EFFECT OF 6-AZAURICIL RIBOSIDE AND PUROMYCIN ON THE TIME COURSE OF BIOCHEMICAL EVENTS IN THE REPLICATION CYCLE OF AVIAN INFECTIOUS BRONCHITIS VIRUS

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ABSTRACT

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By

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The production of avian infectious bronchitis virus (IBV) in chicken embryo kidney cells (CEKC) is inhibited nearly 90% of normal by 50 μg/ml of 6-azauricil riboside (AUR) and 95% of normal by 5 μg/ml of puromycin. When AUR, an inhibitor of pyrimidine nucleotide biosynthesis, is added to IBV infected CEKC at various times after infection, there is a gradual increase in production of IBV until about 3 hours, after which infectious virus production ceases to increase further. Puromycin, a protein synthesis inhibitor, added at various times after infection inhibits virus production through 4.5 hours, after which IBV production sharply increases.

This information implies that synthesis of viral RNA begins shortly after infection and is completed by about 3 hours after infection. It also indicates that

viral protein synthesis is completed between 4.5 and 5 hours, which, according to the results of another experiment, coincides very closely with the length of the replication cycle of IBV.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Since the description of "an apparently new respiratory disease of baby chicks" in 1939 (39), a great deal of information about the disease and the etiologic agent, avian infectious bronchitis virus (IBV), has been collected. Early work was concerned primarily with the disease itself: the clinical symptoms and the treatment. Later work led to a description of the properties of the virus itself, as well as the nature of its component parts. This provided a basis for comparison with other viruses revealing a relationship with the etiologic agents of other diseases, possibly including the human cold virus.

Studies of the replication cycle of IBV have been directed primarily toward the physical and biological aspects. Light and electron microscopy of IBV infected cells have contributed a significant amount of information, as has the use of fluorescent antibodies. However, relatively little has been done to study the replication cycle on a biochemical level. Therefore, a study of the time course of biochemical events in the replication cycle of

IBV was initiated using metabolic inhibitors with known modes of action.

LITERATURE REVIEW

Avian infectious bronchitis virus (IBV) is a member of the Coronavirus group (2,15) which includes viruses causing the human common cold, mouse hepatitis virus, and others (3,24,30). These viruses seem to replicate in a similar manner and have several morphological characteristics in common (6,10).

The protein coat of IBV surrounding the core of ribonucleic acid (RNA) (1) is covered by a lipid envelope (32). The specific gravity of the Beaudette strain is 1.24 as determined by isopycnic density gradient centrifugation in cesium chloride. With a linear density gradient of sucrose, the specific gravity is 1.19. The sedimentation constant is 344 S indicating that the particle is roughly a sphere between 80 and 100 mμ in diameter (14).

Infection by IBV occurs following attachment of the virion to specific receptor sites on the surface of the membrane of the host cell, which nearly always is a chicken cell. The earliest visible immunofluorescence is present 4 hours after infection, primarily in the perinuclear region, but eventually diffusing throughout the

cytoplasm (11,29). Sections of IBV-infected cells fixed between 30 and 40 hours, have viral particles with an overall diameter of 67 to 110 mμ (6,36). The virus buds from the cytoplasm into cisternae of the endoplasmic reticulum (15). During the budding process, the virus acquires the lipid envelope, which is composed, at least partially, of altered host cell membrane material (6,36). Extracellular virions possess club- or pear-shaped spikes, 20 mμ in length, covering the surface of the envelope (7).

Puromycin, an inhibitor of protein biosynthesis, consists of an aminonucleoside linked by an amide bond to the amino acid p-methoxyphenylalanine. A structural analogy to the amino acyl adenosine portion of amino acyl transfer RNA (tRNA) is responsible for its inhibitory activity.

During normal protein synthesis (44), a complex composed of amino acyl tRNA, guanosine triphosphate (GTP), and Transfer Factor I is inserted into the amino acyl ("A") site on the ribosome where anticodon-codon hydrogen bonding holds the amino acyl tRNA in position. Peptidyl transferase, an integral part of the ribosome, removes the polypeptide from the peptidyl tRNA on the peptidyl ("P") site on the ribosome, and catalyzes the formation of a peptide bond between the carboxyl end of the polypeptide and the α-amino group of the amino acyl tRNA. The tRNA left in the "P" site is released and, with the help of GTP

and Transfer Factor II, the ribosomal complex moves up one codon. This allows the translocation of the peptidyl tRNA from the "A" site into the "P" site.

Puromycin will move into the "A" site on the ribosome instead of the amino acyl tRNA. Peptidyl transferase will then form a peptide bond between the carboxyl end of the polypeptide and the free amino group on the puromycin molecule. Since puromycin possesses neither an anticodon to hold it on the ribosome nor a carboxyl group with which to form a peptide bond, the polypeptidyl-puromycin chain is released from the ribosomal complex (19,34,35,40,43,45). By this mechanism, puromycin prevents synthesis of complete proteins.

Puromycin has been used to study various aspects of <u>in vitro</u> protein synthesis including the importance of N-formylmethionyl-tRNA (46) and GTP (21) in peptide initiation, the required presence of ribosomes (33), and the roles of the ribosomal subunits (22).

The effect of puromycin on viral infections in cell and tissue cultures has been used to study the cytopathic effects (CPE) produced by vaccinia virus and the relationship to the replication cycle of the virus (4). The CPE that normally occurred early in infection, as well as viral multiplication, were inhibited by treating infected cells with high concentrations (330 µg/ml) of puromycin. At lower concentrations (33 µg/ml), viral

multiplication was still inhibited significantly, but the CPE was nearly the same as that in infected cells without puromycin. The CPE was interpreted as being caused by viral-induced proteins made early infection.

Synthesis of poliovirus RNA did not occur when puromycin was added within 2 hours after infection of HeLa cells (26). When puromycin was added 2.5 hours after infection, synthesis of significant amounts of viral RNA occurred, but mature virions were not formed. This indicated that a protein required for production of viral RNA was synthesized during the first 2 hours after infection.

Synthesis of pyrimidine nucleotides required for nucleic acid synthesis is inhibited by 6-azauricil riboside (AUR) (20,37). An important precursor in the biosynthesis of pyrimidines is orotic acid. Orotidine-5'-phosphate pyrophosphorylase catalyzes the reversible formation of orotidine-5'-phosphate (05P) from orotic acid and 5-phosphoribosylpyrophosphate (PPRP). Uridylic acid or uridine monophosphate (UMP), a precursor of all pyrimidine nucleotides required for the synthesis of both deoxyribonucleic acid (DNA) and RNA in most types of cells, is formed by the irreversible decarboxylation of orotidine-5'-phosphate by 05P decarboxylase (25,27).

Normal cell enzymes phosphorylate AUR to form 6-azauridine-5'-phosphate, or azauridylic acid, which is the active form of the inhibitor. This form competitively

inhibits 05P decarboxylase, thus preventing formation of UMP and resulting in the accumulation of orotidine. Hence, nucleic acid biosynthesis is inhibited due to a lack of pyrimidine nucleotides (20,37).

The replication of type 5 adenovirus, a DNA virus, was studied by adding AUR to infected cell cultures at various intervals after infection (18). Thymidine and deoxycytidine were also added to allow synthesis of viral DNA. All samples were harvested after 30 hours of incubation and assayed for infectious virus, which was plotted against the time of addition of the inhibitor. The data, when interpreted explicitly, indicated that throughout the first 8 hours of infection the AUR sufficiently inhibited the accumulation of pyrimidine ribonucleotides to prevent synthesis of the RNA necessary for replication of the virus. When the inhibitor was added at any time after 8 hours, however, the pyrimidine nucleotide levels were sufficient to allow synthesis of increasing amounts of RNA which were directly related to the time after infection. By 16 hours, enough nucleotides were present to permit maximal synthesis of viral RNA and, subsequently, maximal production of infectious virus. The implicit interpretation is that synthesis of RNA essential for production of infectious adenovirus began about 8 hours after infection and, by 16 hours, enough viral RNA had been made to allow maximum virus production.

At various intervals after infection, AUR was added to cell cultures infected with an RNA containing virus, dengue-2 virus (41). After 21 hours of incubation, the samples were plaque assayed and the results plotted against the time of addition of AUR. A reversal type of experiment was also performed in which AUR was added to the cell cultures at the time of infection and then, at sequential time intervals, the inhibition by AUR was reversed by the addition of uridine. Again, after 21 hours of incubation, the samples were plaque assayed and the results were plotted against the time of addition of uridine. The data inferred that production of infectious virus depended on a species of RNA whose synthesis began about 6 hours after infection.

METHODS AND MATERIALS

Viruses

The 137th passage of the Beaudette strain of avian infectious bronchitis virus (IBV) adapted to chicken embryo kidney cells (CEKC) was assayed on primary CEKC and found to contain 8.6×10^6 plaque-forming units per milliliter (pfu/ml). The stock extracellular virus from the CEKC was stored in screwcap vials at -90°C.

Cell Cultures

Primary CEKC were prepared from 17-19 day old embryos according to standard methods (13,16). The cells were suspended in Medium 199, Grand Island Biological Company (GIBCO), containing 2 mM glutamine, and supplemented with vitamins and amino acids (GIBCO), 100 units/ml penicillin, 100 µg/ml dihydrostreptomycin, 50 units/ml Mycostatin (GIBCO), and 0.1% sodium bicarbonate. Newborn calf serum (nbcs) was added to a final concentration of 5%.

Cell cultures in 16 x 125 mm tubes (Rochester Scientific Co., Inc.) were inoculated with 1 ml of a 1:200 dilution of packed cells with a final concentration of

5 x 10^6 cells/ml. For plaque assays, 4 ml of a 1:100 dilution of packed cells, final concentration of 10^7 cells/ml, was dispensed into 15 x 60 mm plastic petri dishes (Falcon Plastics). All cell cultures were incubated at 37° in an atmosphere of 6-8% CO_2 and 80-85% relative humidity. Monolayers of cells formed in the tubes in about 48 hours, whereas 3 to 4 days were required for monolayers to form in petri dishes.

Puromycin and 6-Azauricil Riboside

Puromycin dihydrochloride and 6-azauricil riboside (AUR) (Nutritional Biochemical Co.) were prepared as 1 mg/ml stock solutions in Hanks' balanced salt solutions (HBSS) made with double glass distilled water. The solutions were sterilized by filtration (Falcon Plastics, 7103 Filter, 0.22 μ average pore size) and stored at 4°C.

Thymidine and Deoxycytidine

Thymidine and deoxycytidine (Nutritional Biochemical Co.) were prepared as 10^{-4} M and 10^{-3} M solutions, respectively, in HBSS made with double glass distilled water. The solutions were sterilized by filtration and stored at 4°C. Thymidine and deoxycytidine were always used in conjunction with AUR, at a final concentration of 10^{-5} M and 10^{-4} M, respectively, thereby providing the pyrimidine deoxyribonucleotides needed for DNA synthesis.

Inoculation of CEKC Tube Cultures

Extracellular fluid was decanted from the tubes and the cells were washed twice with HBSS. Each tube was inoculated with 0.2 ml of stock IBV containing 1.7×10^6 pfu. This represented a multiplicity of infection of about 0.3. The inoculated cultures were incubated for 1 hour to allow adsorption of the virus to the cells. Following the adsorption period, the inoculum was decanted and the monolayers were then washed 4 times with cold HBSS. Fresh medium was added to each tube and they were then placed in the incubator. This was considered 0 time for all experiments.

Assay of Virus

Extracellular fluid from the infected cultures, containing released virus, was decanted into screwcap vials and stored at -90°C. At the time of use the virus was thawed at room temperature. Monolayers of CEKC in petri dishes were inoculated with 0.5 ml of 10-fold dilutions of each sample, with 1 culture per dilution and 3 plates per sample. The cultures were then treated as described in the plaque assay method (13,16).

Replication Cycle

At various intervals after infection of CEKC tube cultures with IBV, the extracellular fluid from 2 cultures

was pooled in a screwcap vial and stored at -90°C until the assay materials were prepared.

Effect of Various Concentrations of the Inhibitors on the Replication of IBV

At 0 time, various concentrations of the inhibitors were added to tube cultures of IBV-infected CEKC. Two tube cultures per inhibitor concentration were used. The extracellular fluid from each sample was harvested and pooled 12 hours after infection and then assayed for infectivity. The virus yield for each concentration of inhibitor was calculated as per cent of normal yield, which was the yield of infectious virus from cultures not treated with the inhibitor. The per cent yield was then plotted against the concentrations of the inhibitors. The concentration of the inhibitor that was used in subsequent experiments was considered to be the concentration that inhibited IBV replication by about 90% but was not so toxic as to prevent the host cells from surviving during the 12-hour incubation.

Effect of Time of Addition of the Inhibitors on the Replication of IBV

At each of several sequential intervals following infection of CEKC tube cultures with IBV, an inhibitor was added to 2 tubes. The final concentrations of the

inhibitors were 50 µg/ml for AUR and 5 µg/ml for puromycin. The extracellular fluid was harvested from all tubes 12 hours after infection, pooling each sample, and then assayed for infectivity. The relative concentrations of infectious virus were expressed as pfu/ml and then plotted against the time of addition of the inhibitor.

RESULTS

Replication Cycle

A sharp increase in released infectious virus, indicating the end of a replication cycle, occurred between 4.5 and 5 hours after infection (Figure 1). These results agree with previous studies (28,29).

Effect of Various Concentrations of the Inhibitors on the Replication of IBV

The optimum concentrations of the inhibitors were 50 µg/ml of AUR and 5 µg/ml of puromycin (Figures 2 and 3). These were the concentrations used in subsequent experiments.

Effect of Time of Addition of the Inhibitors on the Replication of IBV

There was an increase in the production of infectious virus when AUR was added at anytime during the first 3 hours after infection, and a relatively constant production when AUR was added at anytime after 3 hours and through the last sample at 10 hours (Figure 4). This implies that synthesis of viral RNA began soon after

Figure 1. Replication cycle of IBV. Each point is the average of 3 experiments and represents the concentration of infectious virus in the pooled extracellular fluid from 2 tubes taken at the indicated intervals after infection.

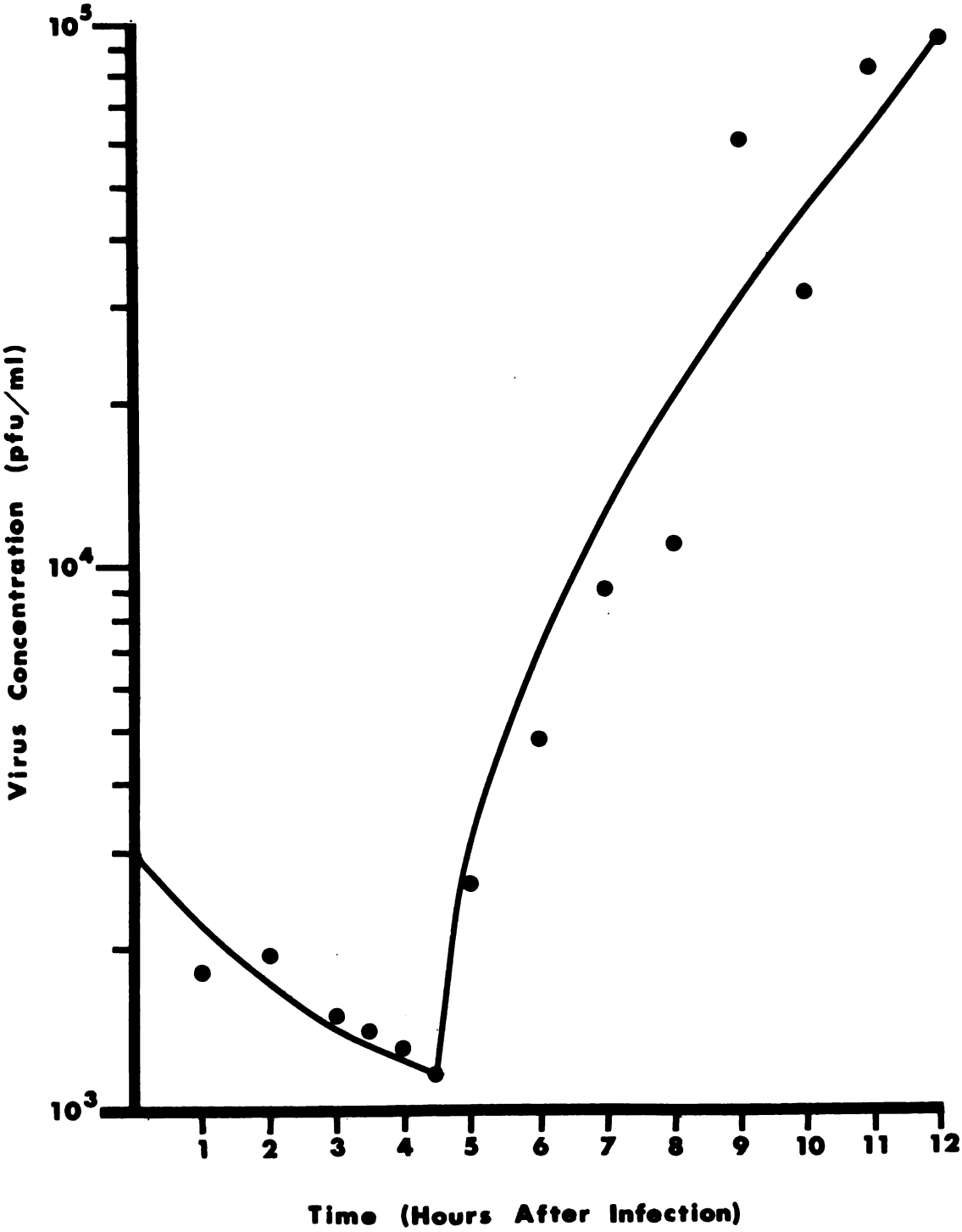

10^5
10^4
10^3
Virus Concentration (pfu/ml)
1
2
3
4
5
6
7
8
9
10
11
12
Time (Hours After Infection)

Figure 2. Effect of AUR on IBV replication. Tube cultures of IBV infected CEKC were treated with various concentrations of AUR for 12 hours. The extracellular fluid was then harvested, pooling each sample, and assayed for released virus. Each point is the average of 3 experiments.

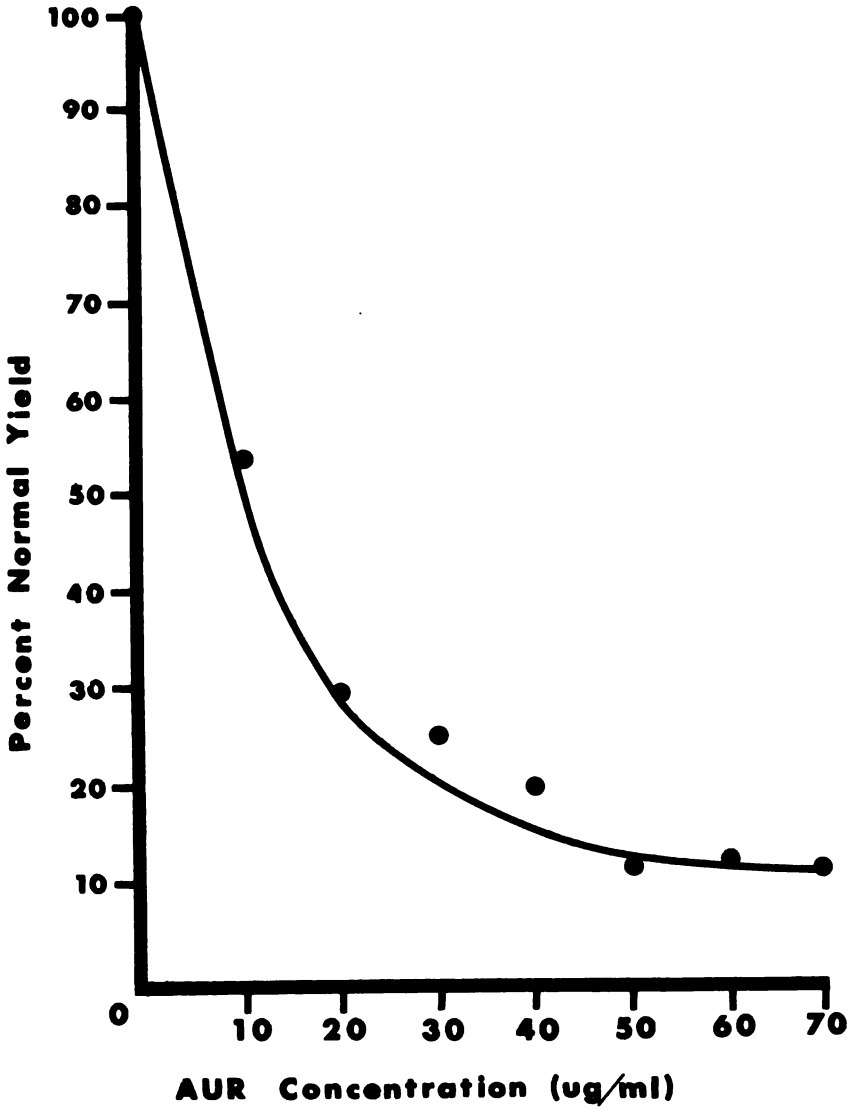

100
90
80
70
60
50
40
30
20
10
0
Percent Normal Yield
10
20
30
40
50
60
70
AUR Concentration (ug/ml)

Figure 3. Effect of puromycin on IBV replication. Tube cultures of IBV infected CEKC were treated with various concentrations of puromycin for 12 hours. The extracellular fluid was then harvested, pooling each sample, and assayed for released virus. Each point is the average of 4 experiments.

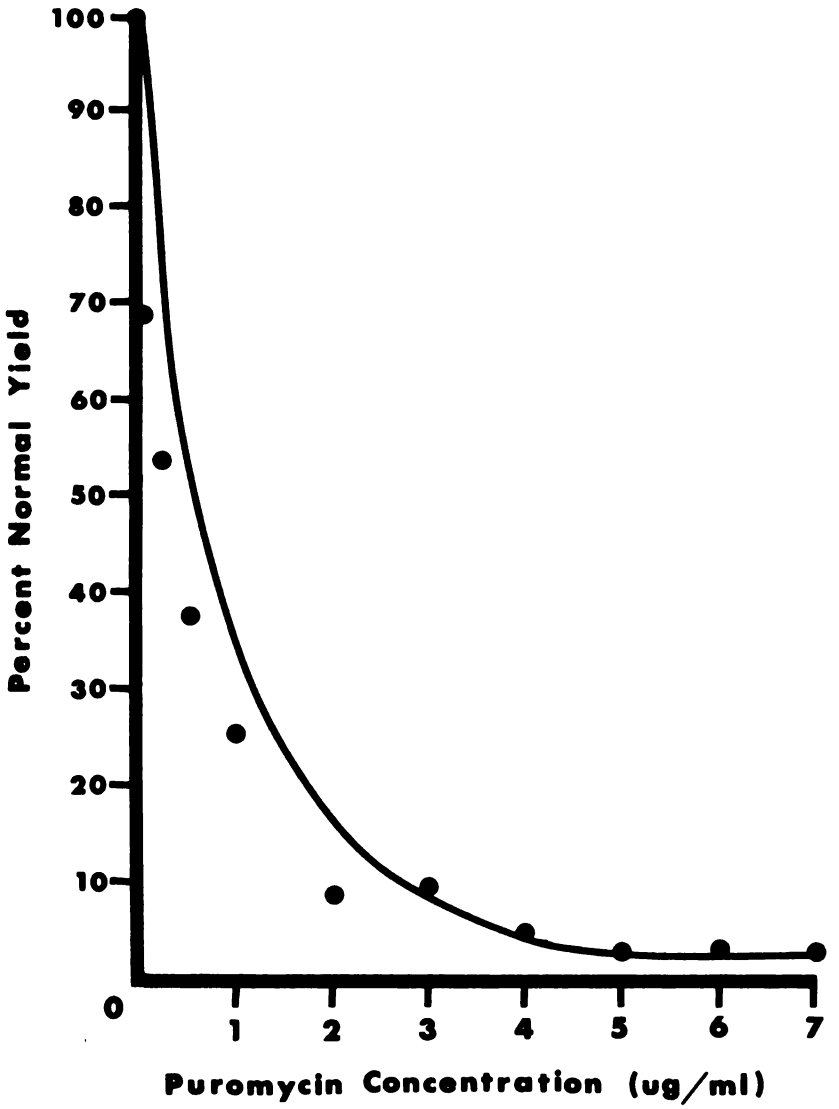
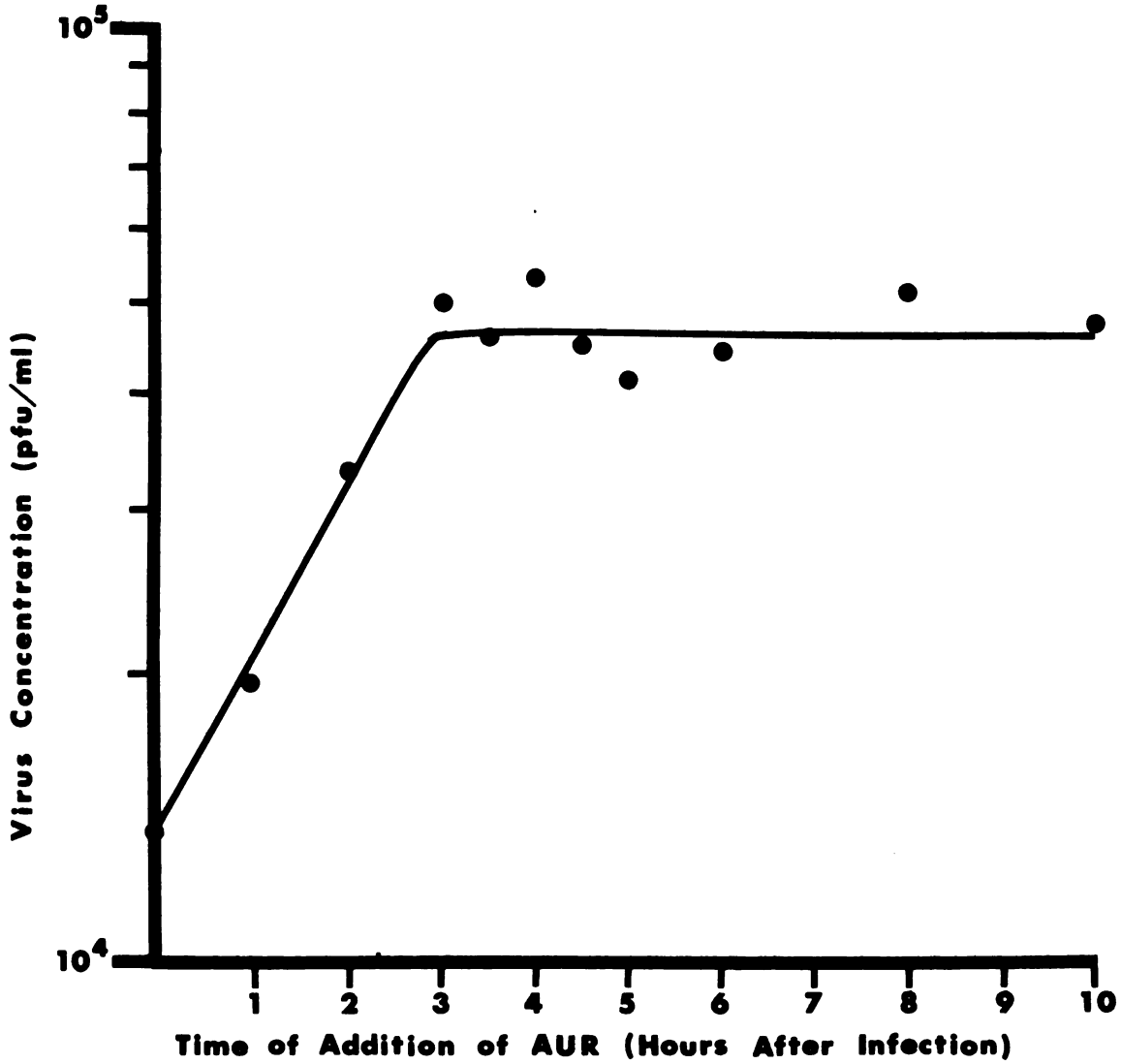

100
90
80
70
60
50
40
30
20
10
0
Percent Normal Yield
1
2
3
4
5
6
7
Puromycin Concentration (ug/ml)

Figure 4. Effect of time of addition of AUR on IBV replication. At each of several intervals after infection, AUR was added to 2 tube cultures of IBV infected CEKC to a final concentration of 50 µg/ml. The extracellular fluid was harvested from all cultures 12 hours after infection, pooling each sample, and assayed for infectious virus. Each point is the average of 2 experiments, each of which were assayed twice with an average variation of ±0.05 log units.

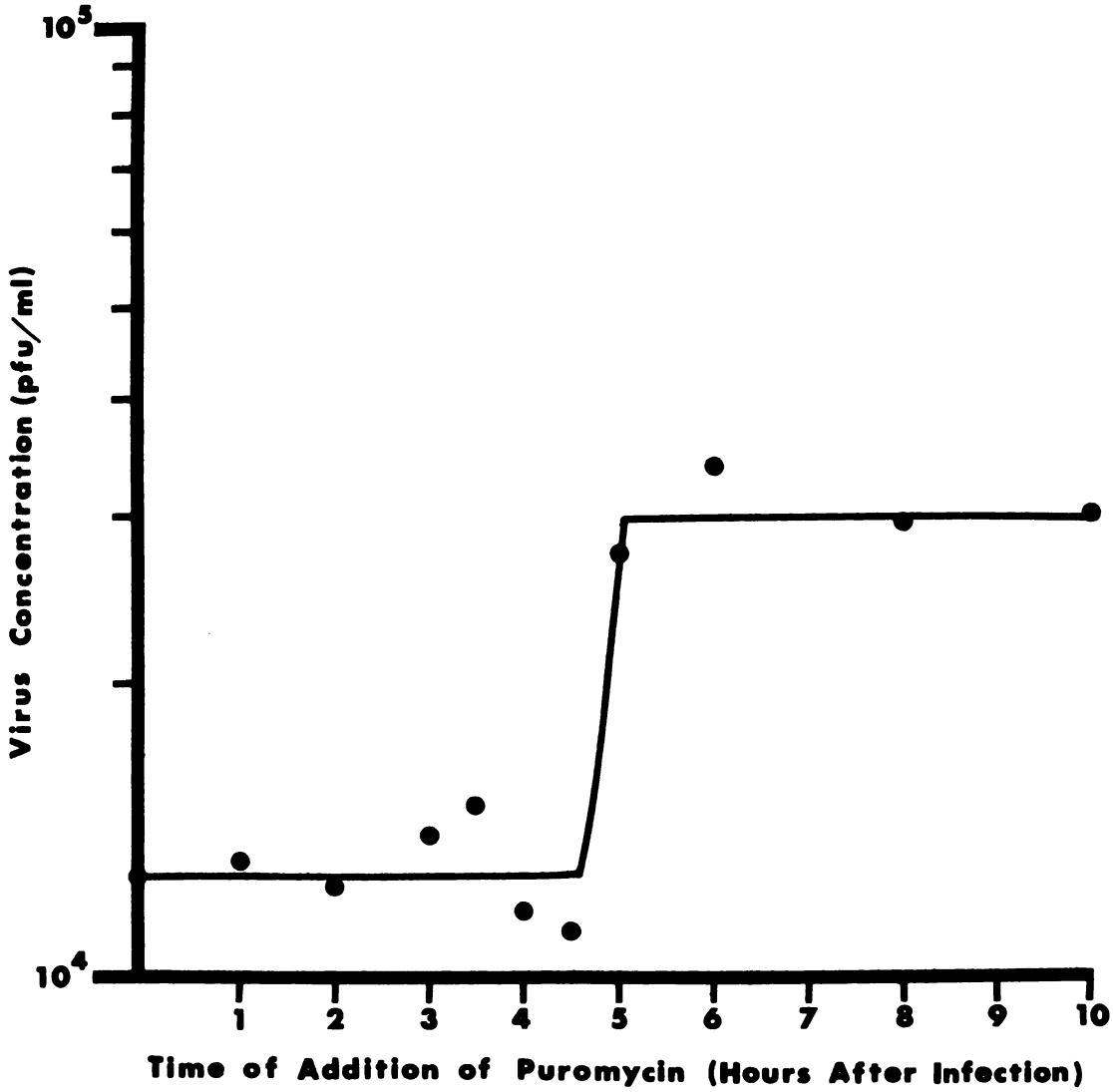

10^5
10^4
Virus Concentration (pfu/ml)
1
2
3
4
5
6
7
8
9
10
Time of Addition of AUR (Hours After Infection)

infection and was completed between 2 and 3 hours after infection.

Synthesis of viral proteins was completed between 4.5 and 5 hours after infection as indicated by the marked increase in virus production when puromycin was added at anytime after 4.5 hours (Figure 5). The time of completion of viral protein synthesis coincides very closely with the end of the IBV replication cycle and the release of infectious virus (Figures 1 and 5).

The lowest yield of virus produced when inhibitors were added early in infection and analyzed 12 hours after infection (Figures 4 and 5) was about 10 times higher than the background level of extracellular virus normally present just prior to the release of new virus in the absence of inhibitors (Figure 1). This difference is due to the production of between 5% and 10% of the normal virus yield with the inhibitor concentrations used, since higher concentrations did not decrease the yield (Figures 2 and 3) and were toxic to the cells.

Figure 5. Effect of time of addition of puromycin on IBV replication. At each of several intervals after infection, puromycin was added to 2 tube cultures of IBV infected CEKC to a final concentration of 5 µg/ml. The extracellular fluid was harvested from all cultures 12 hours after infection, pooling each sample, and assayed for infectious virus. Each point is the average of 2 experiments with an average variation of $\pm$ 0.03 log units.


10^5
10^4
Virus Concentration (pfu/ml)
1
2
3
4
5
6
7
8
9
10
Time of Addition of Puromycin (Hours After Infection)

DISCUSSION

The results from the experiments were based on the assay of extracellular virus which had been released from infected cells. Adding an inhibitor to virus-infected cells at various intervals after infection caused variations in the amounts of virus that were produced. The variation was dependent on two factors: first, the time of addition of the inhibitor and, second, the mode of action of the inhibitor which indicates the viral component whose synthesis was inhibited. The results were then interpreted as to the possible time course of biochemical events in the intracellular replication cycle of the virus.

When AUR was added at the time of infection and at sequential intervals thereafter, the production of infectious IBV increased throughout the first 3 hours after infection and then remained fairly constant. This suggested that synthesis of viral RNA had been completed by approximately the third hour of the 4.5 to 5 hour IBV replication cycle. However, inhibition of infectious virus production was only an indirect effect of the AUR,

since AUR inhibits only the synthesis of pyrimidine nucleotides. Therefore, the most rigorous interpretation of the results is that by 3 hours after infection the nucleotide levels in the infected cells were high enough to permit synthesis of IBV RNA in sufficient amounts for maximum production of infectious virus.

Extrapolating somewhat beyond the rather limited interpretations above, the fact that there was an gradual increase in infectious virus whenever AUR was added during the first 3 hours suggests that much of the RNA that accumulated during that interval eventually became incorporated into the progeny virions and that it began accumulating very shortly after infection. The data do not show what proportion of the RNA made was released in progeny virus, nor how much was used only as messenger RNA (mRNA). Also, it did not indicate how much of the RNA was complementary (-) to the parental or virion (+) RNA, although the RNA that was first made was almost certainly complementary. It is not known whether the (+) strand or the (-) strand acts as mRNA for translation into proteins of IBV, or whether each is translated into different proteins. The use of (-) strands as mRNA has been described for Newcastle disease virus (NDV) and Sendai virus (38), both paramyxoviruses, in which there was a preferential synthesis of (-) strand RNA which then became associated with the polyribosomes of the infected cells.

A prerequisite for early synthesis of IBV RNA would be early synthesis of an RNA polymerase or transcriptase. Synthesis of such an enzyme must, in fact, occur very shortly after the virion enters the cell, since, as the data suggest, production of IBV RNA occurred within the first hour after infection. A speculative, although plausible, explanation would be the existence of an RNA polymerase within the virion. Although an RNA polymerase has not been determined for IBV or other Coronaviruses, such an enzyme has been reported for several other lipid enveloped, single stranded RNA viruses (17,31), namely: Newcastle disease virus (NDV) (23) and Sendai virus (38,42), both paramyxoviruses, influenza A (8,9,12), a myxovirus, and vesicular stomatitis virus (5), a rhabdovirus. The possible presence of this enzyme in the virions of IBV is, then, worth considering in future studies.

Puromycin inhibits the translation of viral proteins, which is a more direct effect on the replication of IBV than is the effect of AUR, and, therefore, the results of the experiments involving puromycin are more conclusive. Although a number of different species of proteins are coded for by the viral genome, the data indicate only the time of completion of the last protein species required for viral replication.

The experimental results indicated that puromycin inhibited virus replication to about the same degree through 4.5 hours of infection. However, production of infectious virus increased sharply at 5 hours and maintained that level thereafter, indicating that puromycin ceased to be effective when added anytime after 4.5 hours. Therefore, synthesis of IBV protein was completed between 4.5 and 5 hours after infection.

The time of completion of the synthesis of the last viral protein coincided very closely with the time of release of mature IBV virions which indicates that release occurred very shortly after the synthesis of viral proteins was completed. This suggests a possible sequence for the last few events in the intracellular replication of IBV. Although it is not possible to identify specific proteins with the techniques employed, the last proteins to be synthesized could be virus specific membrane proteins or, perhaps, structural proteins which are assembled with the viral RNA to form the nucleocapsid. Maturation proceeds as the nucleocapsid buds through the membrane of the endoplasmic reticulum, adquiring its lipid envelope and surface projections, and is released from the cisternae and cytoplasmic vesicles into the fluid surrounding the cell as an infectious virion.

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