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# APPROACHES TO THE BIOCHEMICAL ANALYSIS OF STAGES OF VIRAL DEVELOPMENT

by

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# APPROACHES TO THE BIOCHEMICAL ANALYSIS OF VIRAL DEVELOPMENT

by Hunein F. Maassab

An approach has been made to the problem of determining the biochemical bases which underlie the viral infectious process. Emphasis was directed toward obtaining data pertinent to an understanding of phenomena occurring at the cellular level. Because of the breadth of the problem, the work has been limited exclusively to the immediate problem of developing techniques for the isolation of the stages of viral development.

For a variety of reasons, it is most convenient to use tissue culture maintained in vitro to carry out a study of the type outlined above. At the beginning of the study, there was a practical, sensitive and reproducible method in operation in this laboratory. This technique with some improvement has been used throughout the study. It is a suspended type of tissue culture in which the host tissue is the chorio-allantoic membrane of embryonate eggs. The infecting agent is the PR8 strain of type A influenza virus. It is a stable virus, produces a hemagglutination reaction, grows readily to high titer, and is easy to handle. The suspending medium is a modified Simm's solution containing only inorganic salts and glucose.

A large part of the problem has been to design experiments and develop techniques so that from such a

multicellular system inferences could be drawn of events proceeding at the cellular level.

It is generally believed that viral development proceeds by way of discrete stages. Any effort to study the metabolic sequences involved in a viral infection must be done by isolating these stages. Certain chemical inhibitors which were available at the start of this study and which were known to inhibit viral multiplication were used. An attempt was made to determine the site of action during viral development, i.e., to determine if their actions were restricted to the process of attachment, penetration, maturation, viral release, etc. Three experimental methods were employed:

1) Experiments were carried out in which the relation of inhibitory capacity of these antagonists to the age of infection was followed.

2) The kinetics of viral increase following periods of virostasis induced by the inhibitors were studied.

3) The sensitivity to certain inhibitors of the viral synthesis which followed virostasis induced by other inhibitors was determined.

A number of discrete stages of viral development have been isolated by the use of these specific chemical blocking agents. Some information has been obtained about the interrelationships of these stages. Because of the inhibitor techniques used to isolate these phases, some

biochemical characterization of the stages has been apparent.

The responsible factors producing the pattern of viral development were analyzed and shown in part to be due to the differences in the latent period of individual cells. A technique was developed for synchronization of the infectious process, whereby differences in cellular response could be reduced. The maturation and release of virus in culture were found to occur over a period of time; no burst phenomenon was evident.

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# INTRODUCTION

Green's concept that "the viruses are specialized parasites which have lost a large degree of their synthetic ability and have become dependent on the synthetic ability of the host cells to supply materials for the fabrication of new virus particles" is still faced with the alternative concept that viruses are natural cellular components which find themselves in foreign cells. However, in either case, the biosynthesis of the virus is ultimately dependent and intimately associated with the metabolism of the host cell.

The objective of the plan of investigation and study in this thesis is to learn of the biochemical bases which underlie the host-virus relationship. It should be the goal of a sound biological approach to interpret complex situations in terms of the elementary virus-cell interaction analyzed in simpler situations. The study of the interactions of animal viruses with their hosts is complicated by the high degree of host differentiation. As a rule, manifestations of virus infection at the cellular level can be traced more directly in tissue cultures. Hence, emphasis was directed toward obtaining data which would provide an understanding of phenomena involved at the cellular level.

At the outset of this study, current thought held that virus development proceeded by way of discrete stages. The strongest support of the concept came from kinetic studies of viral growth curves in which the increase in mature virus was followed. However, other lines of evidence were also available at that time. It is obvious that any metabolic study of viral infection must be approached by studying the discrete stages. It is also obvious, particularly after some preliminary studies, that the stages must be isolated at least functionally before any progress can be made.

It is realized that the objective stated above is extremely broad and because of this the work has been limited exclusively to the immediate problem of developing techniques for isolating these stages by means of their sensitiveness to inhibitors and to correlate from the findings any interrelationships of these stages which may be revealed.

# HISTORICAL

During the last few years, knowledge of the influenza viruses and their behavior under carefully controlled laboratory conditions has increased enormously, and today they are the agents most used as models for studying the multiplication of animal viruses.

Up to the present one of the infectious diseases of the most violent pandemic character was the influenza of 1918-1919. Nicolle and Le Bailly (1918) and R. Dujarric de la Riviere (1918) were the pioneers who paved the way for the discovery of influenza virus and demonstrated, by inoculating animals and human volunteers subcutaneously with the filtrate taken from bronchial exudates of persons suspected of having influenza, that the agent which transmits the disease to the human is a virus which passes through a filter.

In 1933 Wilson, Smith, Andrews and Laidlaw had described how they produced an influenza-like disease in ferrets by intranasal inoculation of filtrates from cases of human influenza. The first strain isolated is known today as the WS strain. Transmission of influenza to mice was accomplished by Francis (1934) and Andrews, Laidlaw

and Smith (1934) and made the study of influenza more practical. Francis and Magill (1935) cultivated the virus of influenza <u>in vitro</u> by inoculating lung tissue from infected mice into flasks of Tyrode's solution and chick embryo. They maintained virulence of the agent for twenty subcultures and were also successful in propagating swine and human influenza viruses, obtaining titers of $10^{-3}$ and $10^{-4}$ for ferrets and mice. Smith (1935) reported propagation of influenza virus in Carrel culture in a similar medium used by Francis and Magill. The neurotropic variant of the WS strain was grown in a Maitland type culture by Andrews (1942).

Influenza research took quite a different turn when the ferret and mouse were replaced by the egg. Influenza virus has probably been the most thoroughly studied virus in embryonated eggs. The literature on this subject is voluminous and only a bare outline is given. The virus may be propagated by a number of different methods in the chick embryo. Smith (1935) was the first who tried to cultivate influenza virus in embryonate eggs with no apparent results. However, Burnet (1936) did demonstrate the cultivation of influenza virus in fertile eggs and he was successful in transferring serially and adapting the virus to eggs. Burnet (1940) showed that influenza virus could be readily cultivated by amniotic inoculation of the chick embryos and its growth recognized by the occurrence of large

vacuolated epithelial cells in tracheal smears. Successive cultivation of influenza virus in the allantoic sac of embryonate eggs has been achieved by Burnet (1941); Rickard, Thigpen and Crowley (1944); Eaton, Corey, Van Herick and Meiklejohn (1945); and Henle and Chambers (1941). Yolk sac inoculation of influenza virus was successively demonstrated by Burnet, Beveridge and Bull (1944). The fundamental observation of Hirst (1941) and of McClelland and Hare (1941) that influenza virus agglutinated the red blood cells of a number of animal species was a finding which has been of the greatest theoretical and practical importance.

Today a new strain of influenza virus is usually isolated by amniotic inoculation of chick embryos, then adapted to the allantoic cavity (Nigg, Crowley and Wilson 1945), and studied thereafter by agglutination techniques.

The great simplicity of the newer techniques has led to many important theoretical advances in our knowledge of influenza viruses. The fundamental researches of Hirst (1942) on the agglutination and the enzymic-like elution from red cells stimulated Burnet and his colleagues in Australia to study this reaction as a model for the processes of invasion of susceptible cells by influenza virus (1951). The influenza viruses show many examples of virus variation and such phenomena as the development of neurotropism (Stuart-Harris 1939), the O-D variations (Burnet and Bull 1943), and the adaptation

of virus to kill mice following intranasal inoculation (Hirst 1947, Wang 1948, Davenport and Francis 1951, and Chu 1952) have been fruitful fields of study. The process of interference has interested many investigators as a means to analyze the process of virus multiplication; Henle (1950) has demonstrated the interference phenomenon among influenza viruses, while in Australia a different approach was used to study the process of virus multiplication through the work on recombination by Burnet, Fraser and Lind (1953), in which it was found that following simultaneous infections with two influenza strains, the products of infection showed a cross-over of some of their inheritable characters. Fazekas de St. Groth (1950) and his co-workers in Australia have made detailed investigations of the process of experimental influenza in mice. The techniques of Dawson, Elford (1949) of absorbing influenza virus on red cell ghosts for electron microscopy have been most useful and has led to the striking demonstration of filamentous forms of influenza virus (Chu, Dawson and Elford 1949).

However, the relatively controlled study and manipulation of viruses in tissue cultures make possible certain important investigations. These include investigation of virus propagation and investigation of virus metabolic effects. The role of tissue culture methods in the study of the problem of virus propagation of influenza started in 1933 with Francis and Magill. Tissue

culture other than the suspended cell type have also been employed for cultivation of the influenza virus. Pearson and Enders (1941) used roller tubes. Bernkopf (1949, 1950) obtained unusually high hemagglutination titers with de-embryonated egg preparations, and Fulton and Armitage (1951) have described a simple technique for the cultivation of some viruses in pieces of excised chorio-allantoic or amniotic membrane. Intact chorio-allantoic membrane in Warburg flasks as host cells to grow influenza virus was used by Ackermann (1951) and was found very satisfactory for the study of virus multiplication. Cusning and Morgan (1952) were able to propagate the influenza virus in the presence of minced chick-embryo tissue overlaid with a perforated cellophane disc. In contrast to the earlier work, Lennette and Koprowski (1946) found it is necessary to increase the quantity of tissue from 0.1 gm to at least 0.2 gm in 50 ml. Erlenmeyer flasks before a tissue culture strain of PR8 could be established.

In view of the variety of methods for assaying the influenza virus content of cultures, i. e., egg infectivity, mouse infectivity, and hemagglutinin titers, and the number of strains studied it is difficult to compare different culture methods. The one-hundred thousand fold increase in egg infectivity in 27 hours reported by Ackermann (1951) and the hemagglutinin titers of Bernkopf (1949) and Daniels, Eaton and Perry (1952) suggest the superiority of their respective techniques.

The recent contributions of Ackermann and Kurtz (1952) who propagated the virus of influenza in a new host system (the ascites fluid produced in the presence of Enrlich mouse carcinoma) however, may be considered more of an *in vivo* than an *in vitro* technique.

In the analysis of the nature of virus multiplication there is now on record a large volume of work from different laboratories throughout the world. Hoyle's work in England (1948) assumed that the influenza virus, a particle of about 100 millimicrons in diameter, gains entrance to a susceptible cell and, instead of multiplying by binary fission, the elementary body becomes broken up into particles of about ten millimicrons in diameter and these particles recognized as the soluble antigen multiply within the cell. He presumes that later these small particles recombine to form elementary bodies which are cast out of the cell. Hoyle (1950) suggested that the soluble antigen was an intracellular form of the virus or a virus precursor which preceded the elementary bodies of the virus, but it has been shown that at the same time as the soluble antigen appears the specific complement fixing antigen which is known to be associated only with the elementary body also appears (Fulton 1949, Kirber and Henle 1950). Recently Liu and Henle (1951) and Cairns (1952) have shown that Hoyle's interpretation of his own results is not necessarily correct since in infected tissues treated with RDE, hemagglutinin is demonstrable as early

as the complement-fixing antigen and the discrepancies lie in the sensitivity of the experimental method. Thus from the experimental evidence there is no support for the belief that the soluble antigen precedes the elementary body in time. A majority of opinion seems to exist at the present time which states that before liberation of mature virus from the infected cells, a dormant period is evident in which the soluble complement-fixing antigen and hemagglutinin activities of the elementary bodies of the influenza virus appear simultaneously inside the cell followed by emergence of infectivity properties of the viral particle.

It has been mentioned by Von Magnus (1951) that upon serial passage of undiluted allantoic fluid, containing high titers of influenza seed virus, hemagglutinins are produced to high titers while the infectivity does not develop to a corresponding extent and may not even increase; he called the particle with such properties an "incomplete virus." The evidence of production of incomplete virus in the allantoic membrane is supported by Schlesinger's (1950) work on the central nervous system of mice after injection of large doses of influenza virus. Experiments recently reported by Cairns, Fazekas de St. Groth and Edney (1952) seem to indicate that multiple infection is not a prerequisite for production of incomplete virus. However, the interpretation of the nature of incomplete virus discards the idea that it most likely

represents an intermediate stage in the growth cycle as an immature viral precursor. The current theory proposes that host cell damage by the large virus inocula initiates an aberrant multiplication cycle or effects rapid inactivation of infectious virus as it is synthesized. Ginsberg's (1954) data favors the latter as a working hypothesis for the process of development of non-infectious influenza virus.

Despite the various hypotheses advanced on the nature of viral multiplication in the last decade, a fairly consistent picture has emerged. Infection by influenza virus is initiated by the adsorption of a virus particle to mucoprotein receptors on the cell surface (Stone 1948 and Fazekas de St. Groth 1948). Once the infection has been initiated, there is a latent period of a few hours during which no increase in mature virus is detectable in the extracellular fluid, (Henle and Henle 1949, Ackermann and Maassab 1954) followed by multiplication of virus within the cell, and subsequent liberation of newly formed virus (Cairns, Fazekas de St. Groth, and Edney 1952). After release of the virus from the cells, the virus may attack other susceptible cells and initiate a new growth cycle. Thus virus growth occurs in one or more successive cycles of reproduction and the process involved in each of these cycles may be divided into discrete stages of development (Henle and Henle 1948, Ackermann and Maassab 1954). However,

experiments with one-step growth curves for studies of virus propagation have been achieved, using different technical approaches. To isolate one cycle of growth, Henle, Henle and Rosenberg (1947) utilized the ability of heterologous virus after inactivation by ultraviolet light to suppress reproduction of active virus and to reduce its adsorption onto susceptible cells; a one-step growth curve is evident, apparently virus liberation when started continues for many hours.

From the average infected cell approximately 60-120 infective particles are liberated over a period of two to three hours; fifty per cent of the particles are liberated at approximately eight hours after initiation of infection (Cairns, Fazekas de St. Groth and Edney 1952). Most cells show no sign of serious morphological damage at the end of liberation of virus (Isaacs and Fulton 1952).

The approach followed in this research is based on the assumption that virus proliferates in a succession of steps. Consideration of this process leads to the suggestion that innibition of virus multiplication may be possible at any one of these stages. The destruction of extracellular virus by combination with homologous antibodies effectively eliminates the virus in animals before it can attach itself to susceptible host cells. However, the knowledge of the differences between virus and cell structures is so deficient that no chemical counterparts to the specific action of antibodies have been devised.

Multiplication of influenza virus may also be blocked by biological agents. Numerous examples have been recognized in which one virus interferes with another, but the mechanism for this interference has not been determined (Henle 1950). Entrance of one virus into a cell may alter the wall of that cell so that a virus of another kind cannot enter (Schlesinger 1951), or that incomplete or immature virus, inactive virus or soluble antigens formed during virus infection may compete with infective virus for entrance into susceptible cells and in this way limit the further spread of the infection (Lennette 1951).

Destruction or blocking of receptor areas on susceptible cells may come to prevent the invasion by virus. When a receptor-destroying enzyme (RDE) that occurs in cholera vibrios is allowed to act on red blood cells, it can prevent hemagglutination by influenza virus (Burnet 1951). Moreover, receptor destruction in susceptible mouse lung tissue following intranasal administration of RDE has been shown to protect mice temporarily against infection by influenza virus (Stone 1948). Certain polysaccharides have been demonstrated to inhibit influenza virus. Apparently some polysaccharides possess receptor sites similar to those on susceptible cells; consequently some virus combines with polysaccharide and is not available to infect cells (Green and Woolley 1947). The pre-

vention of invasion by viruses is at best only a partial answer to the problem of treatment of virus infections. The complete solution to the effective treatment of viral infections necessitates the discovery of substances that can inhibit the synthesis of intracellular virus.

Indirect information concerning the significance and nature of the metabolic systems of the host cells which are necessary for influenza virus multiplication has been obtained in recent years in studies with virus grown in tissue culture and de-embryonated eggs, with metabolites and their antagonists. The recent advances in knowledge of enzymatic host-virus cell interactions appear very promising and it seems likely that further studies of this kind may offer results of great value in regard to the nature of intracellular virus growth as well as the control of influenza virus by chemotherapy.

It has been determined that the oxidative processes of the citric-acid cycle are essential for the synthesis of the virus (Ackermann 1951, and Daniels, Eaton and Perry 1952). Using malonic acid and antimycin A, Ackermann (1951) found that propagation of influenza virus was inhibited and it was suggested that this action might be through the inhibition of succinic dehydrogenase. This observation has been confirmed in infection experiments in mice in which the citric acid cycle was partially blocked by administration of sublethal doses of sodium fluoroacetate; in such animals the growth of influenza

virus A was inhibited (Ackermann 1951). Ackermann and Johnson (1953) found that 2,4-dinitrophenol inhibits the propagation of influenza virus in chorio-allantoic membrane. 2,4-Dinitrophenol was shown to have a stimulating effect upon adenosine triphosphate (ATP). It was concluded that the process of biosynthesis of the virus uses the energy derived from the oxidative phosphorylation activities of the host tissue.

The methionine analogues, ethionine and methoxinine, reduce the rate of propagation of the PR8 strain of influenza virus in tissue culture (Ackermann 1951, and Ackermann and Maassab 1954). It was suggested that the amino acid, methionine, normally present in the susceptible cells is involved in the biosynthesis of influenza virus. Eaton, Magasanik, Perry and Koribiau (1951) showed that certain basic amino acids, such as lysine, arginine and ornithine, in high concentrations inhibited influenza and mumps virus in tissue culture of chorio-allantoic membrane. They showed that amino acids containing two amino groups like arginine were the most active inhibitory agents. The reversal of the effect of arginine by egg yolk suggests the possibility of interaction with lipids in the cell membrane.

Synthetic lysine polypeptides have been reported to inhibit the growth of influenza virus (Rubini, Rasmussen and Stanmann 1951). They obtained data which

suggest that the basic polypeptides combine directly with the virus particles to inhibit infectivity. Inhibition was evident only when the polypeptides were injected before or with the virus. Recently, Green and Stanmann (1954) reported that polyvinylamine, a synthetic basic electrolyte, possesses virus inhibitory activity somewhat less than that of polylysine, on the growth of influenza B virus, Lee strain. Ackermann (1952) found that $5 \times 10^{-4}$ molar concentrations of p-amino-sulfonic acid markedly inhibited the propagation of PR8 influenza virus. However, α-amino-p-methoxyphenylmethane sulfonic acid seems to act by affecting the initiation of infection by influenza A virus or impair the release of the virus from the cell (Ackermann and Maassab 1954).

That certain aromatic diamidines were innibitors of influenza virus in eggs or in chick embryonic cultures was shown by McClelland and Van Rooyen (1949). Rassmussen, Stokes, Feldman and Smadel (1947) found that small doses of PR8 influenza A and the Lee strain of influenza B virus were inhibited by nitroakridin 3582, rutenol and quinacrine. More recently, Rasmussen and Stokes (1951) reported that nitroakridin 3582, rutenol, 2-nitro-5-aminoacridine and cobaltous salts retarded and in some instances prevented the growth of small inocula of influenza virus in embryonated eggs. A possible mechanism is suggested in that a specific metabolic system susceptible to poisoning

by nitroakridin is required by both the host cell and the virus. Further observations on the effect of acridines on the influenza virus propagation were reported by Eaton, Cheever and Levenson (1951). They showed that the nitroacridines were effective in inhibiting allantoic infections with influenza B (Lee strain); the possibility of interference by these compounds with some metabolic cellular function is suggested. The acridines are useful despite their toxicities on host cells as an additional tool for studies on the mechanisms of growth of animal viruses. Eaton, Perry, Levenson and Gorke (1952) reported that the viruses of influenza A and B were innibited in tissue culture by 5 to 20 gamma per millliliter of pentamidines and 20 gamma per milliliter of stilbamidine. The inhibitors acted by prolonging the lag period and decreasing the total amount of virus. They attributed the action of these diamidines to a chemical blockage to some tissue component essential to virus synthesis or by a direct action on the virus itself at an early phase of the growth cycle. Briody and Stannard (1951) found by injecting proflavine six hours before or after the virus inoculation, inhibition of multiplication of influenza B in embryonated eggs resulted, while the growth of type A strains was unaffected. One of the hypotheses advanced by the authors on the action of proflavine is that it may function by interfering with the conversion of cellular

nucleoprotein into viral nucleoprotein. Cushing and Morgan (1952) found that the vitamin analogues oxythiamine and desoxypyridoxine inhibit the multiplication of influenza A virus (PR8) in tissue culture. They attribute the action of oxythiamine to the blockage of pyruvate oxidation and entry of pyruvate into the Krebs cycle, while desoxypyridoxine exerts its antagonistic effect after having phosphorylated by competing with pyridoxal phosphate for the apoenzyme. Tamm, Folkers and Horsfall (1952, 1953) have found that 2,5-dimethylbenzimidazole inhibits the multiplication of both influenza A and B viruses. More recently Tamm and Tyrrell (1954) have reported that the glycosides of benzimidazoles are more active in inhibiting multiplication of influenza virus than the 2,5-dimethyl compound. They related its action on a possible interference with nucleic acid metabolism. Takemoto, Robbins and Smith (1954) showed that polymerized sodium salts of substituted benzoic sulfonic acid showed inhibitory action against both influenza A and B viruses and their possible interpretation is that the compounds interfere with influenza virus multiplication at an intracellular site and can prevent infection at a very late stage in the multiplication cycle. The inhibition of multiplication of Lee influenza strain in chick embryo and tissue culture by L-canavanine and its complete reversal by L-arginine was reported by Pilcher, Soike, Smith, Trasper and Folston (1955). They hypothesize that canavanine interferes in some manner

with the formation of new virus.

In actuality there are few aspects of the problem of virus multiplication which are not controversial and there are many facets of the problem which are obscure in detail because of limitations of methodology in protein and enzyme chemistry. Investigators, however, are in agreement with the qualification that all viruses do not have enzymes, commonly associated with metabolic processes. Second, that all virus multiplication appears to be intracellular in nature. Third, if reproduction is occurring the virus must obtain substance and energy; and last, the basic assumption is followed that the true virus represents the ultimate in parasitism and is dependent upon the environment for its survival.

## METHODS AND MATERIALS

The rapid and important developments that have taken place in the field of virology in the last 20 years have closely paralleled the progress in techniques. For this reason considerable attention has been focused in this study to the evaluation and development of techniques. Most of the studies were of virus propagation in a suspended type of tissue culture which uses the chorio-allantoic membrane of embryonated eggs, the fundamentals of which were outlined previously (Ackermann 1951). This technique offers special advantages for the study of the mechanisms of host and virus interactions. The ease and economy with which the tissue can be obtained and the use of a simple synthetic medium are further recommendations. In the following paragraphs, details of the method and materials used are given.

The Virus. The PR8 strain of Type A influenza virus was used as the infectious agent. It is a stable virus, characterized by a reproducible hemagglutination reaction, and grows rapidly to a high titer in a relatively short period. The virus was isolated from man by Francis (1934)

and has undergone seven passages in ferrets, 593 passages in mice and 140 passages in eggs. The preparation of the seed virus involves the use of ten or eleven day embryonate eggs. The proper dilution of infected allantoic fluid was made in a broth medium containing 75 units of penicillin and 240 $\mu$g of streptomycin per milliliter. A $10^{-5}$ dilution was inoculated into the allantoic sac; each embryo was given 0.1 ml. After 48 hours of incubation at 37°C the eggs were chilled for one hour at 4°C. Then the allantoic fluid was removed with a sterile 20 ml. syringe from each egg, pooled and distributed into 3 ml. lusteroid tubes, promptly frozen and stored at -60°C. In this manner it was possible regularly, to obtain a reproducible seed virus with an infectivity titer ranging between $10^{9.3}$ and $10^{9.7}$ $EID_{50}$ per ml.

The Host Cells. The host cells were those of the chorio-allantoic membrane from 14-16 day-old embryonate eggs. The procedure used was to cut with scissors the shell of 14-16 day embryonate eggs at the end opposing the air sac. The contents of the egg then were discarded except for that part which lined the shell. The membrane was peeled from the shell and washed in Petri dishes with cold buffered saline to remove the blood and areas of gelatinous material. The washed membranes were transfered to a 25 ml. wide mouthed bottle containing saline. The bottle was immersed

in crushed ice. Intact pieces of chorio-allantoic membrane were taken out, drained on sterile gauze and weighed with a Roller-Smith balance. These moist pieces were then transferred to individual Warburg flasks containing the culture medium. The weight of each moist intact piece used in each culture flask was 400 mg. On the basis of previous estimates of Fazekas de St. Groth and Cairns (1952) this weight of tissue is equivalent to approximately $5 \times 10^7$ cells.

The Host-Virus System. The host-virus system was cultivated in the Warburg flasks with a modified Simm's solution whose composition is given below.

The Composition of the Culture Media

| | | |
|---|---|---|
| Stock I: | KCl | 200 mg. |
| | $CaCl_2.2H_2O$ | 147 mg. |
| | $MgCl_2.6H_2O$ | 203 mg. |
| | Phenol red | 6 mg. |
| | Penicillin | 100 units per ml. |
| | Streptomycin | 100 g per ml. |
| | Glass distilled $H_2O$ added to a final volume of 650 cc. | |
| Stock II: | $Na_2HPO_4$ | .18% |
| Stock III: | $NaHCO_3$ | 3% |
| Stock IV: | NaCl | 8% |
| Stock V: | Glucose | 6% |

The culture medium used experimentally was prepared from 6.5 parts of Stock I, 1 part of Stock II, 1 part of Stock III, 1 part of glass distilled water and 0.5 part of Stock IV. After mixing, the modified Simm's solution was passed through a Mandler filter, No. 4, 2-1/2" x 5/8", to assure bacterial sterility. In all instances, the solution of glucose was autoclaved at 10 pounds pressure for fifteen minutes. To each culture flask 0.3 ml of the glucose was added separately. Considerable acidity develops in this type of culture during the twenty four hours of incubation, due to the formation of both lactic acid and carbon dioxide. An effective buffering action was essential and in order to maintain such action, $CaCO_3$ of 2 mg/ml concentration was used with the medium. In addition a constant partial pressure of carbon dioxide was maintained using the mixture described by Pardee (1949) in the center well of the Warburg flask. It is a mixture composed of diethanolamine, potassium bicarbonate and hydrochloric acid. The proportions were selected to give an equilibrium concentration of 3% carbon dioxide in the gas phase. The Warburg culture flasks were incubated at 37°C with shaking. In all instances 0.3 cc of virus inoculum was used per flask and the final volume of the medium and tissue was 3.0 milliliters.

The Culture Procedure. The culture procedure was to insert 400 mg of intact moist pieces of chorio-allantoic membrane

into the Warburg flask to which the proper culture media was added. The desired inoculum of the virus was added immediately thereafter. The flasks were put on the Warburg apparatus maintained at 37°C with the proper shaking. After allowing maximum adsorption of one hour and ten minutes, the flasks were taken out and immersed in crushed ice. Each membrane then was washed three times in small Petri dishes filled with cold buffered saline to remove excess virus, then washed once with immune serum, and again three times in buffered saline to remove the immune serum. After each washing, the tissue was drained on sterile gauze to remove excess moisture. The individual membranes were then put back into new Warburg flasks with fresh medium to which no virus had been added and the host-virus system was maintained again in the Warburg apparatus at 37°C as mentioned above. A detailed description of the individual experiments is to be found in the section titled "Experimental and Results."

The Immune Serum. The immune serum used to remove excess virus was prepared by intraperitoneal inoculation of rabbits or nasal instillation of ferrets with allantoic fluid containing the PR8 strain of influenza virus. These animals were bled at intervals and the serum collected and titrated prior to use. The PR8 immune serum used throughout the experimental work had a hemagglutination-inhibition

titer of 4096 and it was diluted ten fold before being used.

Virus Titrations. Samples of fluids from the Warburg flasks were taken at planned intervals for virus titrations. Each sample of 0.2 cc taken out was replaced accordingly with fresh media to assure constant volume. The amount of the virus in the samples was estimated by determining the infectious titer for eggs. For this purpose ten fold serial dilutions were made in broth and four eggs were inoculated with 0.1 ml of each dilution. After forty eight hours of incubation at 37°C, approximately 0.5 ml samples of allantoic fluid were removed from each egg and tested for the presence of virus by the addition of 0.5 ml of 0.5% chicken red blood cells. The 50% end-point was calculated using the method of Reed and Muench (1938).

Receptor-Destroying Enzyme (RDE). The receptor-destroying enzyme was used in the experimental work in some cases where destruction of the receptor-site material was required. The enzyme was a partially purified preparation of cholera vibrio extract containing 32,000 units of receptor-destroying enzyme per milligram of nitrogen. The method of purification used was devised by Dr. R. B. Johnson in this laboratory. The procedure consists of isolating the enzyme

from cholera vibrio filtrate by the addition of acetone to a final volume of 45%. A precipitate is formed which is extracted three times with calcium saline.

The multiplication of the PR8 strain of Type A influenza virus in the chorio-allantoic membrane was studied in the presence of three viral inhibitors which were available at the beginning of the study.

α-Amino-p-Methoxyphenylmethane Sulfonic Acid (AMPS).

This inhibitor was prepared in this laboratory by Dr. W. W. Ackermann (1952) using the procedures described by McIlwain (1941) for the synthesis of α-aminoisobutyric sulfonic acid.

dl-Methoxinine.

This inhibitor was generously supplied by the American Cyanamid Company.

Fluorophenylalanine.

This metabolic inhibitor was obtained through the courtesy of Dr. H. O. Halvorson of the Bacteriology Department of the University of Michigan in Ann Arbor.

The amino acid dl-phenylalanine used as the reversing agent is a product of the Nutritional Biochemical Corporation.

# CHAPTER I

## THE GROWTH PATTERN OF INFLUENZA VIRUS

If a given quantity of virus is used to infect the chorio-allantois, a process is initiated whereby the virus becomes attached to the host cell starting a series of intricate chemical and biological reactions which in time result in increased amounts of virus. The mechanics or kinetics of this process can be considered by following under various conditions the increase or decrease of infectious activity.

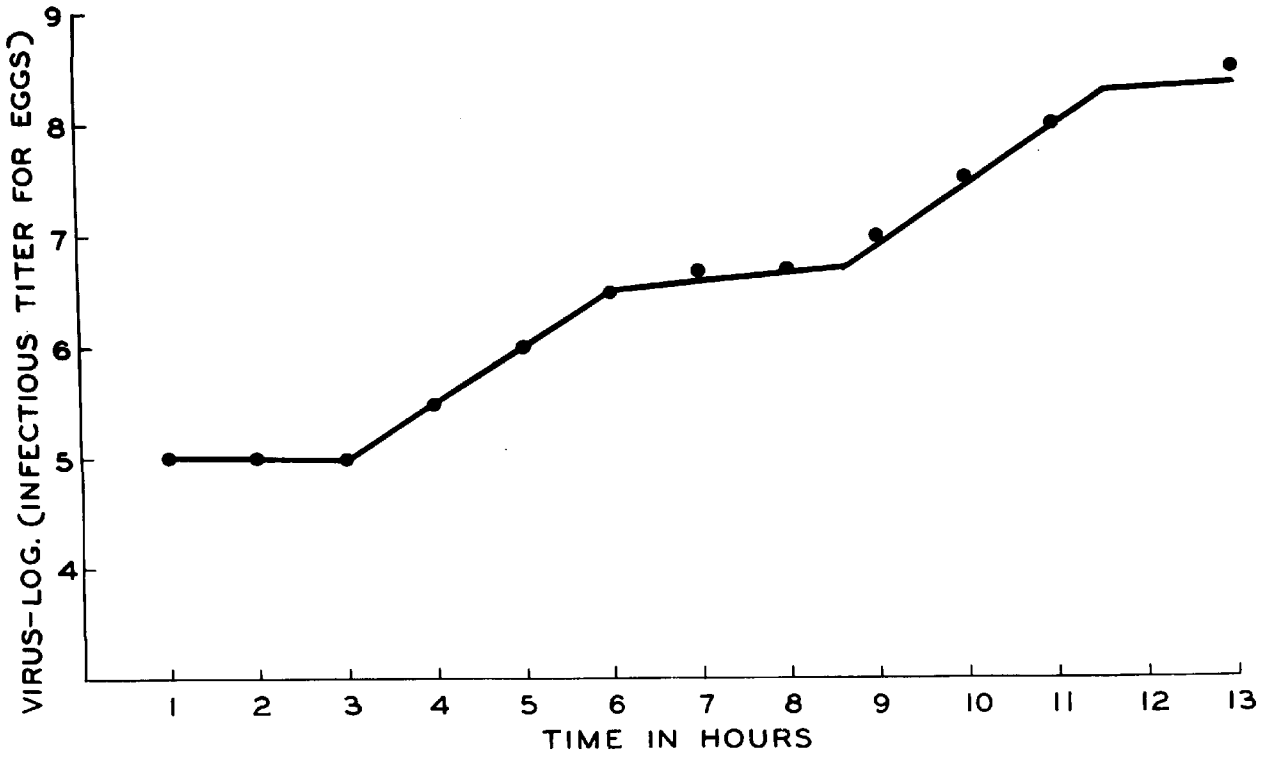
### A. Growth of Influenza Virus Subsequent to a Small Inoculum

A small inoculum of virus ($10^{-6}$) was added to a culture flask containing chorio-allantoic membrane and medium. At hourly intervals, samples (0.1 ml) from the extracellular fluid were removed and assayed for virus in embryonate eggs, after making suitable dilutions. The data and description of the experiment are found in Fig. I. These data show the pattern of release of new mature virus from the tissue. It will be noted that there is a constant period of three to four hours in which there is no virus increase, followed by a four hour period of gradual

FIGURE I

THE GROWTH PATTERN OF INFLUENZA VIRUS

WITH A SMALL INOCULUM




A virus inoculum of the order of $10^{-6.0}$ was used to infect 400 mg. of tissue in a single flask culture. The total volume of the culture was 3.0 ml.

release of new virus. A second lag phase much shorter than the previous one and less distinct clearly exists. It is followed by a new sustained release of mature virus. The maximum yield is reached by fourteen hours.

To attempt an interpretation of such findings, one must assume that with a relatively small inoculum ($5 \times 10^4$ egg infective dose), not all of the cells ($5 \times 10^7$) of the chorio-allantois in the culture flask were infected initially. Because the ratio between the number of infectious doses of influenza virus and the number of cells available is smaller than one, a multiple infectious cycle is possible.

The use of small inoculum does not allow the achievement of the objective outlined in the introduction, so far as a single sequence of infection is desired, for two main reasons. The first reason is that with a small inoculum where the growth of influenza virus is characterized by successive cycles, any attempt to quantitate the release of mature virus will be fruitless due to complications resulting from readsorption of newly released virus to the remaining susceptible cells. The second reason is that the unadsorbed residual virus has a masking effect upon the detection of the earliest releases. It was found that a level of residual unadsorbed virus, which is still infectious upon making suitable dilutions and injecting them into embryonate eggs, remains, above which new virus

must accumulate before it can be detected. This difficulty is inherent in the method of measuring virus by limiting dilutions.

A one-step growth curve was achieved by different methods. For instance, Henle (1949) did achieve a one-step increase of the newly produced virus in the allantoic cavity by addition of a large amount of heterologous irradiated virus which prevented infections of further susceptible cells. The way Stone (1948) achieved the same purpose was by treating the host cells with the receptor-destroying enzyme after infecting with the virus. This prevented any infection of remaining susceptible cells by the released virus. These procedures offer some complications in themselves and fail to solve several problems; for instance, the irradiated homologous virus damages the synthetic capacity of the host cell and reduces the size of the viral yield, probably by virtue of the interfering phenomenon. Further, while RDE prevents infection of new cells it does not prevent adsorption of virus. These complications could be by-passed by proper manipulation of the variables involved. It was considered possible to achieve a single sequence of infection by using a massive inoculum, $5 \times 10^9$ egg infectious doses of influenza virus to infect $5 \times 10^7$ cells, which should reduce the number of susceptible cells to a minimum. The major portion of the unadsorbed and the superficially adsorbed virus could be

removed by extensive washing of the membrane with physiological saline and homologous immune serum one hour after infection. This approach using a massive inoculum was tested and found to be advantageous from many standpoints; on the one hand it offers a single sequence of infection by minimizing the number of available susceptible cells and on the other hand the masking effect of the unadsorbed residual virus could be reduced effectively. The development of this method is considered in the next section.

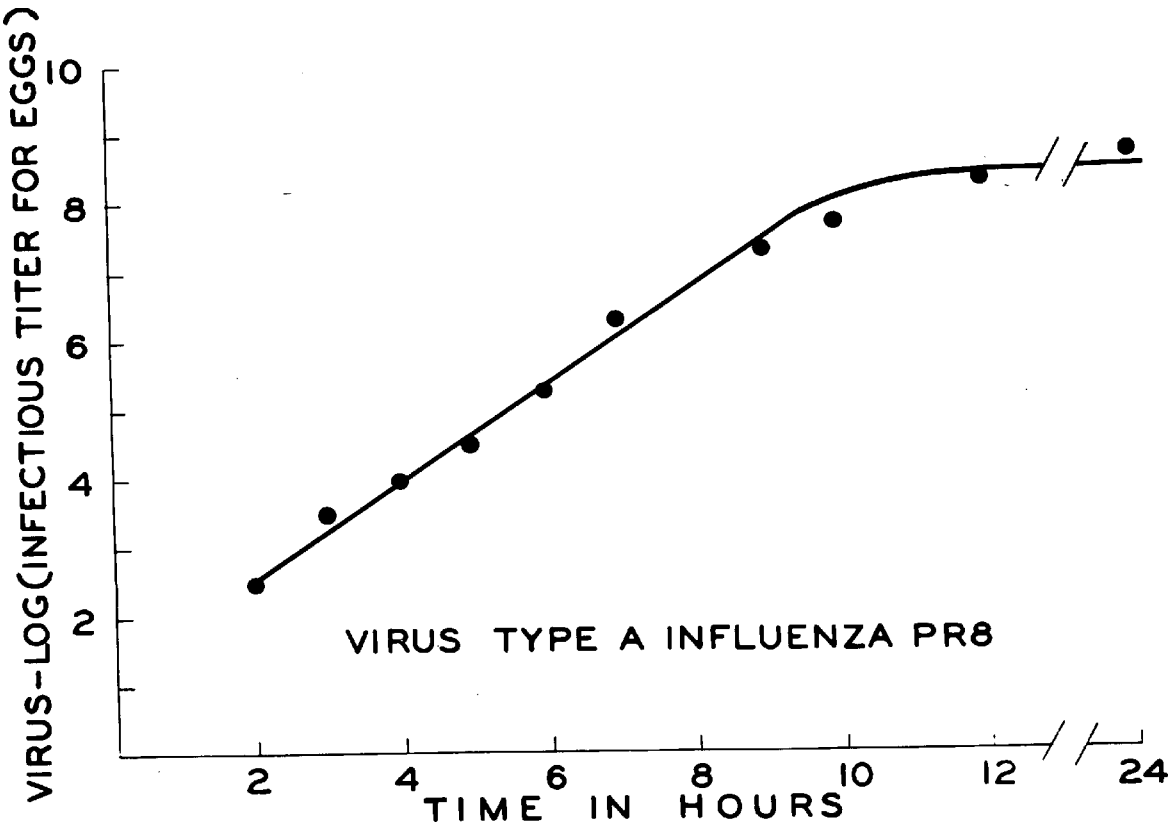
B. Growth of Influenza Virus subsequent to a Large Inoculum.

A single flask culture containing the tissue and the medium was infected with a massive inoculum of influenza virus for the purpose of infecting all the susceptible cells. After one hour of adsorption at 37°C, the residual unadsorbed virus was removed by washing the tissue which was returned eventually to a new culture flask with a fresh medium. Samples (0.1 ml) were taken from the extracellular fluid at hourly intervals and were titrated for the virus in embryonate eggs. The data in Fig. II show the rate at which the newly produced virus is released from the tissue. It is apparent from the results presented that the residual level of viral inoculum can be reduced by washing the chorio-allantoic membrane after adsorption has occurred. The first release of mature virus is between two and three hours. The rise of the virus is exponential until approximately the 9th hour, then the

FIGURE II

A ONE-STEP GROWTH CURVE OF INFLUENZA VIRUS

AS DETERMINED IN A SINGLE FLASK CULTURE




One Warburg flask culture was set up to contain 400 mg. of tissue, 0.3 ml. of 6% glucose, 2.0 ml. of modified Simm's and 0.3 ml. of undiluted allantoic fluid which had a titer of $10^{-9.3}$. The tissue was washed from the residual virus after one hour of incubation at 37°C. The tissue was placed in a new culture flask with fresh medium. Samples were taken at hourly intervals.

growth curve rises more slowly until the maximum yield is reached by the 12th hour; there is no evidence of a burst phenomenon, but rather a sustained release over a period of time. The growth curve obtained by the technique of the experiment seems to be derived from a single sequence of infection, because if the receptor-destroying enzyme, which prevents the adsorption of newly released virus to a new susceptible cell is used after infection, the growth pattern of influenza virus is not altered; also the small amount of virus released at the fifth hour is not sufficient to induce the attainment of $10^8$ $EID_{50}$ viral particles by the tenth hour of the growth cycle.

The nature of the growth curve could be interpreted on the basis of inhomogeneity of the individual cells supporting viral growth. Each infected cell appears to support the replication of the virus differently. Some cells may support viral growth more slowly than others, resulting in variations in the length of the latent period, and hence may begin to release the mature form of the virus at different times, so that a gradual virus production over a period of time results from this multicellular system. Another interpretation, however, is that the cellular system is homogeneous and each individual cell starts to release the virus at approximately the same time and over a period of time. Cairns (1952) and Dulbecco (1954) have offered similar interpretations for the behavior of the infected culture, where growth

pattern of a single infected cell can be obtained.

A single interpretation could emerge from the previous two, contending that the pattern of virus growth in the tissue may be the result of both considerations, a difference in the latent period of individual cells, plus a sustained release over a period of time from each cell.

C. Synchronization of the Infectious Process.

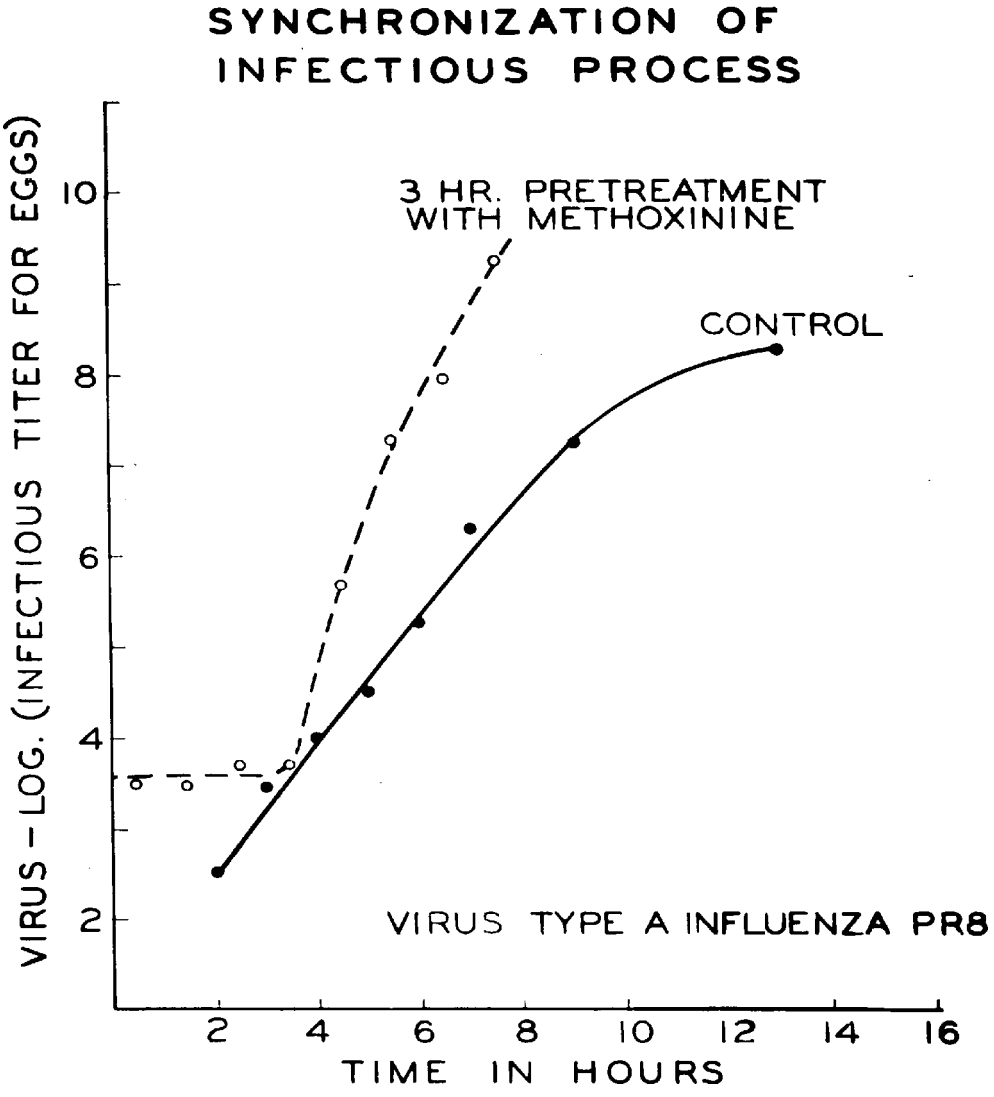
The hypothesis advanced in the previous section was tested by studying the normal growth pattern of influenza virus under the influence of a metabolic block. Methoxinine was chosen as a metabolic inhibitor because it seems to interfere with an early biosynthetic reaction of the virus but not with the initiation of infection. Justification for this statement will be presented in a later section where the action of methoxinine is analyzed.

The cells of the chorio-allantois were treated for three hours with methoxinine (1.3 mg/ml) and a massive viral inoculum in a single flask culture. Upon removal of the metabolic block and residual viral inoculum by washing the membrane thoroughly, the tissue was placed into fresh normal medium and allowed to produce the viral yield. Samples were taken at intervals and titrated to determine the rate of viral increase. By this procedure it was hoped to synchronize virus development in cells of the infected culture by minimizing the individual differences of the host cells. In the cells in which the

infection occurs early, the growth of the virus might be arrested at some point until other cells with a slower response have achieved the same stage of development in the infectious cycle. The details of the experiment and the data obtained are found in Fig. III. It appears that when the infected tissue received prolonged treatment with DL-methoxinine as described above, many deviations from the normal curve were seen. The latent period remained the same but the appearance of the virus was more abrupt and the period of release shorter. The characteristics of this growth curve indicate a greater uniformity of response of the individual cells in the infected tissue, where the cells begin to produce mature virus at more nearly the same time.

The cell suspension type of tissue culture technique has many advantages for the study of the growth of influenza virus and allows the assay of viral increase at any time, whether in the extracellular or intracellular phase. However, much of the information obtained was similar to what was learned previously using the embryonate eggs (Henle, Henle and Rosenberg, 1947; Henle, 1949, Hoyle, 1950; Von Magnus, 1951). Two noteworthy exceptions were the recognition of the absence of a burst phenomenon and the fact that the growth of the virus was exponential. The latent period has been found to be shorter than indicated by the experiments using embryonate eggs. The successive cycles of growth are clearly outlined.

FIGURE III




The cells of the chorio-allantois were exposed at zero time to the inhibitor DL-methoxinine (1.3 mg/ml) and a massive viral inoculum ($10^{-9.3}$). After three hours of incubation at 37°C in the Warburg apparatus, the inhibitor and the residual unadsorbed virus were washed away with cold buffered saline and immune serum. The tissue was returned to fresh medium and samples were taken at intervals.

Analysis of a single sequence of growth of influenza virus substantiates the presence of phases in the growth of the virus. These phases are not listed as being discrete; such may not be the case. For instance, in the studies to be described later, the processes of maturation and liberation are shown to overlap or occur simultaneously, resulting in a sustained liberation of mature virus. This growth pattern of influenza virus appears to be general in the case of animal viruses. The growth of poliomyelitis virus in HeLa cell cultures exhibits such a pattern (Ackermann, Rabson and Kurtz 1954), so does the growth of newborn pneumonitis virus, type Sendai, in the chorio-allantoic membrane of the embryonate egg (Ackermann and Maassab 1954). Newcastle disease virus growth in the cells of the chorioallantois also gives evidence of the presence of these developmental stages (Maassab 1954), so does the WEE virus growth in chick fibroblast culture (Dulbecco 1954).

Biochemical differentiation of these stages is of prime importance to the study of the metabolic sequence of events that underlie the infectious process. The following sections are concerned with the analyses of these problems using the techniques of cultivation described in this section. Specific inhibitors have been used to isolate and define these developmental stages in terms of the metabolic activity of the host-virus system.

# CHAPTER II

## INHIBITION OF INFLUENZA VIRUS MULTIPLICATION BY α-AMINO-p-METHOXYPHENYLMETHANE SULFONIC ACID (AMPS), METHOXININE AND FLUOROPHENYLALANINE (FPA): THE VARIATION IN THE CONCENTRATIONS OF THE COMPOUNDS OR THE SIZE OF INOCULUM AS AFFECTING THE EXTENT OF VIRAL INHIBITION

The concept that multiplication of a virus is intimately associated with the metabolism of the cell invites study of metabolic processes which may be essential for virus replication. Such studies should indicate new approaches to the problem of viral biosynthesis and of the nature and mechanisms of the biological specificity exhibited by viruses.

At the start of this study a number of chemical inhibitors were available; α-amino-p-methoxyphenylmethane sulfonic acid (AMPS), methoxinine and fluorophenylalanine all of which were known to exhibit inhibitory activity toward the growth of bacteria and multiplication of virus without being unduly toxic to animal cells. These compounds were selected for use in this study to define and analyze the stages of viral development. This chapter contains a description of preliminary experiments designed

to obtain the workable range of concentrations of inhibitors, and to demonstrate specificity and reversibility.

A. Effect of the size of viral inoculum on degree of inhibition of influenza virus multiplication produced by AMPS.

The inhibitor AMPS was prepared in this laboratory using the method of McIlwain (1941) who synthesized a series of α-amino-sulfonic acids found to be effective inhibitors of bacterial growth. Furthermore, these sulfonic acids were found to inhibit vaccinia virus in tissue culture (Thomas 1947). Ackermann (1952) tested the effectiveness of the inhibitor AMPS on the growth of influenza virus. The inhibitor AMPS caused marked inhibition of virus propagation in tissue culture; it was not virucidal and was not reversed by any compound, such as tyrosine, to which it was structurally related. The inhibitor AMPS suporessed the multiplication of influenza virus (PR8) strain at a concentration of 0.33 mg per ml., when used with an inoculum concentration of proper size. The sulfonic acid was observed to be less effective as the concentration of the viral inoculum was increased. The effect is shown in the following experiment: Four culture flasks were set up to contain AMPS (0.33 mg/ml), the medium and the tissue. Different inoculum concentrations of virus of $10^{8.3}$, $10^{7.3}$, $10^{6.3}$ and $10^{5.3}$ $EID_{50}$ were added to each culture flask. After one hour of incubation at 37°C, the tissues were removed, washed extensively in saline containing AMPS and returned to flasks containing fresh media and AMPS for

further incubation. Samples of the extracellular fluids were removed and assayed for infective virus in eggs at the third and 24th hour. The data presented in Table I tend to show a relationship between the concentration of the viral inoculum and the inhibiting effect of AMPS. An inoculum concentration of $10^{6.3}$ $EID_{50}$ seems to be affected by an amount of AMPS (0.33 mg/ml) which is not toxic to the tissue or the virus, as infective virus could be recovered at all times.

B. The effect of graded concentrations of methoxinine on inhibition of influenza virus multiplication

Recent observations of several groups of investigators using ethionine and methoxinine as analogues of methionine have established that methionine is essential for normal growth of mice (Sketol and Weiss 1951), for several yeasts (Smith 1953), and for rats (Travers and Cerecedo 1951). Methionine seems to play a central role in both methyl and sulfur metabolism (Cantoni 1952). These studies involving the interference in the function of methionine in biological systems have shed light on its role as an essential metabolite. In addition, studies using ethionine and methoxinine as analogues of methionine have been undertaken to obtain evidence on the role of amino acid methionine in viral infections. Brown and Ackermann (1951) investigated the action of ethionine on poliomyelitis viral multiplication and found an inhibitory

TABLE I

EFFECT OF SIZE OF INOCULUM[a] ON INHIBITION OF INFLUENZA VIRUS MULTIPLICATION BY AMPS

| Time of Samples in Hours | Infectivity Titer[b] in Eggs | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Titer of Viral Inoculum | | | | | | | |
| | Control[c] | | | | Experimental | | | |
| | $10^{8.3}$ | $10^{7.3}$ | $10^{6.3}$ | $10^{5.3}$ | $10^{8.3}$ | $10^{7.3}$ | $10^{6.3}$ | $10^{5.3}$ |
| 3 | 2.3 | 2.0 | 1.0 | 0.5 | 2.5 | 1.0 | 1.0 | 0.7 |
| 24 | 8.7 | 8.0 | 7.3 | 6.0 | 7.0 | 4.0 | 1.0 | 0.5 |

a The titer of viral inoculum represents the values in the culture flasks after making suitable dilutions of allantoic fluid in the Simm's medium. AMPS (0.3 mg/ml) and the virus were incubated with the chorio-allantoic membrane for one hour; the membrane was washed subsequently 6 X in saline containing AMPS and one time with immune sera and returned to new culture flasks containing AMPS.

b The infectivity titer of the extracellular fluid is expressed as the negative log of the limiting dilution 50 per cent infectious for eggs.

c The results are taken from separate comparable experiments.

effect in tissue culture. Ackermann (1951) demonstrated the inhibitory capacity of methoxinine on the growth of influenza virus in chorio-allantoic membrane, a tissue which was found to be unable to synthesize methionine from its immediate precursors, homocysteine and betaine. Methoxinine action is not virucidal and its specificity is demonstrated by the reversal of the innibition by methionine (Ackermann 1951). The antimetabolite is pictured as acting by excluding a structurally related metabolite from combination with its normal specific reactants. The effect of various concentrations of methoxinine on the propagation of influenza virus was investigated in vitro. Six culture flasks were set up which initially contained graded concentrations of methoxinine, the medium and a massive inoculum of the virus ($10^{9.3}$ $EID_{50}$). After one hour of incubation at 37°C, the tissues were washed extensively in saline and immune serum and returned to new culture flasks with fresh medium, the same initial amount of methoxinine and incubated further at 37°C in the Warburg. At the second and the 24th hour, a sample (0.2 ml) from the fluids of each culture flask was removed and titrated in eggs for infectivity, while at the 48th hour, infectivity titrations were performed on the fluids and tissues separately. From the results given in Table II, it can be said that the analogue methoxinine is able to inhibit the growth of the influenza virus over a range

of concentrations, while complete suppression is achieved at a concentration of 1.33 milligrams per milliliter. The dosage of DL-methoxinine used throughout the experimental work is 1.33 mg/ml or that least amount of the inhibitor which elicits maximal response.

C. The effect of prolonged FPA treatment of the normal CAM on its ability to support viral multiplication.

When the structure of phenylalanine (PA) is altered by inserting a fluorine atom in a paraposition of the benzene ring, a compound is produced which antagonizes the function of PA. Halvorson and Spiegelman (1952) demonstrated that the participation of PA in the synthesis of proteins is blocked by the analogue fluorophenylalanine (FPA). Fluorophenylalanine was shown to exhibit antiviral activity in cultures of poliomyelitis virus (Ackermann, Rabson, and Kurtz 1954) and of the agent from Sendai (Ackermann and Maassab 1954). The effect of an antimetabolite may be to slow the rate of a reaction to a point where growth or some other functions of the cell do not occur. However, when the analogue of guanine, 8-azoguanine, was injected into mice, Mitchell (1950) found evidence to suggest that a small part of the analogue passed through reactions normally concerned with the metabolite. Such results have engendered the belief that the altered product which arises is the harmful agent and a permanent functional damage to the biological system results. However, if FPA was incubated with the tissue for 20 hours prior to

TABLE II

THE EFFECT OF VARIOUS CONCENTRATIONS OF DL-METHOXININE ON THE PROPAGATION OF INFLUENZA VIRUS[a] IN VITRO

| DL-Methoxinine mg/ml | Infectivity Titer[b] | | |
|---|---|---|---|
| | 2 hours | 24 hours | 48 hours |
| 0.00 | 4.0, 5.7[c] | 9.3 | 8.7, 8.0[c] |
| 0.66 | 4.3 | 6.0 | 7.3, 7.0[c] |
| 1.00 | 4.3 | 5.7 | 6.7, 6.5[c] |
| 1.33 | 4.0 | 4.0 | 4.7, 4.5[c] |
| 1.50 | 4.5 | 4.7 | 4.5, 4.0[c] |
| 1.66 | 4.0 | 4.3 | 4.3, 4.3[c] |
| 1.83 | 4.0 | 4.7 | 4.3, 4.0[c] |

a Massive inoculum of the order of $10^{-9.3}$ was used to infect the tissue.

b Titers are expressed as the log of the reciprocal of the dilution.

c Titer of the tissue only.

The virus and the membranes were incubated with graded concentrations of DL-methoxinine for one hour, then washed 6 times in saline and one time in immune serum. The washed tissues were returned to fresh media containing DL-methoxinine.

All values are for the extracellular fluid unless stated otherwise.

infection, the mechanism of the cell necessary to support the propagation of the virus is still intact.

Two flasks were prepared to contain the medium and the tissue. Prior to the infection, the control tissue was incubated for twenty hours in Simm's solution at 37°C, while the experimental tissue was incubated in Simm's solution containing FPA. After 20 hours, both tissues were infected with a massive viral inoculum and incubated further for one hour, then removed and washed. The control tissue was returned to fresh normal Simm's solution, while the experimental tissue was incubated at 37°C with the Simm's solution containing FPA and PA. Samples of the fluids were taken from each flask culture at the 22nd and 40th hours, and titrated in eggs for infectious virus. The data in Table III show approximately the same amount of growth of virus in each culture at the 40th hour. No irreversible damage to the ability of the cell to support viral increase resulted from the prolonged treatment of the tissue with FPA.

D. The effect of graded concentrations of FPA on inhibition of influenza virus multiplication.

In this report the inhibition of influenza virus multiplication by FPA is described to determine the importance of PA in viral propagation. Four culture flasks were prepared containing initially graded concentrations of FPA, the tissue, medium and a massive inoculum concentration

TABLE III

LACK OF TOXICITY OF THE ACTION OF FLUOROPHENYLALANINE ON INFLUENZA VIRUS PROPAGATION

| Treatment[a] of Tissue | Time[b] of Treatment[c] | | Viral Titers[d] | |
|---|---|---|---|---|
| | 20 hours | 21 hours | 22 hours | 40 hours |
| Tissue incubated with Fluoro-phenylalanine | Virus | Wash | 3.3 | 9.0 |
| Tissue incubated without Fluoro-phenylalanine | Virus | Wash | 3.5 | 9.3 |

a Each flask contained 400 mg of tissue. Modified Simm's and glucose and to the control no subsequent addition while the experimental flask had fluorophenylalanine (0.2 mg/ml).

b Time measured from original addition of tissue to medium.

c Undiluted seed virus of the titer $10^{9.7}$ $EID_{50}$ was added to the two culture flasks for one hour. The tissues were washed after allowing adsorption of the virus, three times in saline, once in diluted immune serum (titer 1:400), and three times further in saline. The tissues after washing were returned to new culture flasks with normal culture media in the control flask while the experimental flask had fluorophenylalanine and DL-phenylalanine (0.133 mg/ml) added.

d Titers of influenza virus in the extracellular fluid are expressed as log of the reciprocal of the dilution.

of virus. After one hour of incubation at 37°C, the tissues were removed, washed extensively in saline and homologous immune serum. The membranes were placed in fresh medium which contained the same amounts of the amino acid inhibitor. Samples of the fluid were taken at the second, 12th and 24th hours and titrated in eggs for infectivity. It seems that the degree of innibition is dependent on FPA concentrations, with complete suppression of virus multiplication being achieved in the presence of 0.1 mg/ml of FPA (Table IV).

E. The effect of graded concentrations of phenylalanine upon inhibition produced by FPA.

Inhibition by FPA can be completely reversed by PA which indicates that it is some function of PA in viral propagation which is impaired. This is demonstrated in an experiment of similar design in which one concentration of FPA (0.1 mg/ml) was used with graded concentrations of the amino acid PA (Table IV). It is evident from the results that the degree of inhibition is also dependent on the PA concentration with complete reversal of FPA action on viral propagation being achieved with a 0.134 mg/ml concentration. The action of FPA on the viral synthetic mechanism appears to be specifically directed toward PA. In all instances during this study, the concentration of FPA used to give maximal inhibition of influenza virus multiplication was 0.1 mg/ml which is completely reversed by 0.134 mg/ml of the metabolite PA.

TABLE IV

INHIBITION OF GROWTH OF INFLUENZA VIRUS[a]

BY FLUOROPHENYLALANINE AND ITS REVERSAL

WITH DL-PHENYLALANINE

| Fluorophenyl-alanine mg/ml | DL-Phenyl-alanine[c] mg/ml | Infectivity Titer[b] | | |
|---|---|---|---|---|
| | | 2 hours | 12 hours | 24 hours |
| 0.000 | - | 3.7 | 8.7 | 9.0 |
| 0.033 | - | 3.7 | 4.7 | 6.0 |
| 0.050 | - | 4.0 | 4.3 | 5.7 |
| 0.066 | - | 3.7 | 3.7 | 4.3 |
| 0.100 | - | 3.7 | 3.0 | 3.0 |
| 0.000 | 0.000 | 3.7 | 9.0 | 9.3 |
| 0.100 | 0.066 | 3.7 | 7.3 | 8.0 |
| 0.100 | 0.100 | 4.0 | 8.5 | 8.7 |
| 0.100 | 0.134 | 3.7 | 8.7 | 9.3 |

a Massive inoculum of the order of $10^{-9.3}$ was used to infect the tissue.

b The infectivity titers are expressed as the log of the reciprocal of the dilution. All values are for the extracellular fluid.

c Data obtained with DL-phenylalanine were from a separate experiment.

The tissues were exposed to the virus and to the inhibitor or to the analogue-metabolite combination at zero time. After one hour, the tissues were washed six times with buffered saline and one time with immune serum. The washed membranes were returned to fresh media containing only fluorophenylalanine or fluorophenylalanine with phenyl-alanine.

## CHAPTER III

## THE SENSITIVITY OF THE INCIPIENT STAGES OF VIRAL DEVELOPMENT MEASURED BY THE INHIBITORY EFFICIENCIES OF THESE COMPOUNDS RELATIVE TO THE AGE OF INFECTION

The multiplication of influenza virus in host cells is characterized by a number of phases of development which are recognizable from the normal growth curve. The technique used in this study for culturing the virus permits one to determine the effect of an inhibitor on these phases in terms of production of mature virus. It is of very great interest, for instance, to know whether chemical inhibitors interfere with adsorption, influence the latent period, or prevent the release of mature virus. In general, such studies are particularly suitable not only for determining the stages of the viral synthetic process, but also for elucidating the role of the metabolism of the host cell in the biosyntnesis of the virus.

### A. Phases of viral development sensitive to the action of AMPS.

The effect of the time of addition of α-amino-p-methoxyphenylmethane sulfonic acid (AMPS) on viral development was studied. A series of culture flasks were set up to contain the chorio-allantoic membrane (400 mg), a

small inoculum concentration ($5 \times 10^6$ $EID_{50}$) and the medium. AMPS (0.33 mg/ml) was added to the individual flasks at intervals ranging from zero to four hours after virus inoculation. The viral content of the tissue and the extracellular fluid was determined after 12 hours of incubation at $37^0$C. The details and data of the experiment are found in Table V. It appears that when AMPS was added at the zero or 15 minute interval, no increase in virus titer resulted in the extracellular fluid, while cultures receiving AMPS at the 30 minute interval and later, showed viral production. The results point to an effective innibition by AMPS when added between the zero and 15 minute intervals, while later addition gives partial innibition of viral growth. The data presented seem to point to a graded response in lesser innibition as the latent stage proceeds during the infectious cycle. The compound appears to cause innibition to a degree which is inversely related to the time of addition. Due to an effect of AMPS on some process occurring early in the latent period of viral development, it is suggested that its influence is upon the adsorption or the penetration phase. In addition, it seems most likely that AMPS does not prevent the synthesis of the virus but only interferes with the initiation of infection.

B. Phases of viral development sensitive to the action of methoxinine.

The effect of time of addition of DL-methoxinine

TABLE V

SENSITIVITY OF PHASES OF THE LATENT PERIOD TO THE ACTION OF AMPS

| Exp. No. | Control Titers[c] at Various Times | | | Experimental Titers[c] at 12 hours following the Addition of AMPS at various Times[d] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hours | 6 hours | 12 hours | 0.00 | 0.25 | 0.50 | 0.75 | 1.00 | 2.00 | 3.00 | 4.00 |
| I[a] | 4.7 | -- | 9.0 | 4.7 | -- | -- | -- | 5.3 | 5.3 | 5.0 | 6.2 |
| II[a] | 4.7 | 5.5 | 6.7 | 4.5 | 4.7 | 4.5 | 5.3 | 5.6 | -- | -- | -- |
| III[b] | 5.0 | 6.3 | 7.5 | 4.5 | 4.3 | 5.5 | 6.3 | 6.5 | -- | -- | -- |

a Titers of the extracellular fluid.

b Titers of the fluid plus tissue.

c Titers are expressed as the negative log of the limiting dilution infectious for eggs.

d Time of addition of AMPS expressed in hours and measured from the addition of virus.

on viral development was studied to determine the inhibitory capacity on various stages of the infectious cycle and to determine whether it was also phase specific. Seven culture flasks were set up to contain the tissue, the medium and a massive inoculum. After one hour of incubation at 37°C, the tissues were washed in physiological saline and homologous immune serum, and returned to new culture flasks with fresh medium for further incubation. At hourly intervals, the samples of fluid from individual flasks were removed and methoxinine was added. At the time of addition of inhibitor as well as the 3rd, 11th, 12th and 24th hours, samples of fluid were again taken from each flask and titrated in eggs for infectivity. The data of the experiment are presented in Table VI. From the data it was possible to determine if viral growth occurred in any of the flasks before and after the addition of methoxinine. It appears that the longer the addition of the compound is withheld during the latent period, the smaller the extent of inhibition. While the cultures to which the analogue was added, at one or two hours after virus inoculation, failed to support the growth of the agent, all other cultures gave evidence of viral increase. The action of the inhibitor seems to be limited to some interval within the range of the first two hours. It is evident from these data that the utilization of methionine for virus replication is completed before the appearance of new

TABLE VI

SENSITIVITY OF THE PHASES OF THE LATENT PERIOD TO THE ACTION OF METHOXININE

| Hour[a] Methoxinine was added (1.33 mg/ml) | Viral Titers[b] | | | | |
|---|---|---|---|---|---|
| | 1st Hour | When Methoxinine is added | 11th Hour | 12th Hour | 24th Hour |
| 1 | 3.3 | 3.3 | 3.5 | -- | 3.7 |
| 2 | 3.3 | 3.5 | 3.3 | -- | 3.5 |
| 3 | 3.3 | 5.0 | 5.7 | -- | 6.3 |
| 4 | 3.3 | 5.7 | 7.0 | -- | 7.5 |
| 5[c] | 3.5 | 5.5 | -- | 7.0 | 8.0 |
| 6[c] | 3.5 | 6.3 | -- | 9.0 | 9.5 |
| 7[c] | 3.5 | 7.5 | -- | 9.0 | 9.5 |
| none[c] | 3.5 | -- | -- | 9.3 | 9.7 |

a Time is measured from original addition of tissue and virus to the medium. After one hour the tissue was washed to remove the residual inoculum. The infecting dose was of the titer of $10^{-9.3}$.

b Titers of influenza virus in the extracellular fluids are expressed as the negative log of the $ID_{50}$ for eggs.

c These values are from a separate comparable experiment.

infectious virus. The inhibitory activity of methoxinine is not attributable to a direct action on the influenza particle per se as it did not inactivate the virus, nor interfere in the adsorption or penetration, because if the inhibitor is added at the second hour after viral inoculation, inhibition still occurs. Since two hours will provide ample opportunity for virus to infect the cells, this indicates that methoxinine acts by interfering with a phase in viral development dealing with the synthesis of the virus.

C. Phases of viral development sensitive to the action of FPA.

The effect of time of addition of another amino acid analogue, fluorophenylalanine (FPA) was investigated to determine its site of action and to test its phase specificity. In this experiment of similar design to that employed for the study of methoxinine action, the effect of fluorophenylalanine on the age of infection was studied. After infecting the chorio-allantoic membrane with a massive inoculum of influenza virus, the tissue was washed extensively and incubated further in a fresh medium. At hourly intervals samples of the fluid were taken before FPA addition and titrated for infectivity. Titers of the virus were also determined at the 12 and 24 hour periods. This was done in order to determine how late after inoculation of virus, FPA could be added and produce an inhibitory effect. Table VII shows the results of such an experiment, when FPA was added during the latent and pro-

TABLE VII

SENSITIVITY OF PHASES OF THE LATENT PERIOD TO THE ACTION OF FLUOROPHENYLALANINE

| Hour[a] Fluorophenyl-alanine was added (0.1 mg/ml) | Viral Titers[b] | | | |
|---|---|---|---|---|
| | 1st Hour | When Fluoro-phenylalanine is added | 12th Hour | 24th Hour |
| 1 | -- | 3.7 | 3.7 | 4.0 |
| 2 | 3.5 | 3.5 | 3.5 | 4.0 |
| 3 | 3.3 | 4.3 | 4.5 | 4.5 |
| 4 | 3.5 | 4.7 | 5.0 | 5.5 |
| 5[c] | 3.3 | 4.5 | 6.5 | 6.7 |
| 6[c] | 3.3 | 6.3 | 7.5 | 8.5 |
| 7[c] | 3.3 | 7.5 | 9.0 | 9.5 |
| none [c] | 3.3 | -- | 9.3 | 9.7 |

a Time is measured from original addition of tissue and virus to the medium. After one hour the tissue was washed to remove the residual inoculum. The infecting dose was of the titer $10^{9.3}$ $EID_{50}$.

b Titers of influenza virus in the extracellular fluids are expressed as the negative log of $ID_{50}$ for eggs.

c These values are from a separate comparable experiment.

ductive periods of influenza virus. The evidence obtained would indicate that the compound exerted an inhibitory effect after infection of the host. It is apparent that FPA gives complete inhibition if added up to the third hour from inoculation of the virus. However, it inhibits the multiplication to a degree when added between the fourth and sixth hours. The compound seems not to interfere with the adsorption or penetration of the virus into the cell, since it inhibits the multiplication sufficiently long after inoculation of a large amount of virus to assure that the process of multiplication of the agent is well under way. Further, it seems that the mechanism by which FPA achieves an inhibitory effect on the multiplication of influenza virus is through interference with the participation of phenylalanine in the biosynthesis of the virus.

D. The action of AMPS, methoxinine and FPA relative to the action of the receptor-destroying enzyme.

It was suggested previously in this section that AMPS suppresses the multiplication of influenza virus by interfering with the incipient stages of viral development, while methoxinine and fluorophenylalanine do not exhibit such tendencies. It seemed justifiable then to use the receptor-destroying enzyme (RDE) to test the above viewpoint. RDE is believed to act on the incipient stages of

viral development; by destroying the receptor substance on the surface of the cell, it prevents the initiation of infection. Six culture flasks were set up to contain the medium and the chorio-allantois. Flasks 1 and 3 had initial supplement, while the receptor-destroying enzyme was added to the second flask. The 4th, 5th and 6th flasks had AMPS, methoxinine and fluorophenylalanine, respectively. After one hour of incubation at 37°C, all the culture flasks received a small inoculum of influenza virus. At the end of the second hour, RDE was added to the 3rd, 4th, 5th and 6th flasks and incubated further for one hour. At the 3rd hour, each tissue was washed extensively and returned to new culture flasks containing the fresh medium and the receptor-destroying enzyme. The fluids of each individual flask were sampled at the 3.3 and the 11th hours, and tested for infectivity in eggs. By such treatment it was possible to determine whether the tissue, when exposed to the virus under various conditions, allows the establishment of infectivity which would result in an increase of infectious virus. Table VIII presents the summary of such an experiment. Treatment of the tissue with RDE or AMPS prior to infection prevented viral increase, while identical treatment of the tissue with methoxinine and fluorophenylalanine did result in viral increase comparable to the control. Treatment of the tissue with RDE after virus infection gave no adverse effect upon the host cell, and an

TABLE VIII

EFFECT OF AMPS AND METHOXININE ON THE INCIPIENT STAGES OF INFECTION WITH INFLUENZA VIRUS

| Flask No. | Time[a] of Treatments[b] in Hours | | | | Viral Titers[c] | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 3.3 Hours[a] | 11 Hours[a] |
| I | -- | Virus | -- | Wash | 2.0 | 6.0 |
| II | RDE | Virus | -- | Wash | 2.5 | 2.7 |
| III | -- | Virus | RDE | Wash | 2.0 | 6.3 |
| IV | AMPS | Virus | RDE | Wash | 3.0 | 3.5 |
| V | Meth-oxinine | Virus | RDE | Wash | 2.0 | 5.5 |
| VI[d] | FPA | Virus | RDE | Wash | 3.0 | 8.0 |

a Time measured from original addition of tissue to medium.

b Each flask contained 0.4 g of tissue. The tissues were washed three times in saline, one time in diluted immune serum (titer 1:400), and three times further in saline. The tissues after washing were returned to 3 ml of RDE (titer 1:1024), Viral inoculum was 0.3 ml of diluted allantoic fluid (titer $10^{-6.7}$). The concentration of methoxinine was 1.3 mg/ml and that of AMPS was 0.3 mg/ml.

c Titers of influenza virus expressed as negative log of the 50 per cent infectious dose for eggs.

d Taken from a separate comparable experiment in which the viral inoculum was 0.3 ml of allantoic fluid with a titer of $10^{-9.3}$.

excellent yield of virus comparable to the control resulted. These observations indicate that AMPS acts prior to the action of RDE or that the reaction of virus and the host cell did not proceed past the irreversible attachment, where the virus could not be removed by washing or the action of RDE. In the presence of inhibitory concentrations of methoxinine and fluorophenylalanine which will suppress viral replication, viral development proceeded to that stage at which the action of RDE could no longer be effective.

A phase of development was recognized which is sensitive to AMPS but not to methoxinine or fluorophenylalanine. Unfortunately the site of action of AMPS is not known, but it is evident that it has an influence upon the incipient stages of viral development.

It is the purpose of the following studies to determine the influence these phase specific inhibitors have on other stages of viral development and the kinetic aspects of inhibition as measured by infectivity titrations.

## CHAPTER IV

## KINETIC ASPECTS OF INHIBITION BY THE THREE CHEMICAL SUBSTANCES OF THE PRODUCTIVE PHASE OF INFLUENZA VIRUS

In the previous sections of this study, the conditions under which AMPS, methoxinine and fluorophenylalanine would inhibit viral multiplication in vitro were determined. Moreover, the effect of the order of addition of the compounds upon their capacity to inhibit was investigated as well as their activity relative to the receptor-destroying enzyme. It was recognized that these phase-specific inhibitors were powerful tools for examining the productive period of viral development and a wide range of approaches was possible. However, it was considered that the most orderly approach would be to study first the kinetics of viral increase in those cases where partial inhibition resulted, or where growth followed periods of virostasis.

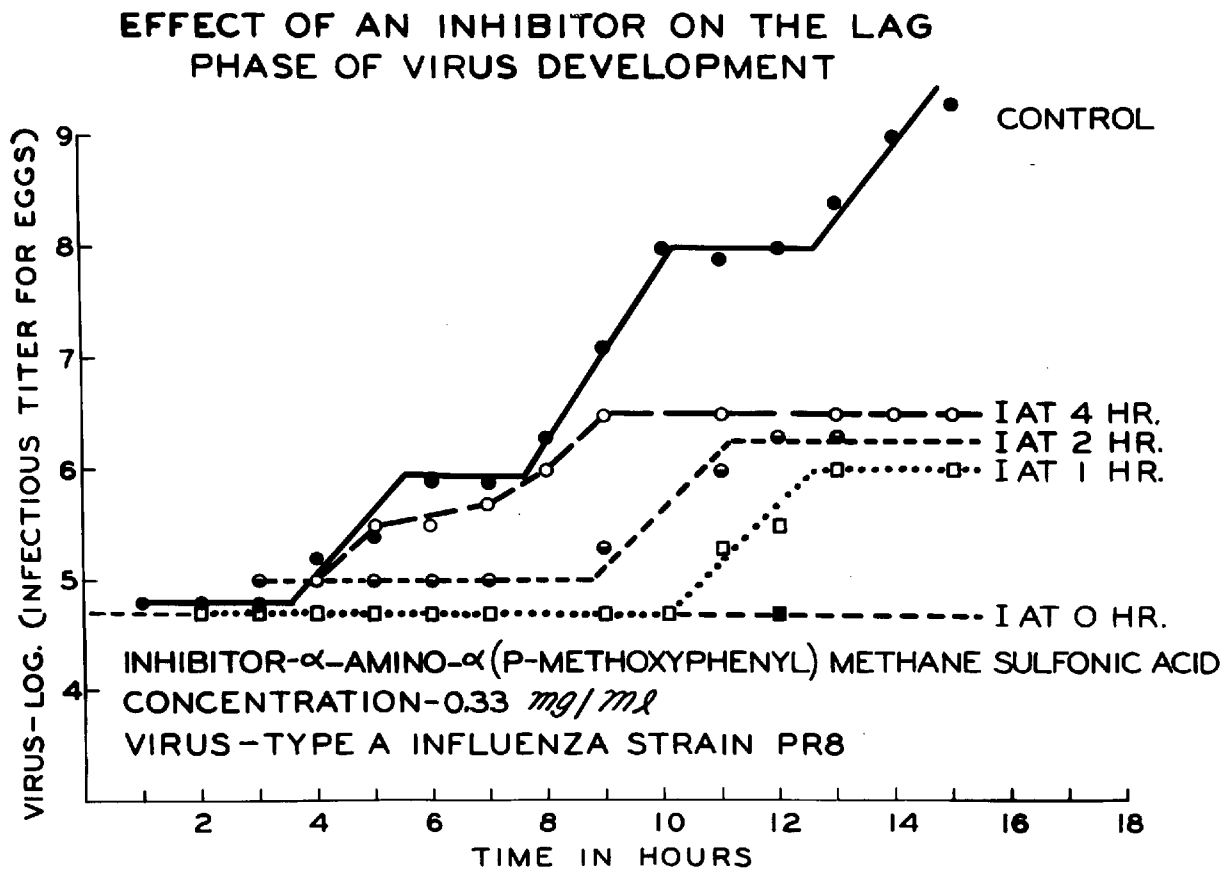
### A. The rate of viral synthesis in the case of partial inhibition produced by AMPS added progressively later during the infectious cycle.

An experiment was carried out in which the time of addition of AMPS to the infected membrane cultures was varied over a wide range from the zero hour to the 4th hour

after infection. This was done in order to determine how late after viral inoculation AMPS could be added and an inhibitory effect obtained (Table V). Addition of AMPS at zero hour produced complete inhibition of multiplication of the virus, but delayed addition at 1, 2, 3, and 4 hours respectively after infection resulted in viral increase. On the basis of the limited growth resulting when AMPS was added during the latent period, the kinetics of the growth cycle were studied upon the addition of the sulfonic acid to the culture at the 1st, 2nd and 4th hour after viral inoculation. In four separate experiments, flask cultures were set up to contain the tissue, the medium and small viral inoculum. In each experiment, two flasks were employed, the first acted as a control while to the second AMPS was added one hour or more after the addition of the virus. Samples of the fluids from each individual flask were taken at hourly intervals and titrated for infectivity in embryonate eggs. The normal growth curve is a composite of four curves in which the value of each point was averaged. Figure IV shows the results obtained.

Addition of AMPS at zero hour produced complete inhibition. However, the addition of AMPS during the latent period permits the multiplication of the virus in the first cycle to proceed, while the development of successive cycles is inhibited, which accounts for the partial growth of the virus obtained at the 12th hour period as

FIGURE IV




Separate culture flasks each had contained 400 mg. of chorio-allantoic membrane. Virus of a titer of $10^{-6.9}$ was added at zero time to each flask. AMPS of a concentration of 0.33 mg/ml was added to the flasks as indicated in the figure. Titers were obtained on the extracellular fluid. The control curve is a composite made from four similar experiments.

compared with the control culture. It will be recalled that a small virus inoculum produces several cycles of multiplication, and when AMPS is added after the sensitive period of the first cycle, it will be present at the beginning of the second cycle, hence producing inhibition. Thus the reduced yield of virus does not necessarily indicate that the inhibitor action is upon the synthetic phase of viral development, but may indicate that it is impairing the appearance of the infectious virus. The latent period was longer, ranging from 11 hours, for the one hour addition to nine hours for the second hour of addition of AMPS.

B. The influence of AMPS on the rate of production of intracellular virus.

Since the results (Fig. IV) represent the infectivity titer of the extracellular fluid, they may not parallel the synthesis of intracellular virus. Does the delay of liberation of the virus induced by AMPS represent a delay in the synthesis of intracellular virus? To determine whether AMPS influences the synthesis of intracellular virus, the development of the virus in the tissue was followed.

Two series of six culture flasks each were prepared which initially contained the tissue, the medium and a small virus inoculum. The first series of flasks received no additional supplement, while to the second series AMPS was added at the second hour to each individual flask. At

hourly intervals from the second to the eighth hour, the tissue from each series was ground with alundum, added to broth and titrated for infectivity. In this way the development of the cellular virus of both the experimental and control series was measured and compared. The results in Fig. V show there was no delay in the production of the cellular virus as compared with the control. The rates of viral synthesis in the infected tissue of both the normal culture and the one in which the inhibitor was added at the second hour are identical.

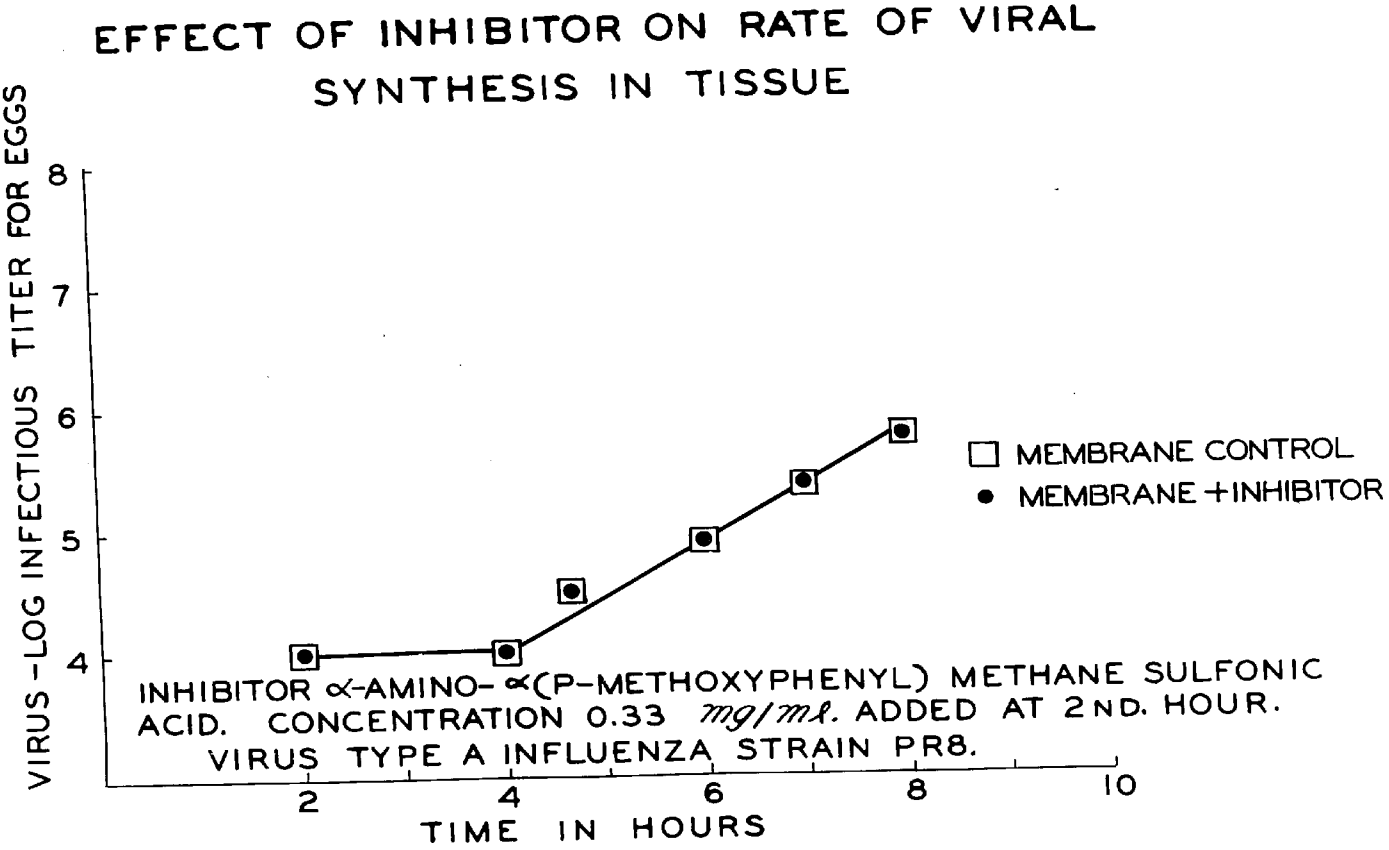
The results shown in Table V and Figures IV and V clearly demonstrate that the very early addition of AMPS prevents the initiation of infection, while later addition interferes with the liberation phase without affecting the synthesis of cellular virus.

C. The rate of viral increase which occurs following the addition of methoxinine at various times during the infectious sequence.

The data presented previously (Table V) showed that inhibition of influenza virus occurred when methoxinine was added up to two hours in the latent period. The compound caused inhibition to a degree which was inversely related to the time of addition. When methoxinine is added at the sixth hour, it could no longer innibit viral development which proceeded to a normal growth.

Attempts were next directed to the study of the effects of methoxinine upon the rate of increase of virus

FIGURE V




Twelve culture flasks were prepared to contain 400 mg. of chorio-allantoic membrane each. The inoculum was added at zero time to the medium with a titer of $10^{-6.0}$. Six flasks had AMPS of 0.33 mg/ml added at the second hour, and the other flasks had no supplement. At hourly intervals, two flasks were removed from one control and one experimental group. The tissues were ground separately with alundum and titrated for infectious virus.

which was incurred when the analogue was added at the zero, fourth and sixth hour of a single infectious sequence. Four culture flasks were set up to contain the tissue, the medium and a large viral inoculum; to one flask, methoxinine was added throughout the experimental period. After one hour incubation at 37°C, the tissue were washed and returned to fresh medium. At successive hourly intervals, samples of the fluids from the individual flasks were removed; however, two of the culture flasks were supplemented with methoxinine at the fourth and the sixth hours. The data in Table IX show a comparative growth of normal culture and of a culture to which the inhibitor methoxinine was added at the fourth and the sixth hour. Some inhibition is evident when methoxinine was added at the fourth hour after the addition of the virus, for the titer increased from $10^{-5.0}$ to $10^{-7.0}$ while that of the control culture and the culture receiving methoxinine at the sixth hour reached approximately $10^{-9.0}$. The partial growth shown when methoxinine was added at the fourth hour may represent the individuality of the infected cells of the chorio-allantois. At the time when the inhibitor was added some cells may have been in the stage when L-methionine was needed for viral synthesis, while others had passed that stage. This further illustrates that the infectious process proceeds not in a homogeneous and well syncrhonized manner, but subject to the individuality of the cells supporting viral multiplication.

## TABLE IX

### THE EFFECT OF METHOXININE ON THE GROWTH CYCLE OF INFLUENZA VIRUS[a] (PR8)

| Time of Samples in Hours | Infectivity Titer in Eggs[b] | | | |
|---|---|---|---|---|
| | Time of Methoxinine Addition | | | |
| | Control | 0 Hours | 4 Hours | 6 Hours |
| 2 | 2.3 | 4.0 | 3.0 | 3.3 |
| 3 | 3.7 | -- | -- | 4.0 |
| 4 | 4.3 | -- | 5.0 | 5.0 |
| 5 | 5.0 | -- | 5.0 | 5.7 |
| 6 | 6.0 | -- | 5.7 | 7.0 |
| 7 | 6.5 | -- | -- | 7.5 |
| 8 | 7.5 | -- | 6.0 | 8.0 |
| 9 | 8.5 | -- | 6.3 | 8.5 |
| 10 | 9.0 | -- | 6.7 | 9.0 |
| 11 | 9.3 | 4.0 | 7.0 | 9.0 |

a Massive inoculum of the order of $10^{9.3}$ $EID_{50}$ was used to infect the four culture flasks which contained each 400 mg of tissue. The residual virus was washed after one hour of incubation at 37°C.

b Titers of influenza virus in the extracellular fluids are expressed as the negative log of the $ID_{50}$ for eggs.

From the data it is apparent that the period of viral development in which methionine is utilized was completed before the appearance of the infectious virus. However, the stage which is sensitive to the inhibitor is poorly defined from that which is refractory. Referring back to Figure III in which the rate of viral increase was measured after a period of induced virostasis, the results illustrate the existence of a latent period of two hours comparable to the control culture, but the rate of viral increase was hastened. The existence of a latent period of two hours comparable to the control may be interpreted on the assumption that the difference in the latent periods of individual cells may be due to the difference in which the incipient stages of viral development are fulfilled. The action of the amino acid analogue seems to be on the earlier biosynthetic process of the virus. A degree of specificity of methoxinine seems certain; the antimetabolite interferes with a narrow segment in the metabolism of the infected cell. The data presented do not prove the exact site of the action of the amino acid analogue, but they do demonstrate that methionine is essential for the synthesis of the virus, and indicate when it functions relative to the appearance of new virus.

Thus, a specific intracellular stage in the development of the virus can be isolated.

D. The extent of viral increase which occurs after various periods of virostasis induced with FPA and terminated with phenylalanine.

The analogue of DL-phenylalanine has been found capable of a reversible blocking of the formation of influenza virus. The evidence obtained has indicated that the compound exerts an inhibitory effect after infection of the host tissue. Moreover, the inhibitory effect is reversible after a period in which viral multiplication was suppressed. In addition, it has been demonstrated that the action of fluorophenylalanine does not damage the entire mechanism of the cell to support viral production.

The results given in Table VII tend to illustrate that fluorophenylalanine (FPA) has inhibitory activity when added during the latent period. During most of the productive period, a partial inhibition of growth is obtained when FPA is added even six hours after addition of the virus. Hence, the relation of the inhibitory effect of FPA to the age of infection is different from that seen with methoxinine. There is apparently a later phase in the development of the virus which is affected by this inhibitor. The addition of fluorophenylalanine at the seventh hour could no longer influence the rate of viral synthesis which then proceeded to the same degree as the control culture. The requirements of the productive phase of influenza virus for phenylalanine were further investigated under conditions of

induced virostasis by FPA. Seven culture flasks were prepared to contain the tissue and the medium. The infection was initiated with a massive inoculum of virus to assure a single sequence of infection. Fluorophenylalanine was added initially to each culture. After one hour of incubation at 37°C, the tissues were removed, washed and returned to fresh medium with FPA. At 3, 7, 8, 10, 11, 12, and 24 hours, a sample of the fluid was removed from each individual flask and phenylalanine was added, respectively. Thus, by titrations of the fluid for virus, it was possible to determine the amount of virus present at one hour and at the time of addition of phenylalanine. At 12, 24, 36 and 45 hours, a sample of the fluid was again taken from each flask culture and titrated for infectivity in embryonate eggs. The data presented in Table X clearly exemplify the requirements of the developmental cycle of the virus for the amino acid phenylalanine. Complete reversal of inhibition had occurred in all the culture flasks to which phenylalanine was added, after varying periods of induced virostasis, and the growth of the virus in all instances was comparable to the control culture. It is, then, possible to demonstrate the ability of the infected cell to retain for one day, in a state of virostasis, its ability to produce mature virus. The evidence of inhibition of virus propagation by fluorophenylalanine and its complete reversal by phenylalanine recognizes the

TABLE X

THE BLOCKED INHIBITION[a] OF INFLUENZA VIRUS BY DL-PHENYLALANINE ADDED AT VARIOUS INTERVALS

| Hour[b] DL-Phenyl-alanine was added (0.133 mg/ml) | Viral Titers[c] | | | | | |
|---|---|---|---|---|---|---|
| | 1st Hour | When DL-Phenyl-alanine is added | 12th Hour | 24th Hour | 36th Hour | 45th Hour |
| 3 | 3.7 | 3.7 | 9.0 | 9.3 | -- | -- |
| 7 | 3.7 | 3.3 | 8.5 | 9.0 | -- | -- |
| 8[d] | 3.7 | 4.0 | 7.0 | 9.5 | -- | -- |
| 10[d] | 3.7 | 4.0 | 6.5 | 8.7 | -- | -- |
| 11 | 3.7 | 3.3 | 4.3 | 8.5 | -- | -- |
| 12[d] | 3.7 | 3.7 | -- | 8.5 | 9.0 | -- |
| 24[d] | 4.0 | 5.0 | -- | -- | 8.7 | 9.0 |
| none[d] | 3.7 | -- | 9.0 | 9.3 | 9.3 | -- |

a All culture flasks except the control flask had the inhibitor Fluorophenylalanine added at zero time.

b This is measured from original addition of tissue and virus to medium. After one hour, the tissue was washed to remove the residual inoculum.

c Titers of influenza virus in the extracellular fluids are expressed as the negative log of the $ID_{50}$ for eggs.

d These values are from a separate comparable experiment.

important fact of the selectivity of action of the compound in suppressing the part of viral synthetic mechanism involving phenylalanine without causing any measurable damage to other synthetic processes of the host-virus system.

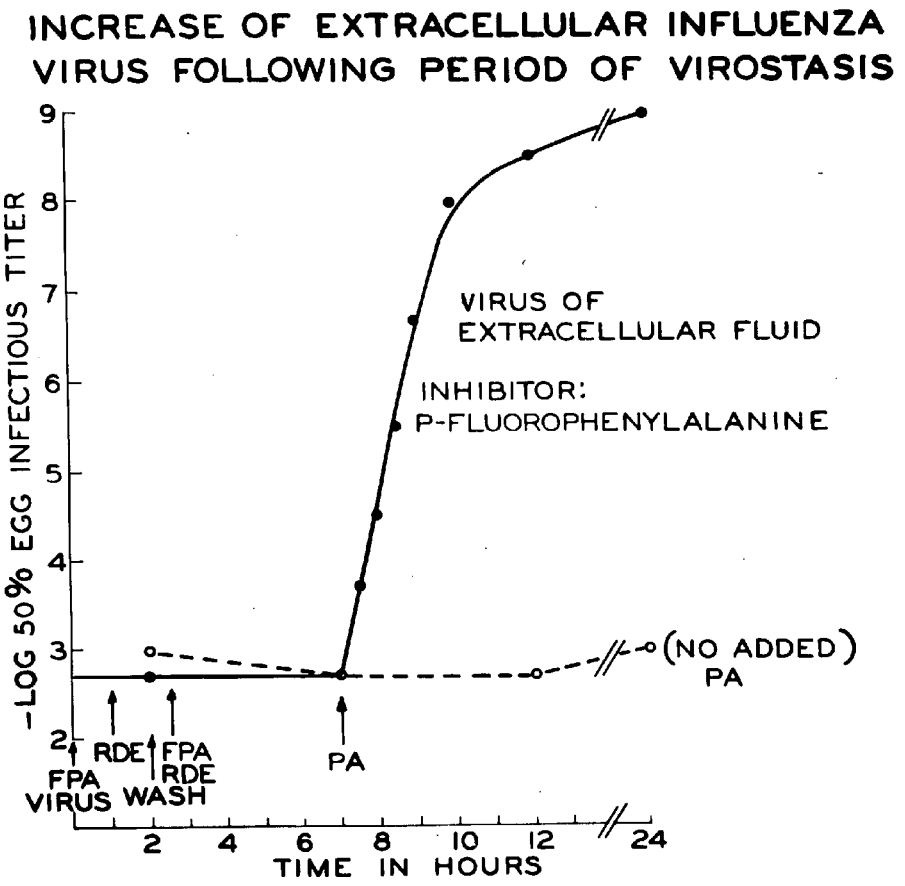
E. The rate of viral increase after a period of virostasis induced by FPA.

The role of phenylalanine in viral development was further analyzed by studying the kinetics of the viral increase which was shown to occur after prolonged periods of virostasis.

Two culture flasks were prepared which contained initially the virus, the tissue, medium and FPA. After one hour of incubation at 37°C, 0.2 ml of a preparation of the receptor-destroying enzyme was added. The tissues were incubated further for another hour, then removed, washed thoroughly and placed into fresh medium containing FPA and RDE. To one flask no further addition ensued, and samples of the fluids were taken at 2.3, 7, 12, and 24 hours, and titrated into eggs for infectivity. The second flask received phenylalanine (0.134 mg/ml) at the 7th hour after the addition of the virus. Samples for virus assay were taken from the fluid at the 2.3 and 7th hours as well as at half hour intervals after the seventh hour.

From the data shown in Figure VI, it is apparant that within thirty minutes from reversing the inhibitor, viral production starts. The rate of viral increase is

FIGURE VI




A massive virus inoculum was used ($10^{-9.5}$) to infect 400 mg. of tissue. Fluorophenylalanine (0.1 mg/ml) was added with the virus. The tissue was washed from the residual virus after one hour of incubation at 37°C. The tissue was placed in fresh medium containing FPA and incubated further. Phenylalanine (0.134 mg/ml) was added at the 7th hour. Samples were taken periodically.

rapid; the maximum titer is reached in about three hours, whereas in the normal growth curve it occurs at nine hours.

F. Comparison of the rates of viral increase after virostasis periods induced by methoxinine and FPA.

In a second single experiment of similar design, both FPA and methoxinine were used to induce a virostatic period of seven hours in separate cultures, the object being to verify the data upon which the existence of two separate stages in the developmental cycle of the virus were postulated.

The data of such an experiment are summarized in Table XI. The results tabulated correlate with the previous individual experiments (Fig. III and Fig. VI) and tend to show that methoxinine and fluorophenylalanine (FPA) act on separate stages in the infectious cycle of the virus. When the tissue was treated with methoxinine for seven hours, an early biosynthetic process in the development of the virus was suppressed and apparently other reactions of the latent period do not proceed during the time of virostasis, hence, it took two hours of latency for virus to appear as in the normal growth of the virus. However, when the infected tissue was exposed to FPA for seven hours and phenylalanine added subsequently, the virus was in an advanced stage of development and no latency in viral production was evident.

## TABLE XI

### VIRAL KINETICS OF EXTRACELLULAR INFLUENZA VIRUS FOLLOWING A PERIOD OF VIROSTASIS

| Time of Samples in Hours | Viral Titers | | | | |
|---|---|---|---|---|---|
| | I[a] | II[b] | III[c] | IV[d] | V[e] |
| 2 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| 4 | 4.0 | -- | -- | -- | -- |
| 5 | 4.7 | -- | -- | -- | -- |
| 7 | 6.3 | -- | -- | 2.7 | 2.5 |
| 8 | -- | -- | -- | 3.5 | 2.3 |
| 9 | 7.3 | -- | -- | 4.7 | 2.5 |
| 10 | -- | -- | -- | 6.7 | 4.7 |
| 11 | -- | -- | -- | 8.0 | 6.5 |
| 12 | 8.7 | 2.7 | 3.3 | 8.5 | 8.0 |
| 24 | 9.0 | 3.0 | 3.5 | 8.7 | 9.0 |

Each flask contained 400 mg of tissue. An undiluted seed virus of titer $10^{-9.7}$ was added (0.3 cc) to each flask at zero time. At one hour the infected tissues were washed accordingly and returned to new culture flasks with the identical composition.

a Control flask, no supplement added.
b Tissue was exposed to fluorophenylalanine (0.1 mg/ml) throughout the experimental period.
c The infected tissue was exposed to methoxinine (1.3 mg/ml) throughout the experimental.period.
d The infected tissue was treated with fluorophenylalanine for seven hours and phenylalanine (0.133 mg/ml) added subsequently.
e The infected tissue was treated with methoxinine for seven hours. The tissue underwent a second washing to remove the innibitor and returned to new culture flask with no supplement.

The viral titers of the extracellular fluids are expressed as the log of the reciprocal of the dilution.

G. The lack of intracellular virus production during prolonged virostasis induced by FPA.

The appearance of the extracellular virus in the above case occurred within 30 minutes of the addition of phenylalanine. Could this have been preceeded by a corresponding increase in the production of the intracellular virus? The following experiment was designed to answer such a question.

A series of culture flasks were prepared to contain the tissue, a large inoculum of the virus, Simm's solution and FPA. After one hour of incubation at 37°C, the tissues were removed, washed and returned to new culture flasks with fresh medium and the antimetabolite. At the seventh hour, all cultures received phenylalanine to allow viral development to proceed. At half hour intervals thereafter both the fluid and the membrane of an individual flask were removed and titrated separately in embryonate eggs. In this manner, it was found possible to determine and to correlate periodically the amount of viral increase in the medium and the tissue after a period of induced virostasis.

The data in Table XII show that when the infected tissue was exposed to FPA for seven hours, no viral increase resulted in the infected tissue as well as in the fluid bathing it. This suggests further that if there is accumulation of viral material during the virostatic period, it must represent some type of non-infective viral precursor. This is also justified by the extremely rapid rate

TABLE XII

THE EFFECT OF INDUCED VIROSTASIS WITH FLUOROPHENYLALANINE ON GROWTH OF INFLUENZA VIRUS

| Experiment No. | Viral Titers[d] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs | 7 hrs | 7.5 hrs | 8.0 hrs | 8.5 hrs | 9.0 hrs | 10 hrs | 11 hrs | 12 hrs | 24 hrs |
| I[a] | 3.3[b] | 3.3 | 4.0 | 4.5 | 5.5 | 6.7 | 8.0 | 8.7 | 9.0 | 9.0 |
| | 4.5[c] | 4.3 | -- | -- | -- | -- | -- | -- | -- | -- |
| II[a] | 3.0[b] | 3.0 | 3.5 | 4.7 | 5.7 | 6.5 | -- | -- | 8.7 | -- |
| | 4.7[c] | 4.5 | 5.0 | 5.5 | 6.0 | 6.7 | -- | -- | 9.0 | -- |

a Separate comparable experiments. Individual flasks were set up to contain 500 mg of tissue. The viral inoculum was 0.3 ml of undiluted allantoic fluid of titer $10^{-9.7}$. Fluorophenylalanine (0.2 mg/ml) was added to each flask at zero time. The tissues were washed three times in saline, once in diluted immune serum (1:400) and three times further in saline. The tissues after washing were returned to new culture flasks with fluorophenylalanine. At the 7th hour all flasks had phenylalanine (0.133 mg/ml) and comparable titrations of the virus were done on the fluid and the tissue at intervals.

b The values in this line represent the extracellular fluid titer.

c The values in this line represent the titer of the tissue.

d Titers of influenza virus expressed as negative log of the 50 per cent infectious does for eggs.

of production of the virus indicating that the rate-determining reaction in the viral biosynthetic process is different from that which operates normally.

A specific intracellular phase in the developmental cycle of the virus seems to exist which is subject to the influence of FPA. This phase seems to begin later in the infectious sequence than that inhibited by methoxinine since the latent period is completed in the presence of FPA. This is also suggested less clearly by the data in Tables V and VI.

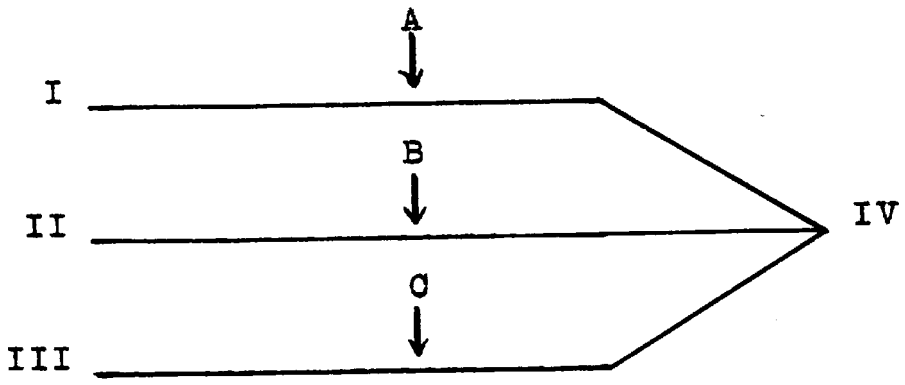
# CHAPTER V

## INTERRELATIONS OF THE DEVELOPMENTAL STAGES OF INFLUENZA VIRUS

The analysis of viral development by the use of metabolic antagonists was extended. The previous approaches have involved the study of the effects of the three chemical inhibitors relative to the action of the receptor-destroying enzyme (Table VIII). Experiments were also carried out in which the relation of inhibitory capacity of these antagonists to the age of infection was followed (Tables V, VI and VII). Furthermore, the study of viral kinetics following a period of virostasis induced by methoxinine or FPA was very suggestive as to the order in which the reactions involving methionine and phenylalanine function in the synthesis of the virus (Figures III and IV, Table XI).

However, on the basis of the data available, one cannot define the limits in which the sensitive phases in the development of virus occur. It is difficult to know the order in which reactions proceed in a single sequence of infection, or learn the interdependence of these reactions. These points will be considered by a different

type of analysis. The following diagram is useful in illustrating the technique to be used in determining whether three reactions, I, II, and III, acted upon by inhibitors A, B, and C, respectively, proceed in a dependent sequence.




For example, to determine if reactions II→IV and III→IV proceed when reaction I→IV is blocked by inhibitor A, one allows the virus and cell to react for a prolonged period in the presence of A. Then after removing the blocking effect of A, the capacity of inhibitors C and B to act upon that culture and to prevent the formation of virus can be determined. If reactions II and III have been completed, blocking them with C or B should not inhibit the appearance of new virus. However, if reaction II or III occurs in a sequence after I, then they may not be completed when reaction I is blocked. In this case, inhibitors B or C may still be effective when used after A.

It is the purpose of the forthcoming chapter to determine whether the incipient stages of viral development

which can be specifically blocked by AMPS, proceed in the presence of methoxinine; hence, the tissue was infected in the presence of methoxinine and, subsequently, incubated with AMPS only. It will also be determined in a single sequence of infection, whether upon treating the infected culture with methoxinine, the reaction involving FPA proceeds independently, or if the reaction sensitive to methoxinine proceeds in the presence of FPA. Hence, the virus development was followed in cultures using the phase specificity of methoxinine or FPA as a marker to describe the relative site of action of either analog.

It was hoped that this combination of various techniques would permit one not only to recognize the various phases in the development of the virus which are sensitive to the three inhibitors, but also to isolate and relate these phases in terms of the metabolic activities of the host cell.

A. The sequential action of AMPS and methoxinine.

The previous sections of this study have defined the site of action of metnoxinine and AMPS relative to the effects produced by the receptor-destroying enzyme. On the basis of those data one would predict tnat the incipient stages are completed in the p resence of metnoxinine and tnat tne process inhibited by metnoxinine is not completed in tne presence of AMPS. Tnis was confirmed by tne sequential type of analysis.

Two series of three flask cultures each were prepared to contain the tissue, medium and the virus. In addition, the cultures of the first series were treated with methoxinine and those of the second series with AMPS. After two hours of incubation at 37°C, the tissues were removed, washed extensively in physiological saline and homologous immune serum. Thereafter, one membrane from each series was placed into fresh medium with no supplement; the second membrane from each series was treated with methoxinine and the third membrane from each series was placed in a medium supplemented with AMPS. The cultures were incubated further and samples of the fluid portions were taken from each flask at the 2.3, 12 and 24th hours respectively (all time is measured from the addition of the virus) and assayed for the infectious form of the virus in embryonate eggs.

The data in Table XIII show that when the infected tissue was exposed to either AMPS or methoxinine throughout the incubation period, no increase in yield of the infectious virus ensues. It is evident that the concentration of the inhibitor used was sufficient to suppress viral production. The other cultures which were incubated after exposure to the virus with a medium containing no supplement showed viral production by the 12th hour, signifying that treatment of the culture did not produce any irreparable effect on the biosynthesis of the

TABLE XIII

SEQUENTIAL ACTION OF AMPS AND METHOXININE

| Flask No. | Pre-Washing Medium[a] | Post-Washing Medium | Viral Titers[b] | | |
|---|---|---|---|---|---|
| | | | 2.3 hrs | 12 hrs | 24 hrs |
| I | Methoxinine | No Supplement | 0.7 | 4.5 | 6.0 |
| II | Methoxinine | Methoxinine | 1.0 | 1.3 | 1.0 |
| III | Methoxinine | AMPS | 0.7 | 4.7 | 5.0 |
| IV | AMPS | No Supplement | 1.3 | 3.9 | 4.3 |
| V | AMPS | Methoxinine | 1.0 | 0.7 | 1.0 |
| VI | AMPS | AMPS | 1.7 | 2.0 | 1.5 |

a Each tissue was incubated with virus (titer $10^{-4}$) and treated with methoxinine or AMPS as mentioned above. After 2 hours of incubation at $37^{\circ}C$, each tissue was washed in saline six times and one time in homologous immune serum before further incubation in fresh medium which contained no supplement, methoxinine or AMPS respectively.

b Viral titers are expressed as the negative log of the 50 per cent infectious titer for eggs and represent the virus titer of the extracellular fluids.

virus. It should also be noted that the infected tissue which was exposed to methoxinine and incubated subsequently with AMPS produced an increase in virus titer by the 12th hour, however, a reverse treatment of the infected tissue with these two inhibitors produced no viral growth. In the presence of metnoxinine, the initial stages of viral development which are sensitive to the action of AMPS are completed, while in the presence of AMPS, it is likely that the stage of infection in which the receptor functions was not completed. Presumably, the phases of development of influenza virus which are sensitive to AMPS or methoxinine occur in an obligate sequence rather than independently and parallel with each other. This seems most logical if one assumes that the methoxinine acts upon an intracellular phase and AMPS prevents the achievement of the intracellular stage. Hence, it is justifiable to conclude, from the study of the inhibitory effect of these two inhibitors relative to the age of infection, from their action relative to RDE and from their sequential action, that three distinct phases could be differentiated in the infectious cycle of the virus: an initial phase which is sensitive to AMPS, insensitive to methoxinine and probably involving the function of the receptor site material of the host cell, a second phase, which is sensitive to methoxinine and insensitive to AMPS, followed by a phase which is insensitive to either inhibitor, and in which mature virus is appearing.

B. The sequential action of methoxinine and FPA.

It is not remarkable to find that AMPS and methoxinine sensitive reactions occur consecutively, as the former interfers with the initiation of infection, while the latter inhibits an intracellular process, but the order in which two analogs act upon intracellular processes is of greater interest.

When the influence of the age of infection upon the inhibitory efficiencies of methoxinine and FPA were studied, there was some indication that methoxinine became less effective at a greater rate than FPA. It suggested that these two structural analogs act on separate phases of development of the virus. The later kinetic studies also confirmed this conclusion. It is the purpose of the forthcoming studies to elaborate on this point using sequential analysis, so that these two stages can be clearly defined in relation to each other.

A group of five flask cultures were prepared to contain the tissue, the virus and Simm's solution. One culture received no supplement, the second and third cultures were treated with methoxinine and FPA, the fourth culture was supplemented with FPA, while the fifth culture received methoxinine. After one hour of incubation at 37°C, the tissues were removed, washed from the excess virus and returned to five culture flasks containing fresh media of the same composition as that in which the tissues were

infected. At the 7th hour, the tissue which had been exposed to virus in the presence of methoxinine, was washed and returned to a new culture vessel with FPA. The culture in which the tissue had been exposed to the virus and FPA received methoxinine and phenylalanine. One membrane was left exposed to both inhibitors throughout the incubation period. The other membrane which received both inhibitors for seven hours, was removed, washed and returned to a new culture vessel with normal fresh Simm's solution. Samples of the fluid portion were taken periodically and titrated for virus.

The results given in Table XIV demonstrate that the concentrations used of both inhibitors were sufficient to suppress the multiplication of the virus during the 24 hour period of incubation. The culture receiving FPA and methoxinine for only 7 hours produced a viral yield comparable to a control untreated culture by the 24th hour period, hence, no irreparable damage to the synthetic machinery of the host cell resulted from a prolonged incubation of the infected culture with the two analogs. It appears also that an initial treatment of the infected tissue with methoxinine, followed by treatment with fluorophenylalanine (FPA) produced no increase in mature virus, while if the infected culture was treated with FPA first, then incubated with methoxinine, an increase in viral yield was evident by the 12th hour.

TABLE XIV

SEQUENTIAL ACTION OF METHOXININE AND FLUOROPHENYLALANINE

| Flask No. | Pre-Treatment[a] Medium | Post-Treatment[b] Medium | Viral Titers[c] | | | | |
|---|---|---|---|---|---|---|---|
| | | | 2 hrs | 7 hrs | 12 hrs | 20 hrs | 31 hrs |
| I | No Supplement | No Supplement | 3.5 | 7.0 | 8.5 | 9.0 | 9.0 |
| II | Fluorophenyl-alanine + Methoxinine | Fluorophenyl-alanine + Methoxinine | 3.5 | 3.3 | 3.0 | 3.3 | 4.0 |
| III | Fluorophenyl-alanine + Methoxinine | No Supplement | 3.5 | 3.5 | 7.3 | 8.5 | 9.0 |
| IV | Fluorophenyl-alanine | Phenylalanine + Methoxinine | 3.5 | 3.3 | 4.3 | 5.5 | 6.7 |
| V | Methoxinine | Fluorophenyl alanine | 3.5 | 3.3 | 3.5 | 3.0 | 3.3 |

a Each tissue was treated 1 hour with virus and the indicated inhibitors; after one hour of adsorption of the virus the tissue was washed three times in saline, one time in immune serum (1:400), and three times in saline. The washed tissues were returned to culture flasks with identical composition and incubated further for six hours.

b At the seventh hour, a new treatment of the infected tissues was followed as indicated.

c The viral titers are expressed as the negative log of the 50 per cent infectious titer for eggs and represent the titer of the extracellular fluid.

However, the limited growth of the virus in the latter case, as compared to the control, implies that some interdependence exists between these two reactions. Hence, an experiment was designed to define the significance of this limited growth by following the kinetics of virus production.

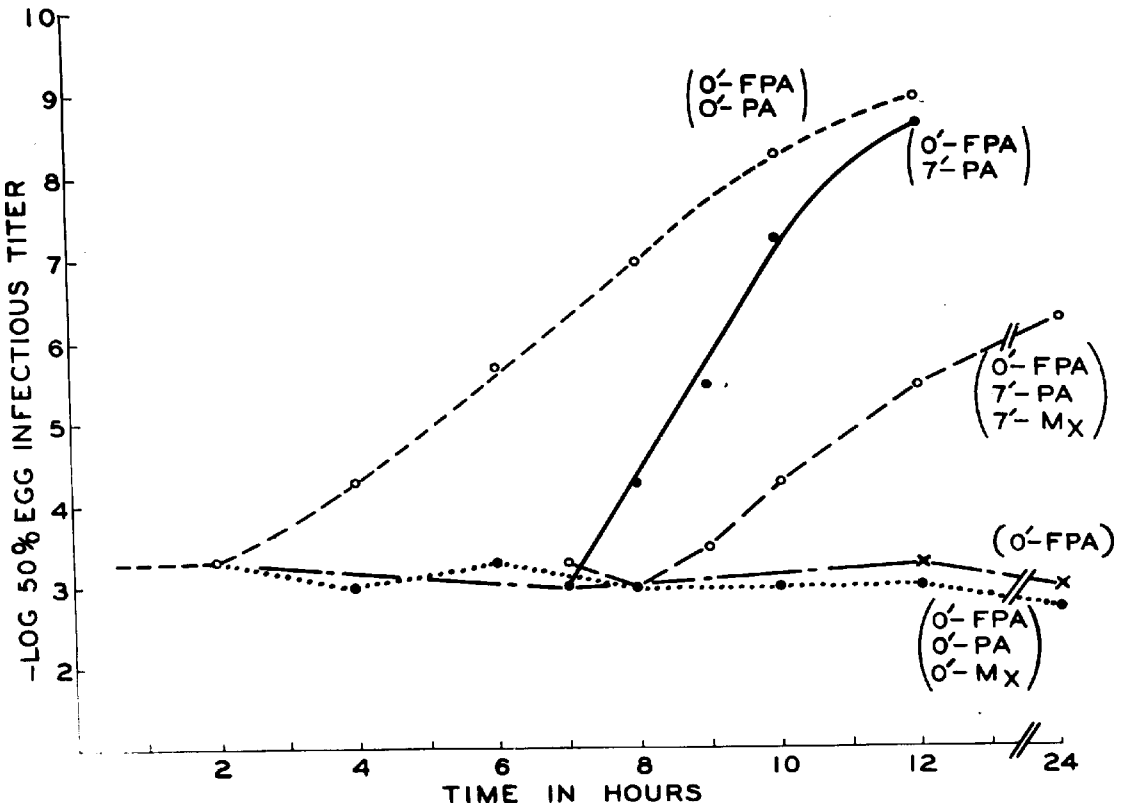
C. The rate of viral increase which results after sequential blocking.

Five culture flasks were set up to contain the tissue, the medium, and a massive viral inoculum and 0.1 mg of FPA. In addition, one of the cultures received 0.134 mg of phenylalanine, while a second membrane culture was given phenylalanine and methoxinine. After one hour of incubation at 37°C, the tissues were removed, washed and returned to new Warburg vessels, containing fresh medium of the same composition as that in which the tissues were infected. At the 7th hour, one of the two initially receiving only FPA received phenylalanine, while the other culture was given phenylalanine and methoxinine. Samples of the fluid portion were taken at short intervals from the 2nd to the 12th hour and titrated for virus in embryonate eggs. The results are illustrated in Fig. VII.

It is again clear from the control cultures that the concentration of FPA used was sufficient to give complete suppression of viral multiplication, since the culture receiving FPA throughout the incubation period showed no increase in virus by the 24 hour period. Also

FIGURE VII

THE RATE OF VIRUS PRODUCTION AFTER SEQUENTIAL BLOCKING BY METHOXININE AND FLUOROPHENYLALANINE



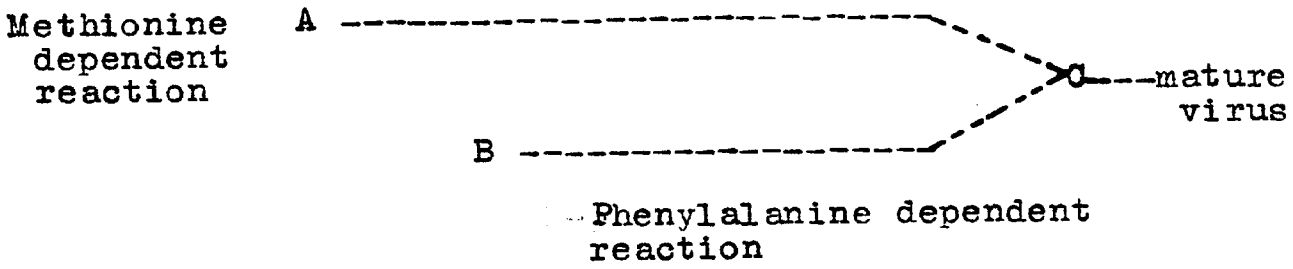

Massive virus inoculum was used to infect 400 mg of chorio-allantoic membrane. The concentration of FPA employed in each flask was 0.1 mg/ml. The concentrations of methoxinine used was 1.34 mg/ml and of phenylalanine was 0.134 mg/ml. After one hour of incubation at 37°C, the tissues were washed from the residual virus and returned to new culture vessels with fresh medium. The details of the experiment are described in the text.

the concentration of the metabolite, phenylalanine, used was sufficient to give complete reversal of the effect of FPA, since the culture receiving both of the compounds at zero hour showed viral production paralleling a control untreated infected culture. The culture to which phenylalanine (PA) was added at the 7th hour showed viral production similar to the culture to which PA was added at zero time, however, the rate of viral increase was rapid and the maximum titer was reached approximately at the same time.

The fourth culture to which FPA and PA were added at zero time, hence capable to supporting normal growth of the virus, received in addition, methoxinine. Since no viral increased ensued, the concentration of methoxinine used at zero time was inhibitory. However, in the fifth culture which was treated with FPA for seven hours, and then phenylalanine (PA) and methoxinine added, a limited growth of influenza virus was evident. Methoxinine was not very effective in its inhibitory capacity when added after a prolonged virostatic period induced by FPA. A one-step growth curve resulted with a latent period and sustained release over several hours, but the final titer of the virus by the 24th hour is lower as compared to a control untreated culture. The data seem to further support the view that some activities dependent on the function of methionine have occurred during the period of virostasis induced by fluoro-phenylalanine. The limited growth in this instance and the

nature of the growth curve might signify that some interrelationship does exist between the FPA and methoxinine sensitive phases, since methoxinine still exhibited some inhibitory action when added after FPA.

The following diagram illustrates the assumption advanced that the actions of methoxinine and FPA are upon separate stages of viral development which occur simultaneously during the latent and productive phase:




Reaction A is methionine dependent, proceeds during the latent period, and is not under the influence of reaction B. Reaction B is inhibited by fluorophenylalanine and occurs later than reaction A; it cannot proceed unless reaction A is occurring, or has operated for some time. The sequential addition of these two inhibitors has demonstrated that if reaction A is suppressed for a period of time and then allowed to proceed in the presence of the analog of phenylalanine, no mature virus appears at the 24th hour. If reaction B is suppressed for a definite period of time, reaction A will proceed regardless, and any subsequent addition of methoxinine will not affect the ability of the developing virus particle to reach reaction C, and, hence,

the appearance of mature virus ensues. However, the limited growth of the virus observed, as compared to the control flasks, indicates probably some secondary effect of reaction B upon A.

It seems logical then to suggest that there are two different dependencies in virus development which are clearly discernible on the basis of their sensitivity to the action of methoxinine and FPA. The data obtained from the various approaches presented imply that in the presence of FPA or methoxinine, infection can be initiated, the function of the receptors sensitive to AMPS or RDE proceed and further the two inhibitors act on the productive phase of the multiplication cycle. In addition, two distinct developmental stages of influenza were recognized which for the most part occur simultaneously during the productive phase of the virus, and which appear to exhibit some degree of interdependence.

# CHAPTER VI

## LIBERATION-INHIBITION PHENOMENON: INDEPENDENCE OF VIRAL MATURATION AND THE RELEASE OF VIRUS FROM THE CELL

In an earlier section of this study, the conclusion was reached that the essential functions of methionine and fluorophenylalanine are not related to the incipient stages of infection, but act during the productive phase of the infectious cycle of the virus. AMPS, on the other hand, seems to interfere with the initiation of infection; added between zero and fifteen minutes of the time of addition of the virus, it will suppress viral multiplication. However, when added to the culture at one or two hours after the virus, it prolonged the constant period of the growth curve (Fig. IV) without affecting the rate of synthesis of virus (Fig. V). This effect upon the appearance of extracellular virus was further investigated.

### A. The impairment of viral release by AMPS.

The measurement of the development of the virus was followed in the tissue and in the corresponding fluid of replicate infected cultures to determine whether AMPS extends the intracellular latent period or merely prevents

the release of the virus from the cells.

A group of culture flasks were prepared to contain each 400 mg. of tissue, a small viral inoculum and the Simm's solution. After two hours of incubation at 37°C in the respirometer, AMPS was added to all the cultures. For each hourly interval from the second to the tenth hour, one culture was used to obtain the yields of the virus separately in the extracellular fluid and in the tissue.

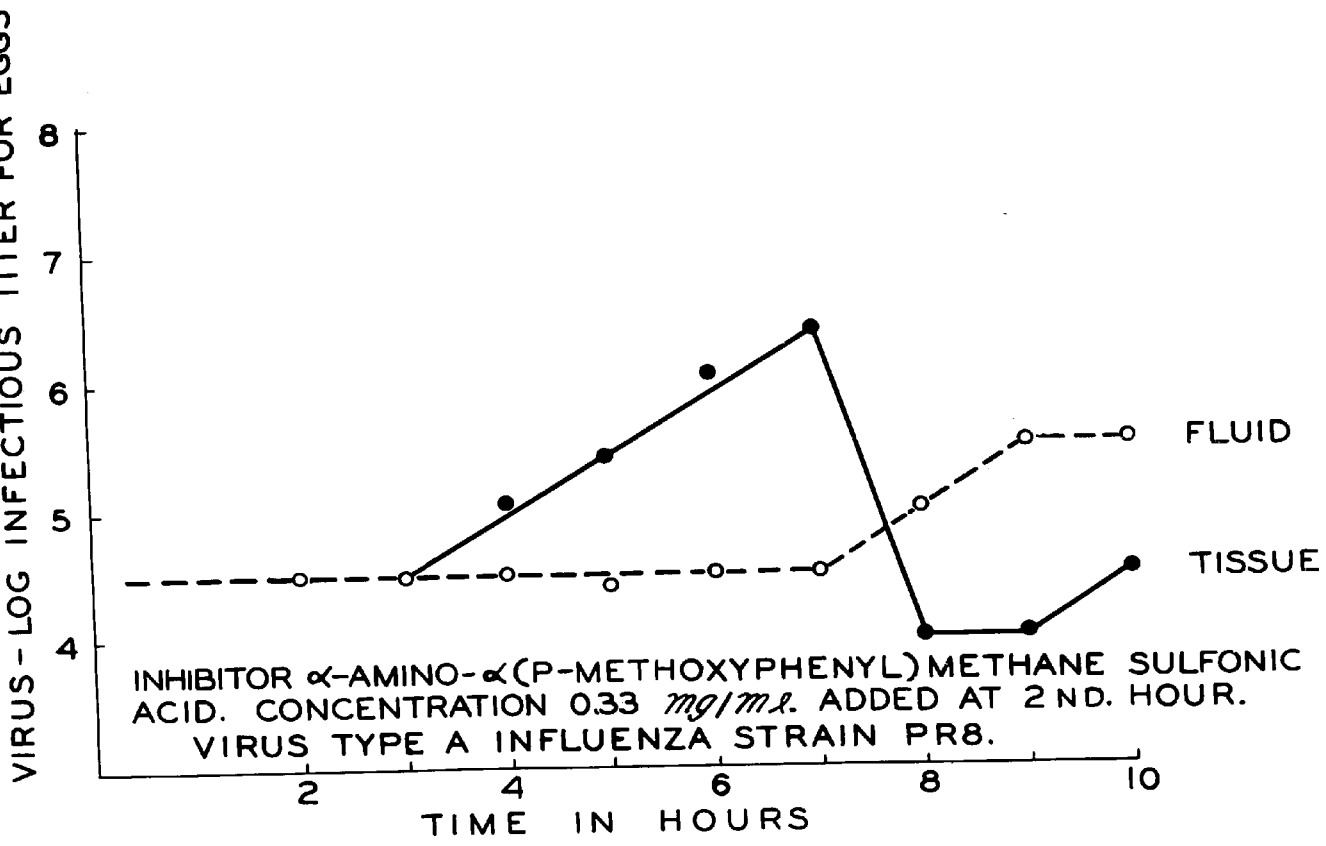
The corresponding data are presented in Fig. VIII. It can be seen that the constant period of the extracellular phase was seven hours followed by a viral increase (from $10^{-4.5}$ to $10^{-5.7}$) which occurred between the seventh and the eleventh hour. In contrast, the viral increase of the cells began at the 4th hour and by the 7th hour it had increased from $10^{-4.5}$ to $10^{-6.3}$. When the titer of the fluid began to rise, there was a corresponding drop in the titer of the cellular virus. It is apparent that the release of the virus from the tissue is impaired by AMPS, and the prolonged constant period of the fluid phase is not paralleled by a corresponding delay in the synthesis of cellular virus.

B. The action of the receptor-destroying enzyme on the liberation-inhibition.

The initiation of infection and the release of the virus from the cell are both influenced by the action of AMPS. This implies that the two processes share some common

FIGURE VIII

THE IMPAIRMENT OF RELEASE OF INFLUENZA VIRUS BY AMPS




Each culture contained 400 mg of tissue. The inoculum was added at zero time and consisted of 0.3 ml of medium with a titer of $10^{-5.7}$. AMPS of the concentraction of 0.33 mg/ml was added to the experimental flasks at the second hour. One culture was used to obtain data for the points at each time interval. The tissues were ground separately with alundum, and the titers obtained represent the infectious titer expressed per gram of tissue. The titers of the extracellular fluids are recorded on a per milliliter basis.

mechanism, both perhaps involving reactions at the receptor site. This assumption was tested in the following experiment:

Two culture flasks were set up to contain the tissue, the medium and the virus. After one hour of incubation at 37°C, the tissues were removed, washed extensively and incubated further with fresh medium. One culture received AMPS while to the second both AMPS and RDE were added. The appearance of the virus in the extracellular fluid was followed at intervals. At the 11th hour, both tissues were ground separately with alundum, combined with the corresponding medium and titrated in eggs ao that the total yield of the virus could be estimated.

The results in Table XV show that by adding AMPS and RDE together after infection, the release of the virus occurred earlier than when AMPS was added alone. The release of the virus in the latter culture occurred between the 6th and 8th hour period, while liberation of the virus from the former culture began at least by the 6th hour. The total yield of virus by the 11th hour was similar in both cultures. However, the amount of fluid virus by the 11th hour was greater in the culture treated with RDE. The receptor-destroying enzyme seems to antagonize the action of AMPS by facilitating release of virus from the tissue. The release of virus seems to be an active process mediated through the eventual destruction of the receptor-site material of the host cell. The results give evidence that loss of

TABLE XV

ANTAGONISTIC EFFECT OF RDE AND AMPS

ON VIRAL LIBERATION

| Time in Hours | Viral Titers[a] | |
|---|---|---|
| | AMPS[b] | AMPS + RDE[b] |
| 2 | 3.5 | 3.7 |
| 6 | 3.5 | 5.0 |
| 8 | 4.0 | 5.7 |
| 11 | 5.0 | 7.0 |
| 11 | 7.0[c] | 7.5[c] |

a Titers of influenza virus found in the extracellular fluid are expressed as the negative log of the $ID_{50}$ for eggs.

b Additions of AMPS (0.6 mg/ml) and RDE (titer of 1:1224) were made one hour after addition of influenza virus to the chorio-allantoic membrane.

c These values are for the total virus found in both the tissues and fluid.

inhibitory effect of AMPS upon release after a certain period of time might be related to the effects produced by the massive amount of enzymic activity which must accompany the accumulation of virus in the cell. One important function of the enzymic activity of virus must be in achieving liberation of virus from the cell.

C. Evidence for the attachment of virus to cells in the presence of AMPS.

The question now arises as to what is the actual function of AMPS? On the basis of the data available, it seems logical to assume that the action of AMPS is upon the interaction of the virus with its host; it impairs two steps in the development cycle of the virus - the initiation of infection and the release upon maturation. The observations also point to the fact that AMPS does not permit the reaction of virus and host-cell to proceed to the irreversible attachment where the virus can no longer be removed by washing or the action of RDE. However, in the presence of AMPS, viral attachment seems to occur since upon removal of the tissue from the culture media after exposure to virus in the presence of AMPS viral production ensues without further opportunity for infection. This is suggested by the following experiment:

Three culture flasks were prepared to contain the tissue, the medium and a small viral inoculum. One flask had no supplement, while the other two received AMPS. After

two hours of incubation at 37°C, the tissues were washed extensively in saline containing AMPS to remove excess virus and then in saline to remove AMPS. Thereafter, two pieces of tissue, one infected in the presence and one in the absence of AMPS were placed into separate flask cultures containing fresh medium with no supplement, while the third piece of membrane was returned to fresh Simm's solution with AMPS and incubated further. At 3, 5, 8, 10 and 12 hours, samples of the fluid portion were taken from each individual flask and assayed for the infectious form of influenza virus in embryonate eggs.

Table XVI shows that if the inhibitor is removed by washing after infection, the cells proceed to produce virus as in the control culture flask; however, the amount of virus in the fluid was greater in the control flask. This result demonstrates that when AMPS is removed, the virus, which was prevented from infecting the cell but was attached to the cell, penetrates the cell and initiates infection. In the presence of AMPS, attachment of virus occurs, but the process does not proceed to the point where the function of the receptor is completed.

The independence of viral maturation from the process of viral release is established on the basis of the differential sensitivity of the processes of AMPS. This is of interest in view of the fact that the maturation and liberation phases operate concurrently.

TABLE XVI

FAILURE OF AMPS TO PREVENT THE ATTACHMENT OF VIRUS TO CELLS

| Flask No. | Pre-Washing[a] Medium | Post-Washing[b] Medium | Viral Titers[c] | | | | |
|---|---|---|---|---|---|---|---|
| | | | 3 hrs | 5 hrs | 8 hrs | 10 hrs | 12 hrs |
| I | Virus | No Supplement | 1.0 | 3.5 | 6.0 | 7.0 | 7.5 |
| II | Virus + AMPS | No Supplement | 1.0 | 3.3 | 5.5 | 6.0 | 6.3 |
| III | Virus + AMPS | AMPS | 1.0 | -- | -- | -- | 1.3 |

a Each flask was set up to contain 400 mg of tissue. At zero time the three culture flasks had virus of a titer of $10^{-6.7}$. The concentration of AMPS added was 0.3 mg/ml. After two hours, the tissues were washed six times with saline containing AMPS and one time with diluted immune serum (titer 1:400).

b The tissues after washing were returned to new culture flasks. Two culture flasks had no supplement, but the third had AMPS (0.3 mg/ml).

c Titers of virus in the extracellular fluid expressed as the negative log of the 50 per cent infectious dose for eggs.

## SUMMARY AND CONCLUSIONS

The work which has been presented in this investigation deals with the growth characteristics of influenza virus and with the approach used to probe the biochemical basis of viral infection at the cellular level.

1. The cultivation technique has been improved so that a single sequence of infection can be produced routinely.

2. The pattern of growth of virus which occurs during a single sequence of infection was determined.

3. The maturation and release of virus in culture was found to occur over a period of time; no burst phenomenon was evident.

4. The responsible factors producing this pattern of viral development were analyzed and shown in part to be due to the differences in the latent period of individual cells. A technique was developed for synchronization of the infectious process, whereby individual differences in cellular response could be reduced.

5. A number of discrete stages of viral development have been isolated by the use of specific chemical blocking agents which were shown to be also phase-specific.

6. Some information has been obtained about the interrelationships of these stages. Because of the inhibitor technique used to isolate these phases, some biochemical characterization of the stages has become apparent.

An early phase in the development of the virus was found to be sensitive to AMPS, and it is by virtue of this that virus multiplication is prevented. This early phase is involved in the penetration of the cell by the virus, since adsorption seems to take place in the presence of AMPS. However, the initiation of infection will proceed under other conditions which do not allow viral increase. The viral inhibitors FPA and methoxinine do not affect this early phase.

A second stage of development was differentiated which is subject to the influence of methoxinine but not to AMPS. It is an intracellular phase corresponding to an interval beginning during the latent period and proceeding into the phase of viral maturation. The latter part of the period in which infectious virus is appearing is insensitive to the action of both inhibitors.

The inhibitory effect of FPA is also dependent upon the age of infection. While it inhibits completely during the latent period, a decreasing effectiveness is observed when it is added at successively later intervals of the maturation period.

The effect of the two inhibitors, fluorophenyl-

alanine (FPA) and methoxinine (Mx), seems to be upon separate phases of viral development, which occur simultaneously but begin at different times. Methoxinine inhibits an early biosynthetic process, while FPA interferes with the one beginning later. These specific phases exhibit a degree of independence and because of this are recognized as separate steps.

The release of mature virus is affected by the inhibitor AMPS. The action of this inhibitor has revealed that the penetration and release of the virus to and from the cell share a common characteristic.

The release phase appears to be an active process on the part of the virus and is accomplished through the destruction of the receptor-site material.

Finally we can say that the phases of maturation and release operate concurrently but are independent processes.

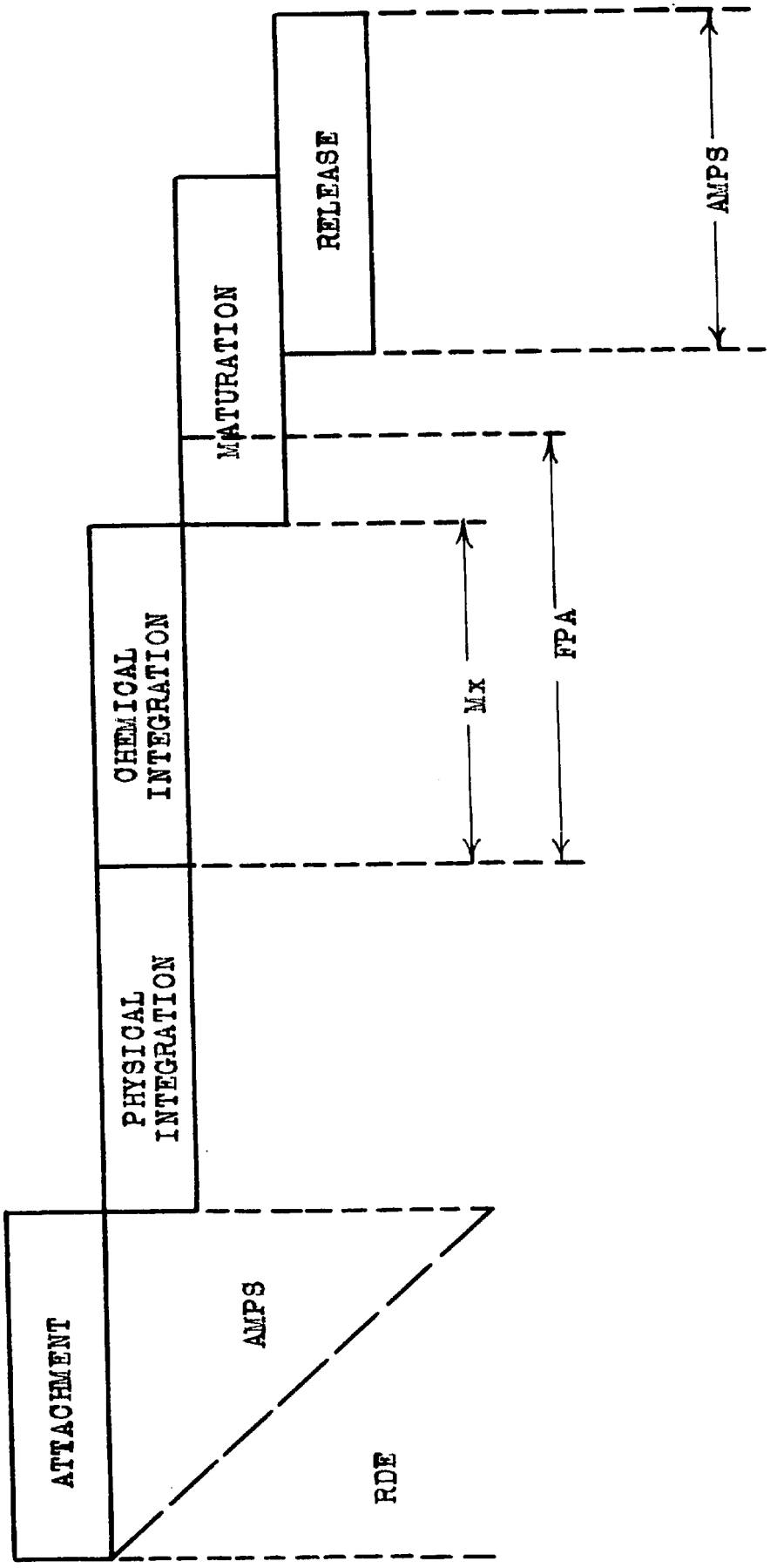
None of the three inhibitors appears to exhibit toxicity on the host cell, or virucidal properties, but they show rather specificity of action directed to particular segments of the developmental process of the virus.

Figure IX summarizes the viral development in a single cell, and the stages in this development which are affected by the three inhibitors.

FIGURE IX

SUMMARY OF THE SITE OF ACTION OF VARIOUS INHIBITORS

ON THE STAGES OF VIRAL DEVELOPMENT IN SINGLE CELL



## BIBLIOGRAPHY


Ackermann, W. W. (1951) Concerning the relation of the Krebs cycle to virus propagation. J. Biol. Chem. 189: 421-428.

Ackermann, W. W. (1951) The relation of the Krebs cycle to viral synthesis. II. The effect of sodium fluoroacetate on the propagation of influenza virus in mice. J. Exp. Med. 93: 635-642.

Ackermann, W. W. (1951) The role of l-methionine in virus propagation. J. Exp. Med. 93: 333-343.

Ackermann, W. W. (1951) α-Amino sulfonic acids and viral propagation. Proc. Soc. Exp. Biol. Med. 80: 362-366.

Ackermann, W. W. and R. B. Johnson (1953) Some energy relations in a host-virus system. J. Exp. Med. 97: 315-322.

Ackermann, W. W. and H. F. Maassab (1954) Growth characteristics of influenza virus: The influence of a sulfonic acid. J. Exp. Med. 99: 105-117.

Ackermann, W. W. and H. F. Maassab (1954) Growth characteristics of influenza virus: Biochemical differentiation of stages of development. J. Exp. Med. 100: 329-339.

Ackermann, W. W. and H. F. Maassab (1954) Alcune caratteristiche dello sviluppo del virus della polmonite nel neonato (Sendai). Minerva Medica Anno XLV. Vol. I, N° 42: 1-8.

Ackermann, W. W., A Rabson, and H. Kurtz (1954) Growth characteristics of poliomyelitis virus in HeLa cell cultures: Lack of parallelism in cellular injury and virus increase. J. Exp. Med. 100: 437-450.

Andrews, C. H., P. P. Laidlaw, and W. Smith (1934) The susceptibility of mice to the viruses of human and swine influenza. Lancet 2: 859-862.

Andrews, C. H. (1942) Interference by one virus with the growth of another in tissue culture. Brit. J. Exp. Path. 23: 214-220.

Bernkopf, H. (1949) Cultivation of influenza virus in the chorio-allantoic membrane of deembryonated eggs. Proc. Soc. Exp. Biol. Med. 72: 680-682.

Bernkopf, H. (1950) Study of infectivity and hemagglutination of influenza virus in deembryonated eggs. J. Immunol. 65:571-583.

Briody, B. A. and C. Stannard (1951) The effect of proflavine on the growth of viruses in the chick embryo. J. Immunol. 67: 423-430.

Brown, G. C. and W. W. Ackermann (1951) Effect of DL-ethionine on poliomyelitis virus growth in tissue culture. Proc. Soc. Exp. Biol. Med. 77: 367-369.

Burnet, F. M. (1936) Influenza virus on the developing egg: 1. Changes associated with the development of an egg passage strain of virus. Brit. J. Exp. Path: 17: 282-293.

Burnet, F. M. (1940) Influenza virus infections of the chick embryo by the amniotic route. 1. General character of the infection. Aust. J. Exp. Biol. Med. Sci. 18: 353-360.

Burnet, F. M. (1940) Influenza virus infections of the chick embryo lung. Brit. J. Exp. Path. 21: 148-153.

Burnet, F. M. (1941) Growth of the influenza virus in the allantoic cavity of the chick embryo. Aust. J. Exp. Biol. Med. Sci. 19: 291-295.

Burnet, F. M., and D. R. Bull (1943) Changes in influenza virus associated with the adaptation to passage in chick embryos. Aust. J. Exp. Biol. Med. Sci. 21: 55-69.

Burnet, F. M., W. I. B. Beveridge and D. R. Bull (1944) Study of a strain of influenza B virus isolated by chick embryo inoculation. Aust. J. Exp. Biol. Med. Sci. 22: 9-16.

Burnet, F. M. (1951) Some biological implications of studies on influenza viruses. Bull. Johns Hopkins Hosp. 88: 119-180.

Burnet, F. M., K. B. Fraser, and P. E. Lind (1953) Genetic interaction between influenza viruses. Nature, 171: 163-165.

Cairns, H. J. F., Fazekas de St. Groth and M. Edney (1952) Quantitative aspects of influenza virus multiplication. I. Production of incomplete virus. II. Heterologous interference. III. Average liberation time. IV. Definition of constants and general discussion. J. Immunol. 69: 155-181.

Cantoni, G. L. (1952) Phosphorus Metabolism, II. McElroy, W. D. and Glass, B., eds., Johns Hopkins Press, Baltimore, Maryland.

Chu, C. M., Dawson, I. M., and W. J. Elford (1949) Filamentous forms associated with newly isolated influenza virus. Lancet, 1: 602-603.

Cushing, R. T. and H. R. Morgan (1952) Effects of some metabolic analogs on growth of mumps and influenza viruses in tissue culture. Proc. Soc. Exp. Biol. Med., 79: 497-500.

Daniels, J., Monroe Eaton and M. E. Perry (1952) Effect of glucose on the growth of influenza virus in deembryonated eggs and tissue cultures. J. Immunol. 69: 321-329.

Dawson, I. M., and W. J. Elford (1949) Electron microscope studies on the interaction of certain viruses with fowl red cell membranes. Nature 163: 63-64.

Dulbecco, R. and M. Vogt (1954) One-step growth curve of western equine encephalomyelitis virus in chicken embryo cells grown in vitro and analysis of virus yield from single cells. J. Exp. Med., 99: 183-199.

Eaton, M. D., M. Corey, W. Van Herick and G. Meiklejohn (1945) A comparison of various methods of demonstrating influenza virus in throat washings. Proc. Soc. Exp. Biol. Med. 58: 6-9.

Eaton, M. D., B. Magasarnik, M. E. Perry and D. Karibian (1951) Inhibition of influenza and mumps viruses in tissue culture by basic amino acids. Proc. Soc. Exp. Biol. Med. 77: 505-508.

Eaton, M. D., F. S. Cheever and C. G. Levenson (1951) Further observations of the effect of acridines on the growth of viruses. J. Immunol. 66: 463-475.

Eaton, M. D., M. E. Perry, C. G. Levenson and I. M. Gocke (1952) Studies on the mode of action of aromatic diamidines on influenza and mumps viruses in tissue culture. J. Immunol. 68: 321.

Fazekas de St. Groth, S. (1948) Destruction of influenza virus receptors in the mouse lung by an enzyme from V. Cholerae. Aust. J. Exp. Biol. Med. Sci. 26: 29-36.

Fazekas de St. Groth, S. (1950) Influenza: a study in mice. Lancet, 1: 1101-1105.

Fazekas de St. Groth, S., A. Isaacs and M. Edney (1952) Multiplication of influenza virus under conditions of interference. Nature 170:573.

Francis, Thomas, Jr. (1934) Transmission of influenza by a filterable virus. Science 80: 457-459.

Francis, Thomas, Jr., and T. P. Magill (1935) Cultivation of human influenza virus in an artificial medium. Science 82: 353-354.

Fulton, F. (1949) Growth cycle of influenza virus. Nature 164: 189-190.

Green, R. G. and C. S. Stulberg (1946) Cell blockade in canine distemper. Proc. Soc. Exp. Biol. Med. 61: 117-121.

Green, R. H. and D. W. Woolley (1947) Inhibition by certain polysaccharides of hemagglutination and of multiplication of influenza virus. J. Exp. Med. 86: 55-64.

Green, M. and M. A. Stahmann (1954) Virus inhibitory activity by polyvinylamine. Proc. Soc. Exp. Biol. Med. 87: 507.

Halvorson, H. O. and S. Spiegelmann (1952) The inhibition of enzyme formation by amino acid analogues. J. Bact. 64: 207-221.

Henle, W. and L. D. Chambers (1941) The serological activity of extra-embryonic fluids of chick infected with virus of influenza A. Proc. Soc. Exp. Biol. Med. 46: 713-717.

Henle, W., G. Henle, and E. B. Rosenberg (1947) The demonstration of one-step growth curves of influenza virus through the blocking effect of irradiated virus on further infection. J. Exp. Med. 86: 423-437.

Henle, Werner (1949) Studies on host-virus interactions in the chick embryo-influenza virus system. I. Adsorption and recovery of seed virus. J. Exp. Med. 90: 1-11.

Henle, Werner (1949) Studies on host-virus interactions in the chick-embryo-influenza virus system. II. The propagation of virus in conjunction with the host cells. J. Exp. Med. 90: 13-22.

Henle, Werner, and G. Henle (1949) Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, hemagglutination and complement-fixation during the first infectious cycle. J. Exp. Med. 90: 23-36.

Henle, W. and O. Liu (1951) Studies on host-virus interactions in the chick embryo-influenza virus system. VI. Evidence for multiplicity reactivation of inactivated virus. J. Exp. Med. 94: 305-322.

Henle, W. (1950) Interference phenomenon between animal viruses: A review. J. Immunol. 64: 203-230.

Hirst, G. K. (1941) The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science 94: 22-23.

Hirst G. K. (1942) Adsorption of influenza hemagglutinins and virus by red blood cells. J. Exp. Med. 76: 195-209.

Hoyle, L. (1948) The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. Brit. J. Exp. Path. 29: 390-399.

Hoyle, L. (1950) The multiplication of influenza viruses in the fertile egg. A report to the Medical Research Council. J. Hyg. (London) 48: 277-296.

Kirber, M. W., and W. Henle (1950) A comparison of influenza complement-fixation antigens derived from allantoic fluids and membranes. J. Immunol. 65: 229-243.

Lennette, E. H., and H. Koprowski (1946) Interference between viruses in tissue culture. J. Exp. Med. 83: 195-219.

Lennette, E. H. (1951) Interference between animal viruses. Ann. Rev. Microbiol. 5: 277-292.

Liu, O., and W. Henle (1951) Studies on host-virus interactions in the chick embryo-influenza virus system. V. Simultaneous serial passage of the agents of influenza A and B in relation to variations in the growth cycle of influenza B virus. J. Exp. Med. 94: 291-303.

Maassab, Hunein F. (1954) Unpublished results.

McClelland, L., and R. Hare (1941) The adsorption of influenza virus by red cells and a new in vitro method of measuring antibodies of influenza virus. Canad. Public Health J. 32: 530-538.

McClelland, L., and C. E. Van Rooyen (1949) Studies on the effect of some aromatic amidines and other chemical compounds on the growth of influenza virus in the embryonated egg. Canad. J. Res. 27: 177-185.

McIlwain, H. (1941) Bacterial innibition by amino sulfonic analogues of some natural amino carboxylic acids. Brit. J. Exp. Path. 22: 148-155.

Mitchell, J. H., Jr., H. E. Skipper, and L. L. Bennett, Jr. (1950) Investigations of the nucleic acids of viscera and tumor tissue from animals injected with radioactive 8-azaguanine. Cancer Research 10: 647-649.

Nicolle and Le Bailly (1918) Quelques notions experimentales sur le virus de la grippe. C. R. Ac. Sci. 167: 601-610.

Niggs, C., J. H. Crowley and D. E. Wilson (1940) On the use of chick embryo cultures of influenza virus in complement fixation tests. Science 91: 603-604.

Pardee, A. B. (1949) Measurement of oxygen uptake under controlled pressures of carbon dioxide. J. Biol. Chem. 179: 1085-1091.

Pearson, H. E., and J. F. Enders (1941) Cultivation of influenza A virus in Roller tubes. Proc. Soc. Exp. Biol. Med. 48: 140-143.

Pilcher, K. S., K. F. Soike, V. H. Smith, F. Trosper, and B. Folston (1955) Innibition of multiplication of Lee influenza virus by canavanine. Proc. Soc. Exp. Biol. Med. 88: 79.

Rasmussen, A. F., Jr., J. C. Stokes, H. A. Feldman, and J. E. Smadel (1947) Effect of Acridines on the growth of influenza A and B viruses. J. Bact. 54: 64.

Rasmussen, A. F.,Jr., a d J. C. Stokes (1951) Chemical innibition of the growth of the virus of influenza in embryonated eggs. J. Immunol. 66: 237-247.

R. Dujarnic de la Riviere (1918) La grippe est-elle une maladie a virus filtrant? C. R. Ac. Sci. 167: 606-607.

Reed, L. J. and H. Muench (1938) A simple method of estimating fifty per cent end points. Am. J. Hyg. 27: 493-497.

Rickard, E. R., M. Thigpen, and J. H. Crowley (1944) The isolation of influenza A virus by the intra-allantoic inoculation of chick-embryos with untreated throat washings. J. Immunol. 49: 263-271.

Rubini, J. R., A. F. Rasmussen, Jr., and M. A. Stahmann (1951) Innibitory effect of synthetic lysine polypeptides on growth of influenza virus in embryonated eggs. Proc. Soc. Exp. Biol. Med. 76: 662-665.

Schlesinger, W. R. (1950) Incomplete growth cycle of influenza virus in mouse brain. Proc. Soc. Exp. Biol. Med. 74: 541-548.

Smith, R., E. E. Anderson, Jr., R. N. Overland and F. Schlenk (1953) The occurrence, formation and isolation of thiomethyladenosine. Arch. Biochem. 42: 72-82.

Smith, W., C. H. Andrewes and P. P. Laidlaw (1933) A virus obtained from influenza patients. Lancet, 2: 66-68.

Smith, W. (1935) Cultivation of the virus of influenza. Brit. J. Exp. Path. 16: 497-499.

Stekol, J. A., and K. W. Weiss (1951) Inhibition of growth of C3H mice by ethionine and its alleviation by methionine or choline. Proc. Soc. Exp. Biol. Med. 77: 213-213.

Stone, J. D. (1948) Prevention of virus infection with enzyme of V. cholerae. I. Studies with viruses of mumps-influenza group in chick embryo. Aust. J. Exp. Biol. Med. Sci. 26: 49-64.

Stone, J. D. (1948) Prevention of virus infection with enzyme of V. cholerae. II. Studies with influenza virus in mice. Aust. J. Exp. Biol. Med. Sci. 26: 287-297.

Stuart-Harris, C. H. (1939) A neurotropic strain of human influenza virus. Lancet, 1: 497-499.

Takemoto, K. K., M. L. Robbins, and P. K. Smith (1954) The experimental chemotherapy of influenza viruses. J. Immunol. 72: 139-145.

Tamm, I., K. Folkers, and F. L. Horsfall (1952) Inhibition of influenza virus multiplication by 2,5 dimethyl-benzimidazole. Yale J. Biol. Med. 24: 559-567.

Tamm, I., K. Folkers, and F. L. Horsfall (1953) Inhibition of influenza virus multiplication by alkyl derivatives of benzimidazole. I. Kinetic aspects of inhibition by 2,5 dimethyl benzimidazole as measured by infectivity titrations. J. Exp. Med. 98: 219-227.

Tamm, I., and D. A. J. Tyrrell (1954) Influenza virus multiplication in the chorio-allantoic membrane in vitro: Kinetic aspects of inhibition by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. J. Exp. Med. 100: 541-561.

Tamm, I., K. Folkers, C. H. Shunk, and F. L. Horsfall (1954) Inhibition of influenza virus multiplication by N-glycosides of benzimidazoles. J. Exp. Med. 99: 227-250.

Travers, J. J. and L. R. Cericedo (1951) Effects of methoxinine in the mouse. Proc. Soc. Exp. Biol. Med. 76: 497-499

Von Magnus, P. (1951) Propagation of the PR8 strain of influenza A virus in chick embryos. I. The influence of various experimental conditions on virus multiplication. Acta Path. Microbiol. Sand. 28: 258-277.

Von Magnus, P. (1951) Propagation of the PR8 strain of influenza A virus in chick embryos. II. The formation of "incomplete" virus following incubation of large doses of seed virus. Acta. Path. Microbiol. Scand. 28: 278-292.