SOME CYTOCHEMICAL STUDIES OF THE MULTIPLICATION OF INFECTIOUS BRONCHITIS VIRUS IN CHICKEN EMBRYO KIDNEY CELLS

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Thomas Gilbert Akers

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THESIS

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ABSTRACT

SOME CYTOCHEMICAL STUDIES OF THE MULTIPLICATION OF INFECTIOUS BRONCHITIS VIRUS IN CHICKEN EMBRYO KIDNEY CELLS

by Thomas Gilbert Akers

The usual virus replication cycle was observed when chicken embryo kidney cell cultures were inoculated with infectious bronchitis virus, strain IBV42-118C. The development of syncytia paralleled the release of infectious virus. Since the formation of syncytia and the release of infectious virus were also simultaneously suppressed by anti-infectious bronchitis virus serum, heat, and ultraviolet irradiation, it was concluded that virus replication takes place within the syncytia.

There were cytoplasmic aggregates of virus ribonucleic acid in syncytia and single cells at 24 and 36 hours, respectively, as revealed by acridine orange staining. This observation confirms the site of virus replication and the nucleic acid character of infectious bronchitis virus.

The release of infectious virus was suppressed when low concentrations of DL para-fluorophenylalanine, a known ribonucleic acid inhibitor, were added to chicken embryo kidney cell cultures infected with the virus. While the formation of syncytia was not suppressed at low concentrations of the inhibitor, fluorophenylalanine at high concentrations inhibited both the formation of syncytia and the release of infectious virus. These two events indicate that multiplication of infectious bronchitis virus is dependent upon ribonucleic acid synthesis. Aminopterin, a folic acid analogue which inhibits synthesis of both cellular and virus deoxyribonucleic acid, had no effect on formation of syncytia or infectious virus release. Virus replication

was independent of host-cell deoxyribonucleic acid synthesis which indicates that the virus is not of the deoxyribonucleic acid type.

Since infectious bronchitis virus is sensitive to ether, the presence of a peripheral structural lipoprotein and peripheral assembly of the virus is assumed. The ratio of cell associated to released virus was less than 1, which indicates maturation of virus at the periphery of the cell.

On the basis of sensitivity to ether, formation of syncytia, ribonucleic acid content, and association with a specific respiratory disease, infectious bronchitis virus may be included in the "syncytial virus" group.

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INTRODUCTION

The cultivation of the Beaudette strain of infectious bronchitis virus (IBV) in chicken embryo kidney cells (CEKC) with the formation of syncytia provides a model system for study of virus-host cell interrelationships. The objective of the present investigation was to determine by cytochemical microscopy and specific metabolic inhibitors the nucleic acid present in IBV.

LITERATURE REVIEW

Infectious Bronchitis Virus

Infectious bronchitis virus is the etiological agent of a world-wide, specific, highly contagious respiratory disease naturally limited to chickens. The virus can be readily propagated in chicken embryos by various routes of inoculation (Beaudette and Hudson, 1937; Delaplane and Stuart, 1941). Strains such as the Beaudette strain which are completely adapted to chicken embryo cultures can kill embryos rapidly but are avirulent and non-immunogenic for chickens. The Beaudette strain can also be propagated in CEKC cultures (Chomiak et al., 1958; Spring, 1960), whole and minced chorioallantoic membrane (Ferguson, 1958; Ozawa, 1959), chicken embryo liver cells (Fahey and Crawley, 1956), and in whole chicken embryo cell cultures (Spring, 1960).

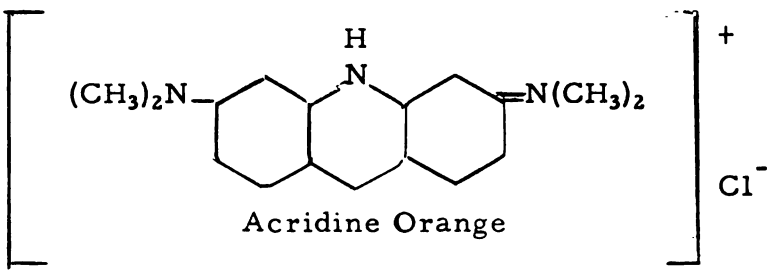
The Beaudette strain is relatively thermolabile having a specific reaction velocity constant, k, of 0.14 hr^{-1} at 37 C and an energy of activation (ΔH for inactivation) of approximately 23 Kcal per mole (Page and Cunningham, 1962). This strain in undiluted allantoic fluid is inactivated within 10 minutes at 56 C, 48 hours at 37 C, 23 days at 25 C, or 27 weeks at 4 C.

Based on electron microscopy, the virus is a sphere with a diameter 60 to 80 mμ according to Nazerian (1960), and 120 mμ according to Buthala (1956). The density of IBV is 1.15 (Buthala, 1956) and the isoelectric point is pH 4.15 (Cunningham and Stuart, 1947; Buthala, 1956).

Fluorescence Technique

Acridine orange (AO), a polychromatic fluorochrome, has an affinity for nucleic acids. In sections of fixed animal tissues stained with

buffered AO, the characteristic fluorescence of nucleic acids can be readily observed by ultraviolet (UV) microscopy with a darkfield condenser and both an exciter filter and a barrier filter (Armstrong and Niven, 1957). This cytochemical technique has become increasingly important because ribonucleic acid (RNA) and double and single stranded deoxyribonucleic acids (DNA) can be simultaneously differentiated. Fluorochromes such as primulin, thioflavin-S, berberine sulfate, trypaflavin, and others have been used as basic fluorescent dyes. The acridine derivatives, AO, and acridine orange-R (AOR) are superior in the intensity of fluorescence (Armstrong, 1956). The structure of AO (2,8 bis dimethylamino acridine (Gurr, 1960), is as follows:



Acridine Orange

Acridine orange-R is the 5-phenyl derivative of AO. Acridine orange is generally used in the range pH 1.5 to 3.5 and AOR at pH 3.5 to 5.0. Both compounds dissolve readily in acetate buffers. The clear orange solution has absorption peaks at wavelengths of 267 mμ and 492 mμ, respectively. The molecules of the dye in dilute solution when bound to polyanions have a green orthochromatic fluorescence, whereas in concentrated solution there is a red or metachromatic fluorescence (Wolf and Aronson, 1961).

With AO, DNA has a characteristic greenish-yellow fluorescence but RNA is an intense red (Schummelfeder et al., 1957). The differences in fluorescence are attributed to the fact that RNA forms polymers of low molecular weight in contrast to DNA which is highly polymerized. When DNA is depolymerized with 1 N HCl, the fluorescence is similar to that of RNA. The specificity of AO for nucleic acids is confirmed by

comparative staining with gallocyanine-chromalum which has a high specificity for nucleic acids (Schummelfeder et al., 1958), and methyl green pyronin which stains DNA green and RNA red (De Bruyn et al., 1953). The same investigators postulated that the nature of the reaction was essentially a chemical one between the basic dye (AO) and the phosphate groups of the nucleic acid. The nucleoprotein complex is considered to be a salt-like combination between negatively charged phosphate groups of the nucleic acid and the positively charged groups of the protein. The chemical affinity of the dyes is due to their strong basic nature which results in displacement of the protein moiety from the nucleoprotein molecule, to the directing effect found in the steric arrangement of the groups in the basic ring structure of the dye, and to the amino groups which exert a strong positive effect. Since a resonance form exists in which there is electron sharing with the ring nitrogen, the ionic form is stabilized. Therefore 2 if not 3 of the nitrogen atoms can fit the spacing of the free phosphoric acid groups. The coplanar configuration of the AO molecule is also considered to enhance its penetration of the cell membrane. This may be one of the reasons why only AO has the uniquely specific affinity for in vivo staining of nuclei (De Bruyn et al., 1950).

The interaction of aminoacridines with nucleic acids was extensively studied employing proflavin as the model (Peacocke and Skerrett, 1956). Two distinct effects were observed; (1) a strong binding which is considered to be a direct combination of single proflavin cations with binding sites on the DNA polymer, and (2) a weak combination which is indicative of aggregates of proflavin bound to other nucleic acid sites, or the binding of more free proflavin to proflavin already bound. The combination of aggregates of proflavin with DNA to the point of electroneutrality occurs at which time one proflavin cation was bound per atom of DNA phosphorus. The initial electrostatic interaction which may also involve van der Waal forces (Schummelfeder et al., 1958; Pearse, 1960) was demonstrated by

salt effects (Peacocke and Skerrett, 1956; Schummelfeder et al., 1958; Pearse, 1960). The DNA anion in solution is considered to be a fairly rigid, highly charged structure which immobilizes sodium ions. Binding of proflavin by DNA simultaneously results in an increase in the concentration of sodium ion and conversely sodium ions added to DNA-bound proflavin cause an increase in concentration of proflavin. The salting effect of magnesium ion is much greater than that of sodium ion.

A spectrophotometrically distinct Complex (I) with an absorption peak at 465 mμ is formed as the result of reactions of AO with the polyadenylic acid bases (Beers et al., 1958) at low concentrations of fluorochrome (base to dye ratio of 1 to 1). With excess polymer (base to dye ratio of 100 to 1), Complex I is replaced by Complex II which has an absorption peak at 502 mμ associated with an intensely green fluorescence. Treatment of the polyadenylic acid with formalin blocks Complex I but not II. The addition of magnesium ion reduces the concentration of Complex II and increases Complex I concentration. Sodium hydroxide, 3×10^{-4} M, disrupts Complex I but not Complex II. With higher concentrations of sodium hydroxide, 3×10^{-3} M, Complex II is changed to the free dye in an alkaline form with an absorption peak at 440 mμ. In view of the insensitivity of Complex II to formalin and its sensitivity to magnesium, it is concluded that AO can be bound by the purine bases of polyadenylic acid as well as by the phosphate groups, although the affinity was far greater for the phosphate groups. Phosphorolysis of polyadenylic acid with polynucleotide phosphorylase results in a gradual decrease in the amount of Complex II formed. No stereospecificity is indicated toward either 3' or 5' secondary phosphates. Adenosine-5-phosphate (10^{-3} M) and adenosine diphosphate (10^{-3} M) do not form Complex II (Beers et al., 1958). While lysozyme and albumin do not form Complex I or II, methylene blue reacts with polyadenylic acid and forms analogous Complexes I and II with absorption peaks at 600 mμ and 670 mμ respectively. Acridine orange

and DNA form a complex with increased viscosity and decreased sedimentation coefficient (Lerman, 1961). These physical characteristics indicate that the AO molecules are intercalated between adjacent nucleotide layers resulting in extension and unwinding of the DNA backbone. The structural change is compatible with the normal restrictions of bond lengths, angles, and maintenance of the hydrogen bonded base pairs perpendicular to the DNA axis. The exceptionally strong interaction of amino acridines with nucleic acids is attributable to the strong cationic charge of the ring nitrogen and to the large flat area of the aromatic rings (Peacocke and Skerrett, 1956). Since the double helix is a fairly open structure, there appears to be no steric hinderance to close approach of a single amino acridine cation to the aromatic rings of the DNA molecule. It is hypothesized that only one proflavin cation is combined for every five nucleotides which corresponds to four proflavin cations per turn of the double helix.

The AO-nucleic acid reaction has been partially explained but the actual mechanism of differential fluorescence of cellular nucleic acids has not been resolved (Anderson et al., 1959). In Carnoy fixed tissues, The DNA reaction with the formation of a fairly stable fluorescing complex is almost instantaneous. The RNA reaction is not as rapid and the fluorescence of the complex diminishes with prolonged activation by UV light and is eliminated instantaneously by alcohol. Ethyl alcohol causes irreversible changes in the structure of isolated DNA as measured by the ability of the nucleic acid to induce dichroism when stained with a variety of dyes (White and Elmes, 1952). Fixatives containing picric acid or osmium tetroxide seriously impair the adsorption of the stain (Anderson et al., 1959). Fluorescence is usually brightest in specimens prepared in Carnoy's fixative (ethanol and glacial acetic acid). The many advantages of the AO fluorescent technique that account for its current popularity are (1) simplicity, (2) high level of sensitivity as compared to other cytochemical methods, (3) simultaneous differentiation of DNA and RNA, (4) specimens

may be stained by other conventional methods after first being used with AO for fluorescence microscopy (Anderson et al., 1959), and (5) the concentration of AO is low (Bertalanffy et al., 1958). This rapid procedure is used with fixed tissues, frozen sections, smears, and touch preparations (Bertalanffy et al., 1958).

The AO fluorescence technique has been applied in plant, animal, and bacterial cytology. The dye has been used as an antimalarial drug, as a bactericidal and mutagenic agent, and as a mitotic inhibitor in animal tissue cultures (Anderson et al., 1959). Fluorescence microscopy has served as a rapid and proved means of detection of cervical cancer (Bertalanffy et al., 1958; Bertalanffy, 1962). Diagnosis is based on the cytoplasmic accumulation of RNA that precedes the nuclear changes associated with malignancy.

Bright field microscopy with Feulgen and Giemsa stains can be used to determine intracellular DNA in bacteria and bacteriophage, but with AO each body emits its own light and produces better resolution. Even though T_2 bacteriophage has a head diameter of 85 mμ and approximately 2×10^{-10} μg of DNA per phage (Hershey, 1953), the fluorescent intensity of the DNA in AO stained preparations is sufficiently strong that each phage particle is a single source of light (Anderson et al., 1959).

In virus research, electron microscopy has been extensively employed to determine sites of virus replication. However, the high magnification required and the multitude of sections necessary per cell, limits this method (Morgan et al., 1958). Feulgen and methyl green pyronin stains have been employed to determine nucleic acids in tissue sections, but in monolayer cell cultures the reactions are weak or equivocal (Anderson et al., 1959).

Infectious nucleic acids have been isolated for poliovirus and Western equine encephalomyelitis (WEE) during studies of the kinetics of viral infections of cells (Wecker et al., 1962), but the procedure is

laborious. On the other hand, the AO technique can be easily and rapidly employed in cytochemical studies of viral nucleic acids in monolayer cell cultures. This method has a limit of sensitivity of about fifty closely packed poliovirus particles (Mayor and Diwan, 1961). The current vogue is to utilize AO staining with other techniques, e.g., fluorescent antibody and electron microscopy to detect nucleic acids and the size and morphology of maturated particles. The AO technique offers a particular advantage in the study of viruses or bacteriophage containing double stranded DNA. These forms of DNA are resistant to deoxyribonuclease prior to fixation, while the normal cellular DNA is not resistant. Viral DNA is susceptible to deoxyribonuclease after prior exposure to pepsin. In contrast, viral RNA cannot be distinguished enzymatically from cytoplasmic host-cell RNA (Anderson et al., 1959). The fluorescence of the single stranded DNA of ϕX-174 bacteriophage with AO is similar to the fluorescence of viral RNA (Mayor and Hill, 1961). Deoxyribonuclease disrupts the DNA strand and fluorescence does not occur. Ribonuclease is without effect.

Among the DNA viruses studied by the AO technique are vaccinia, mouse ectromelia (Armstrong and Niven, 1957), herpes (Ross and Orlans, 1958), adenoviruses (Armstrong and Hopper, 1959; Godman et al., 1960), psittacosis (Pollard and Starr, 1962), and polyoma (Mayor, 1961). The RNA viruses studied include tobacco mosaic and polioviruses (Mayor and Diwan, 1961), mengovirus (Franklin, 1962a), influenza and West Nile encephalomyelitis viruses (Anderson et al., 1959), respiratory syncytial virus (Kirsch et al., 1962), reovirus (Rhim et al., 1962) and vesicular exanthema (Hacket, 1962).

Adenovirus type 6 infection in HeLa and HEp-2 monolayer cell cultures has been studied by the AO technique (Armstrong and Hopper, 1959). The first cytological change which consisted of enlargement of the nucleoli and wrinkling of the nuclear membrane was observed at 25 hours post-infection. After 48 hours intranuclear condensation of yellow fluorescing

material formed irregular shaped masses. A red fluorescing nucleus was present on occasion. The DNA in the cytoplasm after 60 hours was of nuclear origin and was released after disruption of the nucleus. The viral nature of the DNA was confirmed by pretreatment of the specimen with pepsin and deoxyribonuclease. Enlargement of the nucleoli with increased fluorescence was the first indication of infection and was indicative of the fact that the nucleoli may have some role in viral multiplication.

Adenovirus types 5 and 6 in HeLa and HEp-2 cells produce a similar nuclear alteration (Godman et al., 1960). The possible role of the nucleoli was confirmed with P^{32} labeled Sendai virus (Zhdanov, 1962) which reached the nucleus within 10 minutes after contacting the cell. The virus was concentrated in the nucleoli 30 to 60 minutes later.

Herpes virus produces abnormal nucleoli in HeLa cells 12 hours postinoculation (Ross and Orlans, 1958) after which the nucleoli disappear and DNA accumulates near the nuclear membrane. At 16 hours small cytoplasmic lesions appear and cell boundaries lose their identity. Between 24 and 30 hours giant cells appear with intense focal DNA fluorescence.

Replication of psittacosis virus in synovial (McCoy) cells provides a unique system for the study of antimetabolites (Pollard and Starr, 1962). Within 3 hours after inoculation, the DNA psittacosis particles are detected in the cytoplasm. At 14 hours they are covered by a matrix of RNA staining material to give the appearance of a "red ball." These "red balls" enlarge, multiply, and change from red to "DNA green" during the next 24 hours. Only the DNA phase is infective. The "red ball" phenomenon occurs during the so-called eclipse period. Antibiotics and antimetabolites produce fraudulent nucleic acids.

Polyoma, a double stranded DNA virus was purified by cesium chloride isodensity centrifugation (Mayor, 1961). Droplets of purified suspensions were air dried, treated with Carnoy's fixative, and then

stained with AO. Treatment of the dried droplet with pepsin before fixation was necessary for inhibition of fluorescence by deoxyribonuclease. Ribonuclease was without effect.

Influenza was one of the first RNA viruses studied with the AO fluorescence technique (Anderson et al., 1959). The WS strain of type A influenza was cultivated in calf kidney cell cultures and changes following infection were observed. The nucleoli first became enlarged and eventually occupied the entire nucleus. Some cells had red nuclei and there were streaks of cytoplasmic fluorescence. With the MEL strain, intracytoplasmic fluorescence was predominant with little or no intranuclear involvement. With West Nile virus the major site of early infection was in the cytoplasm where RNA became more granular with increased fluorescence and by the fourth day had developed into a prominant mass. Concomitant with the formation of cytoplasmic aggregates of RNA the nucleus and nucleoli enlarged with no other cytochemical changes present.

The formation of poliovirus in monkey kidney cell cultures was characterized by numerous cytoplasmic blebs which stained positively for RNA and appeared at 8 hours postinfection. The blebs also were stained positively by the fluorescent antibody technique. Fifty closely packed particles represented the limit of sensitivity for the AO method. Purified poliovirus, 30 mμ in diameter, fluoresced the brilliant red for RNA and was susceptible to ribonuclease (Mayor and Jordan, 1962). Poliovirus from monkey kidney cell cultures grown in a medium containing AO at a final concentration of 1:200,000 emitted a green fluorescence if the preparation was not fixed. The virus was susceptible to ribonuclease. When the poliovirus was fixed prior to subsequent staining with AO, brilliant red fluorescence occurred. This indicates either surface binding of AO or limited penetration of the viral particle by the stain. Further evidence for the incorporation of AO into poliovirus was that the infective poliovirus from AO containing medium was inactivated by white light (Crouther and

Melnick, 1961). Neutral red was also incorporated into poliovirus when used in the culture medium.

Cytoplasmic RNA of reovirus was present in monkey kidney cell cultures 16 hours after infection (Rhim et al., 1962). At 54 hours a fluorescent ring surrounded the nucleus. Protein in the cytoplasm was antigenic as determined by the fluorescent antibody technique at 12 hours. The nucleus was not involved in synthesis of the virus. Virus from isodensity centrifugation also had identical staining for RNA.

Three to 5 hours after infection of L-cells with mengovirus (Franklin, 1962a), RNA had accumulated in the perinuclear area. At 6 hours the RNA was not affected by ribonuclease unless the cell was pretreated with pepsin. There were no viral antigens or intranuclear inclusions and the nucleoli disappeared at a later stage of the infection.

Formation of Syncytia in Cell Cultures

Multinucleated giant cells or syncytia may be induced by viral infection or by UV irradiation. Virus induced syncytia were first reported to occur in human and monkey kidney cell cultures infected with measles virus (Enders and Peebles, 1954). This cytopathic effect (CPE) was similar to the giant cells in nasal secretions from patients with prodromal measles (Tomkins and Macauley, 1955). Syncytia may also be observed with herpes (Farnham, 1958), mumps (Henle et al., 1954), SV_{40} (Sweet and Hilleman, 1960), parainfluenza (HA-1 or Sendai), (Zhdanov, 1962), and the respiratory syncytial virus (Morris et al., 1956).

The actual mechanism of the formation of syncytia is undetermined and may involve more than a single process or factor. The Sendai virus possesses a lipoprotease capable of lysing cell cultures within 1 hour (Zhdanov, 1962). This enzyme, also referred to as the cell wall destroying enzyme, was considered to be responsible for formation of the syncytium. With measles virus the syncytium was formed by the incorporation of

adjacent cells into a single multinucleated mass (Thomison, 1962). With the respiratory syncytial virus (RS) the production of syncytia was influenced by the basic character of the cell line used, and the composition of the nutrient medium (Jordan, 1961). Simulated syncytia associated with infection of monkey kidney cell cultures by mumps virus were independent of viral multiplication and possibly related to the hemolytic activity of mumps virus (Henle et al., 1954).

Huge syncytia and intranuclear inclusions are the cardinal CPE of measles virus in monkey and human kidney cultures (Enders and Peebles, 1954). With Enders growth medium and HEp-2 cells, characteristic CPE were present from the second to the fourteenth day (Reissig et al., 1956). Using Eagles basal medium there was cellular rounding or elongation. Only a few polynucleated cells contained less than ten nuclei. Supplementation of Enders medium with amino acids, vitamins, and glutamine greatly reduced giant cell formation. Similar differences related to the nutrient medium were also present with two strains of SV_{40} virus (Reissig et al., 1956). Using Enders medium and HEp-2 cultures infected with SV_{40} large syncytial masses were formed. There were few syncytia in cultures maintained in Eagles medium supplemented with glutamine.

Using two strains of human amnion cells (Fernandez and AV-3 strains) and HeLa cells, there was no suppression of the formation of syncytia by measles virus when growth media were supplemented with glutamine (Thomison, 1962). Small and large syncytia of the amnion cells only, were formed by fusion of two or three cells. Growth occurred by rapid incorporation of adjacent cells over a period of 24 to 48 hours. The smaller syncytium usually contained four to ten and sometimes as many as twenty-five nuclei. The larger syncytium contained more than one-hundred nuclei. Sometimes fusion with adjacent syncytia occurred. When not closely approximated to other syncytia, the large syncytium was symmetrical and its outer border resembled a normal flattened cell.

Once an adjacent single cell was incorporated into the syncytium, the relative position of the incorporated cell as determined by the position of the nucleus, remained unchanged. Although considerable cytoplasmic streaming was observed, the nuclei maintained their original positions. The progressive spread of the large syncytium suggested a cell to cell mode of virus infection. The small syncytium was thought to represent a different mode of cell infection, possibly a coalition of simultaneously infected cells. It was also suggested that the two types of syncytia formation may represent a mixed virus population.

With RS virus, the characteristic syncytial change is dependent not only on the composition of the maintenance fluid, but also on the cell line used (Jordan, 1961). Formation of syncytia was striking in HEp-2 cells, minimal in KB and HeLa cells, while rounding was characteristic of DMB cells. Scherer's solution limited the formation of syncytia and delayed CPE, particularly in the HEp-2 cells. The presence or absence of syncytia had little effect on the total yield of infectious virus.

Mumps virus produces CPE that superficially resemble syncytia (Henle et al., 1954). The CPE is not a true syncytium nor the result of virus replication, but is a toxic effect which results from the use of a concentrated inoculum. This phenomenon was characteristic of mumps virus passaged in embryonating chicken eggs and then adapted to tissue culture. However, strains recently isolated from patients and propagated only in tissue culture cells produce cytolytic lesions resembling syncytia. These CPE are thought to be associated with virus replication since dilute inocula were used.

Studies with Antimetabolites

Amino acid analogues and halogenated pyrimidines have been used to suppress the synthesis of viral protein and nucleic acid, and to inhibit maturation of viruses. Such studies are important in order to determine

the nucleic acid precursors required by the virus for replication.

Poliovirus multiplication in HeLa cells was completely inhibited by 0.06 mg per ml of para-fluorophenylalanine (FPA) if added during the eclipse period of infection (Ackermann et al., 1954). Para-fluorophenylalanine did not interfere with the adsorption and initial infection of the chorioallantoic membrane with influenza virus, PR8, but acted during the log period of the infection (Ackermann and Maassab, 1955). The inhibitory effect could be reversed by adding phenylalanine within 7 hours after infection.

Zimmermann and Shäfer, (1960), studied the effect of FPA on fowl plague multiplication. When FPA was added 1 hour after infection no detectable virus was recovered, but the virus had invaded the cell and entered into the eclipse phase. When added at $2\frac{1}{2}$ hours postinfection, FPA did not inhibit production of S antigen but did block production of the hemagglutin and the infective virus. Suppression of hemagglutin and infective virus was related directly to the time of addition of FPA.

Western equine encephalomyelitis virus was inhibited in chicken embryo fibroblast cell cultures by 600 μg per ml of FPA (Wecker and Schonne, 1961). Phenylalanine added within 5 hours after infection reversed the inhibitory effect of FPA. At 15 hours there was some reversal of inhibitory effects. It was concluded that in the presence of FPA new cellular enzymes were not necessary to synthesize RNA.

Interference with the multiplication of psittacosis virus by FPA was studied in cell cultures by Tanami and Pollard (1962). When FPA, 100 μg per ml, was added to cultures not later than 15 hours after infection, intracellular maturation of the virus was suppressed. After 15 hours, FPA did not prevent production of infective virus, thus indicating that the FPA-sensitive viral precursor had already been synthesized (Tanami and Pollard presume the viral precursor to be protein). Although the viral protein may be the affected precursor, it is important to note that

psittacosis virus replication is mediated through RNA (Pollard and Starr, 1962) and FPA may well suppress RNA synthesis.

Wecker et al. (1962), assayed HeLa cell cultures infected with poliovirus or WEE for infectious nucleic acid, viral protein (fluorescent antibody technique), and infective virus. In the presence of FPA at 5 to 10 μg per ml, infectious poliovirus RNA and viral protein synthesis were only 40 per cent of the controls. With FPA at 25 μg per ml, maturation of infective virus was completely inhibited, and infectious RNA was 5 per cent of the controls. Specific viral antigens were no longer detected at 125 μg per ml FPA. Similar results were obtained with WEE infected cultures and FPA at 600 μg per ml. These findings indicate an inverse relation between FPA and viral RNA and protein. The inhibitory effect of FPA may be due to any or all of the following; (1) FPA is incorporated into the protein making it nonfunctional (Zimmerman and Shäfer, 1960), (2) FPA inhibits the synthesis of proteins (Tanami and Pollard, 1962), and (3) FPA can inhibit the synthesis of viral RNA as well as viral protein (Wecker et al., 1962).

Halogenated pyrimidines may be used to determine virus nucleic acid type. The principle differentiation rests on the premise that a DNA intermediate is not a prerequisite for the replication of RNA animal viruses (Simon, 1961). Aminopterin, 10 μg per ml, did not affect replication of Newcastle Disease virus (NDV) or poliovirus, but reduced the replication of vaccinia virus from 73 to 0.1 plaque forming units per cell (Simon, 1961). Aminopterin, 25 μg per ml, interfered with replication of psittacosis virus beyond the "red ball" phase (Pollard and Starr, 1962). Messenger RNA synthesis was not arrested but the psittacosis virus DNA synthesis was completely inhibited.

Halogenated pyrimidines can replace up to 36 per cent of the nucleic acid uracil when poliovirus replicates in the presence of 5-fluorouracil (FU), (Munyon and Salzman, 1962). There were no differences between

poliovirus containing FU and controls in terms of protein composition, infectivity of purified virus, and plaque morphology. Such results suggest that the introduction of FU as a base in poliovirus RNA does not alter its function as the template for synthesis of poliovirus protein.

Whereas FU did not affect the maturation of an RNA virus, it did inhibit the maturation of psittacosis virus (Pollard and Starr, 1962). With FU, the fraudulent RNA produced, inhibited further DNA synthesis. The modified RNA cycle of psittacosis progresses with 5-fluoro-2'-deoxyuridine, but the formation of fraudulent DNA impedes the maturation process.

Interferon

According to Ho (1962);

> Interferon is a type of viral inhibitor receiving increasing attention in the last 5 years. It is unique in that it is a by product of cell-virus interaction and frequently of active virus infection both in vivo and in vitro. It increases resistance of cells by inhibiting intracellular replication. Its exact mechanism of action is unknown but may be through influence on cellular energy metabolism. It is rarely effective, however, at high dilutions, and there is some question of the specificity of its action.
>
> Interferon has been described as an intermediary of certain types of virus interference. It has a role of masking the presence of virus in viral suspensions of high concentrations and in certain attenuated in vitro infections. It may play a part in the recovery and viral prevention in the host. It is being seriously considered as an antiviral therapeutic agent.

Interferon is present in cultural medium collected following inoculation of host cells with infective or noninfective virus. Primary cell cultures are usually employed as continuously cultured cells generally do not produce interferon. The active component in crude interferon preparations cannot be identified either antigenically or morphologically. It cannot be neutralized by specific antiviral serum or be sedimented by ultracentrifugation, and it is not self replicative (Ho, 1962). While specific antiviral

serum will neutralize infective virus but not the infective nucleic acid form of the same virus, interferon can act against infective viral nucleic acid (De Somer et al., 1962). Interferon possesses many properties characteristic of proteins. It is nondialyzable and is inactivated by trypsin. Interferon appears to render cells resistant to viral infection. The degree of resistance depends upon the concentration of virus employed as it is possible to overwhelm the resistance by a massive inoculum (Ho, 1962). Interferon acts within the cell after penetration of the virus and before the formation of mature virus particles. The activity of interferon may be measured by (1) hemagglutination titer of the virus, (2) CPE titer of virus, (3) zone protection on monolayer cultures, and (4) per cent of plaque forming units inhibited (Balducci et al., 1962).

MATERIALS AND METHODS

Viruses

Infectious Bronchitis Virus. The Beaudette strain, adapted to CEKC (Spring, 1960) was used. The virus is identified by the North Central Repository Code Number, and the number of cell culture passages, e.g., IBV42-118C indicates the one hundred eighteenth passage in CEKC. Cultures of confluent 24 hour old CEKC in 30 ml Falcon tissue culture flasks were used to propagate IBV42-117C and IBV42-118C. The cultures were inoculated with 0.5 ml of undiluted IBV42-116C and IBV42-117C. The extracellular fluid was collected at 40 hours, pooled, dispensed in 4 ml portions into 2 dram screw cap vials, and stored at -60 C.

Influenza Virus. A culture of the PR8 strain of influenza virus maintained in mouse lung was propagated in CEKC the same as IBV42-118C. After four passages, the virus caused characteristic CPE. The extracellular fluid from the sixth passage in CEKC, identified as PR8-6C, was used.

Preparation of Chicken Embryo Kidney Cells

The kidneys from 16 or 17 day-old chicken embryos were removed aseptically and washed in Hanks Balanced Salt Solution (BSS) in a sterile petri dish. The kidneys were minced into pieces approximately 0.5 to 1.0 mm^3 and then washed twice with BSS. The minced pieces were then transferred to a 500 ml fluted Erlenmeyer flask containing a "Teflon" covered magnet, and 0.25 per cent trypsin (Difco, 1:250), in BSS, pH 8.0 to 8.2, warmed to room temperature, was added. For each pair of kidneys 10 ml of trypsin was used. The flask was placed over a "Magnestir"

and after trypsinization at 37 C for 30 to 40 minutes, the cell suspension was filtered through four layers of sterile gauze. After centrifugation of the cells at 437 x G for 5 minutes at 4 C, the supernatant fluid was decanted and the packed cells were resuspended in BSS. The procedure was repeated again and the packed cells were diluted 1:200 in growth medium. Fifteen embryos were usually sufficient to produce enough kidney cells to utilize 150 ml of growth medium. The growth medium, pH 7.2, contained 0.5 gm lactalbumin hydrolysate, 5.0 ml of calf serum, 25,000 units of penicillin and 0.5 mg of dihydrostreptomycin in 100 ml of BSS. Leighton tubes, 16 by 125 mm, with and without number 1 glass coverslips (11 by 22 mm), were seeded with 0.9 ml of the cell suspension per tube and sealed with a white rubber stopper. The cell cultures were incubated for 18 to 24 hours at 37 C at which time the monolayers were 75 to 100 per cent confluent. The growth medium was replaced with medium at pH 7.4 to 7.6 and the monolayers were ready for use. Incubation of all cultures was at 37 C for the entire study.

Titration of IBV42 in Chicken Embryo Kidney Cells

Serial tenfold dilutions of the virus were prepared using BSS containing 0.5 per cent lactalbumin hydrolysate as the diluent (Cunningham, 1960). All ingredients were kept in an ice bath during the preparation of the dilutions to prevent possible thermal inactivation of the virus. Each dilution of the virus was used to inoculate three cell cultures, 0.2 ml per culture. The cultures were incubated at 37 C for 48 hours and examined microscopically for CPE. The end point for viral activity was considered to be the highest dilution of virus which produced CPE in two of the three cell cultures.

Acridine Orange Staining Technique

The AO staining technique of Livingston and Moore (1962) was used. Cell cultures on coverslips were fixed in freshly prepared, cold, Carnoy's fixative (2 parts glacial acetic acid and 1 part ethanol). The coverslips were then washed three times, 5 minutes each in citrate-phosphate buffer, pH 3.6 to 3.8, and stained for 5 minutes in 0.01 per cent AO in citrate-phosphate buffer. Excess stain was drained from the coverslips. One drop of buffer was added and the coverslip was mounted on a slide. The edges were sealed with nail polish. Fixed and unfixed cultures were treated with 5 x crystalline ribonuclease (Nutritional Biochemical Corp., Cleveland, Ohio). The coverslips were then washed in buffer, fixed as necessary, and prepared as above for staining.

Fluorescence Microscopy

An American Optical Company, "Fluorestar," microscope with a vertical monocular body, a Mer-Arc Illuminator (Nems Clarke Co.) and power unit, an Osram HBO-200 mercury arc lamp, a heat absorbing filter, and a Corning 5840 exciter filter (55 per cent transmission of the UV rays at a wave length of 365 mμ) were used. A Wratten 2B barrier filter was inserted in the eyepiece. Kodak Super Ektochrome was used with exposure times ranging from 2 to 3 minutes.

Multiplication of IBV42 in Chicken Embryo Kidney Cells

Cumulative released virus. Each of thirty-six cultures in Leighton tubes without coverslips and twelve in Leighton tubes with coverslips were inoculated with 0.2 ml of virus. Twelve uninoculated cultures with coverslips were retained as controls. After adsorption had occurred for 30 minutes, three cultures without coverslips and one culture with coverslip were randomly selected and the extracellular fluids were harvested,

pooled, placed in a 2 dram screw cap vial, and stored at -60 C. The remaining inoculated cultures were washed five times with BSS to remove unadsorbed virus, 1.0 ml of fresh growth medium was added, and the cultures were reincubated. The three cultures without coverslips were then washed five times with BSS. The fifth washings were pooled and stored at -60 C. To each of these cultures, 0.5 ml of growth medium was added. The cultures were frozen and thawed three times in a dry ice-ethanol mixture, transferred to a chilled Tenbroeck tissue grinder, ground for 5 minutes, and stored at -60 C in a 2 dram vial. These preparations were considered to be the zero hour samples. At 1, 2, 3, 4, 5, 6, 12, 24, 36, 48, and 72 hours later, three cultures without coverslips and one with coverslip were processed as above. The remaining inoculated cultures were incubated with no further washing. Titrations were made with cultures grown from the same cell suspension. The coverslip was removed from the single Leighton tube and the cells were stained with AO. A single uninoculated culture with coverslip was stained simultaneously with AO.

Interim released virus. Each of thirty-six cultures in Leighton tubes without coverslips and twelve in Leighton tubes with coverslips were inoculated with 0.2 ml of virus. Twelve uninoculated cultures with coverslips were retained as controls. After adsorption had occurred for 30 minutes, three cultures without coverslips and one culture with coverslip were randomly selected and the extracellular fluids were harvested, pooled, placed in a 2 dram screw cap vial, and stored at -60 C. The cultures without coverslips were processed similarly to the cultures in the cumulative released virus studies. The coverslip was removed from the single Leighton tube and the cells were stained with AO. The extracellular fluid from the remaining cultures was removed and discarded. The cultures were washed five times with BSS, 1.0 ml of fresh growth

medium was added, and the cultures reincubated. The procedure was repeated at 1, 6, 12, 24, 36, 48, and 72 hours. At each sampling time the remaining inoculated cultures were washed five times with BSS, 1.0 ml of fresh growth medium was added, and the cultures reincubated. The coverslip was removed from an uninfected culture and the cells were stained with AO simultaneously with the coverslip from an infected culture.

Antimetabolites

DL-p-fluorophenylalanine, 5-bromouracil, and 6-azauracil (California Corporation for Biochemical Research, Los Angeles, Calif.), were prepared in final concentrations of 500 μg per ml and 6,000 μg per ml in sterile distilled water. To groups of eight cultures without coverslips and four with coverslips, 0.1 ml of the antimetabolite solution was added. The final concentration was either 50 μg per ml or 600 μg per ml per culture. The cultures were incubated for 12 hours after which each culture was inoculated with 0.1 ml of virus. At 12, 24, and 36 hours postinoculation, two cultures without coverslips and one with coverslip were pooled and stored at -60 C. The coverslip culture was stained with AO. Infectivity titrations were made of the pooled extracellular fluids. Four additional cultures with and without coverslips were used for the two concentrations of fluorophenylalanine and were inoculated. After 24 hours extracellular fluids were removed and discarded and the cultures were washed three times with BSS. New growth medium containing 100 μg per ml of phenylalanine was added. At 36 and 48 hours two cultures without coverslips and one with coverslip were selected. The extracellular fluids were pooled and stored at -60 C for later infectivity titration. The coverslip culture was stained with AO.

Ether Sensitivity

A mixture of 4 parts of virus and 1 part of ether in a screw cap vial was incubated at 4 C for 12 hours. The ether layer was removed with a Pasteur pipette. The cap was loosened and the vial placed in a vacuum dessicator jar. Air was evacuated before the jar was sealed and stored at 4 C for 12 hours. The preparation was then removed and titrated for infectivity. Virus controls not exposed to ether were treated in the same manner.

Ultraviolet Light Sensitivity

Approximately 4 ml of virus were dispensed into each of three glass petri dishes without covers 24 inches directly below a Letha Ray (Hanover Chemical and Mfg. Co., Newark, New Jersey) UV light. At intervals of 1, 3, and 5 minutes the covers were replaced and the virus was immediately tested for infectivity.

Interferon

Extracellular fluids from virus infected cultures and normal cultures in plastic tissue culture flasks were harvested after 40 hours. The fluids were pooled from several flasks, centrifuged at 437 x G for 15 minutes to remove cellular debris, and then heated at 56 C in a water bath for 20 minutes. The heated fluids were diluted 1:1 with growth medium. One ml portions were placed in cultures from which growth medium was removed, and then reincubated for 12 hours. The fluids were removed, the cultures were washed twice with BSS, and fresh growth medium was added. Serial tenfold dilutions of PR8-6C were used to inoculate both groups of cultures. Heat inactivated IBV42, 6.0 ml, was treated with 3 ml of 0.25 per cent trypsin for 60 minutes at 37 C. Two ml of 1 per cent eggwhite trypsin inhibitor and 1 ml of calf serum were then added to the mixture.

The mixture was added to cultures for a 12 hour period at 37 C before PR8 challenge. Heat inactivated virus was also treated with ribonuclease at a final concentration of 150 μg per ml for 60 minutes at 37 C. This mixture was added to cultures for 12 hours before PR8 challenge.

PR8-6C was specifically selected as the challenge virus because of its well defined and characteristic CPE. In addition, since PR8 does not form syncytia in CEKC cultures, the appearance of syncytia would indicate viable IBV42 resulting from inadequate heat inactivation.

RESULTS

Multiplication of IBV42 in Chicken Embryo Kidney Cells

Following inoculation of cultures there was a 4 hour period of eclipse of virus followed by a logarithmic increase in both released virus (RV) and cell associated virus (CAV) (Figure 1). The period of greatest virus release, RV of 10^3 per 0.2 ml, occurred between the fifth and sixth hour. The maximum RV, 10^6, and CAV, 10^5 per 0.2 ml, occurred at 36 and 24 hours, respectively. During the next 36 hours the RV decreased to 10^3 and CAV to 10^2.

The interim virus release studies (Figure 2), evinced greatest RV, 10^3, within 6 hours. Maximum RV, 10^5, and CAV, 10^4, was observed at 24 hours. During the next 36 hours there was a decrease in both RV and CAV to 10^1.

Cytopathic effects of degeneration and necrosis were present in unstained, infected cultures as early as 24 hours. The most significant cytological change was the formation of syncytia. Syncytia containing 4 to 6 nuclei were present as early as 6 hours. At 8 hours (Figure 4), the syncytia had enlarged and contained 10 to 12 nuclei, at 16 to 18 hours, 20 to 24 nuclei (Figure 5), and at 18 to 24 hours, 80 to 100 or more nuclei were present (Figure 7). Prior to the twenty-fourth hour viral RNA could not be detected by the AO technique in the cytoplasm of syncytia or individually infected cells. Between the twenty-fourth and thirty-sixth hours there was a progressive increase of viral RNA in the cytoplasm followed by disruption of the syncytia (Figures 8, 9, 10). After 36 hours, syncytia were only occasionally present, some as late as 53 hours. Infected single cells containing RNA (Figures 11, 12, 13), were most

readily detected after 36 hours when the syncytia had disrupted and there were not enough cells adjacent to each other to form syncytia. The viral RNA in syncytia and single cells was susceptible to and removed by ribonuclease after fixation with Carnoy's fixative. Prior to fixation the RNA was resistant to ribonuclease with few exceptions. Results with PR8 virus, which was used as the model and control for specificity of the AO staining, confirmed the results with IBV42-118C.

The formation of syncytia was not affected by the components of the medium in which the cells were propagated. Use of BSS with lactalbumin hydrolysate, Eagle's basal medium (Eagle, 1955), and M-199 (Morgan et al., 1950) incorporating amino acids, vitamins, and L-glutamine (Eagle, 1955), singularly or in combination, had no effect on the formation of syncytia.

Antimetabolites

The effect of FPA was to partially suppress the production of RV, as the maximum titer of virus from cultures with FPA was less than that from cultures without FPA. At 12 hours postinoculation, the RV titer in control cultures was 10^4, and at 36 hours was 10^6. With 50 μg per ml of FPA, the RV titer was 10^4 at 12 hours through 36 hours. At a concentration of 600 μg of FPA per ml, the RV titer was 10^3 at 12 hours and 24 hours, and 10^2 at 36 hours (Figure 15). The formation of syncytia and viral RNA was not suppressed by the 50 μg of FPA per ml but was inhibited by 600 μg per ml of FPA.

Infected cultures containing FPA were washed twice with BSS at 24 hours, and 1,000 μg of phenylalanine (PA) per ml was added. No significant changes in virus release occurred. Cultures originally incubated with FPA at 50 μg per ml before the addition of PA had RV titers of 10^4 at 36 hours and 10^3 at 48 hours. These results are similar to those for cultures with FPA at 50 μg per ml but which did not have PA added.

The RV titer of cultures incubated with FPA at 600 μg per ml prior to the addition of PA was 10^2 at 36 hours, the same as the cultures without PA, but the RV increased to 10^3 by 48 hours. In the latter group, although there was a 10^1 increase in RV titer between the thirty-sixth and forty-eighth hour, there were no syncytia. Phenylalanine at 1,000 μg per ml did not suppress the formation of syncytia in infected cultures.

With 5-bromouracil and 6-azauracil at 50 or 600 μg per ml, there was no effect on the production of RV or the formation of syncytia. Aminopterin, 10, 20, and 30 μg per ml, also had no effect on the production of RV or the formation of syncytia. The cytotoxicity of aminopterin was minimal and did not interfere with the formation of syncytia.

Ether Sensitivity

After exposure to ether for 24 hours at 4 C, the titer of the virus was 10^1 as compared to 10^5 for the control.

Ultraviolet Light Inactivation

The virus was completely inactivated by ultraviolet irradiation within 1 minute.

Interferon

There was a 10^2 reduction in the titer of the released PR8-6C when heat inactivated IBV diluted 1:1 in growth medium was added to cultures 12 hours prior to inoculation with challenge virus. Heat inactivated IBV diluted 1:1 and added to the cultures for 1 hour prior to inoculation with PR8-6C had no effect on the release of virus. The heat inactivated virus was treated with trypsin for 1 hour prior to its addition to cultures 12 hours before inoculation with PR8-6C. No effect on virus release was

observed. There was a 10^2 reduction in PR8-6C release when heat inactivated virus, treated with ribonuclease for 1 hour, was added to cultures for 12 hours and infected with PR8-6C (Table 1).

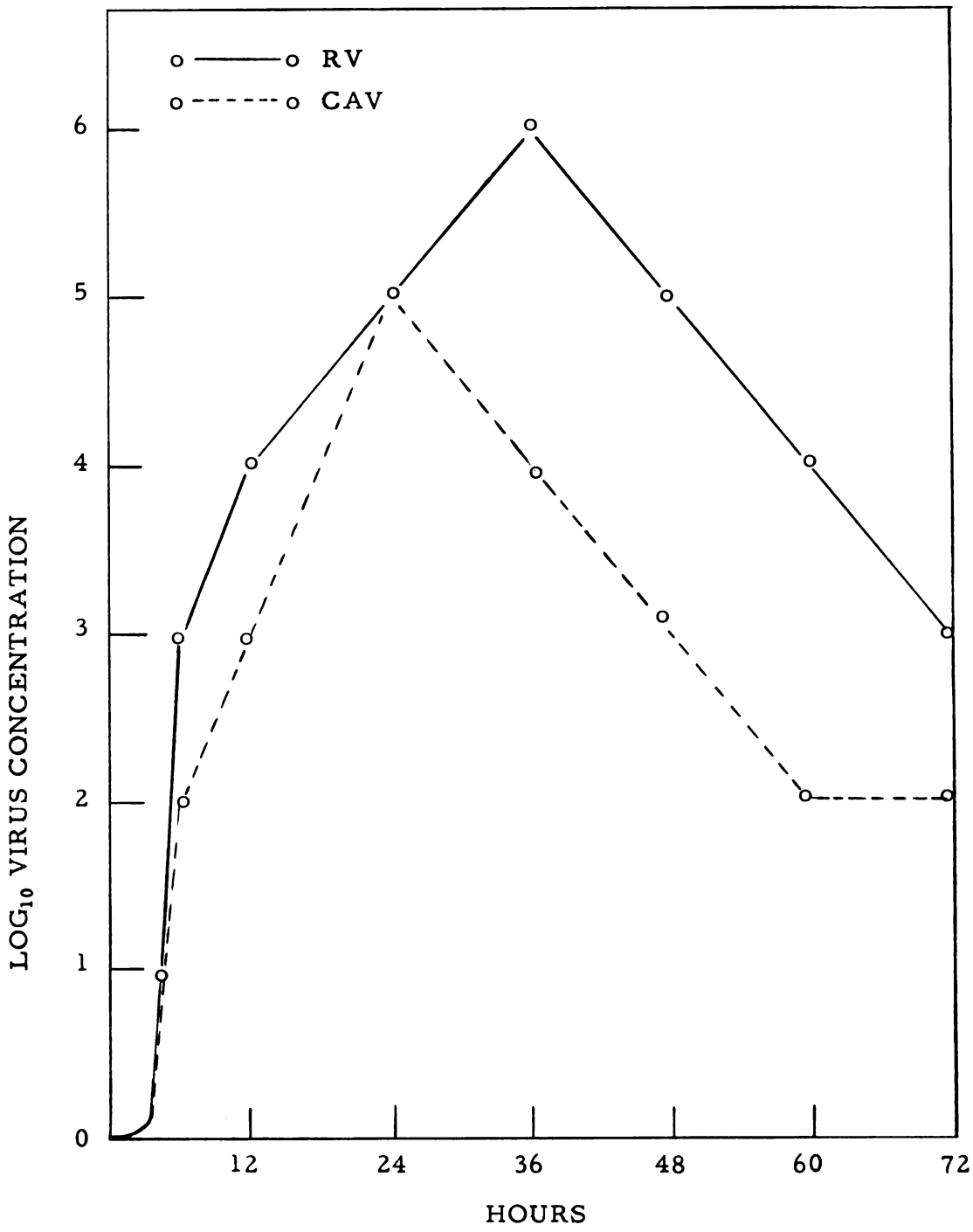


FIG. 1. Cumulative cell associated (CAV) and released (RV) IBV42-118C in chicken embryo kidney cell cultures at 37 C.

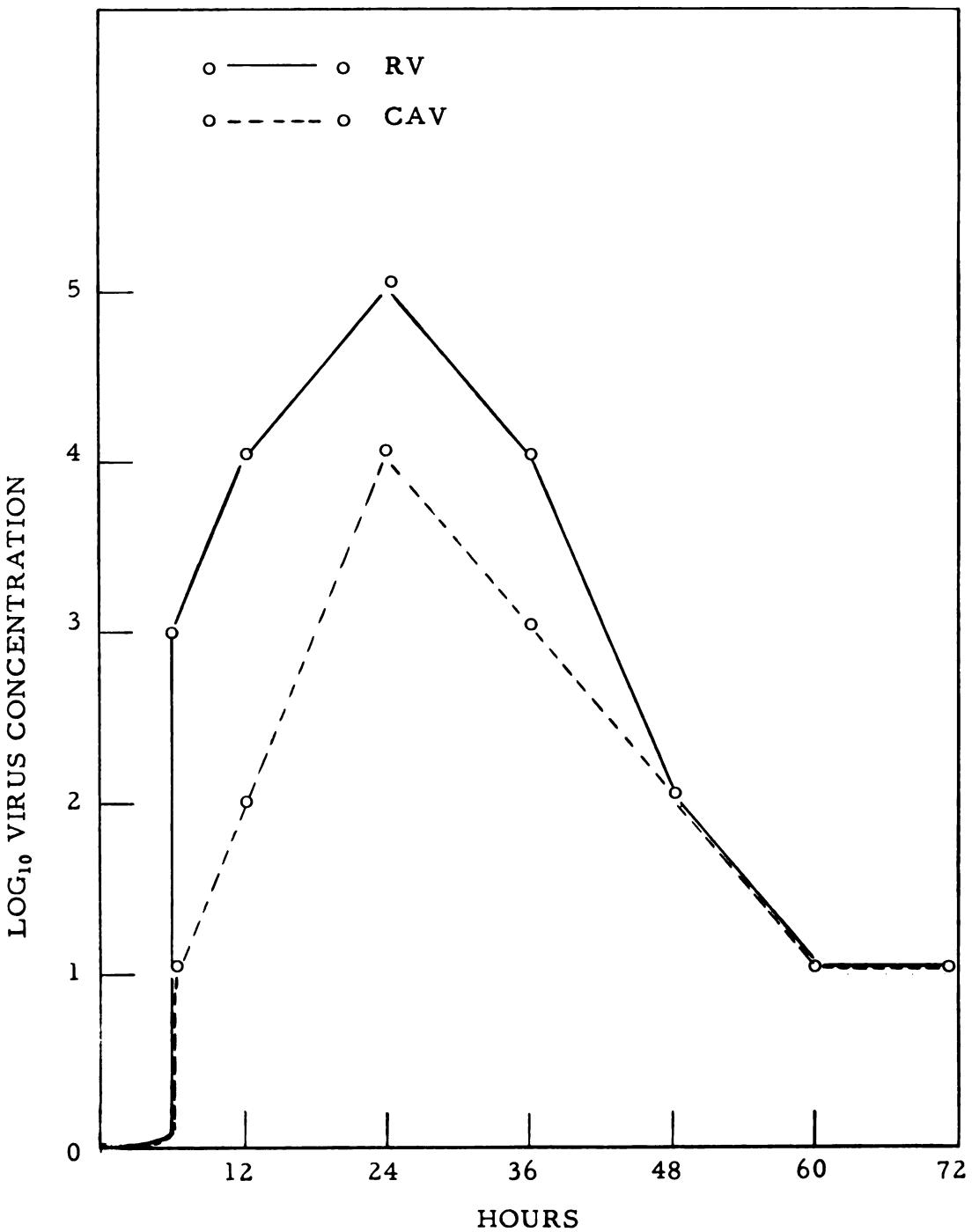


FIG. 2. Interim cell associated (CAV) and released (RV) IBV42-118C in chicken embryo kidney cell cultures at 37 C.

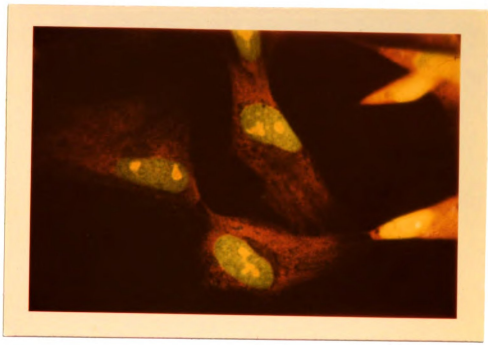


FIG. 3. Normal chicken embryo kidney cells treated with Carnoy's fixative and stained with acridine orange. The red in the nucleoli and cytoplasm indicates ribonucleic acid. Nuclear deoxyribonucleic acid is green. Magnification 970 X, enlargement 6 X.

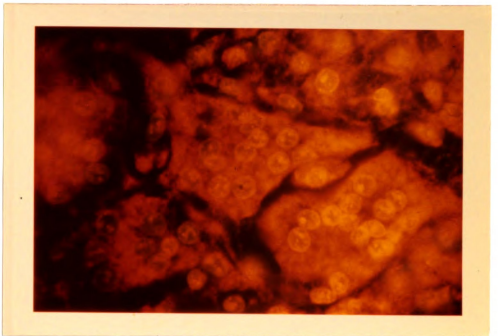


FIG. 4. Chicken embryo kidney cells 8 hours after infection with IBV42-118C treated with Carnoy's fixative and stained with acridine orange. The syncytia contain 10 to 12 nuclei. Magnification 430 X, enlargement 6 X.

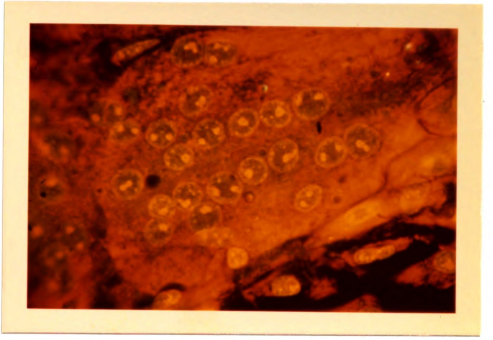


FIG. 5. Chicken embryo kidney cells 16 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. Magnification 485 X, enlargement 6 X.

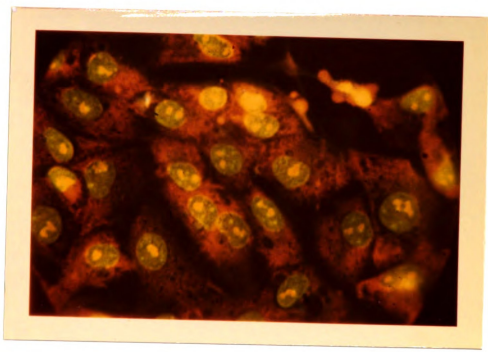


FIG. 6. Normal chicken embryo kidney cells, 24 hour controls, treated with Carnoy's fixative and stained with acridine orange. Magnification 485 X, enlargement 6 X.

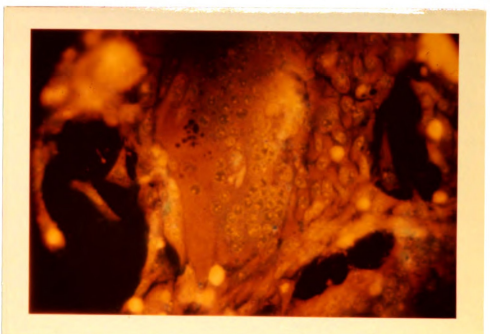


FIG. 7. Chicken embryo kidney cells 24 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. Magnification 215 X, enlargement 6 X.

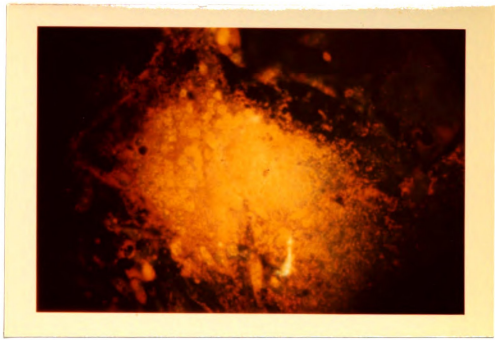


FIG. 8. Chicken embryo kidney cells 30 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. Syncytia with more than 100 nuclei are present. Magnification 215 X, enlargement 6 X.

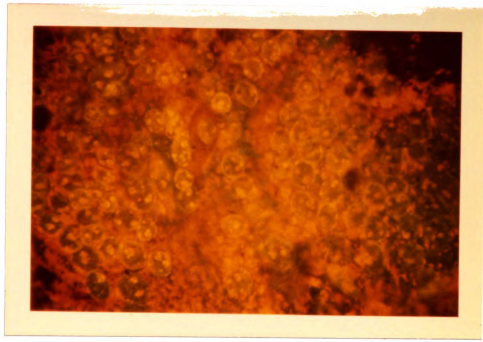


FIG. 9. Chicken embryo kidney cells 30 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. Magnification 430 X, enlargement 6 X.

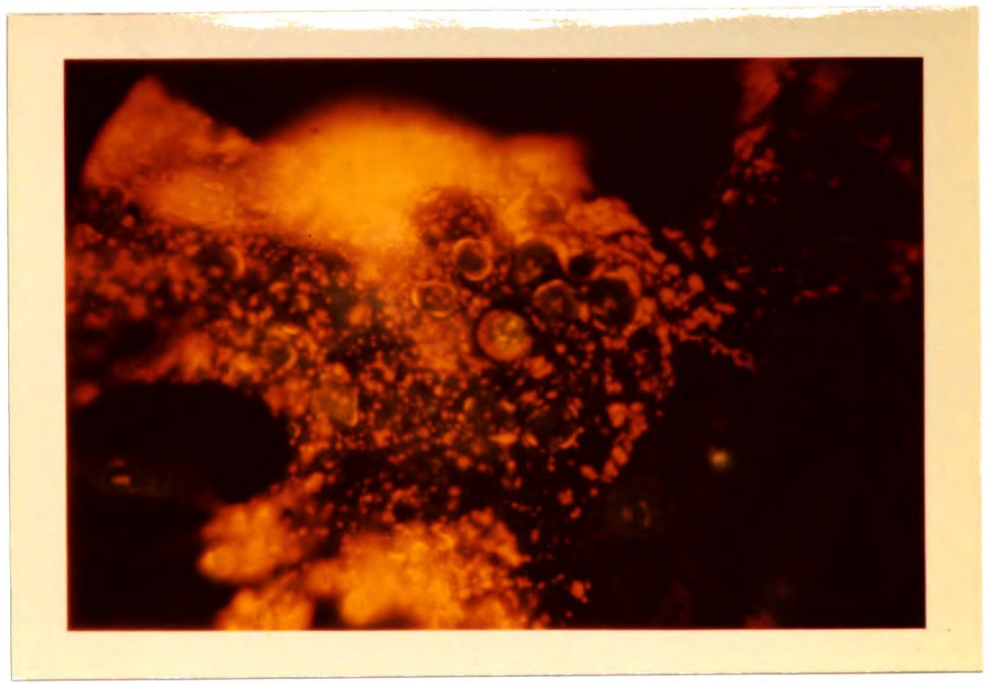


FIG. 10. Chicken embryo kidney cells 30 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. Ribonucleic acid aggregation prior to syncytial disruption is present. Magnification 430 X, enlargement 6 X.

FIG. 11. Chicken embryo kidney cells 48 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. A single infected cell with cytoplasmic ribonucleic acid aggregates is shown. Magnification 485 X, enlargement 6 X.

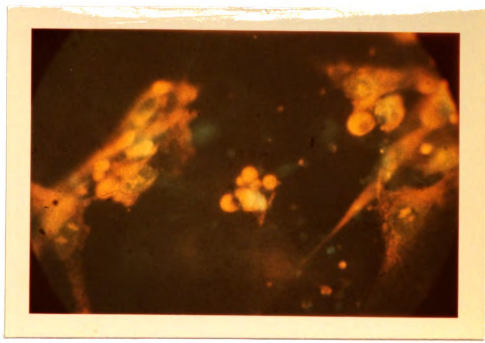


FIG. 12. Chicken embryo kidney cells 48 hours after infection with IBV42-118C, treated with Carnoy's fixative, and stained with acridine orange. Magnification 485 X, enlargement 6 X.

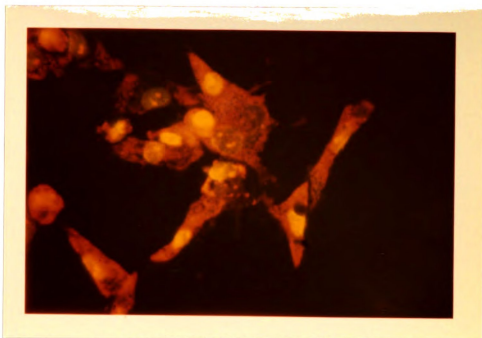


FIG. 13. Chicken embryo kidney cells 60 hours after infection with IBV42-118C, treated with Carnoy's fixative and stained with acridine orange. A single cell containing ribonucleic acid aggregates is shown. Magnification 485 X, enlargement 6 X.

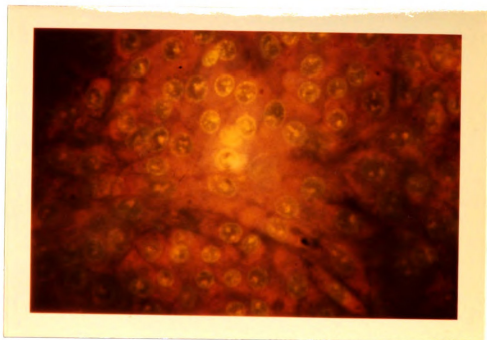


FIG. 14. Normal chicken embryo kidney cells, 60 hour control, treated with Carnoy's fixative and stained with acridine orange. Magnification 430 X, enlargement 6 X.

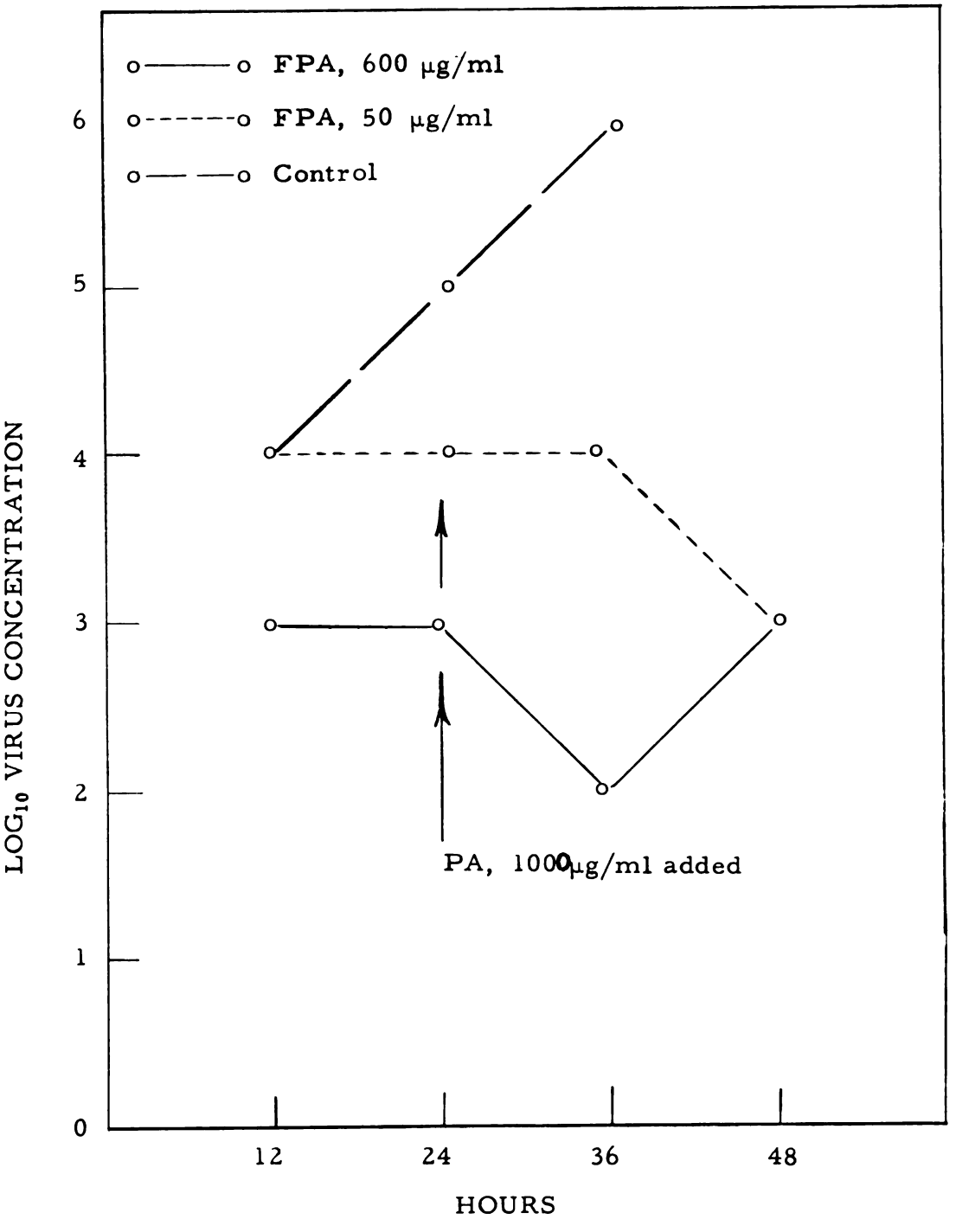


FIG. 15. The effect of DL p-fluorophenylalanine (FPA) and of phenylalanine (PA) on the multiplication and release of IBV42-118C in chicken embryo kidney cell cultures at 37 C. Cultures incubated with FPA 12 hours prior to inoculation with virus, 10^5/0.1 ml.

TABLE 1. Multiplication of PR8-6C in chicken embryo kidney cell cultures to which heat inactivated IBV42-118C was added prior to inoculation.

Preparation[1]	Hours prior to inoculation	PR8-6C titer at 48 hours
None	0	10^3
Cultural medium from cells after incubation for 48 hours	12	10^3
IBV42-118C	1	10^3
IBV42-118C	12	10^1
IBV42-118C treated with trypsin[2]	12	10^3
IBV42-118C treated with ribonuclease[2]	12	10^1

[1]All preparations used were heated at 56 C for 20 minutes.

[2]Incubated with enzyme for 60 minutes at 37 C after heating at 56 C for 20 minutes.

DISCUSSION

The multiplication cycle of IBV42-118C in CEKC includes the eclipse, logarithmic growth, stationary, and decline phases. The 4 hour eclipse phase of the virus is comparable with the myxoviruses, longer than the 2 hour eclipse phase of Eastern equine encephalomyelitis, and shorter than the 9 hour eclipse reported for the RS virus. The initial multiplication cycle of IBV42-118C commences between 4 and 5 hours postinoculation. There is then a sudden increased multiplication rate resulting in a virus titer of 10^3 at 6 hours and followed by exponential increases of virus titer thereafter. A similar phenomenon was reported with poliovirus (Wecker et al., 1962). Decline of RV is exponential between 36 and 72 hours and indicates completion of the cycle.

Syncytia appear during the initial multiplication cycle. Increase in size of syncytia and number of nuclei parallel the increase of RV and CAV. The RV and CAV declined with the disappearance of syncytia. These results indicate that the formation of syncytia is associated with virus replication. This relationship is further confirmed by the fact that IBV neutralized by specific antibody and inactivated by heat or UV irradiation does not produce syncytia or infectious virus. The formation of syncytia is not only a property of IBV cultivated in CEKC as they are also produced by IBV42 in allantoic fluid from embryonating chicken eggs (Cunningham and Spring, 1963). Syncytia are produced by end point dilutions of infective IBV42 and are not related to a cytolytic factor. With mumps virus (Henle et al., 1954) syncytia are produced only with concentrated virus.

Using AO stain, viral RNA aggregates could be detected in the syncytial cytoplasm within 24 hours. The aggregates were susceptible to ribonuclease after fixation. Viral RNA was not detected earlier than 24 hours in either single cells or syncytia even though virus multiplication was exponential. It is concluded that there were not enough total particles or viral aggregates to react with the fluorochrome dye prior to 24 hours. The AO staining technique is not as sensitive as the fluorescent antibody technique. Using the fluorescent antibody technique with RS virus in HEp-2 cells, Bennet and Hamre (1962) reported that fluorescence of single cells occurred 14 hours prior to fluorescence of syncytia. Specific fluorescent antibody reactions precede AO fluorescence of Reovirus by at least 6 hours (Rhim et al., 1962). Stultz (1962), using the fluorescent antibody technique with IBV42 reported the appearance of single infected cells and of syncytia 12 to 20 hours postinfection.

Fluorescence of viral RNA with AO and the absence of fluorescence characteristic of DNA is presumptive evidence for IBV42 classification as an RNA virus. The RNA character of IBV42 is further confirmed by the experiments in which amino acid analogues and inhibitors of nucleic acid synthesis were used. DL p-fluorophenylalanine (FPA) is known to suppress viral or messenger RNA synthesis. Viral RNA synthesis of Eastern and Western encephalomyelitis, foot and mouth disease virus, poliovirus, and certain myxoviruses was suppressed by FPA. Messenger RNA synthesis was suppressed when FPA was added to cultures of psittacosis virus. A virus which is inhibited by FPA can be assumed to be either an RNA virus or a virus in which a messenger RNA cycle is operable. Low concentrations of FPA inhibit maturation of infective virus but do not suppress the synthesis of infective RNA. Typical CPE may be present, but little or no infective virus is released. Higher concentrations of FPA suppress infective nucleic acid synthesis, infective virus maturation, and CPE. Results consistent with the above were obtained

with IBV42-118C. At low concentrations of FPA, syncytia were produced but the titer of RV was reduced. At high concentrations of FPA the formation of syncytia was inhibited. The reduction in the titer of the RV is a reflection of suppression of the multiplication cycle. The addition of phenylalanine (PA) reverses the effects of high but not low concentrations of FPA. It is concluded that at the low concentration of FPA, viral nucleic acid and protein synthesis occur in the syncytia, but the maturation process is not completed. The addition of PA has little effect as the alterations induced by FPA have rendered the syncytia unsuitable for further virus replication. When PA was added there was some reversal of the effect of FPA at high concentration on infected cultures. At high concentrations of FPA, the eclipse of virus is maintained with little or no detrimental effects on the host cell. The addition of PA permits the multiplication cycle to continue. The use of FPA indicates that the formation of syncytia is one characteristic of IBV42-118C and that the virus is either a RNA virus or is dependent on messenger RNA.

There were no differences observed between poliovirus with and without incorporated 5-fluorouracil (Munyon and Salzman, 1962). Infectious bronchitis virus, strain 42-118C was not inhibited by 5-bromouracil or 6-azauracil, and it was concluded that the synthesis of RNA was not altered. However, since Simon (1961) reported that synthesis of DNA by vaccinia is unaffected by 5-bromouracil, the results with IBV42-118C are inconclusive.

Aminopterin (4-aminopteroyl-L-glutamic acid) is an inhibitory analogue of folic acid and suppresses the conversion of pteroyl-L-glutamic acid to its "functional" derivatives. By interfering with the formation of cofactors required in formylation reactions, the conversion of deoxyuridine to thymine is impaired (Fruton and Simmonds, 1960). As a result, the DNA cell cycle is suppressed and viral DNA synthesis

is blocked. Propagation of vaccinia and psittacosis, DNA viruses, is inhibited by aminopterin. Newcastle disease virus and poliovirus, which are RNA viruses, are not affected by aminopterin. Since there was no inhibition of multiplication of IBV42-118C in cell cultures with medium containing aminopterin, it is concluded that viral replication is independent of cellular DNA.

On the basis of the results with AO staining, the inhibitory effects of FPA, and the lack of inhibition by aminopterin, IBV may be classified as an RNA virus.

The sensitivity of IBV42-118C to ether indicates the presence of lipid or lipoprotein on the periphery of the virus which either serves to hold the capsomeres together, as with influenza virus, or to form an envelope as with herpes virus. Viruses with lipids in the periphery are known to be assembled near the cell membrane which in turn is the source of viral lipoprotein (Franklin, 1962b). It may be assumed that IBV42 possesses a lipoprotein and is assembled in the peripheral area of the host cell. This conclusion is supported by the results from multiplication studies in which RV and CAV were simultaneously determined.

According to Franklin (1962b), "as a rule of thumb, if CAV/RV<1, then virus is usually formed at the periphery. If $1<CAV/RV\leq100$, then virus is either formed at the periphery and stored on the external surface or formed elsewhere. If CAV/RV>100, then the virus is usually not formed at a cell surface, peripheral, or otherwise." The CAV/RV ratios reported for some ether sensitive viruses are as follows: fowl plague, 10 (Franklin and Henry, 1960), measles, 1 (Rapp et al., 1960), NDV, 1 or less (Rubin et al., 1957), and arbor viruses, less than 0.1 (Rubin et al., 1955). Ether resistant viruses such as the enteroviruses, adenoviruses, and poxviruses have CAV/RV ratios of 100 or greater. Such high ratios indicate that virus assembly does not occur near the cellular membrane, and that most of the infective virus is completely

assembled before being released. The release mechanism is rapid and characteristic of either a lytic process or budding off of virus-filled blebs (Franklin, 1962b).

The CAV/RV ratio for IBV42-118C was less than 1 during the period of exponential propagation. This ratio is similar to that of ether sensitive myxoviruses or the measles group of viruses. It is also significant that the syncytia continue to increase both in size and nuclei count simultaneously with RV and CAV increases and remain intact for 24 hours or more. This indicates a mode of virus release with only minor damage to the syncytia.

In cultures infected with IBV microscopically normal cells are frequently present. It is possible that due to the asynchrony of these primary cell cultures, all cells are not infected at the same time or that an interferon-like substance may be produced by the cells during the multiplication of the virus. Since trypsin destroyed the activity of this interferon-like substance prepared from infected cell cultures, the substance may be a protein. Insensitivity of the substance to ribonuclease indicates that it is not a nucleoprotein. No interferon-like activity was observed when CEKC cultures were exposed for 1 hour to heat inactivated IBV. Full activity was observed when cultures were exposed to heat inactivated virus for 12 hours. This indicates that viral interference between the heat inactivated preparations and the PR8-6C was not responsible for the interferon-like activity. The 12 hour exposure time is indicative of host-cell participation as in a true interferon reaction.

The "syncytial virus group," of which IBV may now be considered a member, includes mumps, measles, NDV, and rinderpest viruses. All are considered to be etiological agents of respiratory diseases. The host range spectrum of cells susceptible to the syncytial group includes those of the primate, avian, bovine, and rodent species. Viruses of the syncytial group contain RNA. They are good antigens and produce

neutralizing, hemagglutination-inhibiting, and complement fixing antibodies. These viruses are also characterized by epidemic spread of overt infection in primarily immature hosts (Warren et al., 1962). Although considered to be host specific, IBV has good antigenic properties and etiologically causes a respiratory disease.

Information is incomplete for classification of the RS virus, although it also is considered to be of the syncytial group. The RS virus and IBV have many similar properties. Bennet and Hamre (1962) report specific RS virus cytoplasmic fluorescence in HEp-2 cells 9 hours postinoculation. Syncytia appear at 16 to 18 hours, and maximum RV occurs 30 to 36 hours after infection. However, differences in test conditions do not allow specific comparison with IBV42-118C. The HEp-2 cells provide uniform and synchronized cultures while the CEKC cultures used with IBV42-118C are asynchronous but metabolically more active, and cannot be used for as long a period as HEp-2 cultures. The RS virus is ether sensitive, has a diameter of 90 to 130 mμ, a CAV/RV ratio of approximately 1, and forms syncytia. These characteristics are similar to those of IBV42-118C.

SUMMARY

1. The usual viral replication cycle is observed during the multiplication of infectious bronchitis virus, strain IBV42-118C, in chicken embryo kidney cell cultures. A 4 hour phase of eclipse of the virus is followed by a rapid increase in cell associated and released virus at 5 to 6 hours postinoculation. There is a continuous exponential increase in virus release during the next 30 hours. The phase of decline commences at 36 hours and is exponential through 72 hours postinoculation.

2. The formation of syncytia is the characteristic cytological change associated with multiplication of infectious bronchitis virus, strain IBV42-118C. As syncytia and infectious virus production are simultaneously suppressed with specific immune serum, heat, and ultraviolet irradiation, it is concluded that virus replication takes place within the syncytia. Syncytial enlargement and increase in number of nuclei parallel the increase in released virus.

3. The acridine orange fluorescence staining technique indicates aggregates of ribonucleic acid in the syncytial cytoplasm which confirms the site of virus replication and the nucleic acid character of infectious bronchitis virus.

4. DL-parafluorophenylalanine in low concentrations did not alter syncytia formation, but suppressed release of infectious virus. At high concentration, fluorophenylalanine suppressed both syncytia formation and infectious virus release.

5. Aminopterin, 5-bromouracil, and 6-azauracil did not inhibit multiplication of infectious bronchitis, IBV42-118C. The results with aminopterin further confirmed the ribonucleic acid nature of the virus. Infectious bronchitis virus replication was independent of host-cell deoxyribonucleic acid synthesis.

6. Infectious bronchitis virus, IBV42-118C, is inactivated by 20 per cent ether within 24 hours at 4 C. The presence of a peripheral structural lipoprotein and peripheral assembly of the virus is indicated.

7. On the basis of ether sensitivity, syncytia formation, ribonucleic acid content, and association with a specific respiratory disease, infectious bronchitis virus can be considered one of the "syncytial virus group." Further information is required before infectious bronchitis virus can be classified with the multiform viruses of the myxovirus group, the measles virus group, or the respiratory syncytial virus.

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