Chemotherapeutic Experiments with Ribonucleic Acid Containing Animal Viruses

Frank Thomas Frigan
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CHEMOTHERAPEUTIC EXPERIMENTS
WITH RIBONUCLEIC ACID
CONTAINING ANIMAL VIRUSES

by

Frank Thomas Frigan

A Dissertation submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

February
1964
LIFE

Frank Thomas Frigan was born in Zagreb, Croatia, on May 4, 1925. He graduated from I. Real Gymnasium, Zagreb, Croatia, in June, 1944.

From 1946 until 1948 he attended Karl Franzens Universitat, Graz, Austria, where he studied medicine.

In 1951 he came to the United States of America, and in 1956 became a United States citizen.

The author began graduate work in the Department of Microbiology, University of Chicago in 1957, and received a Master of Science degree in 1960. In the same year he continued graduate studies in the Department of Microbiology, Loyola University. In 1962 he was a recipient of a Royal Cabell fellowship.
ACKNOWLEDGEMENT

I wish to express my sincere appreciation to Dr. Thomas J. Bird for his advice and guidance throughout the course of my Ph.D. program and for his assistance during this investigation.

I also wish to thank the other staff members of the Department of Microbiology for their continuous interest and advice.
PURPOSE

The purpose of this work was to study and select from thirty different drugs those with the ability to partially or completely suppress the infectivity of Newcastle disease virus and vesicular stomatitis virus in tissue cultures. Drug(s) showing significant activity in tissue cultures should be tried out in fertilized eggs or animals. Finally, the drug(s) with therapeutic activity in vitro and in vivo should be further studied using biological, biochemical and histochemical techniques, in order if possible, to elucidate the mechanism(s) of its(their) inhibitory action.

The general purpose of this work was to contribute to the field of Chemotherapy of viral diseases, which due to very intricate virus-host cell relationships, has not been achieved on a practical and clinical level.
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CHAPTER I

INTRODUCTION

In 1909 Ehrlich proposed the word chemotherapy to denote the concept of therapy of bacterial diseases by means of drugs selectively toxic for the parasites.

On this basis chemotherapy is defined as the treatment of infectious diseases in vivo by chemical agents which are specifically toxic for the etiological agents without serious toxic or damaging effects for the host.

The discovery by Domagk (1935) that prontosil, an azo dye, significantly inhibited streptococcal infections in mice with no toxic effect for mice, and subsequent findings on the mechanism of its action by Woods (1940), prompted Fildes (1940) to propose a rational approach to research in chemotherapy of infectious diseases. This approach was logical, and synthetic compounds such as sulfones, aminohydroxybenzoic acid and the derivatives of pyridine carboxylic acid were found which are used successfully against infections caused by acid fast bacilli (Burrows, 1955).

The discovery of penicillin and streptomycin and later of broad spectrum antibiotics such as tetracyclines and chloramphenicol greatly contributed to the array of therapeutic agents available for treatment of bacterial diseases.
and also diseases caused by rickettsiae and chlamydozoa.

The studies of the mechanisms of the action of these agents pointed to selective inhibition of various metabolic activities which had a lethal or inhibiting effect on the pathogen. The inability of gram positive bacteria to synthesize their cell wall in the presence of penicillin (Lederberg, 1957), or the inhibition by streptomycin of the Krebs cycle (Oginsky and Umbreit, 1954), and the interference with transamination reactions of Mycobacterium tuberculosis by isonicotinic acid hydrazide are only a few examples of inhibitory mechanisms.

The results of these studies demonstrate that chemotherapy is possible if the parasite exhibits metabolic activity which may be inhibited by a drug.

Chemotherapy today is relatively successful in combating many diseases of protozoan, bacterial, rickettsial and chlamydozoan etiology, whether the parasite is found intracellularly or extracellularly. The only group of obligate intracellular parasites against which chemotherapy has been ineffective is the group of "true" viruses. It becomes clear that the intracellular mode of life of diversified organisms and entities, when examined from the viewpoint of chemotherapy, is graded, and therefore should depend on different factors. The reproduction of viruses and the metabolism of the host cells is such an intimately shared mechanism that selective inhibition of virus is difficult.
CHAPTER II

HISTORY

The word virus comes from the Latin noun meaning venom or similar toxic fluid. It was used from ancient times until very recently, denoting all sorts of noxious and poisonous agents (Fraenkel-Conrat, 1962). Even today the word "virus" is an intractable one. It refers to a characteristic complex of recognizable qualities associated with a natural or experimental disease rather than to the individual unit or microorganism which one tends to speak of as a virus particle (Burnet, 1960).

Viruses probably would never have been discovered if they did not have the property of causing disease. The idea of submicroscopic nonbacterial pathogens was very slow in finding its experimental basis (Luria, 1953). Progress was slow. A century before Iwanovski reported the transmission of tobacco mosaic disease by means of sap filtered through bacteriological filters, Jenner was successful in vaccinating the children of England with "Variolae Vaccinae." Transmission of rabies from a rabid to a normal dog by means of filtered saliva was reported by Zinke in 1804, some seventy-five years before Pasteur's preparation of antirabies vaccine. None of them knew the true nature of these agents. Pasteur might have
perceived the true biological character of the rabies virus, but it remained for bacteriologist Beijerinck (1899), after rediscovering the virus of tobacco mosaic disease, to recognize that he was dealing with a new type of biological agent, and promptly called it, on the basis of its filterability through bacteriological filters, "contagium vivum fluidum."

Following Beijerinck's work, a number of people discovered a variety of filterable infectious agents. Borrel (1904) reported the discovery of the agent of fowl plague; Paschen (1906) rediscovered the elementary particles of vaccinia, which were studied and called by Buist (1887) micrococci of vaccinia; von Prowazek (1907) describes trachoma and Landsteiner and Popper (1909) poliomyelitis virus.

In the three decades following the work of Beijerinck, important progress in the study of the cellular pathology of several viral diseases had been achieved. (Negri, 1903; Councilman, 1904; da Rocha Lima, 1912; Denton, 1925; Lillie, 1930). Despite this, virology as an integrated part within the broader scope of microbiology did not exist.

With the discovery of bacterial viruses by Twort (1915) and d'Herelle (1917), and their subsequent study (Burnet and McKie, 1929; Ellis and Delbruck, 1939; Hershey, 1946), it became clearer that for successful study of the properties of animal viruses a simple host system should be used.

The inability to measure quantitatively viral
infectivity in simple systems on one hand and the need for such studies on the other, disturbed early virologists, as one can conclude from Enders (1959):

It is not surprising that attempts to propagate animal viruses and rickettsiae in isolated systems of cells followed the development by Harrison (1907) of a simple method of tissue culture, for it was early apparent to most observers that these agents, unlike bacteria, fail to multiply in lifeless media. Although the method was applied in the past effectively to the investigation of several fundamental problems relating to animal viruses, during a long period it did not appear to offer advantages for their cultivation and study provided by the susceptible animal, since it was considered that viral increase in cell systems could be demonstrated reliably only by inoculation of appropriate animals with constituents of the culture. Recently, however, tissue culture has assumed the status of a major technic in the virus laboratory.

There are actually three good reasons for this development:

a) use of antibiotics in the medium supporting the growth of cells which are the hosts for a variety of viruses, made it possible to prepare tissue cultures on a large scale and permitted direct isolation of viruses from contaminated materials;
b) use of Rous and Jones (1916) technic of trypsinization of tissues in the preparation of cellular suspensions;
c) observation and recognition that many viruses, when present in tissue cultures and multiplying in the cells are responsible for degenerative changes, which are easily detected and known under general terms of cytopathic effects (Huang, 1942).
With the advancement of modern tissue culture techniques many concepts about viruses were reaffirmed and some had to be changed.

The nature of viruses. Some physical, chemical and biological properties.

Reports on infectious agents passing filters retaining even the smallest bacteria indicated to early workers their diminutive sizes. But it was actually Elford (1931) who published the first reports on the estimation of the sizes of viruses, using graded collodion membranes. Later, the use of the electron microscope and the ultracentrifuge improved these measurements. The application of the electron microscope with newer techniques have given us more details of viral morphology (Howatson and Whitmore, 1962; Brenner and Horne, 1959).

The first work on the chemical properties of viruses was done by Vinson and Petre (1929, 1931) which led to the crystallization of tobacco mosaic virus by Stanley (1935) and, among others, poliomyelitis virus by Schaffer and Schwerdt (1955). Chemical analyses of "purified" virus preparations report findings of nucleic acids, protein, and in some instances, lipids and polysaccharides (Taylor et al., 1942; Smadel and Hoagland, 1942; Ada and Perry, 1954). All viruses contain either RNA or DNA and protein and in some cases (Myxoviruses) exhibit enzymatic properties. Lipid and polysaccharide
materials may or may not be considered integral parts of virus.
Some people believe that the lipids found in some viruses are
acquired by the virus as it emerges through the cytoplasmic
membrane. Treating viruses to remove lipids renders them non-
infective (Theiler, 1957; Franklin, 1962). Viruses when in-
verted into their hosts, stimulate the immune mechanisms to
produce antibodies. This is due to the antigenic nature of
their proteins and nucleoproteins. Since these antibodies are
in many instances protective, inactivated or attenuated viral
preparations are used as vaccines (Schaffer and Zillig, 1954;

Viruses exhibit the properties of genetically indi-
vidual entities. They are subject to genetic mutations. For
example, the recovery of different, but serologically stable
strains of Influenza A virus since the pandemic of 1918 (Fran-
cis, et al., 1953; Hennesy, 1955). Other studies confirm the
ability of viruses to mutate and recombine, giving stable, new
types, which differ from the parent types, either in host
range, virulence or sensitivity to various agents (Berry and
Dedrick, 1936; Luria, 1956).

Rous (1911) showed that viruses are responsible for
some tumor formation in chickens. In recent years, it was
shown by Gross (1956) that mouse leukemia could be transferred
from one animal to another by means of a filtrate of leukemic
tissue. Shope (1933) isolated a virus (Shope papilloma) from
naturally occurring papillomas of wild rabbits. This papilloma is a nonmalignant growth. If filtrates of such tumors are inoculated into the skin of either wild or domestic rabbits, tumors are induced in the animals. Virus could be reisolated from wild rabbits but not from the tumors in domestic animals. Such papillomas are usually benign growths, but after long periods of time (Kidd and Rous, 1940) they may become malignant.

During 1950 it was demonstrated that the nucleic acids of viruses were the carriers of the biological information necessary for their replication. Hershey and Chase (1952) in what are considered classical experiments, demonstrated this with bacterial viruses using radioisotope techniques. Direct evidence for this phenomenon comes from work of Alexander et al., (1958) in which the infectivity of poliomyelitis virus RNA was proven on HeLa cells. Since in nature virus nucleic acids are contained in a protective protein coat, a successful contact between the viral protein coat and the surface of a susceptible cell is a prerequisite for penetration of the cellular membrane by viral nucleic acid. In all systems so far studied specific electrolyte requirements exist, (Kozloff et al., 1957) for the attachment and adsorption of a viral particle to the surface of a cell. The discovery of agglutination of chicken erythrocytes by influenza viruses by Hirst (1941) and the study of its mechanism by S. Gottschalk
(1956) pointed to the phenomenon of adsorption of viral particles to the receptor groups on the surface of susceptible cells, as the first step in subsequent events.

Various histochemical procedures (Coons, 1958, 1959; Armstrong, 1956; Anderson, et al., 1959) confirmed the concept of an eclipse period as a part of the latent period of viral intracellular development. This concept was proposed by Ellis and Delbruck (1939), as a result of their "One step growth experiments," with bacterial viruses and later applied to animal viruses by Dulbecco (1952) and Dulbecco and Vogt (1954).

During this period the first comprehensive definitions of "virus" were proposed. Luria (1956) defined it as follows: "Viruses are submicroscopic entities, capable of being introduced into specific living cells, and of reproducing inside such cells only." Due to quick development of new concepts based on new findings and hypotheses, Luria (1958) defines the "virus" somewhat differently:

Viruses could be considered as genetically specific cell constituents, containing coded DNA or RNA which can, as one of their genetic functions, determine their own incorporation into specific vehicles for transmission to other cells.

**Experimental chemotherapy of viral diseases**

The first attempts at the chemotherapy of viral diseases appeared in the literature in late 1940. There are a number of good reviews on the progress to date in this area. For
example, Matthews and Smith (1955), Hurst and Hull (1956), Horsfall and Tamm (1957) and Staehelin (1959).

A. Amino acids and analogs

Thompson (1947) reported that dl-methoxinine, an analog of methionine, inhibits the growth of vaccinia virus in Maitland type (minced) tissue cultures of chicken embryos. Rafelson, et al., (1950) found that beta-2-thienylalanine inhibited multiplication of Theiler's GD VII virus in minced mouse brains, with no apparent effect on the uptake by this tissue of P\(^{32}\) into its lipid and protein bound fractions. It was also reported in the same paper, that lysine, and to a somewhat lesser extent, tryptophan and histidine inhibited the growth of GD VII virus, and that the inhibition by lysine was reversed by methionine.

Ackermann (1951) showed that methoxinine inhibited the growth of PR 8 strain of Influenza A virus in intact tissue cultures of chicken chorioallantoic membrane (CAM). The inhibitory effect of methoxinine was reversed by L-methionine but not D-methionine, cysteine, creatine, betaine or choline. Methoxinine did not affect the oxygen uptake by CAM. Ackermann and Maassab (1954) found that methoxinine inhibits the growth of Influenza virus if it was added 2-3 hours after viral adsorption on CAM. On the other hand, alpha-amino-p-methoxyphenylmethanesulfonic acid was inhibitory only at the initial stage of infection causing delayed viral release from
the CAM cells.

Inhibition of the Lansing strain of poliomyelitis virus by DL-ethionine in minced human embryonic brain was reported by Brown and Ackermann (1951). In this case methionine competitively inhibited the action of DL-ethionine, which was not virucidal in vitro, and which did not irreversibly affect the tissue. Basic amino acids such as lysine, arginine and ornithine, at concentrations from 1-10 mg per ml of growth medium, retarded the growth of Influenza A and B viruses as well as Mumps virus in minced tissue cultures of chicken CAM, according to Eaton et al., (1951). Ackermann and Maassab (1955) showed that inhibitory properties of 5-fluorophenylalanine (FPA) on the Influenza virus when grown in CAM differ from those of methoxinine. Release of FPA inhibition results in immediate continuation of viral production, whereas in the case of methoxinine at least a two hour lag ensues before any detectable increase in viral infectivity could be measured.

Kundin et al., (1959) reported that several amino acids and their derivatives inhibit the growth of Influenza A and B viruses in chicken embryo lung tissue cultures. Particularly good inhibition was achieved with norleucine, beta-phenylserine, allylglycine and ethylcysteine.

It has been shown that 5-fluorophenylalanine inhibits the growth of foot and mouth virus (Brown et al., 1961), poliomyelitis virus (Levintow and Darnell, 1961), fowl plague
(Zimmermann and Schaffer, 1960) in different host systems and under a variety of conditions.

B. Purines and pyrimidine analogs

Purine and pyrimidine analogs can act in two ways. They either inhibit metabolic steps leading to the synthesis of nucleic acids, or by virtue of their incorporation into virus nucleic acids they can decrease viral infectivity or produce nonfunctional nucleic acids.

It was shown by Matthews (1953) that 8-azaguanine is the most active inhibitor of tobacco mosaic virus in a series of purine and pyrimidine analogs. The activity was probably due to its incorporation into RNA of virus (Matthews and Smith, 1955), because the only other active analogs, 8-azaadenine and 4 (5)-Amino-1H-1, 2, 3-Triazolo-5(4)-carboxamide were also incorporated. Haas and Stewart (1956) reported the synergistic inhibitory effect of 8-azaguanine and amethopterin on lymphocytic choriomeningitis virus in mice.

Thiouracil was reported to inhibit tobacco mosaic virus in tobacco plants (Commoner and Mercer, 1951). The effect of thiouracil could be reversed by uracil (Commoner and Mercer, 1952). This could be done only during early stages of infection as reported by Jeener (1957).

Knox et al. (1957) showed that 2-thiouracil, 2-thio-4-phenyl-6-oxy-pyrimidine, 5-methyl-2-thiouracil and 4,6-diamino-5-nitro-2-thio-pyrimidine completely suppress the
growth of poliomyelitis virus in cultures of monkey testicles for a period up to 12 days. In monkey kidney cultures, growth was delayed for 24 hours and even then it was significantly lower than in controls. Higher concentrations of the drugs were needed to inhibit type 1 than types 2 or 3 virus. This inhibition was reversed by addition of normal pyrimidines. Amos and Vollmayer (1955) reported the inhibitory effects of thiouracil on Influenza A virus in CAM and deembryonated chicken eggs. Infectivity and hemagglutinating activity of the virus was suppressed. Uracil acted competitively, since it could overcome the effect of thiouracil. Minton et al. (1953) showed that phenoxythiouracil protected mice against intranasal or intracerebral infections by vaccinia. The compound could be given intraperitoneally or with the diet, but it was not effective if given 2 days after infection. Fluorouracil (Kaplan and Ben-Porat, 1961) inhibits the formation of infective pseudorabies virus in rabbit kidney cells. This inhibition was overcome by thymidine. The same drug prolongs the life of mice infected intracerebrally with lymphocytic choriomeningitis virus (Levy and Haas, 1958). Rich et al. (1962) found that 5-fluorodeoxyuridine (FUDR) does not inhibit the synthesis of Rous sarcoma virus in chick embryo fibroblasts. FUDR, on the other hand, inhibits the multiplication of DNA containing vaccinia virus (Salzman et al., 1963). Tenfold to hundredfold higher concentrations of FUDR had no
effect on the rate of formation or final yield of RNA containing poliomyelitis virus, and Salzman suggests that FUDR could be used as a means of distinguishing between RNA and DNA containing viruses.

C. Benzimidazole and derivatives

Work on benzimidazole compounds provides one of the more interesting recent developments in animal virus chemotherapy. This compound has some inhibitory effect on a number of viruses (Matthews and Smith, 1955). Extensive screening of benzimidazole derivatives and studies of their action on a number of viruses was started by Tamm in 1953 and continued until this date. Tamm (1956) showed that alkylated and halogenated benzimidazoles are much more inhibitory for influenza virus than benzimidazole, a component of vitamin B₁₂. In another paper, Tamm (1956a) showed that beta-D-ribofuranosyl derivatives of benzimidazole are active at lower concentration and also that they were much more selective in their activity, since the ratio between toxic and inhibitory concentrations was two times higher in the case of 5, 6-dichloro-beta-D-ribofuranosyl-benzimidazole (DRB) and 6 times as high with 4, 5,6-trichloro-beta-D-ribofuranosyl-benzimidazole (TRB) when compared with free benzimidazole. This action was specific for beta ribofuranoside, the natural form in which nucleotides are present in RNA. Influenza virus is an RNA containing virus. Vaccinia virus, a DNA containing virus, is not
inhibited by these benzimidazole derivatives, which is surprising (Tamm and Nemes, 1957). But poliomyelitis virus was inhibited by a D-arabinosyl derivative, and therefore the inhibition of RNA containing viruses in this case is not too clear. This work was done mostly in tissue cultures and eggs, but there was also some activity in animals. Recently Tamm et al., (1961) and Eggers and Tamm (1961) showed that 2(-alpha-Hydroxybenzyl)-benzimidazole (HBB) is perhaps the most selective viral inhibitor among the variety of benzimidazole derivatives which have been so far studied. Structurally HBB is not an analog of any known metabolite, but it inhibits reversibly a late step in replication of many enteroviruses by an unknown mechanism. Kissman et al., (1957) report on the other hand that DRB was inactive against PR 8 strain of Influenza A virus in mice.

D. Miscellaneous compounds

Caprochlorene (Liu et al., 1957) reduced infectivity and hemagglutinin titers of PR 8 influenza virus in demembranated eggs. Twenty hours after infection with virus the compound was active, although not as markedly as in earlier treatment. The compound protected mice almost completely (19 out of 20) against the virus. Hamre et al., (1951) reported the ability of thiosemicarbazones to inhibit multiplication of vaccinia virus in mice. Mepacrine, an antimalarial drug, (Greenhalgh et al., 1956) protected mice against large doses of
a number of arbor viruses when the drug was given orally soon after infection. Xerosine, an antibiotic obtained from an Achromobacter species, showed a number of interesting properties (Groupe, et al., 1952). If mice were infected intranasally with 10,000 LD$_{50}$ of influenza A virus, subcutaneous xerosine was effective even 48 hours after infection of the animals. Orally, xerosine was not effective, and highly toxic when given intraperitoneally. It seems that tissue reactions have an important role in treatment with xerosine, rather than its ability to suppress viral multiplication (Groupe et al., 1954).

Chrowther and Melnick (1961) found that guanidine. HCl inhibited production of poliomyelitis virus in monkey kidney tissue cultures. Rightsel et al., (1961) reported the inhibition of various enteroviruses by this compound. Treatment of monkeys infected with poliomyelitis virus was unsuccessful.

Mitomycin C, an antibiotic product of *Streptomyces caespitosus*, according to Reich and Franklin (1961) does not inhibit the RNA containing Mengo virus at the doses which markedly suppressed DNA containing vaccinia virus. Shatkin et al., (1961) studied the effect of mitomycin C on mammalian cells, and reported that it causes dissolution of the nuclear apparatus in a manner similar to ionizing radiation. Reports by Ben-Porat et al., (1961) on the formation of in-
complete pseudorabies virus in rabbit kidney cells in the presence of mitomycin, and only very slight inhibition (Gomatos et al., 1962) of Reovirus type 3 in L cells, confirms its action on DNA.

Puromycin (Levintow et al., 1962) inhibits multiplication and maturation of poliomyelitis virus in HeLa cells when the infected cells are treated early. Removal of puromycin by washing of the infected cells brings about resumption of normal viral replication. Baltimore and Franklin (1962) and Franklin and Baltimore (1962) reported that puromycin inhibits the formation of Mengo virus RNA in L-fibroblast cells, probably through the mechanism of blocking the formation of new protein in the infected cells that might serve as virus specific RNA polymerases.

A number of basic dyes were studied as possible viral therapeutics (Matthews and Smith, 1955). The findings resulting were not too promising. More recently the inhibitory action of proflavine on fowl plague virus was reported by Franklin (1958).

E. Interferon

Isaacs and Lindemann (1957) reported discovery of a proteinaceous substance which they called interferon, and which is the product of virus-infected cells. According to a number of investigators, interferons from various sources have a wide spectrum of antiviral action and exhibit no particular
specificity for the viruses responsible for their formation. The difficulty in using interferon as a therapeutic agent is the inability to prepare and purify it in the quantities which could be effective in man and animals.
CHAPTER III

MATERIAL AND METHODS

Viruses used in this work were obtained from Dr. F. Deinhardt, St. Luke-Presbyterian Hospital, Chicago, Illinois, and Dr. D. Hamre, University of Chicago, Chicago, Illinois.

The Roakin strain of Newcastle disease virus (NDV) was passed twice in eggs, before stocks were made. Prior to that, virus was passed 7 times at Rutgers University, 17 times at the University of Wisconsin and several times at the University of Chicago. The LD$_{50}$ titer of this strain for chicken embryo can reach $10^9$ and it is uniformly lethal when inoculated intracerebrally. Hemolysin titers of 1:128 were observed and hemagglutinin (HA) titers of 1:1280.

The Indiana strain of vesicular stomatitis virus (VSV) was passed intracerebrally in mice several hundred times as well as numerous foot pad passages in mice, several epithelial passages in cattle and horses and more than 20 passages in eggs. In our laboratory it was passed 3 times through chicken eggs before preparations of viral stocks.

Fertilized, white leghorn chicken eggs were obtained once a week from a nearby hatchery, and incubated at 37°C upon arrival. Ten day-old embryos served as source of primary
embryonic cells, otherwise the eggs were used for preparation of viral stocks and for embryo survival experiments. Several eggs were permitted to hatch, and chickens used as a source of blood for hemagglutination (HA) and hemagglutination-inhibition (HI) tests.

HeLa cells were obtained from Cappel Laboratories, Inc., and continuously cultivated in minimal essential medium (MEM) of Eagle (1959).

**Media and solutions**

Phosphate buffered saline (PBS) was prepared as described by Dulbecco and Vogt (1954). It was used for washings of monolayers and as viral diluent. The composition of PBS is as follows:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>8.00 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.20 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.15 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.20 g</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.10 g</td>
</tr>
<tr>
<td>MgCl$_2$·6 H$_2$O</td>
<td>0.10 g</td>
</tr>
<tr>
<td>H$_2$O Deionized</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

PBS was sterilized by autoclave.

0.5% Lactalbumen-hydrolysate medium (LH) was prepared according to Franklin et al., (1958). It was used for growth of chicken embryo fibroblasts, and contained the
following ingredients:

90% Hanks' balanced salt solution (BSS)
10% calf serum
0.5% lactalbumen hydrolysate (enzymatic)
0.1% yeast extract
100 units of penicillin / ml
50 ug of streptomycin / ml
20 ug of fungizone / ml

Hanks' BSS was prepared as recommended by Hanks and Wallace (1949). Chicken fibroblasts were maintained for viral titrations in a maintenance medium of 0.01% lactalbumen hydrolysate, and 5% serum. Glutamine was added to contain 290 ug / ml.

Trypsin solution was prepared as given by Marcus et al., (1956) and consisted of the following:

NaCl .................. 20.00 g
KCl .................. 1.00 g
Glucose .................. 2.50 g
NaHCO₃ .................. 1.45 g
Trypsin (Difco 1.250) ...... 1.25 g
Versene ................ 0.55 g
Penicillin ............... 0.50 g
Streptomycin ........... 0.25 g

Deionized H₂O to 250 ml

This solution was sterilized by Seitz filtration and stored at...
When needed, it was thawed and diluted 1:10 with sterile deionized water. Trypsinization was done in special flasks designed for this purpose, on a magnetic stirrer.

**Preparation and harvests of viral stock**

Viral stocks were prepared in fertilized, 10-day old chicken eggs by allantoic inoculation of 0.1 cc of $10^{-3}$ dilution of samples. Eggs incubated at 37°C were candled, the air space marked, and an area selected directly above the embryo and showing no blood vessels. The area was swabbed with tincture of iodine, a small slit drilled through the shell carefully, avoiding the piercing of the shell membrane. Virus was inoculated using a 0.5 cc syringe with a 23-gauge, 1 inch needle, by inserting the needle through the slit, through the chorioallantoic membrane (CAM) and away from the embryo toward the shell. Eggs were sealed and incubated at 37°C. The next day the eggs were candled again, and those with dead embryos discarded, assuming that deaths were due to nonspecific causes. After 48 hours the eggs were candled again, chilled in the refrigerator, and allantoic fluid harvested. Harvesting was done by sterilizing the entire shell with 70% alcohol, removing the entire shell above natural air space and collecting the fluid with a syringe, avoiding blood vessels. The allantoic fluids were pooled, distributed in 1 ml volumes in screw
capped tubes, frozen immediately and stored at -20°C. The presence of virus was determined either by hemagglutination or by cytopathic effect (CPE) in chick fibroblasts. Sterility tests for presence of bacteria were done in nutrient broth.

**Preparation of monolayers of chicken embryo fibroblasts**

Monolayers were prepared according to Dulbecco (1952) with small modifications. They were needed for screening of drug toxicities and for the titrations of viral infectivity in the presence or absence of drugs as well as for obtaining NDV hemagglutinin. The following steps were employed:

10-12 day-old embryos were aseptically removed and placed in sterile petri plates. Beaks and eyes were removed, the embryos cut up and these were washed with cold sterile PBS to eliminate as much blood as possible. After washing, fragments were placed in the barrel of a 20 cc syringe and expressed into a trypsinizing flask. Ten ml of trypsin solution was added per embryo and the contents of the flask stirred for about 20 minutes. After trypsinization the contents of the flask were filtered through sterile gauze, discarding undigested connective tissue with the gauze, and the cells in trypsin distributed among several centrifuge tubes. Cells were centrifuged at 600 G for 5 minutes, trypsin discarded and the pellets washed with lactalbumin hydrolysate medium. After two washings, cells were counted in a hemocytometer and diluted in
LH to contain between $3-4 \times 10^5$ cells / ml and finally distributed among bottles or tubes and incubated at $37^\circ$C. It was found that each 0.1 cc of packed cells when diluted with 20 ml of medium gave good results, with monolayers developing 24 hours later. In spite of heterogenic cell origin in these primary cultures, most cells had a fibroblastic appearance, and therefore they are referred to as primary chicken embryo fibroblasts. These were not carried continuously, but were prepared as needed.

**Cultivation of HeLa cells**

HeLa cells were carried continuously. The growth medium actually contained 90% MEM (Eagle, 1959), 10% calf serum and usual amounts of antibiotics. The maintenance medium for HeLa cells differed only in the amount of serum, which was 4-5%. For staining purposes, cells were distributed in 1.5 ml amounts ($2 \times 10^5$ cells / ml) among small Leighton tubes containing small cover slips. When needed, cover slips with attached cells were removed, cells stained by various procedures, mounted on glass slides, and examined. For RNA extractions, HeLa cells were infected with NDV, trypsinized and distributed in $3 \times 10^6$ cells aliquots among tubes and fed with maintenance medium. All the cells, whether normal or infected, in the presence or absence of 5-fluorophenylalanine, were slowly rotated at $37^\circ$C, and after 24 hours in the incubator,
extracted for RNA.

**Purine bound ribose determination**

RNA was extracted from HeLa cells using the method of Schmidt and Tanhauser (1945). Purine bound ribose determinations on RNA extracted from HeLa cells were done as described by Mejbaum (1939) and Ceriotti (1955) using orcinol method. Readings were done in Klett-Summerson colorimeter, using red filter (670μm). For this purpose a standard ribose curve was determined, using the same procedure.

**Drugs**

Drugs were secured from various sources. Information as to solubilities was obtained from Merck's Index, 7th Ed. (1960). Drugs soluble in water were added directly to the medium, and sterilized by either filtration or autoclaving. Drugs soluble in alcohol or chloroform were dissolved in them to the limit of their solubilities, and small aliquots added to the medium to get the desired concentration, and sterilized by autoclave. Occasionally after the addition of the drugs to the media, adjustments to pH 7.3-7.5 were necessary, and this was done with sterile 0.3 N NaOH or 0.3N HCl.

**Assays of drug toxicities**

These assays were done as described by Rightsel et al., (1958). The method was modified in order to obtain the
best possible results in Leighton tubes. Briefly it consisted of exposing monolayers of chick fibroblasts to several concentrations of the particular drugs in maintenance LH and observing the action of the drug on the cells for a period of several days. Rounding of the cells, their detachment from the glass, and the unchanged pH were criteria of toxicity. Only the highest concentrations of drugs tolerated by cells were used in further experiments.

Qualitative assays of viral infectivities in the presence of drugs.

Once the highest tolerated doses of the drugs were determined for the chicken fibroblasts, their potential antiviral activity was ascertained as follows: Monolayers of 24-36 hours-old fibroblasts were infected with approximately 1000 TCID$_{50}$ of each virus. The cells were washed with PBS and the virus adsorbed for one hour at 37°C. The media containing the drugs were then added and the tubes reincubated. Controls were also prepared. Each control tube contained the cells. Cell control contained only LH medium, drug control was prepared with LH medium containing the drug and the virus control contained LH medium and virus. The tubes were examined daily for 3-5 days. Cytopathic effect due to the presence of virus was scored as 0, 1, 2, 3 and 4, depending on cellular damage. Only those drugs which inhibited cytopathic effect to an extent of 3 (lowered CPE from 4 to 3 or lower, as
compared to virus control), and in at least three out of four tubes, were used in further experiments.

Quantitative assays of viral infectivities

These assays were performed using the method of Reed and Muench (1938). Infected allantoic stocks were diluted with cold PBS. Aliquots of 0.1 ml of each dilution were inoculated into monolayers of cells, using for each dilution 5-10 monolayer tubes. The monolayers were washed with PBS, virus adsorbed for one hour at 37°C with occasional tilting of the tubes in order to spread the virus evenly. After adsorption, medium was added and the tubes reincubated. Monolayers were examined daily, CPE scored and the viral titers obtained.

Quantitative assays of viral infectivities in the presence of selected drugs

These assays were done in exactly the same manner as the assays described above but on the virus formed in the chicken cells in the presence of drugs. In order to obtain the virus, the cells in the tubes were frozen and thawed twice and vigorously pipetted, cell debris removed by centrifugation, and the fluid containing the virus titrated as above (Franklin, 1958).
Hemagglutination (HA) and hemagglutination-inhibition (HI)

HA and HI tests (titrations) were done in cupped plastic trays, each cup having a volume of approximately 2.6 ml. Chicken blood for this purpose was obtained by bleeding chickens from the heart and collecting the blood in the following mixture:

- sodium citrate ................. 0.8 g
- sodium chloride ............... 0.4 g
- glucose ......................... 2.0 g
- sterile, dist. water 100 ml

Cells were always kept in sterile refrigerated Alsever's solution. When needed for HA or HI tests, a certain volume was removed, the cells sedimented by centrifugation and the supernatant discarded. The cells were further washed in 2 changes of cold PBS and finally prepared as 0.5% suspension in PBS.

The actual HA tests were run as follows: to each cup in the plastic trays was added 0.5 ml of cold PBS. The infected fluids were diluted in PBS and to each 0.5 ml of diluted fluid 0.5 ml of 0.5% chicken red blood cells was added, covered with a plastic cover and refrigerated. Two hours later the HA titers were read as the highest dilution of fluid which completely agglutinated the cells.

HI tests were performed in a similar way. NDV antiserum previously prepared in rabbits was diluted serially two-fold in 0.25 ml with PBS, and to each cup 4 HA units contained in
0.25 ml and 0.5 ml of 0.5% chicks RBC's were added and refrigerated. Controls were set for HA, antiserum and RBC's. HI titers were determined on the basis of the highest dilution of antiserum completely inhibiting hemagglutination.

Antiserum

The infected allantoic fluids (stock) were prepared in eggs as previously described. Antibodies against NDV were prepared in rabbits. The NDV containing fluids were inoculated intraperitoneally in 2 ml amounts every second day for a period of 14 days. In the third and fourth week the rabbits received 1 ml of the infected fluids intravenously. The rabbits were bled a week after receiving the final inoculation. This immune serum was used in indirect fluorescent antibody techniques.

Staining procedures and use of fluorescent microscopy

Some staining of HeLa cells was done with carmine-fast green stain (Frigan and Bird, 1963). Acridine-orange (Harleco-C.I. No. 46005) staining of HeLa cells was done as reported by Pollard and Starr (1962). This was 0.1% solution in McIlvaine citrate-phosphate buffer pH 3.8. The buffer was prepared as recommended by McIlvaine (1928).

Staining with acridine orange was done as follows: HeLa cells on the cover slips were fixed for 5 minutes in
a glacial acetic acid-ethanol mixture. After fixation, cells were rinsed with the buffer and stained with 0.01% acridine orange for four minutes. They were again rinsed with the buffer and immediately mounted in the buffer on glass slides.

Indirect fluorescent antibody techniques were done according to Tamm and Wheelock (1961). HeLa cells were grown as for acridine orange staining. Cover slips with HeLa cells were washed with PBS, fixed in acetone for 15 minutes and dried. They were again washed in PBS for 15 minutes and a drop of undiluted immune rabbit anti-NDV serum (HI titers was 1:640) placed on the cover slip and incubated for 30 minutes in a humidified chamber at 37°C. After this, the cells were washed for 30 minutes in PBS, a drop of anti-rabbit globulin serum prepared in horses and conjugated with fluorescein isothiocyanate was added and again incubated for 30 minutes. Finally the cover slips were thoroughly washed in PBS for 30 minutes and mounted in 20% glycerol in PBS and examined. Controls were prepared with normal rabbit sera and treated as above. Some of the cover slips with infected HeLa cells were treated with ribonuclease to determine the localization and the synthesis of viral ribonucleoprotein. Therefore, prior to staining with fluorescent antibody, infected cells were treated with 0.04% solution of RNAse in McIlvaine pH 3.8 buffer for 1 hour at 37°C, after which time they were stained and treated as described above.
Photography

Pictures were taken with Kodak Pony IV type of camera. For acridine orange and fluorescent antibody photography, a 35 mm high speed Ektachrome type B film was used. Exposure times were between 2-3 minutes. Carmine-fast green preparations were photographed using Ektachrome X film. Light source for fluorescent microscopy was A.O. Fluorolume. A Spencer microscope equipped with a dark field condenser was used. The following filter combinations were used.

<table>
<thead>
<tr>
<th>Acridine orange</th>
<th>Exciter</th>
<th>Barrier</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corning #5113 (2mm)</td>
<td>Wratten #15</td>
</tr>
<tr>
<td>Fluorescent AB</td>
<td>S-BG #12 (3mm)</td>
<td>Wratten #15</td>
</tr>
</tbody>
</table>
CHAPTER IV

RESULTS

Titrations of viral stocks

Prior to any work, it was important to measure and establish the infectious viral titers and their stability. For this purpose both stocks were titrated 3 times, using 10 tubes of fibroblasts for each dilution. The results in Tables I and II indicate individual infectivity for each titration and also the mean infective titers with standard deviations. These determinations were done in the span of one month; as the work progressed, titrations of both stocks were done with little or no change in viral titers.

The mean TCID$_{50}$ of NDV for chick embryo fibroblasts was 5.2 x 10$^{-7}$ and for VSV 4.0 x 10$^{-6}$.

Drug tolerance assays in chicken embryo fibroblasts

The results of these experiments are contained in Tables III and IV. It can be seen that the spectrum of tolerated concentrations ranges from 0.3 µg/ml for puromycin to more than 500 µg/ml for urethane. Drug tolerance was estimated as described in chapter on "Material and Methods." These concentrations were the maximal tolerated by the cells, and used in assays as described below.
Qualitative assays of viral infectivities in the presence of drugs

The results of these experiments are shown in Tables III and IV. It can be seen that phenoxy derivatives of acetic acid were unable to prevent cytopathic effect (CPE) by either virus. Three indole derivatives of the lowest fatty acids as well as indole itself were also ineffective. Naphthoxyacetic acid, naphthaleneacetic acid, maleic acid hydrazide, gibberelic acid and beta-2-furylacrylic acids belong to the same category in their inability to inhibit either virus. 8-azaguanine showed promise with both NDV and VSV. The other two purine derivatives, namely 8-chloroxanthine and 6-furfurlyaminopurine did not inhibit CPE. A marked effect on CPE was seen with dl-5-fluorophenylalanine (FPA). Both viruses were inhibited by 30 µg of FPA / ml of medium, and in both cases CPE was as low as 1 (Tables III and IV).

Of the three antitumor substances, puromycin showed some promise in the inhibition of NDV, mitomycin C lowered CPE due to VSV and carzinophilin was ineffective.

Guanidine HCl showed inhibitory activity for the CPE caused by both viruses, although the related compounds of urea and urethane had no effect.

As is evident (Tables III and IV), only 5 compounds were active in these tests, and these were 8-azaguanine, FPA, puromycin, mitomycin C, and guanidine HCl.
Quantitative assays of viral infectivities in the presence of selected drugs

Drugs were selected for these experiments on the basis of their ability to partially inhibit viral CPE in previous experiments. The procedure had to account for the time period through which the adsorbed virus was exposed to the action of drugs and the limitation of having available monolayers of fibroblasts in which the measurements of infectivity were done. One wanted to determine the extent of the viral synthesis in the cells exposed to selected drugs, and express this quantitatively as a function of time exposure to the drugs. In this manner one can obtain information on the influence of drugs on viral multiplication (Crowther and Melnick, 1961).

Ten tubes with chick monolayers, were infected with 4 x 10^5 virus TCID_{50}. After adsorption, cells were washed and maintenance media containing the drugs added.

Figure 1 shows that FPA significantly inhibited multiplication of NDV. Five hours after viral adsorption, there were 280 TCID_{50} found in control and none in the tubes with FPA. After 33 hours exposure to FPA the activity was 2.5 x 10^5 TCID_{50} and 3.4 x 10^6 TCID_{50} in controls. This represents an infectivity loss of 92.7%. In other words the infectivity in FPA treated cells represented only 7.3% infectivity of controls. In the same manner the infectivity of VSV
control after 33 hours was $1.6 \times 10^5$ TCID$_{50}$ and only $2.4 \times 10^4$ TCID$_{50}$ in the presence of FPA, a loss of 85% (Figure 5).

Guanidine. HCl (Figures 2 and 6) was more inhibiting in early hours after viral adsorptions. The titers obtained later were comparable to controls.

In the case of 8-azaguanine there is a slight inhibition of virus multiplication. NDV was somewhat more inhibited than VSV. VSV titers are very close to the titers of the control, and probably not significant (Figures 3 and 7).

Puromycin (Figure 4) somewhat suppresses early NDV titers, which later on approach the titers of control. Figure 8 shows clearly that mitomycin C did not influence the synthesis of VSV in chicken embryo fibroblasts.

As it can be seen from these experiments, the only significant inhibition of viral multiplications was caused by FPA.

The influence of FPA on viruses in vitro and measurements of their infectivities when FPA added during different time intervals of latent period. Effect of FPA on free virus

Results of these experiments are contained in Tables V and VI. Control titers in these experiments are similar to viral titers in Tables I and II, which were obtained under completely normal conditions. Titers obtained in monolayers when FPA was added at different times during latent period are
significantly lower from control titers. The significance of this experiment will be discussed in chapter on "Discussion."

The influence of FPA on the formation of NDV hemagglutinin in chick embryo fibroblasts

In these experiments the monolayers of chick embryo fibroblasts were infected with usual doses of NDV. Five HA titrations were done in each case, reciprocal of the titers were averaged, and expressed in percentages of hemagglutinating activity of controls. Average titers as well as percent of HA is given in nearest whole numbers in Table VII.

It can be seen from Table VII that the FPA inhibits hemagglutinin production of NDV. Ten μg of FPA did not have any effect. Twenty μg lowered HA production to only 86% of controls. In the presence of 30 μg of FPA, HA production was only 18% of the normal controls.

The influence of FPA on the survival of chicken embryos infected with NDV and VSV

The results of these experiments are tabulated in Tables VIII and IX. It can be easily seen that in the survival experiments with NDV (Table VIII) there are, after 10 days, 21 dead and 6 survivors in controls, which received the virus but no FPA. Experimental batch on the other hand shows 13 deaths and 13 survivors after the same period of time. In the same manner (Table IX) VSV control shows 21 dead and 4
alive embryos whereas FPA treated batch has 17 deaths and 9 survivors.

The significance of these results has been analysed statistically, using for this purpose $X^2$ test (Batson, 1958) for small numbers.

In the case of NDV, the obtained $X^2$ for one degree of freedom was 4.19 and $p<0.05$.

The $X^2$ obtained from VSV experiments was 1.87, giving a $p>0.30$. This would mean that FPA protected or prolonged the survival time of chicken embryos significantly in the presence of NDV but not when infected with VSV.

The studies on the inhibitory mechanism of FPA
The effect of high concentrations of FPA on the synthesis of NDV in HeLa cells

Results obtained can be seen in Table X. Infectivity of control reached $2.1 \times 10^6$TCID$_{50}$. The extent of infectivity in tubes exposed to FPA inhibition for 8 hours, which was then released by washings and phenylalanine, was $4.3 \times 10^4$TCID$_{50}$. Finally, the entire 24 hour exposure to FPA yielded upon titration only $3.2 \times 10^2$TCID$_{50}$.

The FPA inhibition was so thorough, that 24 hour FPA inhibited samples represent 0.015% infectivity of controls. Tubes in which the FPA inhibition was released after 8 hours still reached only 2% of infectivity in controls.
The effect of FPA on RNA of normal and NDV-infected HeLa cells

From Table XI the following relationship is discernible. If 24 hour normal control is considered 100% then there was increased RNA synthesis in NDV infected cells during this period of time. The samples show approximately 11% more of PBR. Cells exposed to FPA at time 0 show only 58.6%, a few percent lower than normal cells extracted at time 0. Infected cells in the presence of FPA still put together some specific acid insoluble material, which on the basis of ribose accounts for 67.2% of the total that could be expected. Despite this, the PBR in this case is somewhat higher than PBR obtained from normal cells at 0 hour. Cells in which the FPA inhibition was released after 8 hours, resume RNA synthesis at normal rate.

Morphological and histochemical studies

Normal and infected HeLa cells were stained by Carmine-fast green stain (Frigan and Bird, 1963), to determine changes in microscopic appearance of infected cells. Cells infected with NDV (Figures 10-13) show formation of vacuoles and syncytia as compared to normal HeLa cells (Figure 9). Figure 13 shows complete destruction of monolayer of HeLa cells by NDV. Normal and cells infected with NDV were stained with acridine orange (Pollard and Starr, 1962) to study any change in content and distribution of nucleic acids. Figures 15-18 show the changes from orange to deep red fluorescence,
indicating an increase in content of RNA due to NDV. In the presence of FPA this shift in fluorescence was inhibited (Figures 19-21). In the infected cells the syncytia and vacuoles were also present. In the presence of FPA the infected cells did not form syncytial masses or vacuoles, but the cells did show irregular "bumpiness" at the surface. Figure 14 shows normal HeLa cells at time zero, and Figure 22 shows normal cells cultivated through the time period of experiments.

The HeLa cells were stained by indirect fluorescent antibody techniques (Tamm and Wheelock, 1961) to demonstrate the presence and location of specific viral material. Figures 23 and 33 represent controls. Figure 23 represents normal cells stained with anti-NDV antibody and fluorescent tagged antibody, and Figure 33 shows infected cells stained with normal serum and fluorescent antibody. In both there was no fluorescence observed. Figures 24, 26, 28 and 30 show development of specific fluorescent material. Figures 25, 27 and 29 demonstrate inhibition of this specific fluorescent material by FPA. Infected cells treated with RNAse and stained with fluorescent antibody techniques demonstrate that this fluorescent material is ribonucleoprotein (Figures 31 and 32).
### TABLE I

**Titration of NDV Allantoic Stock in Chicken Embryo Fibroblasts**

<table>
<thead>
<tr>
<th>Dil.</th>
<th>Inf.</th>
<th>Not inf.</th>
<th>% inf.</th>
<th>Neg. log.TCID$_{50}$</th>
<th>TCID$_{50}$/ml</th>
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$S^2 = 2.58$

$S = 1.60$

Mean TCID$_{50} = 5.2 \times 10^7 \pm 1.60$
### TABLE II

Titration of VSV Allantoic Stock in Chicken Embryo Fibroblasts

<table>
<thead>
<tr>
<th>Dil.</th>
<th>Inf.</th>
<th>Not inf.</th>
<th>% inf.</th>
<th>Neg.log. TCID₅₀</th>
<th>TCID₅₀/ml</th>
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</table>

\[ S² = 3.81 \]

\[ S = 1.95 \]

Mean TCID₅₀ = \( 4.0 \times 10^6 \pm 1.95 \)
TABLE III

Cytopathic Effect of Newcastle Disease Virus in the Presence of Positive Drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Solvent</th>
<th>Tolerated Conc. µg / ml</th>
<th>CPE Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-naphthoxyacetic acid</td>
<td>2</td>
<td>80</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>0-chlorophenoxyacetic acid</td>
<td>1</td>
<td>50</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>2,4,5-trichlorophenoxyacetic acid</td>
<td>2</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Indole</td>
<td>2</td>
<td>250</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Indole-3-acetic acid</td>
<td>2</td>
<td>220</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Indole-3propionic acid</td>
<td>2</td>
<td>200</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Indole-3butyric acid</td>
<td>2</td>
<td>200</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Napththaleneacetic acid</td>
<td>3</td>
<td>80</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>1,2-benzanthracene</td>
<td>3</td>
<td>250</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>8-azaguanine</td>
<td>4</td>
<td>5</td>
<td>3 2 3 3</td>
</tr>
<tr>
<td>8-chloroxanthine</td>
<td>4</td>
<td>10</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>6-furfurylaminopurine</td>
<td>4</td>
<td>20</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>dl-p-fluorophenylaniline</td>
<td>4</td>
<td>30</td>
<td>2 1 2 2</td>
</tr>
<tr>
<td>formyl-dl-phenylalanine</td>
<td>4</td>
<td>60</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Chloracetyl-dl-phenylalanine</td>
<td>4</td>
<td></td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>4</td>
<td>5</td>
<td>4 3 3 4</td>
</tr>
<tr>
<td>Puromycin</td>
<td>4</td>
<td>0.3</td>
<td>3 2 3 4</td>
</tr>
<tr>
<td>Carzinophylin</td>
<td>4</td>
<td>8</td>
<td>4 2 4 4</td>
</tr>
<tr>
<td>Guanidine. HCl</td>
<td>1</td>
<td>125</td>
<td>3 2 3 3</td>
</tr>
<tr>
<td>Urea</td>
<td>1</td>
<td>450</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Azaserine</td>
<td>1</td>
<td>80</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2</td>
<td>120</td>
<td>4 3 4 4</td>
</tr>
<tr>
<td>Isatin-beta-thiosemi</td>
<td>4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Carbazone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adeno-PAP-#1087</td>
<td>1</td>
<td>150</td>
<td>4 3 4 4</td>
</tr>
</tbody>
</table>

No activity whatsoever was recorded with the following compounds: beta-2-furyl-acrylic acid, 100 µg; p-chlorophenoxyacetic acid, 40 µg; 2,4-dichlorophenoxyacetic acid, 40 µg; maleic acid hydrazide, 150 µg; gibberellic acid, 150 µg; urethane, 500 µg.

Solvents: 1 = water; 2 = 95% ethanol; 3 = chloroform; 4 = 0.1% NaHCO₃
Table IV

Cytopathic Effect of Vesicular Stomatitis Virus in the Presence of Positive Drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Solvent</th>
<th>Tolerated Conc. µg / ml</th>
<th>CPE Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dichlorophenoxyacetic acid</td>
<td>2</td>
<td>40</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>2,4,5-trichlorophenoxyacetic acid</td>
<td>2</td>
<td>40</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>Naphthaleneacetic acid</td>
<td>3</td>
<td>80</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>8-azaguanine</td>
<td>4</td>
<td>5</td>
<td>2 3 3 3</td>
</tr>
<tr>
<td>8-chloroxanthine</td>
<td>4</td>
<td>10</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>6-furfurylaminopurine</td>
<td>4</td>
<td>20</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>dl-p-fluorophenylalanine</td>
<td>4</td>
<td>30</td>
<td>1 2 2 2</td>
</tr>
<tr>
<td>Formyl-dl-phenylalanine</td>
<td>4</td>
<td>60</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>Acetyl-d-phenylalanine</td>
<td>4</td>
<td>60</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>Chloracetyl-dl-phenylalanine</td>
<td>4</td>
<td>50</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>4</td>
<td>5</td>
<td>3 3 3 4</td>
</tr>
<tr>
<td>Puromycin</td>
<td>1</td>
<td>0.3</td>
<td>3 3 4 4</td>
</tr>
<tr>
<td>Carzinophylin</td>
<td>4</td>
<td>8</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>Guanidine HCl</td>
<td>1</td>
<td>125</td>
<td>3 3 3 4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2</td>
<td>120</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>Isatin-beta-thiosemicarbazone</td>
<td>4</td>
<td>100</td>
<td>3 4 4 4</td>
</tr>
</tbody>
</table>

No activity was recorded with the following compounds: beta-2-furyl-acrylic acid, 100 µg; beta-naphthoxyacetic acid, 80; 0-chlorophenoxyacetic acid, 50; p-chlorophenoxyacetic acid, 40; Indole, 250 µg; indole-3-acetic acid, 220 µg; indole-3-propionic acid, 200 µg; indole-3-butyric acid, 200 µg; maleic acid hydrazide, 150 µg; gibberellic acid, 150 µg; 1,2-benzanthracene, 250 µg; urea, 450 µg; urethane, 500 µg; azaserine, 80 µg; adeno-PAP #1087, 150 µg.

Solvents: 1 = water, 2 = 95% ethanol, 3 = chloroform, 4 = 0.1% NaHCO₃.
### TABLE V

The Influence of FPA on NDV Titer When Added During Different Intervals of Latent Period.

NDV Exposed to FPA* in Vitro for Six Hours

<table>
<thead>
<tr>
<th>0.01% LH - FPA Medium Hours After Adsorption</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>1.8 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>3.2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>4.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>control (no FPA)</td>
<td>4.4 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*FPA conc. 30 μg/ml
TABLE VI

The Influence of FPA on VSV Titer When Added During Different Intervals of Latent Period.

VSV Exposed to FPA* in Vitro for Six Hours

<table>
<thead>
<tr>
<th>0.01% LH - FPA Medium Hours After Adsorption</th>
<th>TCID\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.8 x 10\textsuperscript{5}</td>
</tr>
<tr>
<td>1</td>
<td>3.4 x 10\textsuperscript{5}</td>
</tr>
<tr>
<td>2</td>
<td>3.6 x 10\textsuperscript{5}</td>
</tr>
<tr>
<td>3</td>
<td>7.1 x 10\textsuperscript{5}</td>
</tr>
<tr>
<td>control (no FPA)</td>
<td>3.2 x 10\textsuperscript{6}</td>
</tr>
</tbody>
</table>

*FPA conc. 30 µg/ml
### TABLE VII

The Influence of fPA on the Formation of NDV Hemagglutinin in Chick Embryo Fibroblasts

<table>
<thead>
<tr>
<th>μg FPA / ml Medium</th>
<th>HA Titer</th>
<th>% HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>19</td>
<td>86</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>18</td>
</tr>
</tbody>
</table>
### TABLE VIII

The Effect of FPA on the Mortality of Chicken Embryos Infected with NDV. Control Group Received 100 TCID\(_{50}\) of Virus/Egg. Experimental Group Received Virus in the Presence of 75 \(\mu\)g of FPA/Egg. Both Groups Inoculated Allantoically and Experiment Terminated at Hatching Time.

<table>
<thead>
<tr>
<th>Embryos</th>
<th>Alive</th>
<th>Dead</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDV</td>
<td>6</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>NDV</td>
<td>13</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>FPA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>34</td>
<td>53</td>
</tr>
</tbody>
</table>

\[ X^2 (c)_{1} = 4.19 \quad P < 0.05 \]
TABLE IX
The Effect of FPA on the Mortality of Chicken Embryos Infected with VSV. Control Group Received 100 TCID$_{50}$ of Virus/Egg. Experimental Group Received Virus in the Presence of 75 µg of FPA/Egg. Both Groups Inoculated Allantoically and Experiment Terminated at Hatching Time.

<table>
<thead>
<tr>
<th>Embryos</th>
<th>Alive</th>
<th>Dead</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>4</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>VSV FPA</td>
<td>9</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>38</td>
<td>51</td>
</tr>
</tbody>
</table>

$X^2(c)_{1} = 1.87$  \quad P > 0.30
### Table I

**M.T. Infectivity in HeLa Cells Exposed to 500 Ug of EPA For 0, 8 and 24 Hours**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>100%</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls—no EPA</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>24—4 hours</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>24—24 hours</td>
<td>0.015</td>
<td>0.015</td>
</tr>
</tbody>
</table>
TABLE XI

Purine Bound Ribose (PBR) Estimations on RNA Extracted from HeLa* Cells Under Various Treatments. FPA Conc. 300 μg / ml of MEM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μg PBR</th>
<th>% Control at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cells - 0 hour</td>
<td>18.8</td>
<td>62.2</td>
</tr>
<tr>
<td>Normal cells - 24 hours</td>
<td>30.2</td>
<td>100.0</td>
</tr>
<tr>
<td>NDV infected cells - 24 hours</td>
<td>33.5</td>
<td>110.9</td>
</tr>
<tr>
<td>Cells - FPA - 24 hours</td>
<td>17.7</td>
<td>58.6</td>
</tr>
<tr>
<td>Cells - NDV - FPA - 24 hours</td>
<td>20.3</td>
<td>67.2</td>
</tr>
<tr>
<td>Cells - NDV - FPA - 8 hours</td>
<td>26.0</td>
<td>86.0</td>
</tr>
</tbody>
</table>

* 3 \times 10^6 cells per tube
Quantitative assay of viral infectivity in chicken embryo fibroblasts. The influence of the drugs: NDV in the presence of 30 μg of FPA / ml of medium.
Quantitative assay of viral infectivity in chicken embryo fibroblasts. The influence of the drugs. NDV in the presence of 125 μg of guanidine. HCl/ml medium.
Quantitative assay of viral infectivity in chicken embryo fibroblasts. The influence of the drugs. NDV in the presence of 5 μg of 8-azaguanine / ml of medium
Quantitative assay of viral infectivity in chicken embryo fibroblasts. The influence of the drugs. NDV in the presence of 0.3 μg of puromycin/ml medium.
Quantitative assay of viral infectivity for chicken embryo fibroblasts. The influence of the drugs. VSV in the presence of 30 μg of FPA/ml of medium.
Quantitative assay of viral infectivity in chicken embryo fibroblasts. The influence of the drugs. VSV in the presence of 125 μg of guanidine HCl/ml of medium.
Quantitative assay of viral infectivity for chicken embryo fibroblasts. The influence of the drugs. VSV in the presence of 5 μg of 8-azaguanine/ml of medium.
Quantitative Assay of viral infectivity in chicken embryo fibroblasts. The influence of the drugs. VSV in the presence of 5 µg of mitomycin/ml of medium.
Figure 9. Normal HeLa cells. X 670, Carmine - fast green.

Figure 10. HeLa cells infected with NDV. Eight hours after viral adsorption. Beginning formation of vacuoles. x 300, C-Fg.

Figure 11. HeLa cells, 24 hours after NDV adsorption. Large vacuoles in the cytoplasm. x 300, C-Fg.
Figure 12. HeLa cells, 26 hours after viral adsorption. Formation of syncytium in heavily infected cells. x 300, C-Fg.

Figure 13. Complete destruction of HeLa cells by NDV some 48 hours after viral adsorption. x 300, C-Fg.
Figure 12

Figure 13
Figure 14. Normal HeLa cells. Note reddish orange cytoplasm, yellow to green nuclei and orange nucleoli within the nuclei. Cells not very compact, with well defined intercellular spaces. x 670, Acridine orange.

Figure 15. HeLa cells 4 hours after infection with NDV. Inter cellular spaces smaller. Nuclei becoming yellow. x 670, AO.

Figure 16. HeLa cells 14 hours after NDV adsorption. Cytoplasm more red than orange. Formation of syncytium. x 670, AO.

Figure 17. HeLa cells 26 hours after NDV adsorption. Red cytoplasm showing vacuoles. Hyperplasia. x 670, AO.

Figure 18. HeLa cells 48 hours after viral adsorption. Few remaining cells on cover slip show the advancement of infection. Cytoplasm dark red, almost completely vacuolated. Nuclei granular. Cellular disintegration. x 670, AO.
Figure 14

Figure 15

Figure 16

Figure 17

Figure 18
Figure 19. HeLa cells 30 hours after NDV adsorption, cultivated in the presence of 300 ug of FPA / ml of medium. Color of cytoplasm almost normal. Peculiar structures on the cellular periphery. x 670, A0.

Figure 20. Same as Figure 19, but 40 hours in the presence of FPA and after viral adsorption. The peripheral structures are smaller. x 670, A0.

Figure 21. Same as Figure 19, but 50 hours in the presence of FPA and after viral adsorption. x 670, A0.

Figure 22. Normal HeLa cells cultivated in normal medium during viral and FPA studies. Picture taken on 4th day after beginning of studies without change in medium. x 670, A0.
Figure 23. Normal HeLa cells stained by indirect fluorescent antibody techniques. No fluorescence. x 300, FA.

Figure 24. HeLa cells, 4 hours after NDV adsorption. Perinuclear fluorescence. x 300, FA.

Figure 25. HeLa cells, 4 hours after NDV adsorption in the presence of 300 µg of FPA / ml of medium. Faint perinuclear fluorescence. x 670, FA.

Figure 26. HeLa cells, 14 hours after NDV adsorption. Heavy granular, cytoplasmic and perinuclear fluorescence. Cell in mitosis. x 300, FA.

Figure 27. Same as Figure 26 but in the presence of FPA. Less fluorescence. x 300, FA.
Figure 28. HeLa cells 26 hours after NDV adsorption. Very heavy granular fluorescence throughout cytoplasm. Hyperplasia. x 670, FPA.

Figure 29. Same as Figure 28 in the presence of FPA. Somewhat less granular cytoplasmic fluorescence. x 300, FPA.

Figure 30. HeLa cells 48 hours after NDV adsorption. Most of the cells destroyed. More diffuse fluorescence throughout cytoplasm. x 300, FA.

Figure 31. HeLa cells 26 hours after adsorption. Ribonuclease treatment. No fluorescence. x 300, FA.

Figure 32. HeLa cells treated in the same manner as cells in Figure 31, but in the absence of ribonuclease. x 300, FA.
CHAPTER V

DISCUSSION

Of the three general methods available for the study of viral chemotherapy, the tissue culture method and the embryonated egg method were chosen. The tissue culture method is preferable in spite of some criticism that it does not indicate whether an antiviral drug will be active in an infected animal. This same criticism has been raised in the case of in vitro antibacterial antibiotics tests. It is understandable that an in vitro test (Hermann, 1962) can not reflect all the in vivo factors acting on adsorption, excretion, toxicity and detoxification of the test drug. All the drugs, therefore, which exhibit high activity in vitro and low or no activity in vivo, should be returned to the laboratory for the modification of the molecule and further investigation.

The final and crucial tests on the selected antiviral agents in vitro, should be done in laboratory animals.

Tissue culture methods used in this work were of two types. The method of Herrmann et al., (1960) employs agar-diffusion for the detection and bioassay of antiviral drugs. The method was tried at the start of this work and was modified several times. The results obtained were inconsistent and therefore it had to be abandoned. Parallel with
this, a liquid type of tissue culture method was used (Rightsell et al., 1958) which was modified according to our needs. It consisted of preparation of monolayers (Dulbecco, 1952) of chick embryo fibroblasts in which the various drugs in solution were then assayed for antiviral activity. This method gave good and reproducible results. In order to use a liquid type of tissue culture in chemotherapeutic work, two important requirements had to be considered here. The proper solvents must be selected for the drugs to be tested so that they can be removed from the media once the drugs are dissolved.

The second problem was the variation of the pH of the media due to the presence of the test drug. Mammalian and avian cells exhibit normal activities of metabolism and growth in a narrow range of hydrogen ion concentration. The physiological pH for these cells is between 7.2 - 7.6 and it is important that the starting cultures of such cells be within this pH range. The pH of the medium containing the variety of the drugs was adjusted and buffered for this range.

Assays of drug tolerance of chick embryo fibroblasts were done prior to work with viruses. Viral synthesis inside a susceptible host cell is intimately associated with cellular enzymatic processes. It is, therefore, necessary to deal with the highest possible concentrations of the drugs tolerated by the cells in order to expect chemotherapeutic action based on selective toxicity. The therapeutic concentrations in many
instances approach toxic levels, and it is in such situations very difficult to dissociate therapeutic effect of a drug from its toxicity.

Toxic concentrations induce changes in the cells which are difficult or impossible to distinguish from the effects due to the virus.

The effects of viral multiplication in tissue cultures may be detected in a number of ways. Degenerative changes in the cells or pH changes take place as the result of decreased metabolism. One can also harvest viral fluids and inoculate serial dilutions into animals or developing chicken embryos and calculate the 50% infective, lethal or paralytic doses. One can also estimate hemagglutinin (HA) or complement fixing (CF) titers from fluids. In this work the criteria of viral presence was cytopathic effect (CPE), hemagglutinin production and histochemical changes. Huang (1942), and others recognized, that the multiplication of viruses or their presence in tissue cultures brings about degenerative changes, which provide adequate proof of their presence. These changes are generally described as cytopathic effect. Different viruses affect different cells in a variety of ways. The cytopathic effect comprises all the changes in virus infected cells which make them appear granular, rounded up, accumulated in irregular masses without a clear distinction as to individual cells, and finally degeneration and complete
disintegration. These changes had to be specifically induced by viruses in order to decide any therapeutic potential of drugs.

Qualitative assays of infectivity in the presence of the drugs were performed to select the drugs with potential antiviral activities. Even the partial inhibition of CPE in such tests is adequate reason to consider any inhibitive drug as a potential therapeutic. From Tables III and IV it can be seen that only five drugs passed the criteria as formulated in the chapter on "Materials and Methods." These drugs were mitomycin C, guanidine HCl, 5-fluorophenylalanine, 8-azaguanine and puromycin.

But the lowering of CPE per se might not always mean reduction in infectious titer or suppression of viral multiplication. Groupe, et al., (1952, 1954) have shown this in vivo with xerosine.

This possibility was investigated in vitro with drugs in qualitative assays by measuring the infectivities after different time intervals of exposure to the drugs. The lowering of the CPE in such situations with no inhibition of viral multiplication could still be considered therapeutically effective. Whatever the meaning of inhibited CPE in such situations might be, when projected in vivo it can mean two things: Either disappearance of the symptoms or the drug is provoking tissue reactions which are manifested by a decrease
in cellular destruction. In some human diseases, for example influenza, the disappearance of the symptoms in spite of extensive viral multiplication could be regarded as beneficial, (Herrmann, 1962).

From the results (Figures 1-8) of quantitative assays of viral infectivities in the presence of drugs which previously inhibited CPE, only FPA inhibited the multiplication of viruses significantly.

The inability of mitomycin C to inhibit multiplication of VSV (Figure 8) can be explained on the basis of observations of several groups (Reich and Granklin, 1961; Shatkin et al., 1962; Ben Porat, et al., 1961). Mitomycin C apparently acts only on DNA of the cell and the virus, and since VSV is an RNA containing virus (Hossein and Cooper, 1963), mitomycin C does not affect its multiplication. Puromycin did not suppress the multiplication of NDV (Figure 4) at concentration tolerated by cells. Higher concentrations of puromycin (5 μg/ml) Levintow, et al., (1962) inhibit poliomyelitis virus in HeLa cells, but this concentration is extremely toxic for the cells.

Guanidine. HCl (Figures 2 and 6) influences the multiplication of NDV and VSV only early after adsorption. Later, the final titers of both viruses approached the titers of controls. Melnick, et al., (1961) reported that low doses of guanidine. HCl, at least with enteroviruses, select viral
particles with varying degrees of resistance to the drug. This mechanism might explain the early lower titers in our case. Guanidine-HCl inactivated a certain proportion of initial infective dose of each virus giving less infectivity in the beginning, which picked up later and reached the control titers.

8-azaguanine was ineffective with VSV. The titers (Figure 7) were very close to the titers of control. In the case of NDV (Figure 3) 8-azaguanine shows small but definite inhibition. Despite this the viral titers are within one standard deviation of controls. It would be interesting to work with some other guanine derivatives.

The only drug showing significant inhibition of multiplication of both viruses was FPA. FPA (Figure 1) lowers the final NDV titers to approximately 7% of control. At 5 hours after adsorption, titer of the control was on the average $2.8 \times 10^2$ TCID$_{50}$ whereas titer of FPA inhibited test was below $10^2$ TCID$_{50}$ and therefore not measurable using tenfold dilutions. Similar results were obtained with VSV (Figure 5). The differences between both viruses is in their infectivity for chicken embryo fibroblasts. Titration of NDV stocks in chick fibroblasts always gave higher titers than the stocks of VSV. The reason for this probably lies in the nature of host-virus system. Chicken cells provide a natural host for NDV which is not the case with VSV, which is a natural pathogen of horses and cattle.
The lower titers (Franklin, 1958) of both viruses in these tests as compared with the titers achieved in direct titrations of allantoic stocks are without doubt due to some viral inactivation during freezing and thawing of the cells. Despite this fact, the relative inhibition of viral multiplication in the presence of the drug is still evident from these experiments.

Rubin et al., (1957) showed that 95% of cell associated NDV can be neutralized by immune serum. Since it is known that the antibody molecule does not enter the living cell, this neutralizable but cell associated virus (CAV) must be somehow exposed to the action of antibody. NDV is assembled in the cytoplasmic membrane and remains, after maturation, connected with the outer cellular surfaces for approximately one hour, after which it is finally released into the medium. Because of this, there existed a possibility that FPA might be acting extracellularly, namely either on the virus still associated with cell or on free virus in the medium. In order to be sure that FPA does not act as a virucidal agent on the viruses used in this work, experiments were performed to test this possibility. FPA had no effect on the free virus as seen from control titers (Tables V and VI). Nor did FPA exhibit any detrimental effect during the adsorption period.

Ellis and Delbruck (1939) with bacterial viruses and Dulbecco and Vogt (1954) with animal viruses demonstrated that
after penetration of viral nucleic acid into a susceptible cell, a period follows during which the infectivity cannot be measured or recovered, and any remaining measurable infectivity is due to free and unadsorbed virus. Also during this period of time no virus specific material of any kind can be detected within such cells. After this period, which in different systems may take a few minutes, several hours or even days, viral specific materials start to appear, but measurable infectivity, if any, is still due to residual free virus. This time period is called the eclipse phase, and together with the maturation phase, (time interval in which first viral material starts to appear and up to the time when the first increase in infectivity is detected) comprises the "latent period" of viral development.

The latent period of NDV in chick embryo fibroblasts is approximately 3 hours (Levine and Sagik, 1956), of which 2 hours (Tamm and Wheelock, 1961) are taken by eclipse. The VSV latent period in the same host is between 2.5-3 hours, of which 1.5-2 hours belongs to the eclipse (Franklin, 1958). An infected cell culture (low multiplicities) exhibits continuously all phases of viral development; new cells are infected during release of finished virus products; in some of the cells virus is eclipsed, in the others in the process of maturation and so on. (Tables V and VI). The suppression of viral infective titers was evident when FPA was present during
different time intervals of the latent period as well as throughout the extended periods, indicating the intracellular action of FPA (Tables V and VI).

When FPA is present in a glycerol-salt medium during the adaptation of *E. coli* to lactose fermentation, the growth rate of the organism becomes linear rather than exponential (Munier and Cohen, 1959). Such a possibility is discernible from the experiments with NDV hemagglutinin (Table VII). There is, of course, no analogy between *E. coli* and its galactosidase on one side and the synthesis of viral hemagglutinin by mammalian or avian cell on the other. The similarity is the ability of both to synthesize enzyme proteins. If FPA renders a bacterial enzyme nonfunctional by virtue of its incorporation into the enzyme molecule, there is a good possibility that a similar sequence of events will occur in virus infected cell in the presence of FPA. Zimmerman and Schafer (1960) showed in the case of fowl plague virus that the presence of FPA during the eclipse phase inhibits the synthesis of viral protein. Later addition of FPA allows the formation of S antigen but not of viral hemagglutinin. The S antigen of myxoviruses is believed to be a nucleoprotein, which forms before the capsid protein containing HA activity.

In our experience, 30 ug of FPA lowered the HA activity of virus to about 18% of the titer of control. Does FPA cause synthesis of less infectious virus or of nonfunctional
HA? If there is less virus formed there is also less hemagglutinin formed. On the other hand the viral nucleic acid could be infective but HA is not functional.

In order to determine the effect of FPA on viruses under more realistic conditions, namely in a living host that is easily infected and killed by the virus we used embryonated chicken eggs. Embryos are suitable for this purpose, although there are some disadvantages involved. The factors which had to be considered were as follows: toxicity of FPA, dose of the virus which had to be large enough to infect and kill, but also small enough so that it could be acted upon by relatively nontoxic doses of FPA.

In the case of NDV, 75 μg of FPA protected or prolonged the time of survival of infected embryos significantly. This conclusion is based on the outcome of statistical analysis of the results. However, embryos were not protected against VSV as revealed from chi square tests. This difference could be due to the nature of the virus and the response of embryo host. VSV, although "adapted," is not a natural pathogen for chickens. If epidemiological theory is correct, such a relationship is much harder for host than an infection with natural pathogen.

The experiments which were undertaken to study the mechanism of FPA inhibition were of three kinds.

Again the infectivity of virus synthesised during
exposure to a large concentration of FPA had to be measured and compared to noninhibited controls as well as to the infectivity obtained from FPA inhibited cultures in which the inhibition was later released by removal of FPA containing medium and introducing medium rich in phenylalanine (PA). Secondly, an important component of the cells had to be obtained and measured under variety of treatments, a component which could be, during internal virus development, shared with the virus or at least have a biological function similar to one of the viral components. And thirdly, the individual results obtained in the first two steps as well as the conclusions extracted should be then analysed in connection with the results obtained from histochemical experiments. In order to insure as much uniformity as possible, a homogenous cell culture had to be used in these experiments. HeLa cells are preferable over primary chicken fibroblasts. A homogeneous cell line was preferred in histochemical studies because of morphological considerations as well as for analytical reasons in biochemical experiments.

Addition of FPA during early eclipse period and its presence during a 24-hour period completely suppresses the formation of infectious viral particles (Table X). Any infectivity recorded was probably due to some residual unadsorbed virus. The hemagglutinin activity was zero, indicating that there was no synthesis of protein or at least functional
hemagglutinin. Infected cells exposed to FPA for 8 hours and in which the inhibition was then reversed, showed a significant increase in infectivity over 24 hours inhibited samples. But the HA activity of this sample was the same as in the sample inhibited for 24 hours. There was actually a 134 fold increase in the infectivity over the 24 hour sample, whereas HA activity remained the same. Only in the control sample which was not exposed to FPA, HA activity was recorded at 4 HA units. The possibility that FPA inhibits hemagglutinating activity in vitro was tested with the virus from controls. This was not the case, since dilutions were made in medium containing FPA, and the hemagglutinating activity remained steady at 4 HA units. From these experiments we conclude that FPA inhibits both infectivity and HA production acting intracellularly. Reversal of FPA inhibition results in the increase of infectious titer, but there is still not enough virus to give concentrations of enzyme necessary to agglutinate red blood cells.

Since NDV is an RNA containing virus and since viral infection implies the synthesis of viral RNA, we wanted to study the influence of FPA on the RNA synthesis of normal cells, and virus infected cells in order to obtain further information on the mechanism of FPA inhibition.

This was done by the determination of ribose content in RNA extracted from HeLa cells which underwent different
treatments. If the purine-bound ribose from tubes of 24 hours growth of normal HeLa cells represents 100% (Table XI), then there is a net increase of approximately 11% of ribose in the cells infected with NDV despite their progressive destruction. This indicates either viral RNA synthesis, or at least increase in acid insoluble material which is well on its way to incorporation either in cellular or viral RNA. On the other hand, purine bound ribose from infected cells in the presence of FPA was only 67.2% of ribose recorded in normal 24 hour controls. Compared with normal cells which were cultivated through the same time period in the presence of FPA, these infected FPA inhibited cells still show some increase (8.6%) over the PBR value obtained in noninfected FPA inhibited control. This would mean complete inhibition of RNA synthesis in normal cells treated with FPA, but some synthesis in infected FPA treated cells. These PBR values are compared to normal controls at 0 time, which in the terms of 24 hours normal control represents only 62.2% of the net amount. Finally the infected and 8 hour FPA treated cells in which the inhibition was counteracted by washings and phenylalanine gave a PBR value of 26.0 ug which is much higher than the value obtained with infected - FPA treated cells for 24 hours, but still smaller (86%) than value obtained from infected and untreated cells. This indicates that the RNA synthesis in this case continued after release of FPA inhibition.
Histochemical studies done on HeLa cells in connection with above work corroborate these findings, and in some instances expand these results. Acridine orange studies clearly reveal the changes in the infected cells that are due to the viral infection. The gradual accumulation of viral RNA (Figures 14-18) in the infected cells is shown by the shift in color (Armstrong, 1956; Schummelfelder, et al., 1957) from reddish-orange to dark flame red. Infected, but FPA treated cells do not undergo this shift, or at least this is not very pronounced (Figures 19-21). This agrees with the experiments on purine bound ribose obtained from infected-FPA treated cells. In spite of this fact, there appears, on the basis of PBR experiments (Table XI) the possibility of some viral RNA synthesis in such cells. Acridine orange is not sensitive enough to detect this change. Fluorescent antibody techniques (Figures 23-30) reveal the formation of viral specific material in the cells despite the presence of FPA, which from previous experiments, definitely inhibits the production of infective viral particles and hemagglutinin. Viral material is synthesised in such cells although to a lesser extent. In the FPA inhibited cells exhibiting specific viral fluorescence, virus material could be nucleoprotein or S Antigen. Treatment of such cells with ribonuclease (RNAse) for one hour at 37 C removes this fluorescent material (Figures 31 and 32). This material could not be viral nucleic acid
alone, because nucleic acids are not antigenic, and therefore, cannot be responsible for the induction of antibody formation.

It is therefore possible that ribonucleic acid in this case is hapten, which is broken down to oligonucleotides and smaller units through the action of RNAse. Immunologic specificity is lost, and as a consequence, fluorescence also.

The question that arises here is as follows: if high concentrations of FPA inhibit RNA synthesis, a conclusion drawn from previous experiments, how is it possible that viral RNA or viral ribonucleoprotein appears in such cells?! It seems that the assumption of Scholtissek and Root (1961), derived from work on fowl plague virus, is pertinent in the case of NDV, namely that a protein (polymerase), once formed in the early eclipse phase, directs the synthesis of some viral RNA or ribonucleoprotein. This might go on even during the time that FPA inhibits the formation of viral hemagglutinin and viral maturation. In such case, viral protein and nucleoprotein formed contains instead of phenylalanine the analog FPA. This material is immunologically active as shown with fluorescent antibody techniques, but inactive concerning its neuraminidase activity as well as its infectivity.
SUMMARY

Chemotherapeutic experiments involving 30 different drugs and two animal viruses yielded 2 drugs which have the ability to suppress the infectivity of Newcastle disease virus and vesicular stomatitis virus in tissue cultures.

Of the two drugs, 5-fluorophenylalanine, an analog of amino acid phenylalanine, exhibited significant inhibition of infectivity of vesicular stomatitis virus and Newcastle disease virus as well as the inhibition of the formation of Newcastle disease virus hemagglutinin.

The other selected drug, 8-azaguanine, an analog of guanine, had more effect on the infectivity of Newcastle disease virus, although not as significantly as 5-fluorophenylalanine.

In the experiments with fertilized chicken eggs, it has been shown that 5-fluorophenylalanine definitely prolongs the survival time of chicken embryos infected with Newcastle disease virus, which on the other hand cannot be claimed in the case of embryos infected with vesicular stomatitis virus.

The studies on the mechanism of action of 5-fluorophenylalanine were done in HeLa cells and only with Newcastle disease virus. For this purpose 10 times higher concentrations of 5-fluorophenylalanine (300 μg/ml) were used than in
previous experiments, since the criterion of selectivity was
not important in these studies.

The biochemical studies revealed that 5-fluorophenylalanine inhibits general cellular RNA synthesis which would also mean inhibition of protein synthesis, which then reflects itself in the suppression of viral infective titers as well as in the production of enzyme neuraminidase responsible for hemagglutination.

Upon release of 5-fluorophenylalanine inhibition, by washing the cells and introducing into medium normal amino acid phenylalanine, the inhibition of viral infectivity stops, i.e., more infective virus is formed. From these experiments it is concluded that 5-fluorophenylalanine inhibits not only the RNA and protein synthesis, but also the maturation of fully infectious viral particles.

Histochemical studies, using acridine orange and fluorescent antibody techniques, confirm the above findings.
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ABSTRACT

Title: Chemotherapeutic Experiments With Ribonucleic Acid Containing Animal Viruses.

Purpose: The purpose of this work was to select drug(s) which exhibit selective toxicity for 2 RNA containing animal viruses, Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) when grown in primary cultures of chicken embryo fibroblasts, and using biological, biochemical and histochemical techniques, to study the mechanism of action.

Introduction: Chemotherapy of viral diseases is a difficult undertaking. The problem is not so much due to the unavailability of drugs, their solubilities and active or passive transport into virus infected cells as it is due to the intricate relationship of the host-parasite system itself. Upon infection of a cell, viral nucleic acid, which is the carrier of genetic code for the synthesis of its own protein and nucleic acid subunits, reorients the aspect of cellular metabolism in the direction which provides all
necessary enzymes, building blocks and energy for the formation of new viral material. The formation of viral subunits and their subsequent assembly into mature and fully infectious virus is integrated with remaining cellular processes, so that it is difficult to achieve, by use of drugs, a significant toxicity for the virus without disturbing normal cellular metabolism. Usually, a certain drug concentration which is toxic for virus or inhibitive for certain anabolic function(s) leading to the formation of virus is simultaneously toxic for other cellular processes. It is the intimate parasitic relationship at the enzymatic and molecular level in viral diseases which makes it difficult to efficiently separate the antiviral action of drugs from their general toxicity for the infected hosts.

In spite of this, certain drugs show selective toxicity for certain viruses, although almost entirely in vitro. Most of these studies on the chemotherapy of viral diseases had not as yet contributed to any significant practical use, although they were responsible for many interesting findings of the parasitism at the cellular and subcellular levels which
contributed to the general knowledge of virus-cell relationship.

Results:

It was logical, therefore, to assay or estimate in the beginning experiments the highest tolerated doses of various drugs in the cellular system in which most subsequent studies were to be performed. Only at such concentrations can one reasonably expect to achieve selective action. For this purpose, chicken embryo fibroblasts were used and the assays were done using 30 drugs in liquid tissue culture systems. After ascertaining the highest tolerated doses of the drugs by chick embryo cells, the next step was to measure, qualitatively, the ability of these drug concentrations to suppress viral cytopathic effect (CPE) in chick cells. In both instances, 1000 TCID\textsubscript{50} of virus were used to infect cell monolayers, which were then incubated in the presence of medium containing the drugs, and observed for 3 to 5 days for the development of CPE, which was then scored as 0, 1, 2, 3 and 4 when compared to controls. The drugs showing inhibition of CPE under these conditions were used in quantitative assays, to estimate specific loss of infectivity expressed in TCID\textsubscript{50} when compared to controls, as well as
to analyze the significance of this inhibition.

On the basis of CPE inhibition in qualitative tests, 5 drugs were selected for quantitative studies. The drugs were 5-fluorophenylalanine, 8-azaguanine, guanidine-HCl, mitomycin, and puromycin.

Quantitative assays were performed by infecting chicken embryo monolayers with $4 \times 10^5$ TCID$_{50}$ of virus, and after adsorption, media were added containing the above drugs in highest tolerated concentrations. At 5, 10, 23, 28 and 33 hours after viral adsorption, the infected monolayers were frozen and thawed twice, cell debris sedimented by centrifugation, and supernatants titrated in chick cells grown in normal medium; titrations were done using Reed and Muench method. From the results of these studies it could be seen that 5-fluorophenylalanine (FPA) significantly inhibits infectivity of both viruses in chicken fibroblasts. NDV infected cells treated for 33 hours with 30 ug of FPA/ml of medium showed only 7.3% infectivity of controls, whereas in the case of VSV the FPA treated cells showed 15% infectivity recorded in controls.
Of the rest of the drugs only 8-azaguanine showed some inhibition of NDV, which on the basis of statistical approach was not significant.

The addition of FPA containing medium to chick embryo monolayers during different times of latent periods of both viruses significantly lowered both infective titers, confirming above findings, whereas exposure of viruses in vitro to FPA did not have any effect on final titers in chick cells.

It has been shown in the case of NDV that FPA significantly lowers its hemagglutinin titer. In the presence of 30 ug of FPA the NDV hemagglutinin activity was recorded and represented only 18% of HA activity of controls. Since FPA suppresses viral infectivities and in the case of NDV also the hemagglutinating activity, it is reasonable to assume that this is due to FPA incorporation into viral subunits rendering them nonfunctional. The experiments with FPA in embryonated eggs showed on the basis of statistical analysis that 75 ug of FPA significantly prolongs the survival time of embryos in the case of NDV infections. VSV infected embryos
on the other hand did not respond significantly to the treatment with FPA, and the reason for this could be sought in the epidemiological hypotheses.

The studies on the actual mechanism of FPA inhibition were performed with only NDV. For this purpose homogeneous cell cultures (HeLa) were used in order to obtain more uniform results. It is shown that 300 ug of FPA per ml of Eagles MEM maintenance medium completely inhibits the formation of infectious NDV; this inhibition could be released as shown in the experiment with 8 hours FPA inhibited and NDV infected cultures. After thorough washing of such cultures with PBS and addition of MEM rich in phenylalanine synthesis of fully infectious virus takes place as revealed by increased viral titers. It has been further shown in the purine bound ribose experiments that FPA generally inhibits cellular RNA synthesis and that in spite of this fact some RNA is synthesized in virus infected cells. That this RNA could be of viral origin has been shown using histochemical techniques, especially fluorescent antibody procedures. On the basis of these experiments the following conclusions can be drawn:
FPA inhibits viral infectivity and hemagglutinin, and general cellular RNA synthesis. Specific fluorescent material observed in the infected and FPA treated cells is viral nucleoprotein which is nonfunctional due to the presence of FPA moieties. Release of FPA inhibition results in the synthesis of normal virus, meaning that FPA might inhibit also viral maturation.
The dissertation submitted by Frank T. Frigan has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

January 14, 1964
Date

Signature of Adviser