EFFECT OF FLUOROPHENYLALANINE ON THE REPLICATION OF AVIAN INFECTIOUS BRONCHITIS AND NEWCASTLE DISEASE VIRUSES IN CHICKEN EMBRYO KIDNEY CELLS

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ABSTRACT

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By

Lorell Henry Angelety, Sr.

Synthesis of avian infectious bronchitis (IBV) and of Newcastle disease (NDV) viruses in chicken embryo kidney cells was effectively inhibited by p-DL-fluorophenylalanine (FPA). The amount of extracellular virus was not related to efficiency of adsorption to or entry of the host by the virus. Extracellular virus was 1.6 and 3.0 logs less for IBV and NDV, respectively, when compared with virus in the absence of the inhibitor. Inhibition of viral synthesis was reversible by the addition of DL-phenylalanine to the cultural medium.

Differences in sensitivity of viral syntheses to inhibition by FPA may reflect a difference in the ability of either structural (capsid) or non-structural (enzymes) proteins to retain functional integrity following incorporation of the analogue, FPA.

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DEDICATION

I dedicate this thesis to those who made it possible, my wife and my parents.

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INTRODUCTION

It has been demonstrated that viral isolates from apparently typical clinical cases of infectious bronchitis, were in fact mixtures of avian infectious bronchitis virus (IBV) and other viruses, the most common of which was avian adenovirus (IO). Recently it has been demonstrated (21) that laboratory stocks of IBV may contain latent Newcastle disease virus (NDV).

The present study was initiated to determine the effect of p-DL-fluorophenylalanine (FPA) on the replication of IBV and NDV in chicken embryo kidney cells as a possible means for selective separation of the two viruses.

LITERATURE REVIEW

Infectious bronchitis virus has a diameter of 83-100 mμ (29) and bears club-shaped spikes 20 mμ long arranged in clusters (11). The virion possesses an ether-sensitive lipoprotein envelope (27), and a ribonucleic acid core (3). The specific gravity of the particle is 1.24 in CsCl and 1.19 in sucrose. The sedimentation constant of the virus is 344 S which corresponds to a 80-100 mμ sphere (17), and is in agreement with dimensions obtained by electron microscopy. The virion does not normally agglutinate chicken erythrocytes, unless it has been modified by trypsin, ether or DEAE-cellulose treatment (12,13).

The virus has been cultivated in a variety of avian cell and tissue cultures derived from the embryonating chicken egg and the chicken but mammalian cell cultures are generally reported not to support the growth of the virus (16). The cells of choice for propagation of the virus are chicken embryo kidney cells (CEKC)(24).

After attachment of the virion to specific receptor sites at the surface of the host cell membrane, there is an eclipse period of four hours with the maximum titer produced after 36-48 hours (14,24)

Newcastle disease virus is roughly spherical, with a diameter of 100-180 mμ, a particle weight of 800 x 10^6 daltons, and a sedimentation constant of 1,100 S (31). The inner ribonucleoprotein is surrounded by a spiked-armed envelope. The spikes are 8 mμ long and 8-10 mμ apart (39). The inner component has a diameter of 17-18 mμ with a central channel of 5 mμ in diameter (19).

Newcastle disease virus is readily propagated in the chicken embryo (23) and in a wide variety of avian and mammalian cells. There is an eclipse period of two hours with the maximum titer produced after 24 hours in chicken lung cells (18).

Virus specific soluble antigens have been reported for both IBV and NDV. Infectious bronchitis virus possesses at least three antigens separable on the basis of size, buoyant density, thermostability and sensitivity to enzymes (38).

Newcastle disease virus has at least two antigens, which corresponds to a surface component (hemagglutinin) and an inner component (nucleoprotein). The antigens are differentiated by their complement-fixing, hemagglutining and enzymatic activity (31).

Newcastle disease and infectious bronchitis viruses, although similar morphologically and chemically, are members of two distinct groups of viruses. Newcastle disease virus is grouped with the paramyxoviruses by

virtue of its chemical and biophysical properties (42). Infectious bronchitis virus is at present unclassified, but recognition of the similarity between IBV and human respiratory viruses, B814 and 229E, indicated the possibility of a new and previously unrecognized virus group (4,9,26). The morphologic features of these viruses and that of murine hepatitis virus resembles a solar corona, which has prompted the suggestion that the group be termed coronavirus, with IBV as the prototype (5).

Fluorophenylalanine is useful in the study of viral replication as this amino acid analogue is incorporated in place of phenylalanine during the synthesis of protein and causes the production of aberrant and nonfunctional proteins (28). Inhibition by FPA of the synthesis of functional proteins has permitted study of the sequential events of viral replication that would be difficult to follow by conventional methods such as one-step growth curves and others.

Synthesis of poliovirus in HeLa cells is inhibited by the presence of FPA in the growth medium (1). The inhibition is not the result of a toxic effect of FPA on the cells.

The effect of FPA on the replication of polio and Western equine encephalitis (WEE) viruses is a gradient effect as the proteins involved in the maturation of the virus are affected by low concentrations of FPA. The

proteins involved in the replication of the RNA are affected by high concentrations of the inhibitor (22).

Fluorophenylalanine is capable of interrupting RNA synthesis at any time during the multiplication cycle of polio and WEE viruses. The protein affected is RNA dependent RNA polymerase. The polymerase of poliovirus is a labile protein that uses the RNA of poliovirus as a template for the replication of viral nucleic acid (6). The enzyme is rendered non-functional through incorporation of the analogue into it. Decay of functional polymerase, leads to an interruption of RNA synthesis 45 minutes after the addition of FPA to the growth medium.

There is a change in the capsid protein of poliovirus following addition of low concentrations of FPA to the medium of infected HeLa cells. The virus particles produced resemble poliovirus, but have obvious irregularities as determined by electron microscopy. The virus particles are heat labile and are antigenically similar to heat-denatured virions. The general character of the virus particle is thought to be the result of the formation of fraudulent capsid protein, which is incapable of forming the proper three dimensional configuration necessary for production of infectious virus (20).

Inhibition of the replication of influenza virus by FPA, may be reversed by the addition of phenylalanine to the growth medium (2). There is no detectable delay

in viral production if the phenylalanine is added more than seven hours after infection, lack of a delay is not due to the accumulation of mature virus in the host cell, it is due to the accumulation of viral precursors.

Inhibition of influenza virus by FPA has allowed the separation of the replication cycle into two phases. The first phase is inhibited by high concentrations of FPA, whereas low concentrations inhibits a phase following the first by 1-1 1/2 hours (41).

A random delay in the release of infectious virus, the duration of which varies inversely with the multiplicity of infection, occurs early in the replication cycle. Synchronization of viral replication may be accomplished by inhibiting replication and subsequently reversing the inhibition by the addition of phenylalanine to the growth medium (41).

Replication of fowl plague virus in whole chicken embryo and chicken lung cells is inhibited by FPA (45), which does not interfere with the adsorption of the virus to or penetration of the host cell, nor does it alter irreversibly the ability of the host cell to support replication of the virus.

Multiplication of the virus is blocked by FPA in at least two stages. The first stage occurs between adsorption and production of the S-antigen (ribonucleoprotein), and the second stage occurs between production of the

S-antigen and the hemagglutinin and infectious virus. In the presence of FPA the S-antigen accumulates in the nucleus with subsequent inhibition of the production of the hemagglutinin and infectious virus. This separation of the assembly stages is dependent upon the level of the inhibitor and the time at which FPA is added to the growth medium.

Fluorophenylalanine inhibits the synthesis of fowl plague virus RNA, when added to infected cells prior to initiation of nucleic acid synthesis, but not when added later (29). In contrast, synthesis of the RNA of picorna (22) and arboviruses (40) can be inhibited by FPA at anytime during replication of the viruses.

The inhibitor does not interfere with the incorporation of ^{14}C labeled leucine into virus protein, but rather the ^{14}C-leucine labeled proteins are incorporated at about 15% of the normal rate into intact particles.

Multiplication of NDV in chicken embryo fibroblast cells is nearly completely inhibited by FPA at 200 µg/ml. Production of a functional early protein, presumably RNA dependent RNA polymerase, is inhibited when FPA is added up to three hours after infection. After this period RNA synthesis is not affected by the addition of the inhibitor.

According to Akers (3), 600 µg FPA/ml reduced by 2-3 logs the yield of IBV in CEKC, when compared to the virus titer in the absence of the inhibitor.

MATERIALS AND METHODS

Viruses

The Beaudette strain of IBV (IBV-42) adapted to CEKC was used and assayed on CEKC in petri dishes as plaque-forming units (pfu) by the agar overlay method (15). Cells were inoculated with 6.0×10^5 pfu of the 128th passage of the virus. The extracellular fluid was harvested at 48 hours, pooled and centrifuged at 10,000 g for 30 minutes. The supernatant fluid, which contained 2.2×10^7 pfu/ml, was dispensed in 1 ml volumes and stored at -90°C until used.

The Texas Gilbert-Boney (GB) strain of NDV, 1.0×10^7 pfu, was used to inoculate CEKC. After 24 hours the extracellular fluid was harvested and processed as described above for IBV. There were 3.1×10^7 pfu of virus/ml when assayed on CEKC.

Cell Cultures

Primary CEKC were suspended in appropriate concentrations of Medium 199 (Grand Island Biological Company, Inc.) containing 2 mM glutamine, and supplemented with vitamins and amino acids of Eagle's essential medium, 100 units/ml penicillin, 100 g/ml dihydrostreptomycin, 50

units/ml Mycostatin (Squibb) and 0.1% sodium bicarbonate. Newborn calf serum (Grand Island Biological Company, Inc.) was then added to a final concentration of 5%.

Tube cultures were prepared by dispensing 1 ml (1:300 dilution of packed cells) 3.3 x 10^6 cells/ml into 16 x 125 mm tissue culture tubes (Rochester Scientific Company, Inc.). Cells in petri dishes were used to study the effect of FPA on adsorption and the penetration of IBV and NDV and for the assay of the viruses were prepared by dispensing 4 ml (1:100 dilution of packed cells), 10^7 cells/ml into 15 x 60 mm plastic petri dishes (Falcon Plastics). All cell cultures before and after inoculation were incubated at 37°C in an atmosphere of 8% CO_2, 80-85% relative humidity. Confluent monolayers of cells were formed in tubes and petri dishes after 48 hours.

p-DL-Fluorophenylalanine and DL-Phenylalanine

p-DL-Fluorophenylalanine (Sigma Chemical Company) and DL-phenylalanine (Laboratory Park) were prepared as 10 mg/ml stocks in glass double distilled water, sterilized by filtration, and stored at 4°C.

Antiserum

Anti-IBV-41 chicken serum (36) kindly supplied by Dr. Mark F. Stinski, formerly of the Department of Microbiology and Public Health, Michigan State University,

East Lansing, Michigan, was heated at 56°C for 30 minutes prior to use.

Inoculation of Cells with Virus and their Treatment with p-DL-Fluorophenylalanine

After the extracellular fluid was decanted from tube and petri dish cultures of CEKC, the cells were washed with Hanks' balanced salt solution (HBSS). Suspensions of viruses were diluted to the desired pfu/ml at 4°C in phosphate buffered saline (PBS) without Ca^{++} or Mg^{++} and containing 3% new born calf serum.

Cultures of CEKC in tubes were inoculated with IBV, 5.0×10^5 pfu/0.2 ml, or NDV, 6.0×10^5 pfu/0.2 ml, which represents a multiplicity of infection of 1. Incubation for adsorption of the virus was at 37°C for 20 minutes.

Cultures of CEKC in petri dishes were inoculated with 0.5 ml of the viruses and incubated at either 37°C for 90 minutes or 4°C for 30 minutes for certain experiments.

To determine the toxicity of FPA for CEKC without virus, tube cultures were treated with the cultural medium containing graded concentrations of the analogue and were observed microscopically for any cytopathic effects after 24 and 48 hours.

The inhibitory activity of the analogue on viral replication was determined using graded concentrations of the analogue in the cultural medium on IBV and NDV infected cells.

Details as to the treatment of CEKC in petri dishes will be described under Results.

The effect of FPA on cells (toxicity) and on viral synthesis was determined using two and three tube cultures of CEKC, respectively, for each test concentration of the inhibitor.

The effect of FPA on adsorption to or penetration of the host cell, as well as virus concentration was determined using three petri dish cultures of CEKC for each virus sample or test condition.

RESULTS

Toxicity of p-DL-Fluorophenylalanine for Cells

The cells tolerated as much as 600 µg FPA/ml for 24 hours, but they were able to tolerate 300 µg for 48 hours based on cytopathogenicity (Table 1). On the above basis and that the maximum production of IBV occurs 36 and 48 hours (14,24), 300 µg FPA/ml was selected as the maximum concentration to be used to determine the effect of FPA on the replication of IBV and NDV in CEKC.

Effect of p-DL-Fluorophenylalanine on the Syntheses of Infectious Bronchitis and Newcastle Disease Viruses

Cells were inoculated with IBV or NDV, washed with HBSS and then Medium 199 containing 0-300 µg FPA/ml was added to appropriate groups of tube cultures. Non-infected cell cultures were treated with PBS without Ca^{++} or Mg^{++} containing 3% serum, instead of virus during the adsorption period and they served as controls. After 48 hours the extracellular fluids were harvested and assayed for virus. The titer of virus in the absence of FPA was considered to be the normal yield of virus. The titer of virus in the various concentrations of FPA was compared

TABLE 1

Toxicity of p-DL-fluorophenylalanine for CEKC on the basis of cytopathogenicity

µg/ml FPA	24 Hours	48 Hours
0.0	Normal cells confluent sheet	Normal cells confluent sheet
100.0	Normal cells confluent sheet	Normal cells confluent sheet
200.0	Normal cells confluent sheet	Normal cells confluent sheet
300.0	Cell rounding confluent sheet	Cell rounding confluent sheet
400.0	Cell rounding confluent sheet	Extensive cell rounding with detachment of cells
500.0	Cell rounding confluent sheet	Extensive cell rounding with detachment of cells
600.0	Pronounced cell rounding and granular	Extensive cell rounding with detachment of cells

to those in the absence of FPA and expressed as the per cent of normal yield.

The yield of IBV was rapidly reduced as a function of the FPA concentration in the growth medium. As little as 50 µg FPA/ml, which is equal to the concentration of phenylalanine in Medium 199 resulted in a 30% reduction in the titer of IBV. The maximum concentration of FPA employed (300 µg/ml) reduced the titer of IBV by 99.9%. In contrast, 150 µg FPA/ml was required to reduce the NDV titer by 30%, whereas 300 µg FPA/ml, the maximum concentration tested reduced the titer by 96% (Fig. 1).

Effect of p-DL-Fluorophenylalanine on the Adsorption of Infectious Bronchitis and Newcastle Disease Viruses to Cells

Dilutions of IBV and NDV suspensions were prepared in PBS without Ca^{++} or Mg^{++} but with 3% serum. The PBS contained either no FPA or 300 µg FPA/ml. Cultures of CEKC in petri dishes were then inoculated for assay by the plaque method (15).

The efficiency of adsorption of both viruses was not reduced by the inhibitor (Table 2).

Reversal of p-DL-Fluorophenylalanine Inhibition of Infectious Bronchitis and Newcastle Disease Viral Syntheses by DL-Phenylalanine

To determine the specificity of reversal by phenylalanine (PA) of the observed inhibition of IBV and

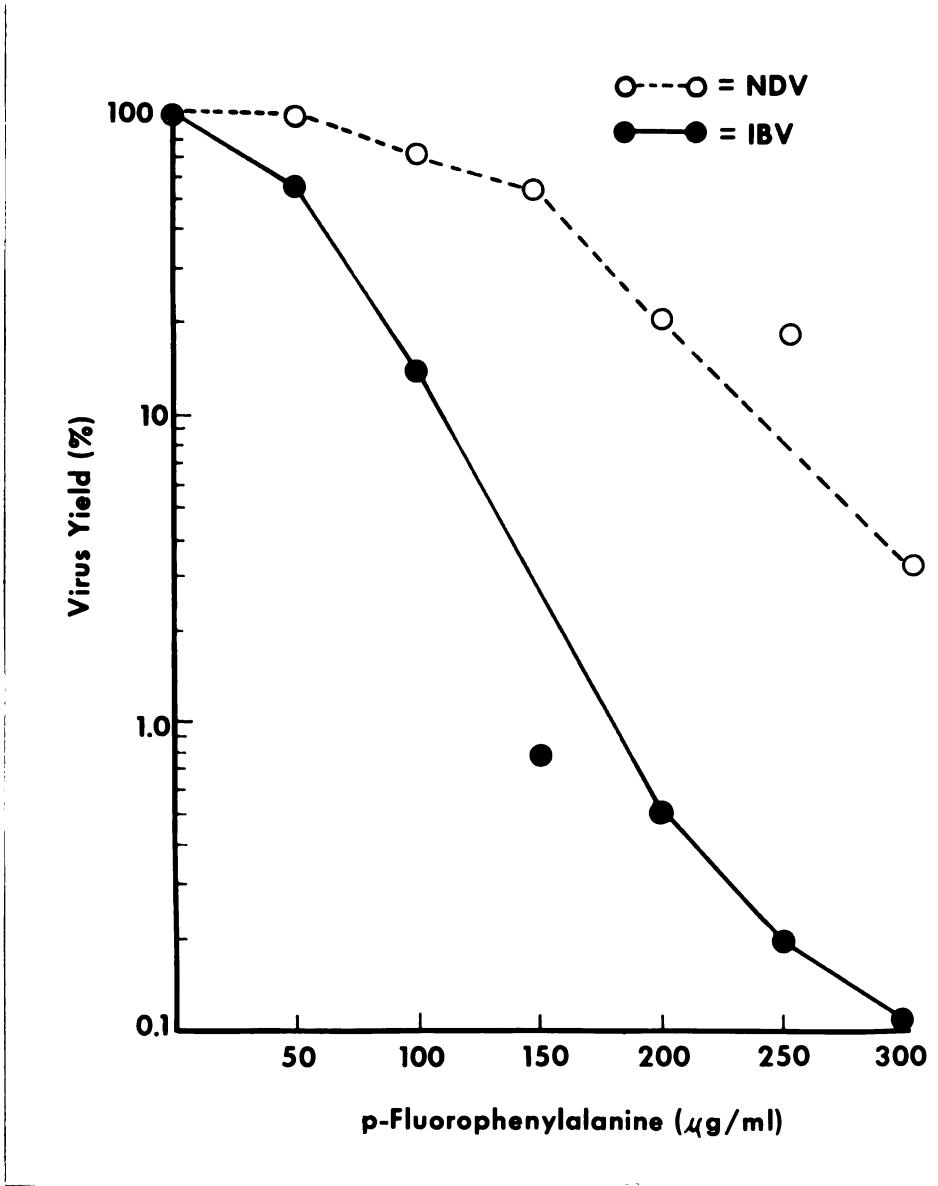

Fig. 1.--Effect of FPA on the synthesis of IBV and NDV.

TABLE 2

Effect of FPA on the adsorption of IBV and NDV onto CEKC monolayers

Diluent	Titer (pfu/ml)	
	IBV	NDV
PBS*	2.1×10^7	2.9×10^7
PBS + FPA‡	2.2×10^7	3.1×10^7

*FPA-free (control) + 3% nbcs

‡300 µg/ml FPA + 3% nbcs

NDV syntheses by FPA, Medium 199 containing 50 µg PA/ml, 300 µg FPA/ml, or 300 µg FPA/ml or 600 µg PA/ml was added to tube cultures to which IBV and NDV, respectively, had been adsorbed. After 48 hours the extracellular fluids were assayed for virus.

Phenylalanine reversed the inhibition of viral synthesis induced by FPA. The titers of IBV and NDV were 3.1 and 1.5 logs greater, respectively, in the presence of the higher concentration of PA, than the titer in the absence of high concentrations of PA (Table 3).

Effect of Pre-Treatment of Cells with p-DL-Fluorophenylalanine on the Entry of the Host Cell by Infectious Bronchitis Virus

Intracellular IBV is not neutralized by extracellular specific antibody. The virion adsorbs to but does not penetrate the cell at 4°C (37). Accessibility of virus to antibody was studied in cells treated with FPA and the rate of penetration of the cells was compared with that obtained under FPA-free conditions.

Medium 199 which was either FPA-free or contained 300 µg FPA/ml was added to petri dish cultures of CEKC. After incubation at 37°C for 2 hours, the cells were washed with cold PBS, re-incubated at 4°C for 30 minutes, and then inoculated with 8.4×10^3 pfu per culture. After adsorption at 4°C for 30 minutes the inoculum was decanted

TABLE 3

Reversal of FPA inhibition of IBV and NDV synthesis by phenylalanine

Growth Medium	Log_{10} Titer	
	IBV	NDV
Medium 199*	6.2	7.1
Medium 199 + 300 µg/ml FPA	2.2	5.2
Medium 199 + 300 µg/ml PFA + 600 µg/ml PA	5.3	6.7

*Medium 199 contains 50 µg/ml PA

and the cells were washed with cold PBS. After incubation at 37°C for 5, 15, 30, and 45 minutes, the cells were washed with cold PBS and treated with 0.5 ml of anti-IBV-41 antiserum (1:20). The antiserum treated cells were then incubated at 4°C for 30 minutes and then overlaid with Medium 199 containing 0.9% agar (Difco-Noble) and 5% serum. The plates were processed as outlined in the plaque assay method (15).

Pre-treatment of the host cell with FPA did not alter the efficiency of the virion to adsorb to or penetrate CEKC (Fig. 2).

Effect of the Time of Addition of p-DL-Fluorophenylalanine on the Synthesis of Infectious Bronchitis and Newcastle Disease Viruses

The synthesis of mature virus is dependent upon the synthesis of early and late proteins. An experiment was performed to determine the latest time at which FPA could be added to infected cell cultures and achieve maximum inhibition of viral synthesis.

Tube cultures of CEKC infected with IBV or NDV were treated after 0, 2, 4, 6, 8, 12 and 24 hours at 37°C with Medium 199 which contained 300 µg FPA/ml. A duplicate set of infected cell cultures were processed in an identical manner, with FPA-free medium. The extracellular fluid was collected after 48 hours and assayed for virus.

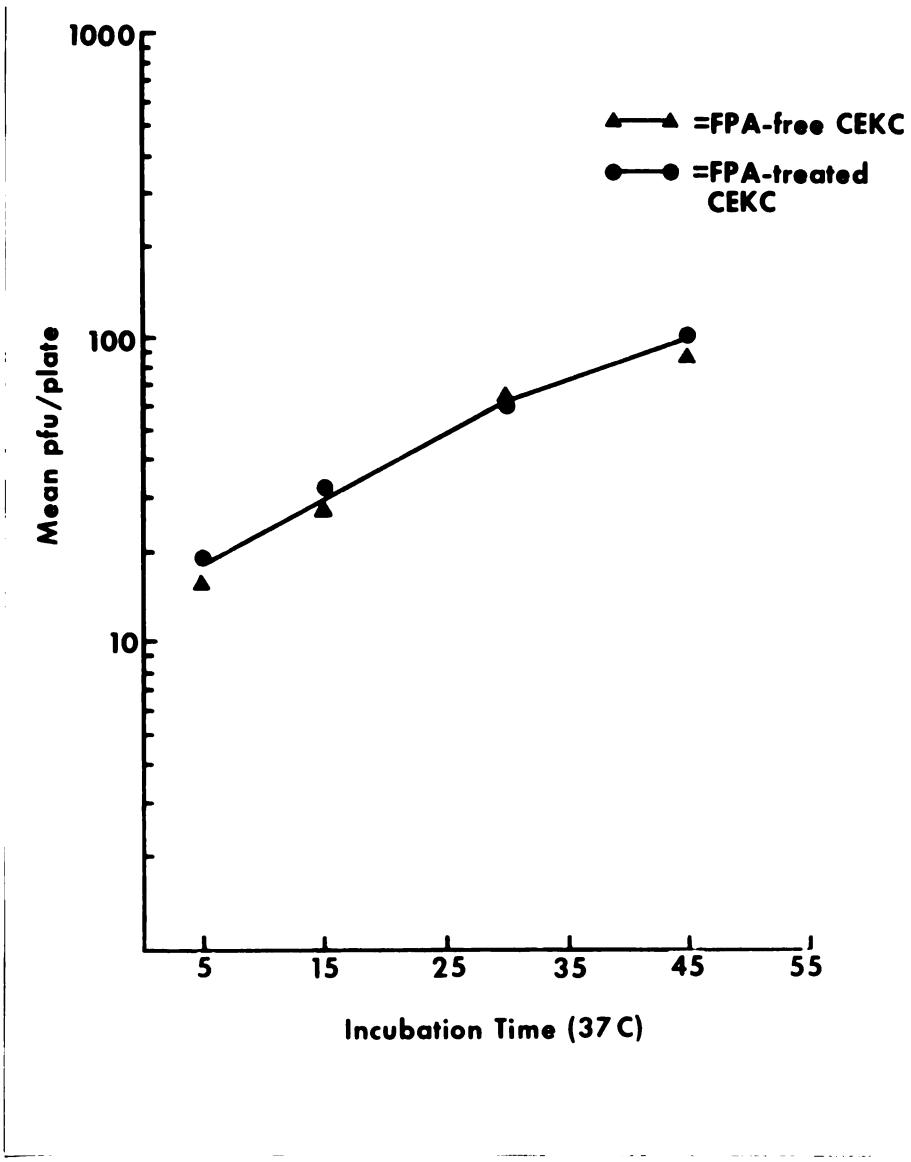

Fig. 2.--Effect of pre-treatment of CEKC cultures with FPA on the penetration of the host cell by IBV as measured by the inability of antibody to neutralized intracellular virus.

Virus titers in the presence of the inhibitor at the various time periods, were compared to those in the absence of FPA and expressed as the per cent of normal yield.

Maximum inhibition of IBV and NDV occurred when the inhibitor was added to infected cultures no later than 4-6 hours (Fig. 3), which is approximately the eclipse period reported for the synthesis of these viruses. Incorporation of the FPA probably interfers with the formation of early proteins that are essential precursors necessary for the synthesis of mature virus.

The amount of virus produced when the inhibitor was added after 4-6 hours, was only about 0.5% and 3% for IBV and NDV, respectively, when compared to the controls. The quantity of virus produced when the inhibitor was added as late as 24 hours was only about 50% and 3% for IBV and NDV, respectively, when compared to the controls.

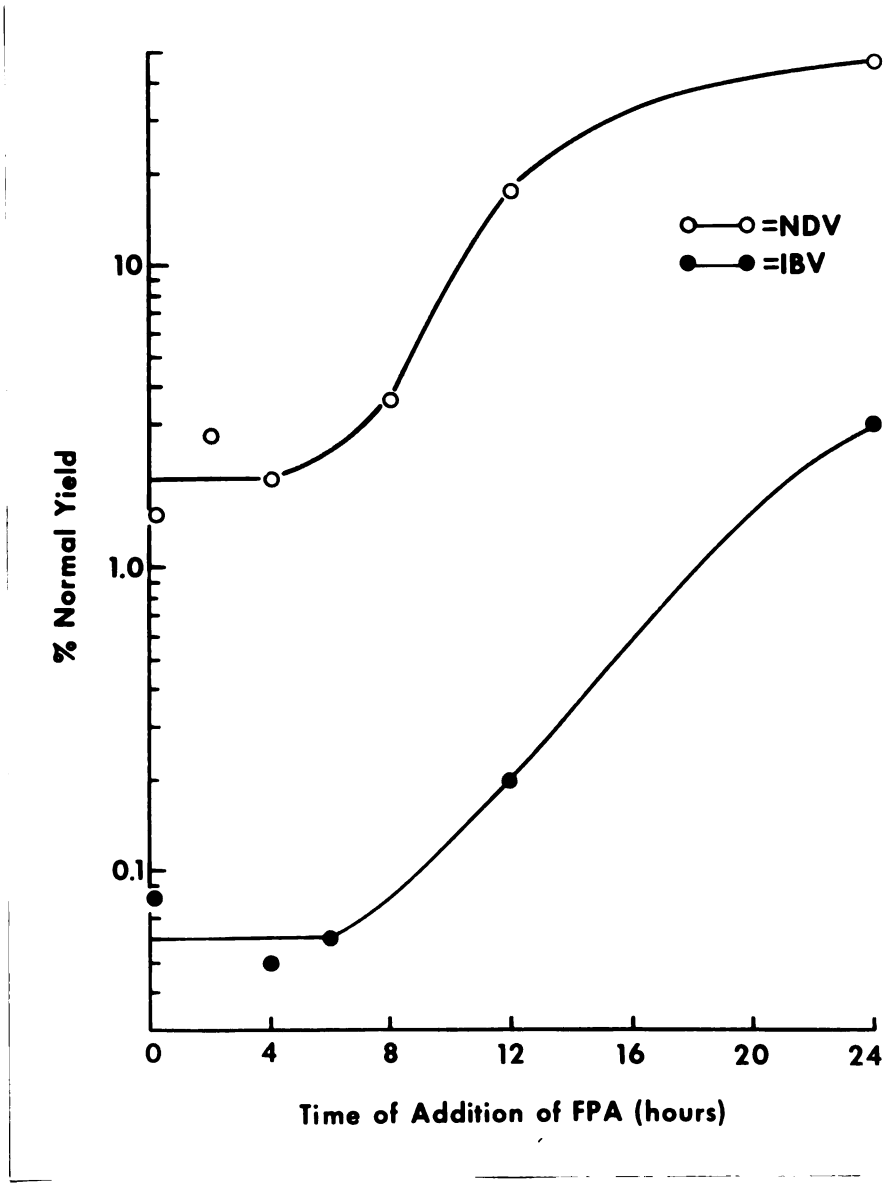

Fig. 3.--Effect of the time of addition of FPA on the synthesis of IBV and NDV.

DISCUSSION

Adsorption of IBV and NDV to the host cells was not affected by the FPA. The results clearly indicate pronounced differences in the effect of FPA on the synthesis of IBV and NDV, which might be utilized for the differential separation of these two viruses in mixed populations.

Inhibition of viral replication by FPA appears to be due to the production of aberrant or non-functional proteins following incorporation of the analogue (20). Of particular importance is the report that incorporation of FPA into α amylase of Bacillus subtilis does not change its biophysical properties, e.g. sedimentation velocity, but there was a reduction in its enzymatic activity (44). Proteins participating in the synthesis of mengo and polio viruses differ in their ability to remain functional following the incorporation of FPA (8,22). The capsid protein(s) become non-functional in the presence of low concentrations of FPA without the cessation of RNA synthesis; RNA synthesis is inhibited and RNA-dependent RNA polymerase is rendered non-functional in the presence of high concentrations of the inhibitor. In the presence of low concentrations of the analogue protein synthesis is

not affected but the production of infectious virus is inhibited (20).

It is probable that the differences in sensitivity of IBV and NDV to FPA during replication are related to the ability of proteins of the viruses to maintain enzymatic and structural integrity following incorporation of the analogue.

Replication of viruses requires the synthesis of structural (capsid) and non-structural (enzymes) proteins. A non-structural protein (RNA Nucleotidyl Transferase) necessary for the synthesis of RNA, has been reported for mengo (7), polio (6), Semliki Forest (25), foot-and-mouth disease (30), vesicular stomatitis (43), fowl plague (34), and ND (35) viruses. Although evidence of this enzyme has not been reported for IBV, the fact that it is an RNA virus would suggest the existence of the enzyme. Synthesis of RNA of NDV is inhibited by FPA no later than two hours after infection (33). This would indicate that RNA synthesis requires an early protein, presumably RNA-dependent RNA polymerase.

If the differences in the sensitivity of IBV and NDV is reflected in the polymerases of the viruses, it would appear that the enzyme of NDV is able to maintain its activity to a greater degree following incorporation of FPA residues into the molecule.

Similar considerations may also apply to the structural proteins of the virus particle. Proteins of fowl plague virus formed in the presence of FPA are incorporated into intact particles at only 15% of the normal rate (32). This would indicate that the capsid proteins containing FPA do not have the proper configuration necessary for ready incorporation into the viral particle. Incorporation of capsid proteins containing FPA into virions without the proper configuration may result in a virion that is more sensitive to heat or nuclease inactivation, as proposed for poliovirus (20).

Critical evaluation of these hypotheses for the observed difference in the ability of the proteins of IBV and NDV to retain enzymatic or structural integrity must await the isolation and analysis of amino acids of the individual proteins of the viruses.

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