wilzbach method did not
uniformly label the compounds treated. Also, due to the long incubation period, many compounds would lose their biological activity before sufficient labeling could be completed.

White and Riesz (1968) developed the free-radical interceptor technique as a means for preparing tritium-labeled proteins. Their method depended upon forming carbon free-radicals by bombarding lyophilized protein with gamma-radiation. The lyophilized gamma-irradiated protein was then exposed to tritiated hydrogen sulfide gas, whereupon the carbon free-radicals became labeled by the tritium from the gas. The specific activity of tritium-labeled lysozyme using this method was 0.77 µCi/mg protein.

Zull and Repke (1972) have labeled protein by preparing an acetamidino derivative of the parent compound using methyl ³H-acetamidate as the radioactive label. In this process free amino groups in the parent protein received the radioactive label. The molecular weight of the target compound was increased by 74 for each acetamidino group formed.

A fourth method used for introducing tritium into proteins was developed by Cooper and Reich (1972). In this exchange reaction the parent protein was oxidized with pyridoxal phosphate and then reduced with tritiated sodium borohydride. This method produced a specific activity of 2 Ci/m mole of protein.

Rifkin et al. (1972) successfully used the borohydride exchange reaction to specifically label the outer surface proteins of influenza virus. The specificity of this reaction was due to the fact that
phosphate esters, such as pyridoxal phosphate, are unable to penetrate intact viral membranes.

Another radioactive isotope, $^{14}C$, has also been used to label proteins. Oliphant and Brackett (1972) have labeled antibody with $^{14}C$ through reductive alkylation using $^{14}C$-formaldehyde as the isotope source and sodium borohydride as the reducing agent. The label remained stable for more than seven months so that the same antibody preparation could be used for many experiments. McMillen and Consigli (1974) have extended the usefulness of this reaction by labeling all of the proteins of polyoma virus. The labeling of both external and internal virion proteins was accomplished without loss of viral activity.

Radioactive iodine has also been incorporated into proteins. The method of Phillips and Morrison (1970) has been used extensively to incorporate $^{125}I$ into different proteins. The iodination depended upon the following reactions. Lactoperoxidase reacted with $H_2O_2$ to form a lactoperoxidase-0 complex; this then reacted with $^{125}I$ to form a lactoperoxidase-$^{125}I$ complex; this complex iodinated proteins with exposed tyrosine groups. This method has been used to specifically label the outer surface of membranes since the lactoperoxidase-$^{125}I$ complex will not penetrate cell membranes (Phillips and Morrison, 1970; Tsai et al., 1973; and Walter and Mudd, 1973). The lactoperoxidase labeling technique has also been extended to include the labeling of antibodies (Marchalonis et al., 1971). One of the disadvantages associated with iodine-labeled tracers has been the relatively short half-life of the isotopes involved:
60 days for $^{125}\text{I}$ and 9 days for $^{131}\text{I}$. Thus there is a self-imposed time limit on any experiments to be completed with a particular batch of tracer.

**Radioimmunoassay**

With the advent of iodine labeled antibodies, techniques have been developed which can detect nanogram quantities of a specific antigen within a serum sample. Radioimmunoassays have been extensively used for the detection of hepatitis B antigen in serum. These assays are based primarily on the procedure developed by Catt and Treager (1967). Plastic tubes were coated with non-radioactive anti-hepatitis B antibody. To each tube a sample of serum was added and incubated for several hours. After incubation the sample was removed and the tubes washed. Radioactive anti-hepatitis B antibody was then added to the tubes. The tubes were counted in a gamma radiation counter after unreacted labeled antibody was removed.

Competition type radioimmunoassays have been developed to monitor viral antigens in cell system (Stephenson et al., 1973). In this technique an unknown amount of antigen was incubated with a known amount of non-radioactive antibody. After incubation and washing, a known amount of $^{125}\text{I}$-labeled antigen was added to the system. A decline in the amount of labeled antigen bound was indicative of antigen presence in the test sample.

Hayashi et al. (1972) have developed a direct antibody-binding assay for viral antigens in tissue culture. Vaccinia virus infected monolayers
of rabbit kidney cells were reacted with $^{125}\text{I}$ labeled anti-vaccinia virus antibody. The cells were assayed for bound radioactivity after excess unreacted antibody was removed. This assay proved to be a sensitive measure of vaccinia infection of the host system.

**Electron Microscopic Autoradiography**

The first pictures combining the use of autoradiography and the electron microscope were produced by Liquier-Milward (1956). However, it wasn't until 1961 that resolution higher than that achieved with light microscopic autoradiography was produced (Van Tubergen, 1961). With the introduction of very fine grained emulsions applied to thin sections, electron microscopic autoradiography became an analytical tool (Salpeter and Bachmann, 1964). The developed grain size has now been reduced, with gold latensification, to a diameter of 50 nm (Salpeter and Bachmann, 1964).

One of the major problems of autoradiography with the electron microscope has been the long incubation times needed to activate the silver grains of the emulsion. By using $^{125}\text{I}$, a gamma emitter, instead of $^{3}\text{H}$ which emits beta radiation, Gonatas et al. (1974) have been able to reduce exposure time to 30 days. However, the resolution obtained was comparable only to the peroxidase labeled antibody technique due to the high energy of the gamma radiation.

A new technique has been developed at the light microscopic level which has reduced exposure time to a few hours for isotopes of high specific activity. Durie and Salmon (1975) have mixed scintillator with the photographic emulsion and applied this mixture to smears of cells
containing tritiated thymidine. When the emitted beta particles struck the scintillator, photons were emitted which activated silver grains in the emulsion. Thus sensitivity was increased with both the beta and light emissions activating the silver grains. This method has yet to be applied at the electron microscopic level but holds great promise for lowering exposure times.
MATERIALS AND METHODS

Water

Water used in cell culture work was distilled and then passed through a Millipore deionizing filter system (Millipore Corporation, Bedford, Mass.). Glass double-distilled water was used in electron microscopic procedures.

Growth Medium 1X, 2X

Dry powdered tissue culture medium, BME-Hanks base with L-glutamine and without sodium bicarbonate, was the base for growth medium (KC Biologicals, Kansas City, Kan.). To 10.3 g dry base was added 5% v/v calf serum, 5% v/v tryptose phosphate broth, 100 units/ml K penicillin G, 100 μg/ml streptomycin Sulfate, 9.53 g HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) buffer, and water to provide 1 liter of 1X growth medium. Growth medium was adjusted to pH 7.2 with 5 M NaOH, filter sterilized, and frozen at -20 C until used.

Growth medium 2X contained the same components as the growth medium 1X except that the total volume was adjusted to 500 ml instead of 1000 ml.

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

BSS-10X was made by combining 80 g NaCl, 4 g KCl, 3.5 g NaHCO₃, 10 g glucose and 100 ml of 0.2% phenol red. The total volume was brought to 1000 ml with water. The 10X stock solution was stored at room temperature with the addition of a small amount of chloroform.
The stock was diluted to a 1X solution and autoclaved at 121 C, 15 psi for 15 min before using as a wash for either whole lung tissue or monolayer tissue cultures.

Trypsin-EDTA Solution

Trypsin-EDTA solution used for the dissociation of BHK Cells had the following composition: 0.6 g of 1:250 trypsin, 0.02% w/v EDTA, and 1 liter of BSS. Before using, the solution was filter sterilized and frozen at -20 C.

Pronase Solution

Pronase solution used for the dissociation of chick embryo lung cells had the following composition: 0.25% pronase and 0.02% EDTA in 0.04 M HEPES buffer, pH 7.2.

Chick Embryo Lung Cells (CEL Cells)

CEL cells were derived from 14-15 day-old embryonated chicken eggs. Candled eggs were swabbed with alcohol and the embryos were removed to a sterile petri plate. The embryos' lungs were removed with sterile forceps and placed in a sterile petri plate containing BSS. Each lung was then cut into 2-4 mm pieces with two sterile scalpels using a scissoring motion. Pieces of lung were then transferred to a sterile trypsinizing flask containing 25 ml cold pronase solution. The tissue was stirred with a magnetic stirring bar for 25 min or until the tissue was dissociated. The pronase solution containing the CEL cells was filtered through a double layer of sterile cheese cloth.
CEL cells were removed from the solution by centrifugation at 440 x g for 5 minutes. Cell pellets were resuspended and adjusted to a cell density of $10^6$ cells/ml with BME.

Various tissue culture vessels received the following amounts of cell suspension:

- 500 ml glass prescription bottle: 50 ml
- 60 x 15 mm plastic petri dish: 4 ml
- 16 mm Linbro multi-dish disposo tray: 1 ml per dish.

All cells were initially cultured at 37°C under controlled humidity and CO$_2$ tension. Monolayers were acclimated to 31°C before infection with Sendai virus.

**BHK-21 Cells**

BHK-21 cells were a gift of Dr. Arnie Matchett, Veterinary Biologics Laboratory, Ames, Iowa. BHK-21 cells were maintained and passaged in 500 ml prescription bottles under 50 ml BME at 37°C. BHK-21 cells were subcultured by first washing the monolayer with 20 ml BSS and then trypsinizing with 5 ml trypsin solution. BME was added to the trypsin cell solution to stop further cell digestion. Enough BME, 100-150 ml, was added to make either a 1-2 or a 1-3 split of the cells. Cells were then transferred to new culture bottles in 50 ml volumes.

**Overlay Medium**

The overlay medium was designed for plaque assays of Sendai virus infected cells. Purified agar, BBL, was made 2X at 1.3% and sterilized
by autoclaving. The melted 2X agar was cooled to 45°C and added to an equal volume of 2X BME at the same temperature. Just prior to use, 10 μg/ml of sterile 1:300 trypsin in water was added according to the method of Shibuta et al. (1971).

Neutral Red Agar Overlay

Neutral red agar was used to develop the Sendai plaques. Neutral red overlay had the same composition as the overlay medium except that a 1:13000 dilution w/v of neutral red was added to the 2X agar prior to autoclaving.

Plaque Assay

Sendai virus was serially diluted in BME and 0.2 ml of each dilution was added to a confluent monolayer of CEL cells and allowed to attach at 4°C for 45 minutes. Five ml of overlay medium was added to each plate. Plates were incubated at 31°C for 96 hr at which time 4 ml neutral red overlay was added. Plaques could be observed by 108 hr post infection and were fully developed by 120 hours.

Alsevers Solution

Alsevers solution was used to preserve chicken red blood cells. To 100 ml water was added 2.05 g glucose, 0.8 g sodium citrate, 0.42 g NaCl, and 0.02 g citric acid. The solution was stored at 4°C until used. Chicken blood was preserved with an equal volume of Alsevers solution.
Phosphate Buffered Saline (PBS)

PBS was made by combining 10.57 g Na$_2$HPO$_4$·7H$_2$O, 2.7 g KH$_2$PO$_4$, 4.1 g NaCl, and 1000 ml H$_2$O. The pH was adjusted to 7.2.

Hemagglutination Titration (HA)

Hemagglutination titers of Sendai virus were determined in plastic disposo trays (Linbro Chemical Company Inc., New Haven, Conn.). PBS was dispensed in 0.2 ml volumes to each well of the tray to be used. A virus sample of 0.2 ml was added to the first well and serial 2-fold dilutions were made. Usually a series of 12 wells was enough to titer a virus sample using two additional wells as controls. A 0.6% solution of washed chicken red blood cells in PBS was used as the indicator for the test. A 0.2 ml volume of the RBC suspension was added to each well. The trays were covered and incubated for 1 hr at room temperature. The viral HA titer was considered to be the inverse of the dilution of virus in the well showing 50% hemagglutination.

Hemagglutination-Inhibition Titration (HAI)

Antisera to Sendai virus was diluted serially with two-fold dilutions in PBS using plastic disposo trays. To each well containing 0.2 ml PBS and antiserum was added 4 HA units of Sendai virus. Two control wells were also prepared: one containing PBS only and one containing PBS and 4 HA units of Sendai virus. The trays were then allowed to incubate at room temperature for 30 min. Each well then received 0.2 ml of 0.6% chicken red blood cell suspension. The trays were covered and incubated for an additional hour. The antiserum titer
was reported as the highest dilution of antisera showing hemagglutination-inhibition.

**Virus Stock**

Sendai virus was provided by Dr. Donald P. Durand, Dept. of Bacteriology, Iowa State University. To verify that the virus was indeed Sendai virus, 0.2 ml of virus stock was neutralized in an HAI titration with Sendai virus typing antiserum provided by the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. Purified preparations of Sendai virus were also examined with the electron microscope to verify size and morphology.

Sendai virus was propagated in 10-day-old embryonated chicken eggs. A small hole was placed in the alcohol-swabbed blunt end of the egg. Two-tenths ml of Millipore filtered infectious allantoic fluid was injected into each egg through the air sac. The eggs were then incubated at 31 C for 48 hours. The eggs were refrigerated at 4 C for 2 hr before the allantoic fluid was harvested. The crude allantoic fluid was stored at -40 C. When sufficient allantoic fluid was accumulated, a virus purification run was made. One liter of allantoic fluid was clarified by low speed centrifugation at 16,000 x g, 4 C, for 30 min in a Sorvall RC-2 centrifuge. The clarified allantoic fluid was then either mixed with polyethylene glycol or recentrifuged at high speed.

Sendai virus was precipitated from allantoic fluid with 6% polyethylene glycol, 6,000 mw, in the presence of 0.5 M NaCl at 4 C for 24 hours.
Precipitated virus was collected by low speed centrifugation at 12,100 x g, 4 C, for 20 minutes. Pellets were resuspended in PBS followed by 90 sec sonication with a Bromwell Biosink II microprobe. The polyethylene glycol was removed from the virus suspension by ultrafiltration using an XM 50 filter in an Amicon positive pressure ultrafiltration chamber.

Sendai virus was also pelleted directly from allantoic fluid using a Beckman type 42 rotor at 20,000 RPM, 4 C, for 2 hours. Pellets were resuspended in 0.01 M phosphate buffer pH 7.2 overnight at 4 C.

Virus concentrated with polyethylene glycol was used for infection while virus pelleted directly was used for antigen preparation.

Plaque-Purified Virus

Sendai virus was plaque-purified by stabbing a single plaque with a flattened inoculating needle and transferring the stab to 10 ml sterile EME. This material served as the egg stock inoculum. The allantoic fluid collected from the first inoculation of eggs was Millipore filtered and served as the inoculum for all viral production. The plaque-purified inoculum was divided into 10-ml portions and stored at -40 C.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was used to monitor the purity of viral antigens and tritiated antibody.

A standard 0.1 M phosphate tank buffer, pH 7.2, was made by combining 140 ml of 0.2 M monobasic sodium phosphate and 360 ml of
0.2 M dibasic sodium phosphate and diluting with water to a total volume of 1000 ml.

Running gels, for both slab and disk PAGE, contained 7.5% polyacrylamide gel and were composed of the following reagents:

- 25 ml acrylamide:bis-acrylamide in a ratio of 30.0 parts acrylamide to 0.8 parts bis-acrylamide in 100 ml water
- 74 ml tank buffer
- 50 μl TEMED (N,N,N',N'-tetramethylethylenediamine)
- 1 ml 10% ammonium persulfate.

Glass tubes with an inside diameter of 7 mm and capable of containing a 10 cm gel column were dipped in a solution of Kodak Photo Flo and allowed to dry. A double layer of parafilm was used to seal the bottom of each tube in preparation for disk electrophoresis. A Buchler vertical disk gel apparatus was used to carry out electrophoresis (Buchler Instruments, Ft. Lee, New Jersey).

An E-C vertical Gel Electrophoresis Cell (E-C Apparatus Corporation, Philadelphia, Pa.) was used for forming and running slab gels. Slab gels were poured according to the directions in the E-C technical Bulletin 128.

The components of the gels were mixed just prior to use, being careful to avoid air bubble formation. Each tube received 10 ml acrylamide and the slab required 100 ml of the gel solution. Two ml of tank buffer was carefully layered over each column of gel in the disk tubes to insure a flat surface for sample entry. After polymeri-
zation the parafilm was removed from the disk tubes while in place, tips submerged in buffer, to insure that no air space would exist between gel and buffer.

Staining and destaining of gels was according to the methods for Coomassie brilliant blue staining outlined in the Ortec Application Note AN 32 A (Ortec Incorporated, Oak Ridge, Tenn.).

Not more than 0.2 ml of purified antigen was mixed with 0.05 ml 40% sucrose containing a small amount of brom-phenol blue, 0.05 ml 2-mercaptoethanol, and 0.05 ml 10% SDS. Before electrophoresis, the antigen samples were heated to 100 C for 1 minute. Antibody samples were mixed with only the sucrose solution before electrophoresis.

Gels were pre-electrophoresed for 1 hr at 7 mA per slab or 1.5 mA per tube gel before the sample was added. Samples were then added to the gels, not to exceed 0.2 ml per gel or per well in the slab gels. Samples were electrophoresed at 4 mA per tube and 25 mA for the slab. After the brom-phenol blue dye front had moved about 8 cm, the gels were removed, fixed, stained, and destained according to the procedure outlined above. Gels which were counted by scintillation counting were fixed and then sliced into 1 mm slices. Slices were placed into scintillation vials and eluted overnight in 0.2 ml water. Ten ml of Beckman Cocktail "D" was added for counting in a Beckman DPM-100 liquid Scintillation System.

Preparation of Antigens

Whole Sendai virus antigen was purified by discontinuous gradient centrifugation on sucrose step-gradients of 60%, 30%, and 15% sucrose
in PBS. Virus samples were layered on top of the gradients and were centrifuged for 2 hr at 20,000 rpm, 10 C, using a Beckman SW 27 rotor in a Beckman L2-65B ultracentrifuge. Purity of the antigen preparation was determined by PAGE.

Sendai virus nucleocapsid antigen was isolated from either whole Sendai virus or from infected BHK-21 cells. Whole virus was fractionated by a modification of the method of Scheid and Choppin (1973) using the detergent triton X-100. After treatment with triton X-100 the virus suspension was centrifuged at 20,000 rpm, 4 C, in a SW 27 rotor for 2.5 hours. The nucleocapsid pellet was resuspended in 0.01 M phosphate buffer pH 7.2 and layered over potassium tartrate step-gradients of the following composition: 50%, 40%, 30% potassium tartrate layers and a top layer of 5% sucrose for detergent removal. The gradients were centrifuged at 20,000 rpm, 10 C, in a SW 27 rotor for 2.5 hours. The viscous looking band appearing in the 40% region was removed with a needle and syringe through the side of the centrifuge tube. This fraction was dialyzed overnight against 0.01 M phosphate buffer pH 7.2, 4 C, and checked for the presence of nucleocapsid with uranyl acetate negative-staining and the electron microscope. Upon confirmation of nucleocapsid, the fractions were centrifuged to equilibrium twice in continuous potassium tartrate gradients of density 1.21 to 1.35 including a cushion of density 1.40 in a SW 27 rotor at 20,000 rpm and 10 C. The final band collected was again dialyzed overnight against 0.01 M phosphate buffer at 4 C. PAGE and the electron microscope were used to analyze final purity.
Sendai nucleocapsid was also prepared by infecting confluent BHK-21 monolayers and incubating them for 48 hr at 31 C. The method of Compans and Choppin (1967) was followed with modification for isolation of nucleocapsid from the infected cells. Cells were removed from culture bottles with a 0.05% EDTA solution and pelleted at 400 x g for 5 min in a clinical table top centrifuge. The pelleted cells were resuspended in water and homogenized with 12 strokes of a tight-fitting glass Dounce homogenizer. Cell debris was removed from the homogenate by centrifugation in a Sorval RC2-B Centrifuge using a SS34 rotor at 8,000 rpm, 4 C, for 20 minutes. The supernatant containing nucleocapsids was monitored with the electron microscope. The nucleocapsids were banded on tartrate gradients as described for fractionated whole virus.

Antibody Preparation

Antigen preparations, whole Sendai virus and Sendai nucleocapsid, containing at least 1 mg/ml protein were mixed with an equal amount of Freund's complete adjuvant and injected sub-scapularly at weekly intervals into rabbits and guinea pigs. Rabbits received 1 mg viral protein per injection and guinea pigs received 0.5 mg. After 5 injections, the hyperimmune sera was collected by cardiac puncture. The gamma globulins were non-specifically precipitated with 0.5 volume of saturated ammonium sulfate under slightly alkaline conditions, pH 7.4-7.8. The precipitate was collected by centrifugation at 1,000 x g for 15 minutes and resuspended in 0.1 M PBS to its original volume. This procedure was repeated twice with the final precipitate resuspended
to one-half its original volume and dialyzed for 48 hr against 0.1 M PBS. The anti-Sendai globulins were titered using the hemagglutination-inhibition (HAI) method of Durand et al. (1973). The anti-Sendai nucleocapsid globulins were titered by passive hemagglutination.

**In Vitro Tritium Labeling of Antibody**

Fifteen mg of antiviral or anticapsid antibody in 0.05 M HEPES buffer pH 7.5 were reacted with 60 μl of 0.01 M pyridoxal-5'-phosphate for 30 min at 37 C as described by Rifkin et al. (1972). The reaction mixture was cooled to 0-4 C for 15 min in an ice salt bath. It then received 7.85 μmole of cold NaB\(^3\)H\(_4\) (Specific activity 5.9 Ci/m mole, Batch 37, and 10.5 Ci/m mole Batch 46, Amersham Searle) in 0.01N NaOH and was incubated at 0 to 4 C for 15 minutes. The reactions were monitored spectrophotometrically with a Beckman DB spectrophotometer and a Beckman 10" recorder. The IgG was precipitated from the reaction mixture with two volumes of ice-cold 70% ethanol. The precipitate was collected by centrifugation, 500 X g for 10 min, resuspended in 0.1 M PBS, and then reprecipitated twice with cold 70% ethanol. The labeled antibody was resuspended to its original volume with PBS and frozen at -20 C until used. The labeled IgG was analyzed for \(^3\)H incorporation, HAI titer, reactivity with goat-source anti-rabbit IgG, PAGE pattern, ability to clump antigen, and for stability of label.

**Preparation of CEL Cells for High Resolution Immunocautoradiography with Anti-Sendai Antibody**

CEL monolayers, 48 hr old, in Linbro multi-dish disposo trays were incubated at 4 C with 200 HA units of Sendai virus for 45 minutes.
The monolayers were then washed once with 2 ml PBS. To each monolayer, 1 ml of a 1/100 dilution of tritiated anti-Sendai virus antibody was added. This represented the addition of approximately $10^5$ CPM per monolayer. The monolayers were further incubated for 1 hr at 4 C. The monolayers were then washed 6 times with cold 0.1 M cacodylate buffer. The cells were then fixed for 60 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 at 4 C, followed by two washes of 0.05 M cacodylate buffer. Cells were then post-fixed for 30 min with 2% OsO$_4$ in 0.05 M cacodylate buffer. Post-fixation was followed by a wash of 0.85% NaCl. Monolayers were then stained with 0.5% uranyl acetate in 0.85% NaCl for two hours. Dehydration was by an alcohol series of 25%, 50%, 70%, 95% and 100% ethanol. After dehydration, the monolayers were embedded in plastic using the following plastic changes: 88% Araldite, 502 or 6005, and 22% absolute ethanol for 10 min; 40% Araldite 60% DDSA two changes of 15 min; 20% Araldite, 20% Epon 812, 58% DDSA and 2% DMP 30. All changes of plastic were 1 ml in volume. Following the addition of the last mixture of plastic, the cluster plates were placed in a 60 C oven for 36 hr for polymerization. The embedded tissue blocks were separated from the plastic culture plates with a plyers. Two blocks were glued together, monolayers touching, with the same epon-araldite mixture and polymerized for 36 hr at 60 C. Blocks were cut and faced so that two vertical tracks of monolayers appeared in the section when cut with a diamond knife. This arrangement gave a top and bottom view of each monolayer, providing a good view for the penetration of virus.
High Resolution Immunomunopulography (HRIM)

Autoradiography at the EM level was accomplished by a modification of the method of Salpeter and Bachmann (1964). The sections, silver interference color, were picked up on 100 mesh formvar coated grids. The sections were stained in 20% methanolic uranyl acetate for 10 min and washed by 40 dips in each of the following solutions: absolute methanol, absolute ethanol, 50% ethanol, double distilled water two changes. Grids were then floated on drops of lead citrate for 8 min and washed by 40 dips in two changes of double distilled water. After the grids had dried, they were secured flat, section side up, to glass slides by touching their edges to a strip of double stick tape. A layer of carbon was then evaporated over the grids. Kodak NTE emulsion was prepared by the method of Salpeter and Bachmann (1964) and applied to the grids by letting a large drop of the emulsion flow over the grids at a 45° angle. When the emulsion had dried, the grids were stored in a light-tight box containing a dehumidifying agent, Dryrite, at 4°C. At weekly intervals, slides were removed and developed by the following sequence:

- Dektol 1:2: 2 minutes
- Rinse: double distilled H₂O: 15 seconds
- Stop: 2% acetic acid: 15 seconds
- Rinse: double distilled H₂O: 15 seconds
- Fix: 20% sodium thiosulfate
  2.5% sodium metabisulfite: 1 minute
- Two rinses double distilled water: 2 minutes.
Tritiated Antibody Binding Studies

Sendai virus infection of tissue culture was monitored using antibody binding techniques. CEL cells were used as the host system for a productive infection, while BHK-21 cells were used as a non-productive system (Darlington et al. 1970, and Compans et al. 1966).

CEL and BHK-21 cells were seeded in multi-dish dispo trays, as previously described, and allowed to form monolayers at 31°C. Just prior to infection each monolayer was washed once with 1 ml sterile PBS. The PBS was aspirated off the monolayers and 128 HA units of Sendai virus, in a volume of 0.1 ml, was added to each well. The cells were incubated at 4°C for 10 min, to allow for viral attachment without penetration. Unattached virus was removed with a single wash of 1 ml BME. The cells were then incubated at 31°C under 1 ml fresh BME.

At hourly intervals, post infection, four wells were washed once with PBS and fixed with either 2% paraformaldehyde or 70% ethyl alcohol. At the end of a 17 hour run, all monolayers were washed three times with PBS to remove excess fixative. Monolayers fixed with paraformaldehyde were reacted with 0.2 ml tritiated anti-Sendai virus antibody for 45 min at room temperature. The short antibody incubation time and paraformaldehyde fixation was designed to limit the antibody-antigen reaction to the cell surface. The ethanol fixed cells were reacted with 0.2 ml tritiated anti-Sendai virus nucleocapsid antibody for 45 min at room temperature as suggested by Girardi et al. (1973) for the location of intracellular antigens.
All monolayers were washed 5 times with 1 ml volumes of PBS. Following removal of the unreacted antibody each monolayer was digested overnight with 0.2 ml 40% NaOH. After neutralizing the NaOH with 0.1 ml glacial acetic acid, each well was scraped with the large end of a flat toothpick to suspend any non-digested tissue.

The digested monolayers were transferred to scintillation vials with tuberculin syringes equipped with 26 gauge, 3/8 inch, needles. Two processed monolayers were combined in one scintillation vial with 10 ml Beckman cocktail "D" for counting in a Beckman DPM 100 liquid scintillation system. Mean CPM/monolayer were plotted against time for both the productive and non-productive infections.

Ten monolayers of each cell type were infected with Sendai virus, fixed at 0 time, and reacted with tritiated antibody as described above. The standard deviation from the mean for a single reading was determined. Control cultures were treated exactly as experimental cultures except that no virus was added.

Electron Microscopy

All transmission electron microscopy was done with a Hitachi HU-11C electron microscope at an accelerating voltage of 75 kV. Scanning electron micrographs were taken on a Jeol JSM-S1 scanning electron microscope at an accelerating voltage of 10 kV.

Uranyl acetate stock solution was made by dissolving 10 g hydrated uranyl acetate \( (UO_2(CH_3COO)_2H_2O) \) in 50 ml of absolute methanol. The stock solution was diluted 1:10 with water for use as a negative stain. Negative stained preparations of Sendai virus and nucleocapsids were
made by placing a drop of sample on a formvar coated grid for 1
minute. A drop of uranyl acetate solution was placed on the grid
after drawing off the excess sample with a small piece of filter
paper. A staining time of 45 sec was found to provide the best
resolution for both virus and nucleocapsid samples. All samples were
either dialyzed against two changes of water or diluted in water to
minimize precipitation of the uranyl acetate.

Scanning electron microscopy was used to determine if Sendai virus
infection could be visually monitored at the cell surface. CEL cells
were seeded on 60 mm X 15 mm plastic petri plates as reported above.
Just before confluent monolayers were formed, the cells were infected
with Sendai virus at 4 C for 45 minutes. Each plate received
approximately 10^3 HA units of Sendai virus. Control plates received
no virus. The plates were washed once with 4 ml PBS to remove unattached
virus. Cells were then fixed with 2% glutaraldehyde for 1 hr at 4 C.
After two 4 ml washes with PBS, small 5 mm squares were cut from the
petri plate bottoms with a razor blade. The squares containing tissue
culture were then processed for scanning electron microscopy by the
method of Beltz (1974). Care was taken to insure that the cell side
of the squares always remained in an upright position.
RESULTS

Virus Characterization

Sendai virus stock was initially obtained from Dr. D. W. Kingsbury and was characterized, in our laboratory, by electron microscopy and serology to insure that it was Sendai virus.

Anti-Sendai virus typing-antiserum neutralized the stock virus in an HAI test. The typing-antiserum, provided by the National Institute of Allergy and Infectious Diseases, was designed for the identification and confirmation of research viral stocks.

Electron micrographs of negatively-stained stock virus particles were made. The virion morphology ranged from spherical to pleomorphic, Figure 1. Each virion was covered with spike-like projections. The virion envelope was also evident as a layer directly below the spikes. In some cases, the envelope layer seemed to be extremely dense, Figure 1c. Helical nucleocapsids were noted within the particles, Figure 1a. The average diameter for 60 randomly-selected virions was 190 nm with a range in diameter from 65 nm to 304 nm.

Scanning Electron Microscopic Study

Scanning electron micrographs were made of CEL cells before and after Sendai virus attachment. Control cells, before viral attachment, were not smooth surfaced. These cells had numerous projections on the upper surface with the edges of the cells containing fiber-like extensions, Figure 2. The cell surface, at higher magnification, seemed smoother, Figure 3. Virus infected cells, at low magnification, appeared no
Figure 1. Negatively-stained preparations of Sendai virus

la and lb, typical Sendai virions with spike studded envelopes and helical nucleocapsids. lc, virion with dense envelope layer. ld, pleomorphic Sendai virions. Magnification, 230,000X.
Figure 2. Scanning electron micrograph of control CEL cells

Rough surface and fiber-like projections are evident.

Magnification, 1,722X.
Figure 3. Relatively smooth rolling surface of a non-infected CEL cell as shown by scanning electron microscopy

Magnification, 5,600X.
different in surface morphology than the control cells, Figure 4. However, at a higher magnification, the virus infected cell surface contained more small projections than the control cells, Figure 5. Unfortunately resolution was not enough to determine if the projections were viral particles or just part of the host cell.

Sendai Virus Antigens

Sendai virus nucleocapsids were isolated from whole virus and from infected BHK-21 cells. Nucleocapsids were separated from cell and viral debris by centrifugation on discontinuous tartrate step-gradients. Final purification was by centrifugation to equilibrium on continuous tartrate gradients. Multiple bands were obtained on the discontinuous tartrate gradients, Figure 6a. The lowest band from the discontinuous tartrate gradients was removed and centrifuged to equilibrium, Figure 6b. The single band obtained from equilibrium centrifugation was dialyzed against distilled water and portions were negatively stained for electron microscopy, Figure 7. Intact nucleocapsids were seen as well as several fragments. The nucleocapsids were quite flexible as seen in Figure 7.

Purity of the final antigen preparation was monitored by PAGE. The nucleocapsid preparation migrated as a single band which matched the protein #4 band of whole Sendai virus. Only five of the eight Sendai virus proteins, reported by Lamb and Mahy (1975), could be resolved on the PAGE system used.
Figure 4. Scanning electron micrography of Sendai virus infected CEL cells

The same general surface topography is noted as in the non-infected cells. Magnification, 900X.
Figure 5. High magnification view of the surface of Sendai virus infected CEL cell

Many small lumps are noted on the surface that were not evident in the non-infected cells. Magnification, 28,000X.
Figure 6. Results of tartrate gradient purification of Sendai virus nucleocapsids

6a, bands observed after discontinuous centrifugation. Bottom band in 6a was removed and centrifuged to equilibrium. 6b, single band of Sendai nucleocapsid after equilibrium centrifugation.
Figure 7. Negatively-stained preparation of Sendai virus nucleocapsid from equilibrium centrifugation

Note the flexible nucleocapsids with their typical herring-bone pattern. Magnification, 128,000X.
Tritiated Antibody Preparation

Antibodies against Sendai virus and the viral nucleocapsids were tritium labeled by the borohydride exchange reaction.

The reaction was monitored spectrophotometrically to determine if changes might occur to the antibody during the reaction. No spectrum changes were noted in the absorbance of the antibody throughout the reaction sequence, Figure 8 and Figure 9. There was, as expected, a change in the pyridoxal spectrum after the addition of the tritiated sodium borohydride.

The tritiated anti-Sendai antibody was compared to pre-tritiated antibody with PAGE. Slices of the gels were also made and counted by scintillation counting to locate the radioactivity in the gels, Figure 10. The radioactivity was located in the single band of protein representing the gamma globulin fraction of the antibody preparation. From this analysis it was found that the borohydride exchange procedure labeled the antibody with tritium. The alcohol precipitation step removed other contaminating serum components leaving a pure preparation of IgG labeled with tritium.

The specific activities of the antibody preparations after tritium labeling were as follows: anti-Sendai virus IgG reacted with batch 37 $^3$H-sodium borohydride, 28.5 μCi/mg; anti-Sendai virus IgG reacted with batch 46 $^3$H-sodium borohydride, 7.9 μCi/mg; anti-nucleocapsid antibody reacted with batch 46 $^3$H-sodium borohydride, 5.9 μCi/mg.
Figure 8. Spectrum of the borohydride exchange reaction

\(8a,\) pyridoxal phosphate in HEPES buffer. \(8b,\) pyridoxal phosphate and antibody in HEPES buffer.
Figure 9. Spectrum of the borohydride exchange reaction

9a, pyridoxal phosphate and antibody after 30 min at 37 C.
9b, antibody and pyridoxal phosphate after the addition of sodium borohydride.
Figure 10. Slab polyacrylamide gel electrophoresis of antisera to Sendai virus before (10b) and after (10a) tritiation

Samples were run from left to right on 7.5% gels for 13 hr with a 200 mA current. The gels were fixed in 12% trichloroacetic acid and either stained with Coomassie brilliant blue or sliced into 1 mm slices. Slices were then counted by scintillation counting. A plot of the scintillation data is matched to the stained gels.
To insure that the product was IgG, it was reacted with goat-source anti-rabbit IgG antibody. No HAI could be found when the neutralized anti-Sendai IgG was used with stock Sendai virus in an HAI titration.

It was found that the HAI titer of the anti-Sendai virus IgG dropped from $10^4$ HAI units/ml to $10^3$ HAI units/ml after the tritiation reaction. Sendai virus was reacted with tritiated antibody and prepared for negative staining by the method of Almeida and Waterson, 1969. Electron microscopic examination showed that Sendai virus was clumped by the tritiated antibody, Figure 11.

After 1.5 years of storage at -20 C, with several freezing and thawings, tritiated anti-Sendai antibody had retained 27% of the tritium originally incorporated. About 6% of the radioactivity lost was attributed to radioactive decay alone.

High Resolution Immunoautoradiography (HRI)

Sendai virus was attached to both BHK-21 cells and CEL cells at 4 C for 45 minutes. The cells were washed and then reacted with tritiated anti-Sendai antibody for 1 hr at 4 C. After washing 6 times, the cells were fixed and processed for HRI.

Control cells were prepared just as the antibody treated cells were, but without the addition of the labeled antibody. Virus-like particles were found attached to the cell membrane, Figure 12 and Figure 13. In most cases the host cell membrane showed some response to the presence of the virus, Figure 12 and Figure 13b.

Grids with emulsion coated sections were incubated at 4 C within a light-tight box. At weekly intervals the autoradiograms were developed.
Figure 11. Two Sendai virions clumped with anti-Sendai virus antibody

Antibody seemed to have coated half of the lower virion. Negatively-stained preparation. Magnification, 264,000X. This micrograph was taken by Charles F. Amstein when he was a graduate student in the Dept. of Bacteriology, Iowa State University, Ames, Iowa.
Figure 12. BHK-21 control cells with attached Sendai virions

In 12a and 12b, the host membrane has started to envaginate. Magnification, 119,600X.
Figure 13. CEL control cells with attached Sendai virions

In 13b, the cell membrane is completely absent at the site of virion penetration. Magnification, 119,600X.
After four weeks incubation time, developed grains were found in association with virus-like structures on the cell surfaces, Figures 14 and 15. Developed silver grains appeared either above or adjacent to the virus-like particles. In Figure 14a, the viral particle penetrated the cell membrane with a developed grain marking its location. The host cell membrane seemed to be disrupted at the site of particle entry.

Fifteen grids containing 15 to 20 sections per grid were observed. Approximately 90% of the virus-like particles contained no developed silver grains while only 10% were found in association with developed grains. A few grains were found associated with the cell membrane in the absence of a recognizable virus particle. The number of background grains produced in the Kodak NTE emulsion was 1 grain/30 µm².

Tritiated Antibody Binding Studies

An antibody-binding assay was developed for cell associated Sendai virus antigens using tritium labeled antibodies. Host cells were infected with the virus, fixed at hourly intervals post infection, and reacted with either anti-Sendai or anti-nucleocapsid tritium labeled antibodies. The cell-antibody preparations were counted by scintillation counting. Ten replica samples of infected CEL cells and tritiated antibody were prepared to determine standard deviation from the mean for a single reading. The standard deviation was found to be 52 CPM.

Productive and non-productive host systems were analyzed to determine if the assay could denote any differences in antigen production between the two cell types. Surface-associated antigen was monitored with the
Figure 14. HRI of Sendai virions on CEL cells

14a, the host cell membrane is disrupted above and to the left of the virion which has penetrated the cell. A silver grain marks the location of the virus particle. 14b, an isolated virion is marked by a developed silver grain. Magnification, 119,600X.
15a, a single silver grain locates two virions. Either particle or both may carry the antibody label. 15b, a virion attached to the cell is marked by a developed silver grain. Magnification, 119,600X.
tritiated anti-Sendai antibody while intracellular nucleocapsid antigen was assayed with the tritiated anti-nucleocapsid antibody.

A decrease in viral antigen at the cell surface was noted until 4 hr post infection in the CEL system, Figure 16. At this point the bound antibody increased until, at 7 hr post infection, it almost matched the amount bound at 0 time. Other peaks of surface bound antibody were found at 10 and 12 hr post infection. The intracellular bound anti-nucleocapsid antibody showed slight fluctuation between 0 and 8 hr post infection. At 8 hr post infection the amount of nucleocapsid antibody bound to the cell began to increase. A slight plateau of bound antibody was evident between 12 and 13 hr with a steady accumulation until termination of the experiment.

In the case of the non-productive BHK-21 system, Figure 17, surface bound antibody showed a slight initial decline. The first increase appeared at 3 hr post infection. Two other major peaks of bound antibody appeared at 12 and 15 hours. Bound anti-nucleocapsid antibody decreased until three hours post infection. A small rise at 4 hr preceded a very large peak at 8 hr post infection. There was no corresponding peak for the surface bound antibody during this time period. After 9 hr the nucleocapsid antibody curve paralleled the whole virus antibody curve.
Figure 16. Scintillation counts of tritiated antibody bound to Sendai virus infected CEL cells at various times post infection

The top curve, small dashes, represents bound anti-Sendai antibody. The bottom curve, large dashes, represents intracellular bound anti-nucleocapsid antibody.
Figure 17. Scintillation counts of tritiated antibody bound to Sendai virus infected BHK-21 cells at various times post infection

The top curve, small dashes, represents bound anti-Sendai antibody. The bottom curve, large dashes, represents intracellular bound anti-nucleocapsid antibody.
DISCUSSION

It was evident from the virus characterization studies that the virus stock was Sendai virus. Anti-Sendai virus antiserum, provided by the National Institute of Allergy and Infectious Diseases, completely neutralized the virus in an HAI test. Furthermore, the morphology of the virions fully matched descriptions of Sendai virions as presented by Horne et al. (1960) and Choppin and Stoeckenius (1964).

The range of virion diameters was greater than the 120 nm to 300 nm size range reported by Hosaka et al. (1966). This difference in range might be due to actual size differences in various strains of the virus or in the way the diameters of pleomorphic virions were measured. I found many very small particles which may have been incomplete virions, a common occurrence with the paramyxoviruses. These were included in my measurements while they may not have been measured by other investigators.

I observed that some virions, removed from the lower bands on discontinuous sucrose gradients, had a dense layer underlying the envelope (Figure 1c). This condition has not been reported in previous descriptions of Sendai virus. The dense layer may indicate increased amounts of M protein in these virions or it may be an artifact of staining. I feel that the virions probably do contain excess M protein, increasing particle density, since they were found only in the lower bands of virus purified on sucrose gradients. If this condition were strictly an artifact of staining one would have also expected to find these particles in other bands of the gradients.
Hosaka et al. (1966) found that different fractions of Sendai virus removed from sucrose gradients after rate zonal centrifugation gave varying fusion results when added to tissue culture. The virus removed from the lowest bands, the denser particles, had the highest fusion ability. It is possible that the dense layers I observed could relate in some way to the cell fusing ability of the virus.

Scanning Electron Microscopy

It was hoped that scanning electron microscopy could be used as a tool to monitor the activity of Sendai virus on the cell surface during the infection process. Virus infected cells had the same general morphology as the control cells until greater magnification was used. Control cells then had a smoother appearing surface than the infected cells. Many small projections on the infected cells could have been virus particles. With the low resolution obtained with the scanning electron microscope, no positive virus identifications could be made. This will continue to be a problem until the methodology of scanning electron microscopy is improved. One method of locating viral antigens on the surface of cells might be to use ferritin-conjugated antibodies and the elemental analysis mode of certain scanning scopes. The iron in the ferritin-conjugated antibodies could serve to differentiate between cell projections and virus particles. However, the need to coat specimens with metal would probably eliminate the chance of resolving virus-host cell membrane interactions.
In my work the scanning electron microscope helped by providing a topographical view of the host system but failed to resolve Sendai virus on the cell surface.

Sendai Virus Antigens

Sendai virus nucleocapsid was successfully isolated from whole virus and infected BHK-21 cells by combining the methods of Compans and Choppin (1967) and Scheid and Choppin (1973). Their methods included CsCl gradients for purification of nucleocapsid preparations. However when I tried to duplicate their purification procedure I was only able to isolate very short fragments along with many disrupted nucleoprotein-like aggregates.

I felt that the CsCl might be somewhat harsh on the nucleocapsid, causing fragmentation and dissociation. By replacing the CsCl with potassium tartrate, resulting nucleocapsid preparations proved to be far superior. Longer nucleocapsid strands and very few aggregates of disrupted nucleoprotein were found.

Compans et al. (1972) have reported that Sendai virus nucleocapsid is rigid and tends to remain tightly coiled. Sendai nucleocapsids, banded on CsCl, and negatively stained for electron microscopy have supported this contention (Hosaka, 1968).

My preparations show that the nucleocapsid is not rigid but rather flexible (Figure 7). This would seem to be the more natural state for the nucleocapsid since both negatively-stained preparations and thin sectioned whole virus showed a coiled arrangement within the virion (Figure 1 and Figures 12 - 15).
The purity of the nucleocapsid and whole virus preparations used as antigens was monitored with PAGE. The nucleocapsid preparation showed one band which co-migrated with the nucleocapsid protein of whole Sendai virus. PAGE of whole Sendai virus resolved only five of the eight reported proteins of the virus. The three bands that were not resolved represented proteins in the lowest concentration in Sendai virions. These proteins might have been resolved if the gels had been scanned with a spectrophotometer which could have detected small amounts of the stain bound to the proteins but not visible to the unaided eye.

Tritium Labeled Antibodies

Anti-Sendai virus and anti-nucleocapsid antibodies were tritium labeled by the borohydride exchange reaction. This method was a fast and gentle procedure for the incorporation of tritium into immunoglobulins.

The addition of the alcohol precipitation step insured the purity of the final product and removed the labeled IgG from the radioactive reactants.

Labeling antibody with $^3$H-sodium borohydride, batch 46, produced lower specific activities in the final product that did batch 37. I feel that this difference was due to the accidental loss of some of the $^3$H-sodium borohydride before it could be added to the appropriate reaction vessel. The difference between the specific activities of the two antibodies labeled with batch 46 was also probably due to unequal amounts of $^3$H-borohydride which were added to each reaction vessel.

After the reactions were complete and the IgG was isolated, only minimal loss of antibody titer could be noted. This loss was attributed
to loss of antibody during the precipitation and centrifugation steps rather than the harshness of the reaction itself.

Tritium is more unstable than hydrogen and tends to exchange with stable hydrogen atoms; this was the basis for the Wilzbach reaction (Wilzbach, 1957). Similarly, compounds labeled with tritium tend to lose their label because of this exchange phenomenon. During a storage period of 1.5 years the tritium labeled antibody I prepared lost 73% of its radioactivity. Six percent of this loss was attributed to radioactivity decay. The rest of the loss was due to exchange with hydrogen in the environment. The loss was probably accelerated by the several freezings and thawings that occurred as portions of the antibody were used for experiments. The loss observed at the end of 1.5 years seemed excessive and indicated a rather severe instability of the label. However, iodine labeled antibodies would lose 75% of their radioactivity due to decay in the following times: 12 days for $^{131}$I and 90 days for $^{125}$I. Thus the tritium labeled antibodies retain their radioactivity much longer than the iodine labeled antibodies. This would make tritium labeled antibodies more desirable for long range research problems in which the same labeled antibody preparation was used.

One of the problems researchers have had in using antibodies labeled with radioactive iodine has been that the short half-life of the compound prevents experimentation over extended periods of time with one antibody preparation (Gonatas et al., 1974; and McMillen and Consigli, 1974). The tritium labeled antibody seems to be an answer to this problem. A modification of the borohydride reaction allowed McMillen and Consigli
(1974) to label antibody with $^{14}$C. Thus two methods are now available to label antibody with long lasting labels.

**High Resolution Immunooautoradiography**

Sendai virions were located on the surface of infected cells with the aid of tritium labeled antibody and autoradiography.

A modification of the method of Salpeter and Bachmann (1964) was used to prepare the sectioned material for autoradiography. Their autoradiograms were processed on glass slides and then transferred to grids for viewing in the electron microscope. This method proved unsatisfactory for me since I could not successfully transfer the processed material from the slides to the grids. I feel that their method probably provided the best coating by the emulsion, yet this value is lost if one cannot view the final product. By adding the emulsion to the sections after they were on the grids I was able to view many more grids.

After four weeks exposure time I was able to find developed silver grains associated with virus particles on the cell surface of Sendai virus infected cells. The developed grains were located either directly over or next to the virus. According to Caro (1962) 75% and 87% of the silver grains associated with a radioactive source will be found within 0.1 μm and 0.2 μm of that source, respectively. These distances are equal to 1.28 cm and 2.56 cm, respectively, on the micrographs in Figures 14 and 15. The silver grains shown in my results are all within 1.28 cm of a virus particle.

I was disappointed that so few virus particles were found associated with silver grains. This problem could be due to a short exposure
period, a nonuniform emulsion layer or an insufficient amount of antibody binding to the virus. Of these three possibilities I feel that the exposure time might be the most probable cause of the problem. Two ways might be used to overcome this situation. First, exposure time might be increased to 60 days or longer. This however does not facilitate rapid processing of materials. A second method would be to increase the specific activity of the antibody, providing more radioactivity for grain development. According to Rifkin et al. (1972) the amount of tritium incorporated into the molecule may be increased by 60% when the reaction is carried out at pH 9. The tritium incorporation can be further increased by raising the pyridoxal phosphate concentration (Rifkin et al., 1972).

The emulsion thickness seemed to conform to the criteria of Salpeter and Bachmann (1964) in that it produced a gold interference color when viewed in reflected light, indicating a monolayer of silver grains. Certainly local areas might not have received their quota of silver grains but this should not have been a phenomenon associated with over 100 sections that I viewed.

If the amount of antibody had not been sufficient to react with most of the virus particles, the graphs of the antibody binding studies (Figures 16 and 17) would not have turned out as they did. One would have expected a plateau at the beginning of each experiment until the excess virus was either taken into or was released from the cells. This did not happen; thus it may be assumed that the antibody was in excess.
I feel that HRI has an excellent future as a research tool. Gonatas et al. (1974) have already shown that intracellular antigens may be located with radioactively labeled antibody and autoradiography. Their preparations were shown at relatively low magnification thus the quality of ultrastructural preservation could not be determined. Even though they achieved excellent cell penetration with their labeled Fab fragments, their resolution undoubtedly suffered by using \(^{125}\)I as their radioactive label. One advantage to be realized by using HRI over enzyme labeled antibodies is that the antigen is not completely occluded by the labeled antibody as in the case of peroxidase labeled antibody. As soon as a method is developed for incorporating a scintillator into the photographic emulsion, incubation times will be cut to a few days or even hours as has been done at the light microscopic level (Durie and Salmon, 1975). This would put HRI on a time sequence compatible with many diagnostic procedures now in use.

Tritiated Antibody Binding Studies

A tritiated antibody binding assay was developed and successfully used to monitor cell-associated Sendai virus antigens throughout the first 17 hr of the infection process. The large size of the antibody molecule prevents it from rapidly penetrating formaldehyde fixed cells (Gonatas et al., 1974). Thus in my binding assay I was able to limit the antigen-antibody reaction to the surface of the infected cells by first fixing the cells with formaldehyde and then using a short incubation period of 1 hr, for the labeled antibody. Girardi et al. (1973) have shown that
alcohol-fixed cells are readily penetrated by peroxidase-conjugated antibody. Furthermore, the antigenicity of the system was not destroyed by the alcohol fixation. Thus by using alcohol fixation in my binding assay, intracellular nucleocapsid antigens were labeled after the cells were incubated with tritium labeled anti-nucleocapsid antibody.

A standard deviation from the mean of 52 CPM was determined for a single reading in the binding assay. This deviation is not excessive considering that each monolayer was washed 5 times to remove excess labeled antibody, scraped with a toothpick to remove non-digested cells, and finally removed with a syringe to a scintillation vial for counting.

The methodology of this portion of the binding assay could be improved by using circular coverslips to grow the cells on in the culture dishes. After washing, the coverslips could be transferred directly to the scintillation vials for digestions and counting. This could prevent any losses due to syringe transfer alone.

A similar binding assay has been developed by Hayashi et al. (1972) using radioactive iodine labeled antibodies against vaccinia virus. Their method was limited to using live cells and antibody against the whole virus. Also they did not adapt their technique to small tissue culture vessels.

My antibody binding assay is not limited to studying virus-cell interactions but could be easily modified to quantitate antibodies to virus in serum samples. This could be done by labeling the serum with the radioactive tag and measuring the radioactivity bound to infected
cells, or by quantitating the amount of unlabeled serum required to block the attachment of a standard amount of labeled IgG.

Analysis of the Results of the Tritiated Antibody Binding Studies

My results show that for the productive CEL system Sendai virus disappears from the cell surface until approximately 4 hr post infection. From this time until the completion of the experiment at 17 hr, three major peaks of surface associated Sendai virus antigen appear. These peaks may represent parental viral envelope proteins being inserted into the host membrane, progeny virus envelope proteins being inserted into the host membrane, progeny virus at the cell surface before release, or a combination of these factors.

The intracellular nucleocapsid antigen showed a slight decrease at the initiation of the experiments followed by a slight peak at 4 hr post infection. The major rise in nucleocapsid antigen began to appear at 8 hr post infection. The early changes seen in bound nucleocapsid antibody are at the 50 cpm limit for a single measurement; thus, their significance may be suspect. After 8 hr there was a rapid increase in the amount of nucleocapsid produced with a small plateau between 12 hr and 13 hr post infection.

The following statements are an attempt to explain the events happening during the early portions of Sendai virus infection of host cells based on the results of Figure 16 and 17 and what is already known about the process. The early decrease of surface associated antigen indicates
that the virus particles are entering the host cell. Some of the attached
virus may undergo spontaneous elution from the host cell as described by
Dales (1973). The bound anti-nucleocapsid antibody, during the early
portions of the experiment, remains relatively uniform indicating that
the amount of virus added to the cell remained with the host system. The
slight decrease in bound nucleocapsid antibody between 2 hr and 3 hr post
infection might indicate that a small fraction of adsorbed virus might
have eluted from the system. Early activity of the virus-host system has
recently been shown by Durand et al. (1975) and Kohn (1975). Parental
viral materials was found to enter the host cell during the first few
hours post infection and was then released from the cell at about 8 hr
post infection (Durand et al., 1975, and Kohn, 1975). When inactivated
virus was taken into cells the amount of resurfacing parental components
was reduced yet the time of resurfacing was the same (Durand et al., 1975,
and Kohn, 1975). It is interesting to note that at 7 hr post infection
there is an increase in surface associated Sendai virus antigen which
corresponds to the release of parental viral components at 8 hr in the
experiments of Durand et al. (1975). Thus the decrease in cell surface
associated antigen at 8 hr post infection represents release of viral
antigen from the host rather than reentry into the cell.

The slight rise in bound nucleocapsid antibody at 4 hr might indicate
that the nucleocapsids are completely released from the parental virion
making them somewhat more accessible to the antibody. This is consistent
with the findings that the amount of newly synthesized viral RNA does not
begin to increase until after 4 hr post infection (Zhdanov and
Bukrinskaya, 1970). Bukrinskaya et al. (1969b) have found that parental Sendai virus ribonucleoprotein remains within the host cell for at least 6 hr post infection. During this time the nucleocapsid partly deproteinizes and becomes associated with virus specific polysomes.

Thus it seems that the first 4 or 5 hours of the infection process are a tooling-up stage for the production of viral components. This can be inferred from Figure 16 since there is really little in the way of antigen production noted.

Kingsbury (1974) has suggested that capsid proteins are the components which control the transcription and replication of the viral genome. As capsid proteins are manufactured they associate with the virion RNA and block attachment sites for the transcriptase. Thus as the infection process progresses and more capsid proteins are produced the system shifts to replication as opposed to transcription. The system presented by Kingsbury is a cyclic one, switching back and forth between translation and replication depending upon the amount of nucleocapsid protein available. As more and more capsid proteins are produced one would expect the cyclic phenomenon to shift in favor of replication. This shift may be expressed in the results shown in Figure 16. The first peak of surface associated antigen may be the result of a first round of transcription followed by translation and the production of envelope proteins. The interval between 8 hr and 10 hr post infection may represent the shift back to the replication phase of the cycle with little product being formed. With the increase in nucleocapsid production the
replicative phase of the cycle becomes favored. Once the virus replicating system has been set up one would expect it to become more efficient. This might be a reason why there is only one hour time difference between the second two peaks of surface associated antigen while the first two are separated by three hours.

I feel that by 14 hr post infection the cyclic phenomenon would become less noticeable due to the overwhelming amount of viral components being produced. Viral envelope protein, inserted into the host membrane, would no longer need to "wait" for nucleocapsid and may be released as whole virus within a few minutes of their insertion.

The non-productive BHK-21 cell line presented a different picture for virus-host interaction. A decrease in nucleocapsid bound antibody was noted until 3 hr post infection. At this time there was a corresponding peak of surface associated virus antigen. At 4 hr post infection there was an increase in the amount of nucleocapsid antibody bound to the cell. This may add support to the concept that after the virus was uncoated the nucleocapsids were more accessible to the antibody. It seems that the envelope proteins were inserted into the host membrane. This is consistent with the results reported by Durand et al. (1975) and Kohn (1975). It should be noted that the first nucleocapsid peak in the BHK-21 system coincides with the small peak at 4 hr in the CEL system. Large amounts of surface associated antigen are never found in the non-productive system.

An interesting phenomenon was noted in the production of nucleocapsid antigen in the BHK-21 system. Nucleocapsid production reached an
early peak at 8 hr post infection with no concurrent peaks of surface associated antigen. From this point on production of nucleocapsid antigen seemed to parallel that of surface associated antigen.

In the CEL system the nucleocapsid production showed a rapid increase at 8 hr and continued to rise. This may indicate that the nucleocapsid production was premature in the BHK-21 system. Also there was no surface associated antigen to coincide with this early rise in nucleocapsid. It is possible that if newly synthesized nucleocapsid does not receive an envelope it may be recycled back into the system since the nucleocapsid has been shown to be infective (Kingsbury, 1974). Inside the cell there would be nothing to stop a nucleocapsid from "reinfecting" a cell except envelopment and release.

Since the labeled antibodies measured changes in the infection process in the BHK-21 system it seemed that some virus was produced but very inefficiently. Exactly why the system was inefficient cannot be determined with the techniques used. The only conclusion I would make concerning the non-productive system was that in general, viral antigens were not made in as large a quantity as in the productive system and that the sequence of production of viral components could be responsible.
A new method was developed in which cell associated Sendai virus antigens were monitored during the infection process using tritium labeled antibodies in both antibody binding studies and high resolution immunol autoradiography.

The identity of Sendai virus was confirmed by electron microscopy and serology. Some of the virions removed from the lower bands, after rate zonal centrifugation, were found to have a previously undescribed dense layer beneath the virion envelope. It was postulated that this layer might be excess M protein and could contribute to the increased fusing ability of these particles reported by other investigators. Sendai virus nucleocapsids were isolated from both whole virus and infected cells and purified by centrifugation on potassium tartrate gradients. This purification procedure was found to be more gentle than the use of CsCl gradients, producing intact, flexible nucleocapsids as opposed to short, non-flexible, and often disaggregated pieces of nucleocapsid removed from CsCl gradients. Sendai virus and Sendai virus nucleocapsids were used as antigens to produce specific antibodies.

The borohydride exchange reaction was used to label antibodies with tritium. This procedure incorporated high specific activities into anti-Sendai virus antibodies without the addition of large complex groups which could interfere with antigen-antibody reactions. The tritium labeled antibodies retained radioactivity six times longer than the predicted radioactive decay of iodine labeled antibodies. I found the borohydride
exchange reaction to be a fast and gentle method of incorporating tritium into antibodies with little loss of anti-viral titer.

Tritium labeled antibodies were used, in antibody binding studies and high resolution immunoautoradiography, to follow the infection process of Sendai virus in productive and non-productive host-cell systems. High resolution immunoautoradiography was developed to locate viral particles on the surface of infected cells. Sendai virus particles, interacting with host cell surface membranes, were found associated with developed silver grains after processing for high resolution immunoautoradiography and examination with the electron microscope. The developed silver grains clearly located the viral antigens without the occlusion found with enzyme labeled antibody procedures.

Tritiated antibody binding studies monitored surface associated and intracellular Sendai virus antigens in both productive and non-productive host systems. The results confirmed work by other investigators that parental viral components are reinserted into the host-cell membrane before progeny virus is released. Also the results suggested that during the early phases of infection, production and release of viral antigens is a cyclic phenomenon. The non-productive host system did not follow the same pattern as the productive system in that there was no corresponding increase of surface associated antigen matching the major production period of the nucleocapsid. I feel this factor would be the key to the differences between productive and non-productive virus-host cell systems in general.
BIBLIOGRAPHY


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