THE UNIVERSITY OF MICHIGAN
INDUSTRY PROGRAM OF THE COLLEGE OF ENGINEERING

THE INTRACELLULAR SYNTHESIS AND MATURATION OF A VIRAL PRECURSOR

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan 1957.

June, 1957

IP-222

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. W. W. Ackermann for his interest in this work and for the sedulity with which he edited the manuscript. What has been more important, however, is the general stimulating association and exchange of ideas between this graduate student and Dr. Ackermann and his colleagues, for it has been through this association that the writer has gained an insight into the scientific approach to a problem.

The writer also wishes to thank Dr. Thomas Francis, Jr., who made available the facilities of the Virus Laboratory of the School of Public Health for the present doctoral work in Biophysics.

He is indebted to the Industry Program of the College of Engineering, The University of Michigan, for support in the final stages in the preparation of this dissertation.

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INTRODUCTION

At the Second Symposium of the Society for General Microbiology held at Oxford University, in 1952, on The Nature of Virus Multiplication, Hoyle said:

> "It is very probable that all biological multiplication is fundamentally similar in nature, and that a complete understanding of the multiplication of a simple living agent would be of the greatest help in understanding the behavior of more complex forms. It is for this reason that a study of virus multiplication is of more than ordinary importance and interest."

This statement provides a justification for studies on virus multiplication, but, the possibility of finding that viral synthesis utilizes metabolic pathways not common to the uninfected host cell provides additional interest and incentive, for it is at such a dichotomy that the application of a chemotherapeutic agent will be practical.

The process of multiplication can be represented as a series of events which begins with the exposure of susceptible cells to infectious virus and ends with the liberation of new virus that is usually indistinguishable from the infecting agent. This series of events constitutes an infectious cycle. It can be divided into the following phases: (1) attachment of the virus onto the surface of the host cell, (2) penetration of all or part of the virus into the cell, (3) the synthesis of new virus within the cell, and (4) the release of new virus from the cell. The division into these phases is rather arbitrary and the extent of each phase is not well defined.

Attachment of virus can be demonstrated experimentally and appears to involve the union of specific sites on the virus and on the cell. Until

recently (with influenza) the penetration of viral material into the cell was only inferred as a step necessary for the intracellular synthesis of virus and by analogy with studies on bacterial viruses. Hoyle and Frisch-Niggemeyer (1955) provided experimental evidence of penetration by showing that fifteen minutes after exposure of the chorio-allantoic membrane to labeled influenza virus radiophosphorus could be recovered from cell fractions which do not contain intact virus.

It is generally believed that during the "eclipse" period after penetration the nucleic acid of the infecting virus comes to hold a key position in the mechanisms which govern the synthetic activities of the host cell. Thus the cell is changed from a characteristic host cell to a biochemically different "organism" which synthesizes more of the substance that produced the biochemical alteration. A virus, then, can be thought of as part of a complex living entity which does not exist as such in the absence of the virus, but does have an alternate existence as a normal animal cell. The virus, when it is outside of the cell, is a nonliving thing which has the ability to gain entrance into the normal animal cell and change it into the complex living entity. The events involving the early stages of virus-host interaction will be reviewed in the Historical Survey.

This dissertation concerns primarily the synthetic processes taking place in the virus-infected cell. The host cells used for this investigation were the entodermal cells lining the chorio-allantois of the chick embryo, and the infecting agent was the PR8 strain of type A influenza virus.

Viruses are particulate in nature. Associated with particles of influenza virus there are several activities which can be used for the

determination of the relative concentration of the virus particles. Two of these are the ability to infect the embryonate chicken egg and the capacity to agglutinate red blood cells. The titration for infectivity is the more sensitive method, one infectious particle theoretically being detectable, whereas about ten million particles per milliliter are required for detection by hemagglutination titration. In these studies quantitative determination of virus was done exclusively by titration for hemagglutination. There were several reasons for this: (1) the hemagglutination titration is more accurate; (2) the sensitivity of the infectivity titration is not required, since the chorio-allantois contains about 100 million susceptible cells and therefore the production of one particle per cell corresponds roughly to the threshhold of detection by hemagglutination titration; (3) the property of hemagglutination is more stable than that of infectivity; (4) the production of hemagglutinin probably reflects more realistically the synthetic processes of the infected cell, for the difference in an infectious and noninfectious virus particle may be subtle, and, therefore, if we do not include noninfectious virus in our measurements we may receive a false impression of the cell's synthetic activities; and (5) hemagglutination titrations are less time-consuming and less costly.

There is a concept of "stage-wise" development of influenza virus. This concept was presented independently by Hoyle and by Henle and was based on the findings that complement fixing antigens (Hoyle, 1948, 1950) and hemagglutinin (Henle and Henle, 1949, and Henle et al., 1950) precede a rise in infectious titer. It has not been shown, however, that noninfectious hemagglutinin is a stage through which each virus particle must

pass before becoming infectious. That the synthesis of influenza virus takes place by a series of biochemical stages has been shown by Ackermann and his colleagues. The differentiation of these stages does not depend upon the isolation or detection of incomplete forms, but upon the characterization of the stages in terms of sensitivity of the stages to various inhibitors. An inhibitor-sensitive stage is manifest in reduction of the viral yield. Specifically, Ackermann and Maassab (1955) recognized two biochemical reactions, one inhibited by methoxinine and the other by p-fluorophenylalanine. They found that the first reaction began early in the latent period and proceeded independently of the second, but that the second reaction could not proceed unless the first had already begun. It was possible, therefore, to place these two reactions in a definite time sequence. In a similar manner, Ackermann and Maassab (1954a) defined four stages of development (including initiation of infection and release) on the basis of sensitivity to methoxinine and α-amino-p-methoxyphenylmethanesulfonic acid. In the present dissertation emphasis was placed on a detailed study of the p-fluorophenylalanine-sensitive reaction. In addition the effect of specific antiviral immune serum on the infected cell was studied and by this means the product of the p-fluorophenylalanine-sensitive reaction was characterized.

HISTORICAL SURVEY

Prior to the development of bacteriologic techniques most disease-producing agents were called "viruses" (virus is the Latin word for poisons). With the development and application of pure-culture techniques by Koch (1877, 1882) it became evident that some diseases were caused by bacteria, some by protozoa and some by mycotic organisms. These organisms could be visualized with the aid of the optical microscope, possessed definite morphology, and usually could be grown in pure culture. It was soon recognized, however, that many common diseases, for example, the pock diseases of man and lower animals, poliomyelitis, yellow fever, and rabies, were caused by agents which defied cultivation in bacteriologic media and were submicroscopic in size. Loeffler and Frosch (1898) demonstrated that the "virus" of foot and mouth disease of cattle passed through bacteria-retaining filters, and thus the term "filterable viruses" came into current usage. The only significant virus research in the 19th century was done by Pasteur (1884), who modified the pathogenicity of rabies virus by serial passage in the rabbit. He demonstrated that injection of this attenuated or "fixed" virus protected dogs from the natural or "street" virus. By injecting "fixed rabies virus" Pasteur (1885) protected a boy who had been bitten by a rabid dog. Pasteur (1881) postulated the ultramicroscopic nature of the infectious agent of rabies, and twenty years later Remlinger (1903) showed that the agent passed through filters which retained bacteria.

The modern era of animal virology began in the 1920's. From that time to the present, as in all experimental sciences, rapid advances were

made following the development and refinement of experimental techniques. The progress in animal virology since 1925 is exemplified by the work on the influenza viruses.

Epidemics of influenza have occurred since ancient times in all parts of the world. The most severe of these was the pandemic of 1918-1919, which was responsible for the death of an estimated 20 million persons. Research concerning the etiologic agent, however, could not begin until a method was found for the propagation of the virus in the laboratory. As the techniques of propagation became more convenient, the literature on influenza became voluminous. Today the influenza viruses are probably the most studied animal viruses.

Smith, Andrewes, and Laidlaw (1933) were the first to demonstrate that the virus could be isolated from throat washings of patients with the disease. They showed that the disease could be produced in ferrets by intranasal instillation of virus-containing throat washings. These authors also demonstrated that during convalescence from influenza a patient developed neutralizing antibodies against the virus. The virus they isolated was the WS strain of type A influenza.

Francis (1940) and Magill (1940) recovered type B influenza virus from throat washings and showed that the neutralizing antibodies developed in convalescing patients neutralized type B but not type A influenza virus. It has been shown that after passage in experimental animals type A influenza virus (Smith and Stuart-Harris, 1936) and type B influenza (Francis et al., 1944) maintain their ability to induce the typical disease in man. Taylor (1949) isolated an antigenically distinct strain of influenza virus. Francis et al. (1950) recovered a strain which they showed was serologically different from types A and B influenza virus, but similar

to the strain isolated by Taylor. These two strains were classified as type C influenza virus (Francis et al., 1950). Many of the type A strains isolated since 1947 have been called A-prime strains (Salk and Suriano, 1949) because of the degree of immunologic differences between these and the early type A strains.

Francis (1934) and Andrewes, Laidlaw, and Smith (1934) successfully transmitted the disease to mice by the intranasal route. Although freshly isolated strains multiply in the lung of the mouse, lesions are not produced in this organ until the virus has undergone several passages in the mouse. Smith (1935) demonstrated that influenza virus could be propagated in the embryonate chicken egg and that multiplication occurs in the chorioallantoic membrane. Burnet (1940) discovered that inoculation into the amniotic sac of the embryonate chicken egg is the most successful method of isolating new strains from throat washings. The use of the embryonate chicken egg has become increasingly important in the study of influenza virus. It is a method which is simple and less costly than that of mouse or ferret inoculation; the extraembryonic membranes are uniformly highly susceptible to infection, and the virus appears in high concentration in the relatively cell-free extraembryonic fluids. Techniques for the cultivation of influenza virus in cultures of chick-embryo tissue were developed by Francis and Magill (1935) and Smith (1935). Francis (1947) maintained influenza virus in tissue culture for more than 700 transfers.

Much of the early research on influenza virus concerned the serological, biochemical, and biophysical properties of the extracellular virus.

All strains of influenza virus can be divided into three distinct serologic types, i.e., types A, B, and C (Horsfall et al., 1940; Taylor, 1949, 1951; Francis et al., 1950). These three types have no known common

antigenic component. Animals which are immune to one type are susceptible to the other two types. When one type of virus is mixed with its homologous hyperimmune serum, the resulting antigen-antibody reaction is inferred from the fact that the mixture fixes complement, fails to agglutinate red blood cells, and does not produce infection in the embryonate egg or in the mouse. There is no evidence for antigen-antibody reactions in mixtures of one type of virus with hyperimmune serum prepared against another type. The strains of one type of influenza virus are not antigenically identical. Recently Jensen and Francis (1953) showed that each of the 29 strains of types A and A-prime influenza virus they studied could be represented antigenically by the relative amounts of 18 different antigenic components.

Hirst (1941) and McClelland and Hare (1941) observed that influenza virus in sufficiently high concentration agglutinated the red blood cells of the chicken and several other animals. This finding has been of the greatest practical and theoretical importance. Previous to 1941, the infective principle of influenza virus was assayed by inoculation of mice. Evidence of infection was the finding of specific lesions in the lung. Titration for infectivity could not be carried out by inoculation of embryonate eggs, for no lesions were produced in the embryo or the membranes. The finding that influenza virus agglutinates red blood cells made possible infectivity titrations in embryonate eggs, since the allantoic fluid of an infected egg contains enough virus to agglutinate red blood cells.

The hemagglutination reaction between influenza virus and red blood cells has been thoroughly studied. The basic observable phenomena of this hemagglutination are the following: (1) upon mixing virus-containing fluid with a suspension of chicken red blood cells in a tube, macroscopic

aggregates are formed, which settle to the bottom of the tube and form a distinctive pattern; (2) the fluid above the sedimented cells has decreased in virus activity; (3) after a short time at 37°C or a longer time at a lower temperature, the aggregates break up, the distinctive pattern disappears, and full virus activity returns to supernatant fluid, i.e., the virus elutes from the cell; (4) the virus is not altered with respect to infectivity, hemagglutinin, or antigenic properties; and (5) the red blood cells are modified in that they no longer can be agglutinated by the same strain of virus and a marked change occurs in the electrophoretic mobility (Hanig, 1948). These facts suggested, and subsequent research supported, the idea that virus attaches to the red blood cells and causes aggregation by forming bridges between the cells. The attachment of the virus to the red blood cell is that of an enzyme on the surface of the virus with its substrate on the surface of the cell, and spontaneous elution of the virus is due to the enzymatic action of the viral enzyme on the substrate.

Certain bacterial enzymes destroy the capacity of red blood cells to adsorb and to be agglutinated by influenza virus (Burnet, McCrea, and Stone, 1946). An enzyme derived from Vibrio cholerae is very active in this respect. Stone (1947) demonstrated that the enzyme is not virucidal or toxic to the chick embryo, but can prevent infection of the chorioallantoic membrane if it is administered before or at the same time as the virus. Stone (1947) was the first to refer to this enzyme as RDE or receptor-destroying enzyme. Burnet, McCrea, and Anderson (1947) suggested that the substrate of RDE is a mucin. Numerous inhibitors of hemagglutination by indicator virus (virus whose enzymatic activity has been destroyed by heat) have been found in biological fluids and tissues, such as ovalbumin,

human urine, ovarian cyst fluids, extracts of salivary glands, extracts of red blood cells, and serum. In all cases the inhibitors are mucoid in nature though not chemically identical. These inhibitors presumably act by competing with the receptors on red blood cells for the viral enzyme. The inhibitors in these naturally occurring substances are destroyed by treatment with RDE or with active influenza virus. There exists a close analogy between the reactions of (1) RDE on red blood cells, (2) RDE on mucoid hemagglutination-inhibitors, (3) active virus on inhibitors, and (4) active virus on red blood cells. The work on inhibitors of hemagglutination has been reviewed by Burnet (1951). Recently Gottschalk (1953) isolated a split-product, namely the O-glucoside of 4-hydroxy-2-carboxy-pyrroline, obtained by the enzymatic reaction between the viral enzyme or RDE on mucoprotein analogs of the red cell receptor. He believes that the glucoside is joined to a hexosamine unit by an amide linkage between the carboxyl group of the pyrroline derivative and the amino group of the hexosamine residue, and that this amide link is broken by the enzyme. This receptor-destroying enzyme and the adenosine triphosphate dephosphorylating enzyme of the virus of avian erythromyeloblastic leucosis (Mommaerts et al., 1952; Sharp et al., 1954; and Eckert et al., 1955) are the only complete enzymes that are known to be closely associated with a virus.

The chemical and physical properties of influenza virus have been studied for the most part on purified preparations. The first step in purification usually consists of adsorption of the virus onto red blood cells or red blood cell stromata, followed by centrifugation and elution of the virus into a smaller volume (Hirst, 1941, 1942; McClelland and Hare, 1941; Francis and Salk, 1942). Other methods have been used, however, for

preliminary concentration and partial purification. Hare et al. (1942) and Hirst et al. (1942) found that when infected allantoic fluid is frozen and then slowly thawed and kept cold, a precipitate forms which contains 90 to 95 percent of the viral activity. Chambers and Henle (1941) precipitated virus and only four percent of the original nitrogen by adding spermine to the allantoic fluid. Bodily et al. (1943) used alum ($Al_2(SO_4)_3K_2SO_4 \cdot 12H_2O$) to precipitate the virus which was then redissolved in a solution of twenty percent sodium citrate. Cold methanol was used to precipitate virus from allantoic fluid by Cox et al. (1947) and Moyer et al. (1950). Adsorption followed by elution from cation exchange resins was used by Muller and Rose (1952) to concentrate and partially purify influenza virus. Following one or more of these methods several cycles of ultracentrifugation are employed to sediment extraneous material and then the virus.

From sedimentation studies of PR8 influenza virus Sharp et al. (1945) have found the following physical properties: sedimentation constant, 742; hydrated density, 1.104; partial specific volume, 0.882; and particle diameter, 116 millimicrons. By electron microscopy of freeze-dried preparations, Williams (1953) determined the mean diameter of influenza virus to be around 80 millimicrons. Electron micrographs of infected allantois show many bizarre forms which supposedly possess all the characteristics of influenza virus (Eddy and Wyckoff, 1950). These forms range from short rods of partially fused spheres to long filaments with terminal swellings. By filtration through collodion membranes of graded porosity (gradocol membranes) Elford et al. (1936) estimated the diameter of type A influenza virus to be 80 to 120 millimicrons.

With respect to the chemical composition of influenza virus some of the data in the literature are of questionable value because of the probable impurity of the virus preparations. The lipid content appears to be around 30 to 40 percent by dry weight and to be composed of phospholipid, cholesterol, and neutral fat (Taylor, 1944). Knight (1947b) found both RNA and DNA in influenza virus, but later workers (Ada and Perry, 1954a, 1954b) found only RNA. The amino acid composition of influenza virus is quite similar to that of the host tissues in which it has multiplied (Knight, 1947a).

Various means of degradation of influenza virus have yielded information about its structure. By ether treatment of influenza type A virus Hoyle (1952) obtained two units: the soluble antigen and the red cell agglutinin. The soluble antigen was a ribonucleoprotein, contained the type specific antigen, and was identical with that obtained from infected tissue. The agglutinin was a protein and contained the strain specific complement-fixing antigen, which was identical to that of the intact virus, and a nonspecific antigen. The agglutinin contained no nucleic acid or carbohydrate. Frisch-Niggemeyer and Hoyle (1956) also using ether for the degradation of influenza type A virus, found that the soluble antigen contained 5.3 percent ribonucleic acid and that this represented all the nucleic acid of the virus. The soluble antigen was 12.0 millimicrons in diameter and was 14 percent by weight of the virus particle. In contrast to the earlier finding of Hoyle (1952) they found 4.2 percent polysaccharide in the hemagglutinin. Tyrrell and Horsfall (1954) degraded influenza virus by repeated freezing and thawing and obtained a soluble blocking antigen which was highly strain specific and gave little activity in complement fixation tests. These authors concluded that the "soluble

blocking antigen ... is protein in nature and represents the essential antigenic material of the intact virus."

Schlesinger (1950) found that nonneurotropic influenza virus undergoes a single cycle of infection in the mouse brain. This infection is recognized by an increase in hemagglutinin and complement fixing activity but no increase in infectivity. Henle and Girardi (1955) observed a similar phenomenon in tissue cultures of human carcinoma cells, strain HeLa (Gey et al., 1952). In this case the hemagglutinin and complement fixing antigen was not spontaneously released from the cells, but were observed after disruption of the cell by freezing and thawing or by sonic vibrations.

Schlesinger (1953) believes that the products of incomplete growth cycles of influenza virus do not represent a phase in the normal production of the virus, but that they have gone through the entire developmental cycle only to emerge "malformed and deficient in one cardinal attribute: ability to reproduce." Ada and Perry (1956) found that "incomplete virus" contains the same proportion of bases in its nucleic acid but that the content of nucleic acid was lower than in infective virus.

It was shown by Henle and Henle (1943, 1944, 1945), Ziegler and Horsfall (1944) and Ziegler et al. (1944) that either active heterologous, ultraviolet-irradiated, or heat inactivated homologous and heterologous influenza virus diminished or prevented the growth of active influenza virus. It was observed that either homologous or heterologous inactivated virus completely inhibited the growth of active virus if added simultaneously with, or up to 96 hours before, the active virus. If, however, inactive heterologous virus was added shortly after the active virus the infectious cycle already started would go to completion but infection of

new cells would be prevented, while inactive homologous virus completely inhibited viral production. Although the mechanism of interference between the influenza viruses in the chorio-allantoic membrane is not well understood, most investigators agree that it is an intracellular phenomenon and probably involves "blocking of a key enzyme or enzymes" (Burnet, 1953).

Genetic recombination between influenza viruses was demonstrated by Burnet and Lind (1951). By simultaneous inoculation of the neurotropic NWS strain and the nonneurotropic MEL strain into the brains of mice, these investigators were able to isolate an "NM" strain which was neurotropic but had the serologic character of the nonneurotropic strain. Later, Fraser and Burnet (1952) obtained the same results by inoculation into the allantoic cavity of embryonate eggs. In neither case could the reciprocal recombinant be demonstrated. The hypothetical nonneurotropic NWS was obtained by Lind and Burnet (1954) by the interaction of neurotropic NWS with nonneurotropic WSM. In each example above, the genetic interaction occurred between two strains of type A influenza virus. Recently Baron and Jensen (1955) and Burnet and Lind (1955) demonstrated recombinant forms between types A and A-prime influenza virus. Recombination between types A and B has not been shown.

The nature of the process of multiplication of influenza virus in the cells of the chorio-allantoic membrane has been studied by many investigators. During the first hour after exposure of the chorio-allantoic membrane to virus, 50 to 70 percent of the virus is adsorbed to the cells (Cairns and Edney, 1952). It is commonly assumed that adsorption is mediated by a specific combination of the mucinolytic viral enzyme with the mucoid receptors on the surface of the cell. The union requires the

presence of ions and probably involves electrostatic forces (Puck et al., 1951). The mechanism of penetration is not known; it is clear, however, that enzymatic action is unnecessary, since treatment of the receptors with metaperiodate, which prevents degradation of the substrate by the viral enzyme - and also elution of the virus from red blood cells so treated - does not prevent the initiation of infection (Fazekas de St. Groth, 1949; Fazekas de St. Groth and Graham, 1949). Teleologically speaking, the main function of the enzyme may be to elute the virus from cells which are incapable of supporting multiplication. It is clear that a large portion of the inoculated virus cannot be recovered (Henle, 1949; Ishida and Ackermann, 1956). In addition, it will be shown in this dissertation that treatment with antiviral immune serum is without effect for a period of approximately two hours after initiation of infection.

In a study of the neutralization of Newcastle disease virus by immune serum, Rubin and Franklin (1956) found that several molecules of antiviral antibody were required to prevent adsorption of a virus particle into a cell, but that penetration could be prevented by a single antibody molecule.

New virus is first detectable two or three hours after the initiation of infection (Hoyle, 1950). The subsequent increase of virus will be in steps if the original inoculum is small; or it will be in a single step if the inoculum is large, and also if it is small, provided reinfection is prevented by destroying (or covering up) the receptors on uninfected cells with receptor-destroying enzyme or inactive virus. It is now generally agreed that liberation of new virus from infected cells occurs over an extended period of time.

The multiplication of influenza virus in the chorio-allantoic membrane can be modified by affecting (1) the virus particle, (2) the interaction of the virus with the host cell, (3) the metabolism of the host cell, and (4) the release of new virus. Examples of the modification produced by chemicals directed against these points are given below.

In addition to the virucidal effects of formalin, etc., and the inhibition caused by naturally occurring hemagglutination inhibitors, discussed earlier in this section, treatment of the virus with high concentrations of metaperiodate (Fazekas de St. Groth and Graham, 1949) alters the viral enzyme to the extent that adsorption cannot take place and treatment with lower concentrations of metaperiodate changes the virus so that it attaches but cannot elute from the receptors on red blood cells. Destruction of the cellular receptors by receptor-destroying enzyme (Stone, 1948) or metaperiodate prevents adsorption and thus the initiation of infection. Ackermann and Maassab (1954) demonstrated that α-amino-p-methoxyphenylmethanesulfonic acid interferes with adsorption or penetration of the virus into cells.

Inhibition of the oxidative process of the host-cell metabolism has been shown to inhibit viral synthesis. For example, Ackermann (1951a) demonstrated that a decrease in oxygen tension of the gas phase in flask cultures of the chorio-allantoic membrane inhibits viral production. Ackermann and Johnson (1953), Ingraham et al. (1953), and Eaton and Perry (1953) have shown that 2,4-dinitrophenol inhibits the growth of influenza virus in the chorio-allantoic membrane. The action of this substance appears to be due to an uncoupling of phosphorylation from oxidation. Butyl-3, 5-diiodo-4-hydroxy-benzoate, which is chemically related to thyroxine, has a similar effect on phosphorylation and viral growth (Eaton et al., 1953).

The presence of an energy source, such as glucose, is necessary for synthesis of virus in the chorio-allantoic membrane suspended in a synthetic medium (Ackermann, 1950).

Ackermann (1951a, 1951b) revealed that the functioning of the Krebs cycle is essential to the multiplication of influenza virus. He showed that administration of fluoroacetate or malonate, which inhibit the oxidation of citrate, and the dehydrogenation of succinate, respectively, inhibits viral synthesis.

Analogs of several amino acids have been shown to inhibit the multiplication of influenza virus in the chorio-allantoic membrane. Examples of these are the methionine analogs, ethionine and methoxinine (Ackermann, 1951c, and Ackermann and Maassab, 1954b), and the phenylalanine analog, p-fluorophenylalanine (Ackermann and Maassab, 1955).

Ackermann (1952) showed that each of the three α-aminosulfonic acids (α-aminophenylmethanesulfonic acid, α-amino-β-phenylethanesulfonic acid, and α-amino-p-methoxyphenylmethanesulfonic acid) inhibited the multiplication of influenza virus in the embryonate egg.

The vitamin analogs, oxythiamine and desoxypyridoxine,were shown to inhibit the growth of influenza virus in tissue culture (Cushing and Morgan, 1952).

Recently it has been shown that 2,5-dimethylbenzimidazole (Tamm et al., 1952, 1953), 4,5,6- or 5,6,7-trichloro-1-β-D-ribofuranosylbenzimidazole (Tamm, 1954), and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (Tamm and Tyrrell, 1954) inhibit the multiplication of influenza virus. It is believed that their inhibitory action is due to interference with nucleic acid metabolism. The inhibitory action of proflavine (Briody and Stannard, 1951) is also related to nucleic acid metabolism.

Ackermann and Maassab (1954) reported that the release of virus from the cells of the chorio-allantoic membrane is inhibited by α-amino-p-methoxyphenylmethanesulfonic acid.

METHODS AND MATERIALS

Many of the methods employed during this work are standard in the field of animal virology. However, since most of the present studies were of a quantitative nature and necessitated the obtaining of data with precision, the minutiae of the experimental techniques are of prime importance and the methods will, therefore, be discussed in detail.

The Virus—The PR8 strain of type A influenza virus was used; it was derived from a strain isolated by Francis (1934). Since its original isolation from man it has undergone several passages in ferrets; 593, in mice; and 140 to 149, in embryonate eggs. During this time there has been no deliberate selection for virus with certain properties, i.e., it is the wild type; nevertheless, this strain has characteristic properties which do not vary significantly upon passage. Von Magnus (1951) reported that after 193 passages in embryonate eggs PR8 virus was serologically indistinguishable from the mouse passage line from which it was obtained.

Pools of virus were prepared in the following manner. Allantoic fluid from the previous passage was diluted to 10^{-6} in broth containing 500 units of penicillin and 500 micrograms of streptomycin per milliliter. One tenth milliliter of this was inoculated into the allantoic cavity of each of two dozen eggs containing 11-day old embryos. After 48 hours of incubation at 37°C, the eggs were candled. If no movement of an embryo could be detected it was presumed to be dead, and the egg was discarded. Dead embryos were seldom found. The eggs then were chilled at 4°C for not more than 12 hours, cleaned with 70 percent alcohol and their allantoic fluids were harvested. This was done with a 20-ml syringe fitted

with an 18-gauge hypodermic needle. The fluid was centrifuged in 40-ml conical centrifuge tubes at 2000 rpm for 15 minutes. The supernatant fluids were pooled and distributed in 2-ml aliquots into screw-capped lustroid tubes. The entire pool then was frozen by lowering a rack containing the lustroid tubes into a 95 percent alcohol—dry-ice mixture. The tubes were stored in a deep freeze at about minus 45°C. Upon thawing, a virus pool prepared in this manner had an infectivity titer of $10^{10.1}$ to $10^{10.3}$ egg infective doses per milliliter and a hemagglutinin titer of 4000 to 6400 hemagglutinin units per milliliter. The ratio of the infectivity titer to the hemagglutinin titer was thus $10^{6.5}$. Aseptic technique was used throughout and the pools were tested for bacterial contamination by inoculation into thioglycolate broth.

Embryonate Eggs—Fertile eggs from Barred Rock hens were obtained from one supplier only. The age of the embryos at which the eggs were used will be specified for each experimental procedure. Eggs containing 11-day-old embryos, for example, will be referred to as 11-day embryonate eggs.

The Host-Virus System—The host-virus system was a modified form of that described by Ackermann (1951). The components of the system are the PR8 strain of type A influenza virus and the chorio-allantoic membrane of a 14-day embryonate egg suspended in 3-ml modified Earle's saline medium. These components, contained in a 25-ml Erlenmeyer flask, were incubated in a room kept at 37°C. During incubation the flasks were shaken at the rate of 50 cycles per minute by a lapping machine converted for this purpose. This rate of shaking was sufficient to slosh the medium and gently agitate the tissue. The flasks were kept rubber-stoppered to control

evaporation and evolution of carbon dioxide. At appropriate times the membranes were washed free of virus or inhibitor-containing medium. This was done by swirling the membrane in one to three changes of 50-ml phosphate-buffered saline contained in 125-ml flasks. After washing, a particular membrane was drained on gauze and either returned to another 25-ml flask for further incubation or else put into a small tube and stored at 4°C pending extraction. Unless otherwise specified the membranes were washed at 20°C and the time required for washing (10 to 15 minutes for washing four membranes three times each) was not included in the time scale of the experiment.

Modified Earle's Saline Medium (ESM)—This medium is a simple carbonate-buffered balanced salt solution described by Earle (1943) modified to contain twice the amount of glucose and in addition horse serum, antibiotics, and phenol red. The composition is given below.

The working solution was made immediately before use by adding 1.8 ml of the glucose solution and 3.6 ml of the horse serum mixture to 100 ml of the salt solution. The resulting medium had a pH of about 7.4, glucose and horse serum concentrations of 0.2 percent, and 3 percent, respectively, and contained 100 units of penicillin and 100 micrograms streptomycin per milliliter. Twice-glass-distilled water was used as diluent.

The Composition of Earle's Saline Medium

Salt solution:	NaCl	6.80 g/liter
	KCl	0.40 "
	$MgSO_4$	0.10 "
	$NaH_2PO_4 \cdot H_2O$	0.144 "
	$NaHCO_3$	2.20 "
	$CaCl_2$ (anhyd)	0.20 "

Glucose solution:		11.7 %
Horse serum mixture:	Horse serum	48.0 ml
	Penicillin (100,000 U/ml)	1.60 "
	Streptomycin (250,000 µg/ml)	0.64 "
	Phenol red (0.5%)	4.16 "

For expediency all the salts except the calcium chloride were kept in a 10-times concentrated stock solution and the calcium chloride in a 10 percent solution. Two hundred milliliters of the stock salt solution were diluted to about 1900 ml, four milliliters of the calcium chloride solution added,and the volume was brought up to 2 liters. This usually resulted in a turbid solution due to the precipitation of calcium carbonate. Enough phenol red was added to give a tinge of color, usually pink at this stage. By bubbling carbon dioxide into the solution, the precipitate was dissolved and the color changed to orange (pH, 7.2-7.4). The solution was chilled and then sterilized by filtration under pressure through a Selas No. 03 procelain filter candle. It was distributed into milk dilution bottles in 100-ml aliquots, the solution being withdrawn through a tubulation at the bottom of the receiving flask. The bottles were sealed with rubber stoppers and stored at 4°C.

The glucose solution was sterilized by autoclaving for 15 minutes at 10 lbs pressure.

The horse serum was obtained from the Difco Laboratories, Detroit, Michigan. It was inactivated by heating at 56°C for 30 minutes.

Phosphate-Buffered Saline (PBS)—This 0.01 M phosphate-buffered physiological saline was taken from the general laboratory supply. The pH was adjusted to 7.2.

The Chorio-Allantoic Membrane (CAM)—Fourteen-day embryonate eggs were used as a source of CAM. According to Needham (1950) the allantois reaches its maximum wet weight at 10 days, after which time the weight remains roughly constant. It is seen, however, by opening eggs of 10- to 16-days' incubation age that the portion of the allantois which is fused to the serosa (chorion), forming the chorio-allantoic membrane, increases up to at least 15 days. After 14 days of incubation insoluble deposits of uric acid are formed in the allantoic sac. Eggs incubated 14 days seem to be ideal with respect to the size of the CAM and the absence of uric acid crystals. It is interesting to note that the capacity to support the growth of PR8 influenza virus is high at 14 days. This is shown by the data in Table I, from experiments in which CAMs were harvested from eggs of various incubation ages, weighed, infected with $10^{8.68}$ egg infectious doses in 3-ml Earle's saline medium, and allowed to produce virus for 23 hours. The hemagglutinin (HA) figures are averages from three experiments.

TABLE I

HEMAGGLUTININ PRODUCTION AS A FUNCTION OF AGE OF CHORIO-ALLANTOIC MEMBRANE

Age of CAM	HA/CAM	HA/400 mg CAM
12 days	1040[a]	933
13 "	1460	1276
14 "	1502	1341
15 "	1100	1013
16 "	974	502

a. Hemagglutination units per milliliter of medium; total volume, 3 ml.

Early in these studies it was discovered that there was a variation in the quantity of virus produced by CAMs obtained from different eggs. Even when weighed amounts of CAMs were used, the variation in the rate of virus production was as great as fourfold. At first, replicate membranes were selected on the basis of the amount of hemagglutinin they produced during the first four hours. This procedure necessitated not only beginning an experiment with at least twice as many flasks as were actually used but also a rapid titration of the 1 to 4 hour yield of hemagglutinin. This technique was soon abandoned in favor of a composite CAM technique, which was used for the majority of the experiments. Using composite CAMs no measurable differences could be detected in the rates of hemagglutinin production.

The composite CAM was prepared as follows. To prepare, for example, four replicate membranes, four 14-day embryonate eggs were scrubbed with 70 percent alcohol, and the albumin end of the shell was cut off. The incision was made through the membranes, thus separating the chorio-allantois from the visceral portion of the allantois, the hole being made just large enough to allow the embryo to pass through. The allantoic raphae and large blood vessels were severed close to the CAM before the embryo and yolk sac, etc., were shaken out. The CAM remained adherent to the inner shell membrane and was washed twice by filling and emptying the CAM-lined shell with phosphate-buffered saline. The egg shell with the CAM was then cut longitudinally into four equal pieces, the sections of CAM were removed from the shell and put into four petri dishes containing 40 ml PBS. The other three eggs were treated in the same manner, the CAM sections being placed in the same four petri dishes. Each petri dish then contained one replicate composite CAM. The membranes were drained on

gauze before placing them in ESM at the beginning of an experiment.

DL-p-Fluorophenylalanine (FPA)—Two different lots of this amino acid analog were used. The one was generously supplied by Dr. Maurice D. Armstrong at the University of Utah, and the other was a product of the California Foundation for Biochemical Research, Los Angeles, California.

DL-Phenylalanine (PA)—This amino acid, used as a reversing agent for its analog FPA, was produced by the Nutritional Biochemicals Corporation, Cleveland, Ohio.

Immune Serum (IS)—Hyperimmune rabbit serum was used as a source of antibodies against PR8 virus. For the preliminary experiments pools of immune sera were prepared from several bleedings of a single rabbit which had received repeated intraperitoneal injections of allantoic fluid from virus-infected eggs. The injections and bleedings were at approximately six-week intervals. For the majority of the experiments, however, a single IS pool was used. It was obtained from one bleeding of each of three rabbits which had received the following injections of virus: on the first day, an intravenous injection of 2.5 ml, as well as an intraperitoneal injection of 5 ml, and on the second day, an intraperitoneal injection of 5 ml. After three weeks 20 ml of blood were removed from the heart of each rabbit. The IS obtained by pooling the sera from these three animals proved to be about 10 times more effective than the IS pools used in the preliminary experiments. The first sera pools were stored undiluted at 4°C. The high-titered pool was diluted to 20 percent in ESM and stored at 4°C. This pool had a hemagglutination-inhibition titer of 10,240 hemagglutination-inhibition units per milliliter of undiluted serum. Control

preparations of normal sera were prepared from pre-immunization bleedings. All sera were inactivated at 56°C for 30 minutes.

Normal-CAM Adsorbing Antigen—In order to remove antibodies against the host cells the IS was adsorbed by an antigen prepared from normal CAM. Using aseptic technique the CAMs from four dozen 14-day embryonate eggs were harvested, pooled, and washed in four changes of 500 ml PBS. The membranes then were drained on gauze, washed quickly in distilled water, and deposited in 250-ml suction flasks, each flask receiving about twelve washed membranes. Ten milliliters of distilled water were added to each flask, and the contents slowly frozen and thawed three times. The membranes then were lyophilized, i.e., dried under vacuum while in the frozen state. The dried membranes were triturated in a porcelain mortar. This was done without the use of an abrasive. The yield of powdered CAM averaged 36 to 40 mg per egg.

PR8 Adsorbing Antigen—Formalized human type-O red blood cells coated with PR8 virus were used to adsorb from IS antibody against PR8. These cells as well as uncoated formalized red cells were kindly supplied by Dr. Keith Jensen, formerly of this laboratory, and now at the Communicable Disease Center in Alabama. They were supplied in the form of a 50 percent suspension in PBS. In order to avoid the dilution effect observed when using packed, wet, red cells, they were washed with distilled water and then lyophilized. A dry powder was obtained which, when reconstituted in saline or in distilled water, appeared under the microscope as a suspension of single cells and did not hemolyse in saline or in water, although hemolysis was observed in serum. A powder was prepared from the control cells in the same manner.

Receptor-Destroying Enzyme (RDE)—This enzyme is obtained from cultures of Vibrio cholerae and has the property of reacting with the mucoid receptors of chicken red blood cells in such a way that hemagglutination of the cells by influenza virus is prevented. The proper application of this substance will also prevent the initiation of infection in the CAM (Stone, 1948). The RDE used in the present studies was prepared in this laboratory in the following manner. The allantoic cavities of 10 dozen or more 11-day embryonate eggs were inoculated with 0.1 ml of a 24-hour broth culture of the 4Z strain of the cholera vibrio. After 48 hours of incubation at 37°C, followed by thorough chilling, the allantoic fluid was harvested from the eggs, taking care not to contaminate it with yolk. The enzyme was extracted from the allantoic fluid and partially purified by the method of Ada and French (1950), which involved precipitating the enzyme with cold methanol and with ammonium sulfate, dialysis, and centrifugation. The final precipitate was redissolved in 0.05 percent calcium chloride and lyophilized. The reconstituted RDE was titrated by the method of Burnet and Stone (1947). In this assay procedure serial twofold dilutions of the enzyme are allowed to react with chicken red blood cells at 37°C in the presence of calcium ions, after which 4 HA units of virus are added. Where RDE is in sufficient concentration to destroy - or cover up - the receptors of the red blood cells, hemagglutination does not take place. The end-point of a titration is the highest final dilution of RDE which completely prevents hemagglutination by 4 HA units of virus. RDE prepared in this manner had an activity of 25,600 units per milliliter of reconstituted material.

Hemagglutination (HA) Titrations—The pattern method of Salk (1944) was used. The titration for hemagglutinin activity of influenza virus prep-

arations as it is routinely done in the laboratory is regarded to have an accuracy or reproducibility of not more than twofold. This was found to be true when performing two titrations of the same fluid at different times and with different lots of chicken red blood cells (CRBC), no matter how carefully the reagents were made and the test was performed. However, in assaying the HA activities of several fluids, differences much smaller than twofold were found to be significant if the tests were carried out carefully, if only one batch of CRBC was used, and if each titration was read after the same elapsed time.

The HA titrations were performed according to the following technique. The CRBC were prepared by the method proposed by the Committee on Standard Serological Procedures in Influenza Studies (1950), except that the cells were diluted to 0.5 percent in PBS containing two percent sodium citrate rather than in plain PBS. Citrate saline was used as a diluent throughout the test. The use of this solution inhibits the enzymatic activity of the virus (Ada and French, 1950), preventing or delaying elution of the virus from the CRBC and thus stabilizing the pattern of agglutinated cells. This was found to be especially true when assaying CAM extracts. One-half-milliliter quantities of the diluent were distributed into Kahn tubes by use of a Brewer Automatic Pipetting Machine for which, according to its manufacturer, the "...average error in consecutive deliveries is less than 1 percent." The samples were diluted in twofold steps with a Hilleman type diluter (Hilleman and Blumberg, 1952) fashioned from a Cornwall automatic syringe. An individual pipette was used for each dilution series. One-half-milliliter quantities of 0.5 percent CRBC were added by the Brewer machine. The test was read when the negative control exhibited a small button of nonagglutinated cells. This occurred in 40 to

60 minutes, for different batches of CRBC. The test was carried out in an air-conditioned room at 20-22°C, and the reagents were at this temperature when added to the tubes. All the samples from a given experiment were titrated in the same test. If there were several racks of tubes, the time of addition of CRBC was staggered so that each rack could be read after the same elapsed time.

If the test was performed as outlined above, it was found, for example, that in a set of 12 replicate titrations of one sample the pattern of agglutination in the last tube containing agglutinated cells was the same in all 12 titrations. The degree of agglutination in the last tube was designated as plus (+) if the cell pattern was in the form of a disc covering most of the bottom of the tube, as plus-minus ($\pm$) if the pattern was that of a ring with rough edges, and as minus-plus ($\mp$) if the pattern was a button of apparently nonagglutinated cells which did not flow upon tilting the rack. If the button of cells did flow it was scored as negative (o). Obviously, there were patterns which were intermediate between these, i.e., a pattern designated as (+) might contain a suggestion of a ring, the ring in a ($\pm$) pattern might have fairly smooth edges, etc., but when comparing several rows of tubes the patterns were divided easily into the three groups. Occasionally a pattern was recorded as half way between two of these. It was decided to let the intervals between (+) and ($\pm$) and between ($\pm$) and ($\mp$) represent a $2^{1/3}$ (approximately $10^{0.1}$) dilution factor. It was some time after this decision that these arbitrary end-points were compared to end-points obtained by the Horsfall and Tamm (1953) fractional dilution method which calls for 1.0 percent cells and a decilog dilution factor. Titers obtained by the two methods were different by a constant factor; therefore, in the method

used the intervals were the same as those of the decilog dilution method, i.e., about 26 percent. Horsfall and Tamm claim for their method that a difference of 35 percent is significant at the 95 percent confidence level. Although a statistical study was not carried out on the method used in this work it is believed to have an accuracy similar to that of the Horsfall and Tamm method. The titers recorded are numerically equal to the final dilution (after adding red cells) of the original fluid which would produce a (+) agglutination in the last tube showing agglutinated cells, and this then is the number of HA units per milliliter of the original material. For an illustration let us consider the following example.

Final dilution of sample	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	titer in HAU/cc
	+	+	+	+	+	+	+	+	o	256
	+	+	+	+	+	+	+	±	o	200
	+	+	+	+	+	+	+	-+	o	160
	+	+	+	+	+	+	+	o	o	128

Calcium Borate-Buffered Saline (CBS)—The calcium ion and a pH near neutrality are requisite for optimal activity of RDE and of the virus enzyme on their substrate, the mucoid receptors of red blood cells and of the cells of the CAM (Burnet and Stone, 1947). This saline was employed as a diluent in titrations of RDE and in the extraction of virus from the CAM, in both of which cases enzymatic activity is involved. The composition is given below.

The Composition of Calcium Borate-Buffered Saline (CBS)

$CaCl_2$ (anhyd)	1.0 g
NaCl	8.5 "
H_3BO_3	1.203 "

$Na_2B_4O_7 \cdot 10H_2O$	0.052 g
Glass distilled water to make	1000 ml

The pH was adjusted to 7.0 with M/10 HCl or M/10 NaOH.

Hemagglutination-Inhibition (HAI) Titrations—The HAI titer of immune serum was determined according to the method used in this laboratory, i.e., 0.25-ml antigen diluted to contain 16 HAU per milliliter is added to each 0.25-ml twofold dilution of serum. The mixture is incubated for 30 minutes at room temperature; 0.5 ml of 0.5 percent chicken red blood cells is added and the test is read when the cell control tube shows a small button of nonagglutinated cells. The serum dilutions were made by mixing 0.5-ml amounts, rather than the usual 0.25-ml amounts. One quarter of a milliliter of each dilution was transferred to another set of tubes as the dilutions were made, a separate pipette being used for each dilution. The HAI titers were calculated in a manner similar to that used for the calculation of the HA titer and were recorded as HAI units per milliliter of original material.

Infectivity Titrations—As a measure of infectivity the EID_{50} (50 percent egg infectious dose) was determined. This is the dilution of the original material which will infect exactly one half of the eggs inoculated with that dilution. The sample being tested was diluted in plain broth, containing 500 units of penicillin and 500 micrograms of streptomycin per milliliter, by tenfold steps to the lowest dilution to be inoculated and then by two-, four-, or tenfold steps to the highest dilution. One-tenth milliliter of each of the latter dilutions were inoculated into the allantoic sac of each of 4 or 6, 10- or 11-day embryonate eggs. After an incubation period of 48 hours at 37°C, the eggs were chilled and their

allantoic fluids tested for presence or absence of hemagglutinin. The end-point was calculated by the method of Reed and Muench (1938). The titers are expressed as egg-infective doses (EID_{50}'s) per milliliter of original material.

As an illustration, below are the results of an egg infectivity titration of the 147th passage of the PR8 strain of type A influenza virus in embryonate eggs. This passage was designated PR8-E147. In this case each of six 10-day embryonate eggs were inoculated with 0.1 ml of each twofold dilution from $10^{-8.0}$ to $10^{-10.4}$.

Egg Infectivity Titration of PR8-E147

Dilution[a]	Infected	Not Infected	Σ Infected[b]	Σ Not Infected[c]
$10^{-8.0}$	6	0	27	0
$10^{-8.3}$	6	0	21	0
$10^{-8.6}$	5	1	15	1
$10^{-8.9}$	3	3	10	4
$10^{-9.2}$	3	3	7	7
$10^{-9.5}$	2	4	4	11
$10^{-9.8}$	1	5	2	16
$10^{-10.1}$	1	5	1	21
$10^{-10.4}$	0	6	0	27

a. One twofold dilution equals $10^{-0.30103}$ or approximately $10^{-0.3}$.
b. Summation from greatest to lowest dilution.
c. Summation from lowest to greatest dilution.

The end-point of this titration is at the dilution $10^{-9.2}$ or, in terms of concentration, $10^{9.2}$ EID_{50} per one tenth-milliliter or $10^{10.2}$ EID_{50} per milliliter.

Extraction of Virus from the CAM—At various times after initiation of infection of the CAM and at the termination of each experiment (usually at 24 hours), the amount of virus in (or closely associated with) the membrane was determined. This virus is referred to as pre-emergent virus (PEV). The name is not to be taken as indicating that this virus is necessarily all intracellular. It is defined operationally as the amount of virus which has not "emerged" into the suspending medium during incubation and is not removed by repeated washings in PBS at 20°C. More will be said concerning the location of PEV in a later section. The quantitative determination of PEV presented somewhat of a problem. It was necessary, of course, to extract the virus from the membrane into a liquid so that it could be measured by the conventional dilution technique. The problem was to establish with certainty that the extraction was quantitative.

In all cases the CAM was ground in a porcelain mortar using a minimum quantity of alundum as abrasive. Grinding was continued until the membrane was reduced to a smooth cream, which then was diluted with 3 milliliters extractant. After extraction the brei was centrifuged at 2000 rpm for ten minutes and the supernatant fluid preserved.

A series of experiments was performed in order to determine the best conditions for complete extraction. In the following description "infected CAM" refers to one infected with $10^{8.68}$ EID_{50} virus in 3 ml ESM, freed of residual inoculum after one hour by washing once in 50 ml PBS, returned to ESM, and incubated for 5 more hours at 37°C, at which time the CAM was washed in two changes of 50 ml of PBS at 20°C.

A. To determine the best extracting medium: Eight infected composite CAMs were ground as outlined above and the breis were extracted for four

hours, two each in four different fluids—plain broth, PBS, ESM, and CBS (calcium borate saline)—one at 20°C and the other at 37°C. After centrifugation the supernatant fluid was diluted serially in 1.5-ml amounts by twofold steps in 2 percent citrate saline. One-half milliliter of each dilution was then transferred to each of two sets of tubes. Prior to adding red blood cells, one set was incubated at 20°C and the other at 37°C for one hour. The results are shown in Table II.

TABLE II

THE EFFECT OF EXTRACTANT AND TEMPERATURE ON THE EXTRACTION OF VIRUS FROM THE CHORIO-ALLANTOIC MEMBRANE

Incubation Temp.		Extractant			
Brei[a]	Dilutions[b]	Broth	PBS[c]	ESM[d]	CBS[e]
20°C	20°C	200[f]	200	200	400
20°C	37°C	256	320	400	640
37°C	20°C	320	320	400	800
37°C	37°C	400	512	640	800

a. The brei was extracted for 4 hours at the temperature in this column.
b. The dilutions were incubated for 1 hour, prior to adding red cells at the temperature in this column.
c. Phosphate-buffered saline.
d. Earle's saline medium.
e. Calcium borate-buffered saline.
f. Hemagglutinating units per milliliter of supernatant fluid; total volume, 3 ml.

The results indicated that incubation at 37°C was preferable to incubation at 20°C, that CBS was the best of the four extractants tested, and that in this case further incubation of the dilutions at 37°C did not result in a more complete extraction. These results were to be expected since enzymatic action is involved in the elution of virus from the cell—in this case from the cell fragments—and calcium ions and incubation at 37°C both favor this action.

B. To determine the time required for maximum elution and to determine whether the addition of RDE increased the degree of elution: For this experiment two sets of eight infected composite CAMs were used. They were ground and then extracted by incubation at 37°C in CBS and in CBS containing 2,560 units of RDE per milliliter. One set of replicate membranes was extracted for 1, 2, 3, and 4 hours, the other set for 6, 8, 12, and 24 hours. The breis were centrifuged after incubation and the supernatant fluids stored at 4°C, so that all the fluids could be tested together. The results of this experiment are shown in Table III. From these data it is seen that maximum extraction is obtained in four hours of incubation at 37°C in CBS alone and in three hours of incubation in RDE-containing CBS.

TABLE III

THE EFFECT OF INCUBATION TIME AND RECEPTOR-DESTROYING ENZYME ON THE EXTRACTION OF VIRUS FROM THE CHORIO-ALLANTOIC MEMBRANE

Extractant	Incubation Time in Hours[a]							
	1	2	3	4	6	8	12	24
CBS[b]	200[c]	320	512	640	640	640	640	640
CBS + RDE[d]	400	512	640	640	640	640	640	640

a. The length of time the brei was extracted at 37°C.
b. Calcium borate-buffered saline.
c. Hemagglutinating units per milliliter of extractant; total volume, 3 ml.
d. Receptor-destroying enzyme - 7,680 units.

C. To determine whether the residue of an extracted infected CAM has the ability to combine with HA-inhibiting antibody: Four composite CAMs, two infected and two not, were prepared. One infected and one normal CAM were extracted at 37°C in CBS for 7 hours, and the other pair at 4°C. The breis were centrifuged, the supernatant fluids tested for HA activity,

and the sediments washed with cold PBS and tested for their ability to remove HA-inhibiting antibody from immune serum. For this determination the washed sediments were resuspended in 3 ml of 1:80 IS, incubated for 20 minutes at 20°C, centrifuged, and the supernatant fluids titrated for HAI activity. The results are shown in Table IV. The supernatant fluid from the infected membrane extracted at 4°C contained submaximal HA activity, and the sediment from this extracted membrane removed HAI antibody from the immune serum. Extraction at 37°C for seven hours left no detectable HAI antibody-combining activity in the sediment.

TABLE IV

ABILITY FOR EXTRACTED CHORIO-ALLANTOIC MEMBRANES TO COMBINE WITH HEMAGGLUTINATION-INHIBITING ANTIBODY

CAM	Temperature[a]	HA of Extract[b]	HAI Titers of IS[c]
Normal	4°C	0	128
Normal	37°C	0	128
Infected	4°C	128	<8
Infected	37°C	640	128

a. Temperature at which the membranes were extracted for seven hours in calcium borate-buffered saline.
b. Hemagglutinating units per milliliter of supernatant fluids; total volume, 3 ml.
c. Hemagglutination-inhibiting units per milliliter of 1:80 immune serum after incubation for 30 minutes at 20°C with the washed sediment of the extracted membranes.

The results of the last experiment indicated that incubation at 37°C for seven hours in calcium borate-buffered saline gives complete extraction of virus from the CAM. This procedure was adopted.

D. The lack of hemagglutination inhibition by extracts of normal chorio-allantoic membrane: To determine whether the hemagglutinin

inhibitor present in normal chorio-allantoic membranes (Schlesinger and Karr, 1956a) interfered with the measurement of HA in the CAM extract, a normal CAM was triturated and extracted into virus-containing ESM. The HA titer of this was compared to that of the original virus-containing medium. Although the patterns of agglutination were somewhat different the end-point was the same.

EXPERIMENTS AND RESULTS

As mentioned in the Introduction, the scope of this investigation was confined to a study of the intracellular stages of infection of the chorio-allantoic membrane (CAM) with PR8 influenza virus. Information about these stages was obtained by studying the effect (on the production of virus) of p-fluorophenylalanine (FPA), which inhibits the biosynthesis of protein in the host cells, and that of immune serum (IS), which is directed specifically against viral protein. For these investigations to be meaningful it was imperative to study first the production of virus by normal tissue, and also to perform experiments using FPA and IS separately, to serve as controls.

This section will be divided into five parts:

I. The production of virus by the normal CAM,

II. The effect of FPA on the production of virus,

III. The effect of IS on the production of virus,

IV. The ultracentrifugal sedimentation of extracted and spontaneously released virus, and

V. Summary and conclusions.

I. The production of virus by the normal chorio-allantoic membrane.

In order to gain an understanding of processes occurring in the individual cells from the data on the infection of the CAM, all the cells of the infected CAM had to be in the same stage of the infectious process at any one time. This was done by synchronizing the initiation of infection of the cells by using a large inoculum of virus. The inoculum required to give a single cycle of infection was $10^{8.68}$ EID_{50} in 3 ml.

This was obtained by diluting the stock pool of virus to 10^{-2} in Earle's saline medium (ESM).

That this concentration of virus produced a single cycle of infection is shown by data from the following experiment. Eight replicate composite CAMs were infected in duplicate with inocula of $10^{6.68}$, $10^{7.68}$, $10^{8.68}$, and $10^{9.68}$ EID_{50}. After one hour the membranes were washed in 50-ml phosphate-buffered saline (PBS) and then the two groups were further incubated in 3 ml ESM containing either 7,680 units RDE or no RDE. (The presence of RDE prevented infection by virus released during the first cycle.) After 24 hours the yield of virus was determined by hemagglutination (HA) titration. The use of 2 percent citrate-PBS as diluent prevented interference by RDE. The results of this experiment are shown in Table V.

TABLE V

THE ONE-CYCLE AND MULTIPLE-CYCLE YIELD OF VIRUS FROM CHORIO-ALLANTOIC MEMBRANES INFECTED WITH INOCULA OF DIFFERENT SIZES

Inoculum Size		Virus Not Adsorbed[d]	Virus Adsorbed[e]	Yield of Virus[a]	
EID_{50}[b]	HA[c]			With RDE[f]	Without RDE
$10^{6.68}$	<2	<2	<2	20[g]	400
$10^{7.68}$	4	<2	4	400	1280
$10^{8.68}$	40	20	20	3200	3200
$10^{9.68}$	400	256	144	3200	3200

a. Virus appearing in the fluid from 1 to 24 hours.
b. Egg-infective doses in 3 ml.
c. Hemagglutinin units per milliliter; total volume, 3 ml.
d. Hemagglutinin remaining in the inoculum after removal of the membrane.
e. Activity of original inoculum minus that remaining after one hour.
f. Receptor-destroying enzyme (7,680 units) in 3 ml present after one hour.
g. All titers in hemagglutinin units per milliliter; total volume, 3 ml.

It is evident that all the cells of the CAM were infected with the inocula of $10^{8.68}$ and $10^{9.68}$ EID_{50}, for the presence of RDE did not affect the yield of virus in these cases. The inoculum of $10^{8.68}$ EID_{50} provided a total exposure of the CAM to 0.693* x $10^{8.68}$ or approximately 3.3 x 10^8 egg-infective particles. Cairns and Edney (1952) concluded that the 11- to 13-day old allantois (in situ) contains 1.6 x 10^8 susceptible cells. As mentioned before, the allantois obtains its maximum wet weight at 10 days (Needham, 1950), and since at 14 days about two-thirds of the allantois is represented in the CAM, there are probably about 10^8 cells in the 14-day CAM. In other words, the inoculum of 3.3 x 10^8 egg-infective particles per milliliter in the above experiment represents a ratio of 3.3 infectious particles per cell. While the higher concentration probably shortened the time required to infect all the susceptible cells (Ishida and Ackermann, 1956), this inoculum resulted in the adsorption of a large excess of virus (124 HAU per milliliter) over that required to give complete infection (20 HAU per milliliter).

The time required for infection of all the susceptible cells of the CAM.—Four replicate composite CAMs were prepared and infected with an inoculum of $10^{8.68}$ EID_{50} of PR8 virus. After 15, 30, or 60 minutes, three of them were washed with 50 ml PBS and then further incubated in ESM containing 7,680 units of RDE in order to prevent further infection by the inoculum virus. The fourth was washed at one hour and then incubated in ESM with no RDE present. Incubation was continued for 24 hours, at which time the HA activities of the fluids were determined. In addition, a

*From statistical theory, the dilution which infects 50 percent of the eggs inoculated with that dilution contains 0.693 infectious particles per milliliter.

fifth whole CAM was infected and incubated the entire 24 hours in the presence of RDE. The results of this experiment are given in Table VI.

TABLE VI

THE YIELD OF VIRUS FROM THE CHORIO-ALLANTOIC MEMBRANE AFTER VARIOUS TIMES OF CONTACT WITH THE INOCULUM

Time RDE[a] Added	Yield of Virus[b]
None	2560
60 minutes	2560
30 "	2560
15 "	2560
0 "	32

a. Receptor-destroying enzyme (7,680 units in 3 ml) added at the designated time after addition of inoculum containing $10^{8.68}$ EID_{50} of PR8 virus in 3 ml.

b. Yield of new virus from the CAM in hemagglutinin units per milliliter; total volume, 3 ml.

The data indicate that 15 minutes or less was sufficient time for the inoculum of $10^{8.68}$ EID_{50} to initiate infection in all susceptible cells of the CAM, since a higher yield was not obtained after a longer contact of the CAM with the inoculum.

The rate of release of virus from the chorio-allantoic membrane.— The rate of release was obtained by following the appearance of virus in the fluid phase of the culture, i.e., the release of virus from the CAM. A study of this kind does not yield information concerning the rate of production ("growth") of virus, as will be seen later.

The kinetics of release were established by using three experimental procedures, which are outlined below:

(1) Sampling technique: A single entire CAM was incubated at 37°C for one hour in an inoculum of $10^{8.68}$ EID_{50}. The residual inoculum was removed by washing in 50 ml PBS; and the CAM was returned to 3 ml ESM. Thereafter, at hourly intervals up to 10 hours and then at 12, 15, 18, and 24 hours, the medium was sampled by removing one milliliter. The volume was maintained at 3 ml by adding one milliliter fresh prewarmed ESM after each sampling. At 24 hours the CAM was washed in two changes of 50 ml PBS and then extracted. (See extraction procedure in Methods and Materials section, page 33.) All the samples including the CAM extracts then were tested for HA activity.

(2) Complete change of the medium: This procedure gave the incremental yield. A single CAM was infected as in the sampling technique, and then at intervals it was removed from the flask, drained on gauze, and placed in a new flask containing fresh ESM. The time intervals were the same as those given above under (1). At 12, 15, and 18 hours, the CAM was washed in one change of 50 ml PBS at 20°C and drained before placing in the new flask. The CAM was extracted at the termination of the experiment. All the fluids were tested for HA activity.

(3) Use of a nonreplicate composite CAM for each time interval: The data were obtained from 15 experiments, each performed on a different day. (The experiments were conducted about a year later than those described under (1) and (2). The CAMs involved were actually control membranes for a series of experiments in which the amino acid analog p-fluorophenylalanine and immune serum were used.) For each experiment six replicate composite CAMs were employed. They were infected as above. The control membranes then were incubated for various lengths of time (a different time for each experiment) in 3 ml ESM. At appropriate times the CAMs were

removed from the suspending medium, washed in two changes of PBS at 20°C, incubated for 15 minutes at 37°C in fresh ESM, washed, and returned to fresh prewarmed ESM for the remainder of the 24-hour period. At the termination of each experiment the control CAMs, as well as the others, were washed and extracted. All the fluids from one experiment were tested at the same time, which means that the titers used to determine the rate of release were obtained by several different tests performed on different days and with different lots of reagents. From each experiment the virus produced up to a certain time, as well as the virus produced from that time to 24 hours, was determined. The time intervals were the same as those of procedures (1) and (2).

A curve representing the rate of release of the virus from the CAM as a function of time could be constructed from the data obtained by each of these procedures. However, since the total virus produced in each case varied within fairly wide limits owing to the differences in the CAMs and the differences in the lots of reagents used for the HA tests, the curves would not be subject to direct comparison. By taking the total virus produced in each case (including that in the CAM extract at 24 hours) to be 100, and expressing all the fractional yields as percent of the total yields, the data are easily interpreted. The HA titers and their conversion to percentages are tabulated in the Appendix, pages 100, 101, and 102. The converted data are plotted in Fig. 1. (See also curve B in Figs. 2 and 3.)

It is remarkable that a single smooth curve could be drawn through a plot of the converted data obtained by the three procedures. This indicates that the rate of release of virus (on a percentage basis) is a fixed characteristic for the host-virus system used in these studies,

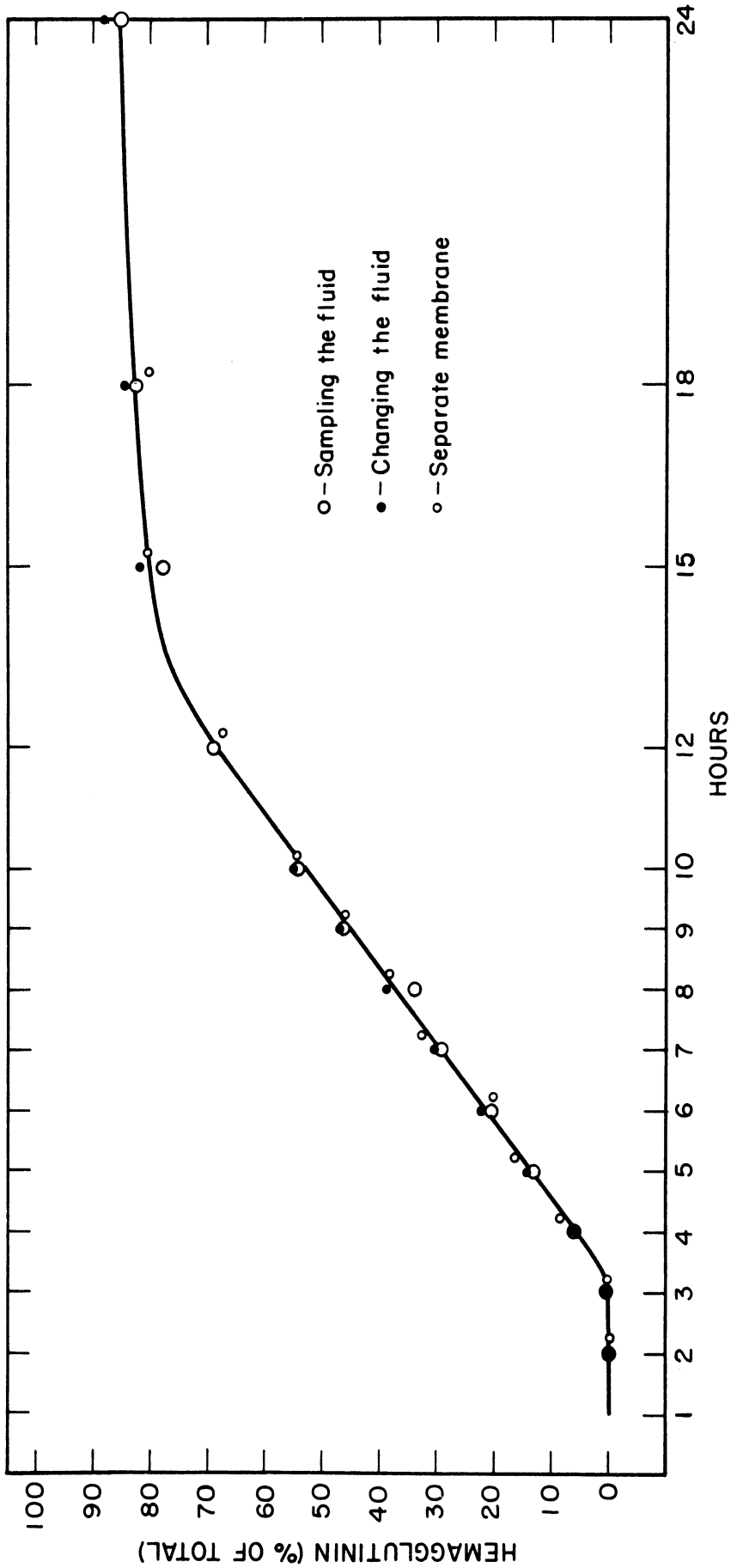


Fig. 1. The release of virus from the chorio-allantoic membrane.

for it is not affected by manipulation of the CAM, or by accumulation of virus or products of metabolism, or by pH, which is fairly acid after an incubation of 24 hours in the same medium due to solution of evolved carbon dioxide. The release of virus begins at about 3 hours after initiation of infection and is constant from about 3-1/2 hours until 12 hours. At about 10 hours 50 percent of the virus has been released. After 12 hours the rate of release decreases so that at 24 hours only 85 percent of the total virus has been released.

The variation in the amount of pre-emergent virus (PEV) with time of incubation.—The PEV is defined operationally as the amount of virus (HA) in the infected CAM after repeated washing at any time during the period of incubation. It is measured by HA titration of the CAM extract. (See extraction procedure in the Methods and Materials section, page 33.) Data for the variation of PEV with time were obtained from the same series of experiments referred to in the third method of determining the rate of release (see page 42). The infected CAMs were removed from incubation and washed as above, but, instead of incubating them in ESM for the remainder of the 24-hour period, they were stored at 4°C until the termination of the experiment, and then extracted. The extracts were tested for HA activity, and the titers were converted to percentage of total virus produced. The data are tabulated in the Appendix, page 102. The PEV plotted against time is shown in Fig. 2 (curve A).

The growth curve.—By adding the amounts PEV and virus released, at any time, a curve was constructed which represents the rate of production of hemagglutinating virus. This curve appears in Fig. 2 (curve C) along with the curves representing the rate of release of virus (curve B) and the pre-emergent virus (curve A). In Fig. 3 the slopes of these

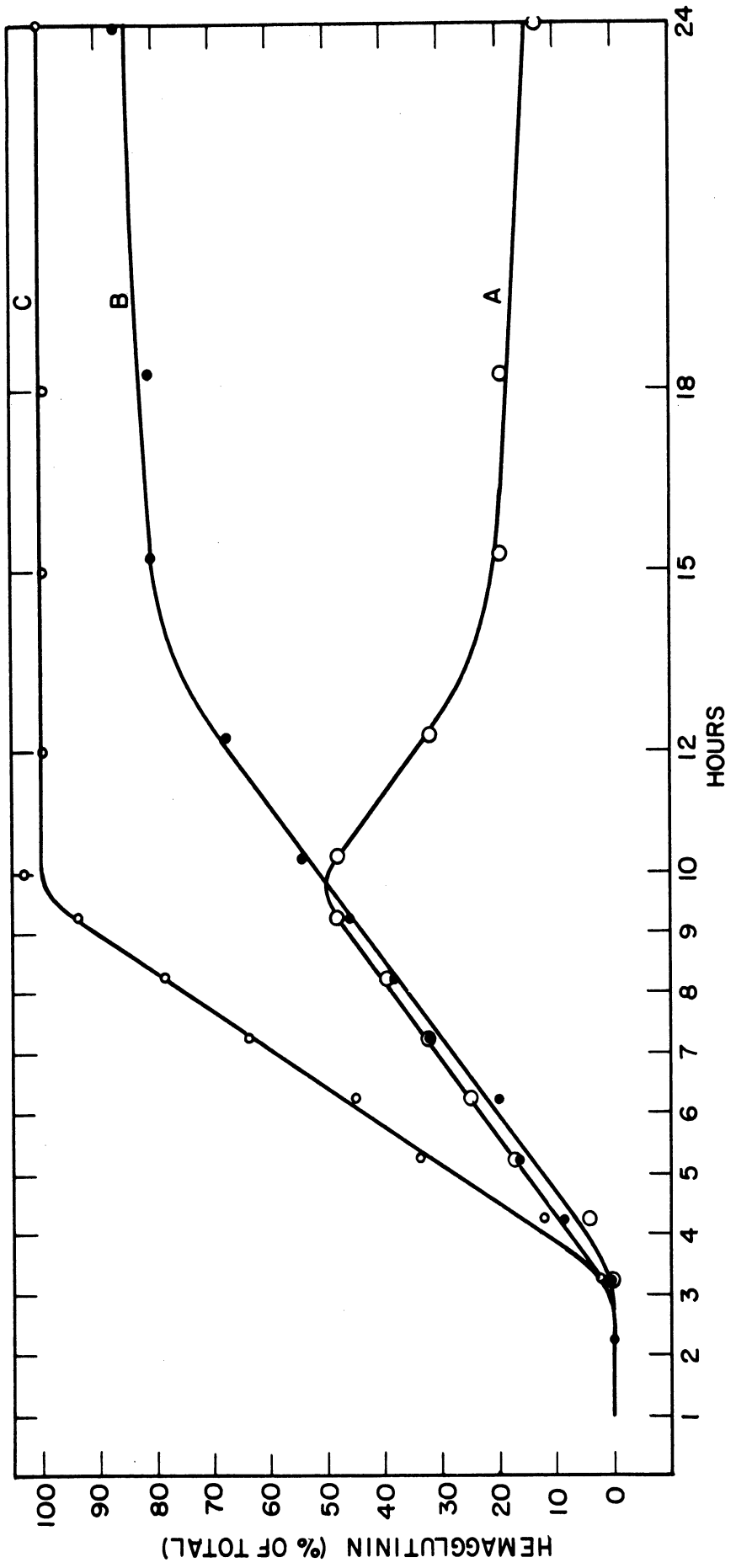


Fig. 2. The production and release of virus.—Curve A represents the pre-emergent virus (PEV) in the chorio-allantoic membrane. Curves B and C show the release of virus from and the total virus produced by the chorio-allantoic membrane, respectively.

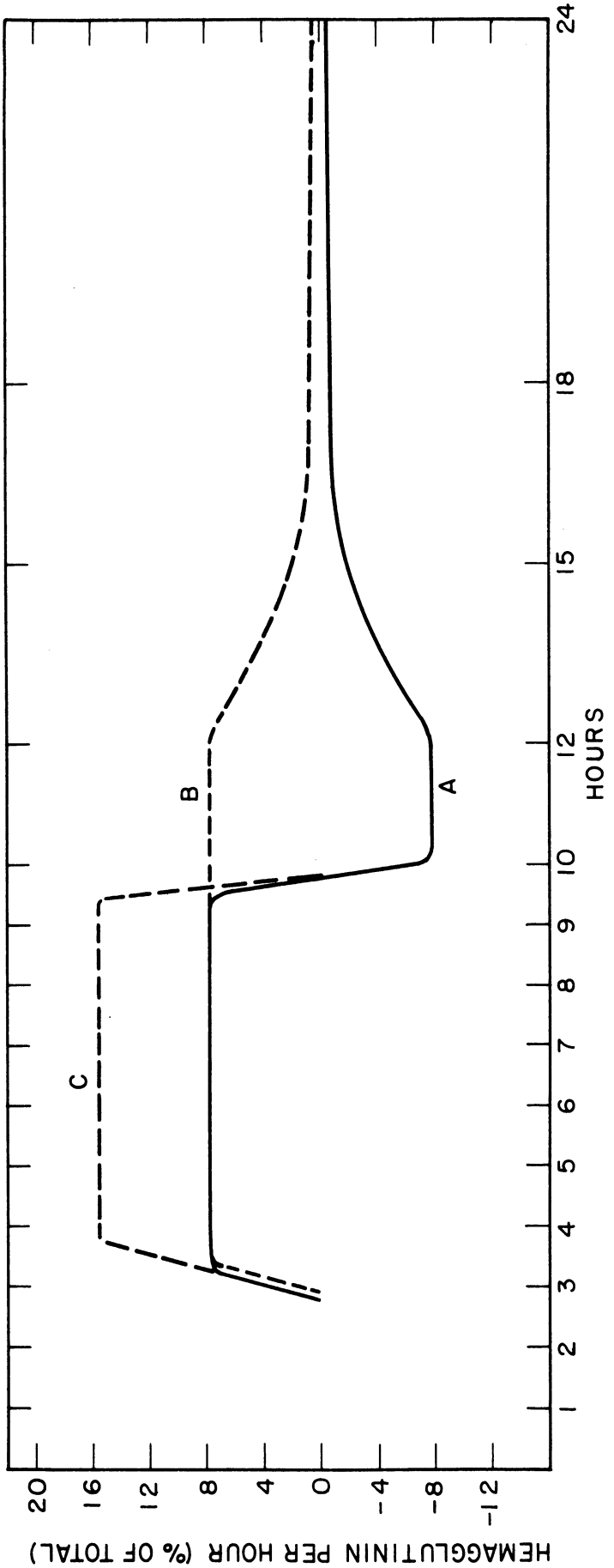


Fig. 3. The rates of production and release of virus.—Curve A represents the rate of change in the amount of pre-emergent virus. Curves B and C show the rates of release of virus from and production of virus in the chorio-allantoic membrane, respectively.

curves are plotted, showing the rate of change of the PEV content of the CAM and the variation in the rates of release and production of virus, curves A, B, and C, respectively.

These curves reveal several interesting characteristics of the production of PR8 influenza virus by the CAM. The first detectable virus appears in the membrane between 2-1/2 and 3 hours but it is not until about 3 percent (of total virus) has accumulated that release begins. This may indicate a sort of pressure that is needed before release can take place. In about one-half hour after each process begins the rates of accumulation of PEV and of release become constant and equal. (Compare curves A and B in Figs. 2 and 3.) Until about 10 hours the CAM contains a fixed greater amount of virus at any time than has been released. This results from the lag of first release behind the first appearance and from the fact that both these processes reach their constant rate in about the same length of time. At about 10 hours the production of virus is complete, for the membrane and fluid phase each contain 50 percent of the total virus.

As a consequence of the rate of release and rate of increase of PEV being constant and equal, the rate of production (see curve C in Figs. 2 and 3) is also constant and twice the rate of release, except for a short time at its beginning and end.

After the production of virus is complete the PEV which has accumulated in the CAM continues to be released at the same rate for about two hours, which means that the PEV is decreasing from 10 to 12 hours at the same rate at which it was increasing before 10 hours. After 12 hours the rate of release falls off rather rapidly.

These characteristics of the production and release of virus from the CAM indicate that the replication of the PR8 strain of influenza virus in a single cell of the CAM may proceed by the following scheme. Soon after the initial interaction of virus and host cell, a sequence of biochemical reactions is initiated, the end product of which is mature virus, mature being defined here as having the ability to agglutinate red blood cells. At some time between 2 and 3 hours after infection, the synthetic machinery turns out the first virus particle. For the next 7 hours or so mature virus is produced at a constant rate of about 15 percent per hour. At about 10 hours the production of virus stops.

> The total virus produced by each of the 17 CAMs used in the foregoing experiments ranged from 6,360 to 14,400* hemagglutinating units. With the technique of titration for hemagglutinin used for these studies, the conversion factor for hemagglutinating units to infectious particles was found to be $10^{6.5}$ or 3.16×10^6 (see page 20). We obtain, then, a total yield of $(6{,}360 \text{ to } 14{,}400) \times 3.16 \times 10^6$ or 2.01×10^{10} to 4.55×10^{10} particles.† If we assume that there are 10^8 susceptible cells in the CAM (see page 40), then the total yield of virus was 201 to 455 particles per cell. Fazekas de St. Groth and Cairns (1952) found 67 to 120 particles per cell released by 10 hours in ovo.

Thus mature virus is being produced at the rate of 30 to 68 particles† per cell per hour, or for the average cell about one particle every 45 seconds. There is a limit to how fast the cell can release this virus. The maximum rate is one half the rate of production, or, for the average cell, one particle every 1-1/2 minutes. This maximum

*In the tabulation in the Appendix (page 102, column H) the yields are recorded as 2120 to 4800 HAU per milliliter. The total yield is obtained by multiplying these titers by three since the total volume in each case was 3ml.

†This is assuming that all the virus particles are infective. Although a single infectious particle is sufficient to produce infection in the embryonate egg, there is evidence (Evans, 1956) that on the average about ten infectious particles must be inoculated into an egg for a successful infection.

rate of release continues for about 2 hours after the last mature virus particle has been produced. After 12 hours the virus pool that has accumulated in the cell is released more slowly. This slow rate of release after 12 hours may be due to permeability changes in the cell membrane caused by the infection, or simply to the aging of the cell in an unnatural environment; or it could reflect a nonreleasable fraction that is perhaps bound to injured or imperfect receptors on the cell. It has been shown, for example, by Ackermann, Ishida,and Maassab (1955) that under certain conditions infectious virus is bound to the CAM, that this bound virus does not spontaneously elute,and that it is not removed by washing in saline. They used infectivity as a measure of virus activity and worked with inoculated virus rather than with released virus and found that the ratio of the bound fraction to the unbound fraction was about one to one thousand. If we assume that about 14 percent of the virus is nonreleasable, the rate of release after 12 hours appears to be proportional to the releasable fraction remaining in the membrane.

II. The effect of p-fluorophenylalanine (FPA) on viral synthesis.

The effect of the para-fluoro derivative of the amino acid phenylalanine has been studied in several biological systems. Creaser (1955) demonstrated that the induced adaptive biosynthesis of β-galactosidase in Staphylococcus aureus is inhibited by FPA and that the inhibition can be reversed by phenylalanine (PA). Chantrenne and Courtois (1954) presented evidence that the oxygen-induced formation of catalase in baker's yeast is inhibited by FPA. In an assay of fifty amino acid analogs for toxicity to tissue cultures of mouse heart and sarcoma T241, Jacquez and Mottram (1953) found that selective toxicity was rare and that of the halogenated phenylalanines only the fluoro derivatives were competitive

antagonists of phenylalanine. Bergmann et al. (1953) showed that FPA inhibits the growth of Escherichia coli (wild type). On the basis of experiments using the tryptophaneless, phenylalanineless, and tyrosineless mutants they concluded that p-fluorophenylalanine blocks the conversion of phenylalanine to tyrosine. Thus, it appears that FPA acts by inhibiting protein synthesis and that its mechanism of action is that of interfering with the utilization of the amino acid phenylalanine. FPA has been shown to exert an inhibiting effect upon the synthesis of influenza A virus in the cells of the chorio-allantoic membrane (Ackermann and Maassab, 1955). These authors have shown that FPA does not interfere with the adsorption of virus, with the initiation of infection, or with the release of virus, and that its inhibitory action could be demonstrated over a large part of the viral-productive period. They noted, however, that its effectiveness decreased as the inhibitor was added later during this period. In the present study it will be shown that the kinetics of inhibition of viral synthesis by FPA is consistent with the known mode of action of the inhibitor. Also, data acquired by the use of FPA will be utilized to elucidate the nature of an intracellular stage in the synthesis of virus.

The complete suppression by p-fluorophenylalanine of viral synthesis.— For each of two experiments four replicate composite CAMs were infected with an inoculum of $10^{8.68}$ EID_{50} in the presence of 0.3 to 3.6 mg FPA. After one hour the membranes were washed free of residual inoculum and further incubated in the same concentrations of the inhibitor. A membrane receiving no FPA was included in each experiment. After 24 hours the fluids were tested for HA activity. The results, shown in Table VII, indicated that although 0.3 mg of FPA produced over 80 percent inhibition, eight times this amount (2.4 mg) was required to produce complete inhibition.

TABLE VII

THE YIELD OF VIRUS FROM CHORIO-ALLANTOIC MEMBRANES INFECTED AND INCUBATED IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF p-FLUOROPHENYLALANINE

FPA, mg in 3 ml[a]		Yield of Virus in HAU/ml[b]		Percent Inhibition	
0.0[c],	0.0[c]	4096,	1600	0 ,	0
0.3		800		80.47	
0.6		200		95.12	
1.2,	1.2	10	3	99.76,	99.81
	2.4		0		100
	3.6		0		100

a. Amount of p-fluorophenylalanine in which the membranes were infected and incubated.
b. Yield of virus from 1 to 24 hours; in hemagglutinin units per milliliter; total volume, 3 ml.
c. Two experiments.

The effect of p-fluorophenylalanine added at six hours on viral synthesis.—Four CAMs, infected with $10^{8.68}$ EID_{50} in 3 ml, were incubated for six hours in ESM, and washed in two changes of PBS at 20°C. One CAM was stored at 4°C, one was incubated until 24 hours in 3 ml ESM, and the other two, in 3 ml ESM containing 2.4 and 3.6 mg FPA, respectively. At appropriate intervals they were washed and returned to fresh media. The four membranes were extracted at the end of the experiment. The fluids and extracts were tested for HA activity. The results appear in Table VIII.

It is evident that 2.4 mg FPA added at six hours gave maximal suppression of subsequent viral yield. (See last column of Table VIII.) The CAM treated with this concentration of FPA produced 1,794 HAU, while the membrane contained only 1,024 HAU at the time the inhibitor was added,

TABLE VIII

THE YIELD OF VIRUS FROM THE CHORIO-ALLANTOIC MEMBRANE SUBSEQUENT TO ADDITION OF p-FLUOROPHENYLALANINE AT SIX HOURS

FPA[a], mg in 3 ml	Yield of Virus							
	During Time Intervals[b] (In Hours)						In CAM[c]	Total 6—24
	1—6	6-9.5	9.5-13	13-17.5	17.5-20	20-23.5		
0	800[d]	1280	1024	320	80	32	640	3376
2.4	800	1024	512	160	32	16	50	1794
3.6	800	1024	512	200	32	16	50	1830
0	800	In CAM at 6 hours - 1024						

a. Fluorophenylalanine present in media after 6 hours.
b. Membranes incubated in a separate flask with fresh media during each time interval; washed in two changes physiological buffered saline at 20°C between intervals.
c. Membranes extracted at 24 hours; titer of extract.
d. Hemagglutinin units per milliliter; total volume, 3 ml.

i.e., addition of FPA at six hours did not produce complete suppression of virus production. It will be shown, however, that this concentration of FPA did completely inhibit the subsequent synthesis of viral protein.

The prevention by phenylalanine (PA) of inhibition caused by p-fluorophenylalanine.—Four replicate composite CAMs were infected by $10^{8.68}$ EID_{50} in 3 ml ESM, or in 3 ml ESM containing FPA, PA, or both FPA and PA. They were washed after one hour and incubated until 24 hours in media of the same respective composition. Four additional CAMs were infected for one hour, washed, further incubated for 5 hours in 3 ml ESM, washed again, and then incubated from 6 to 24 hours in media containing FPA and PA as above, the membranes being washed and the media renewed at 12 hours. At 24 hours the latter four membranes were extracted in the fluids in which they were incubated from 12 to 24 hours. The amount of FPA used was 2.4

mg in 3 ml and that of PA was 4.4 mg in 3 ml, the latter being twice the former on a molar basis. The results are shown in Table IX.

TABLE IX

PREVENTION BY PHENYLALANINE OF INHIBITION CAUSED BY p-FLUOROPHENYLALANINE

Treatment		Yield of Virus			
0 to 24 hr	6 to 24 hr	1 to 24 hr	1 to 6 hr	6 to 12 hr	12 to 24 hr[a]
-		1024[b]			
PA[c]		1024			
FPA[d] + PA		1280			
FPA		0			
	-		256	640	512
	PA[c]		256	640	512
	FPA[d] + PA		256	640	512
	FPA		256	400	128

a. The 12- to 24-hour titers include the virus content of the membrane at 24 hours.
b. Hemagglutinin units per milliliter.
c. Phenylalanine; 4.4 mg in 3 ml in each case.
d. p-Fluorophenylalanine; 2.4 mg in 3 ml in each case.

The data indicate that the inhibition due to FPA did not occur in the presence of PA. This result was significant because it showed that the effect of the inhibitor preparation was due entirely to the interference with the utilization of phenylalanine by FPA. If, for example, free fluoride ions had been present in the FPA solution in high enough concentration, they would be toxic to the tissue and might inhibit viral synthesis. This noncompetitive inhibition would not be prevented by PA.

The incremental release of virus subsequent to addition of p-fluorophenylalanine at six hours.—Two nonreplicate CAMs, were infected for one hour with an inoculum of $10^{9.68}$ EID_{50}, were incubated in 3 ml ESM at 37°C until 6 hours. From 6 to 24 hours one was further incubated in 3 ml ESM and the other in 3 ml ESM containing 3.3 mg FPA. The CAMs were washed and the media renewed at hourly intervals until 12 hours. All fluids were tested

for HA activity. The HA titers are tabulated in the Appendix (page 103), and the data are plotted in Fig. 4. Curves A and B show the incremental yields of virus, and curves C and D show the accumulative yields (obtained by successive summation of the incremental yields) for the inhibited and control membranes, respectively. It can be seen that while the rate of release of virus from the control membrane remained constant until 12 hours, that from the membrane receiving FPA decreased after addition of the inhibitor. The rate of decrease was exponential (first-order kinetics), suggesting that at any time the rate of release was proportional to the amount of virus remaining in the CAM at that time.

Production of virus subsequent to administration of the maximal inhibitory level of p-fluorophenylalanine: its variation with time of addition of the inhibitor.—Data relevant to the variation of inhibition with the time of addition of FPA were obtained from the same series of experiments utilized for the third procedure for determining the rate of release and for the study of the variation of the pre-emergent virus. (See pages 42 and 45.) After appropriate periods of incubation in normal medium the membranes were incubated for the remainder of the 24-hour period in 3 ml ESM containing 2.4 mg FPA. At the termination of the experiment the CAMs were extracted. HA titrations were performed on the fluids and extracts. The data and their conversion to percentages are tabulated in the Appendix, page 104. In Fig. 5 the yield of virus (the virus released plus that in the CAM at 24 hours) subsequent to addition of FPA is plotted against time of addition of FPA, for the inhibited membrane (curve A) and the control membrane (curve B). Curve C shows the variation of the percentage of inhibition produced by addition of FPA at these times.

It is seen that if FPA is present from the first hour there is no release of virus (100 percent inhibition) and that if FPA is not present until

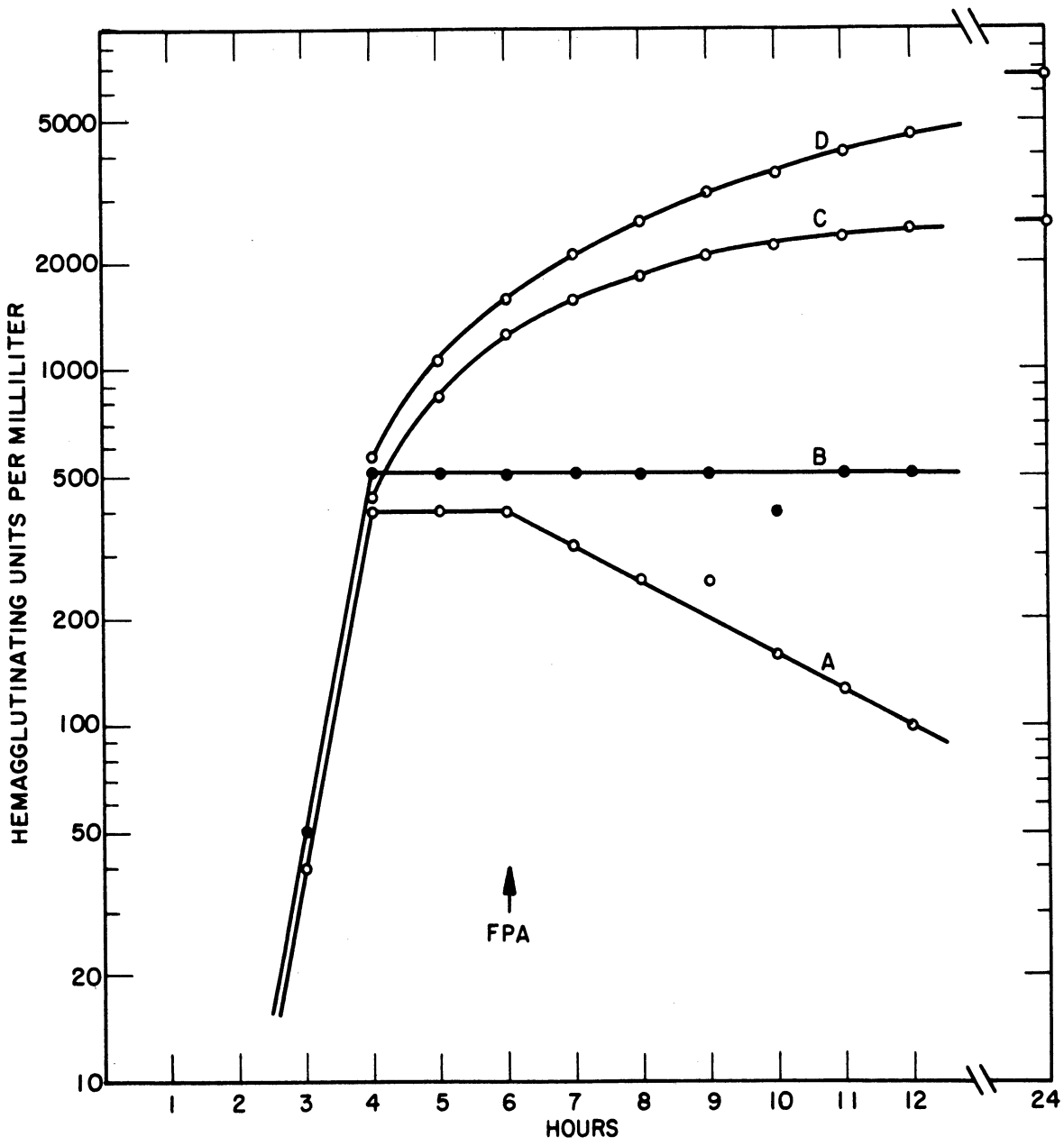


Fig. 4. The effect of p-fluorophenylalanine on the production of virus.— Curves A and B show the rate of release of virus from a membrane which received 3.3 mg FPA at 6 hours and the control membrane, respectively. Curves C and D show the cumulative release of virus from these membranes.

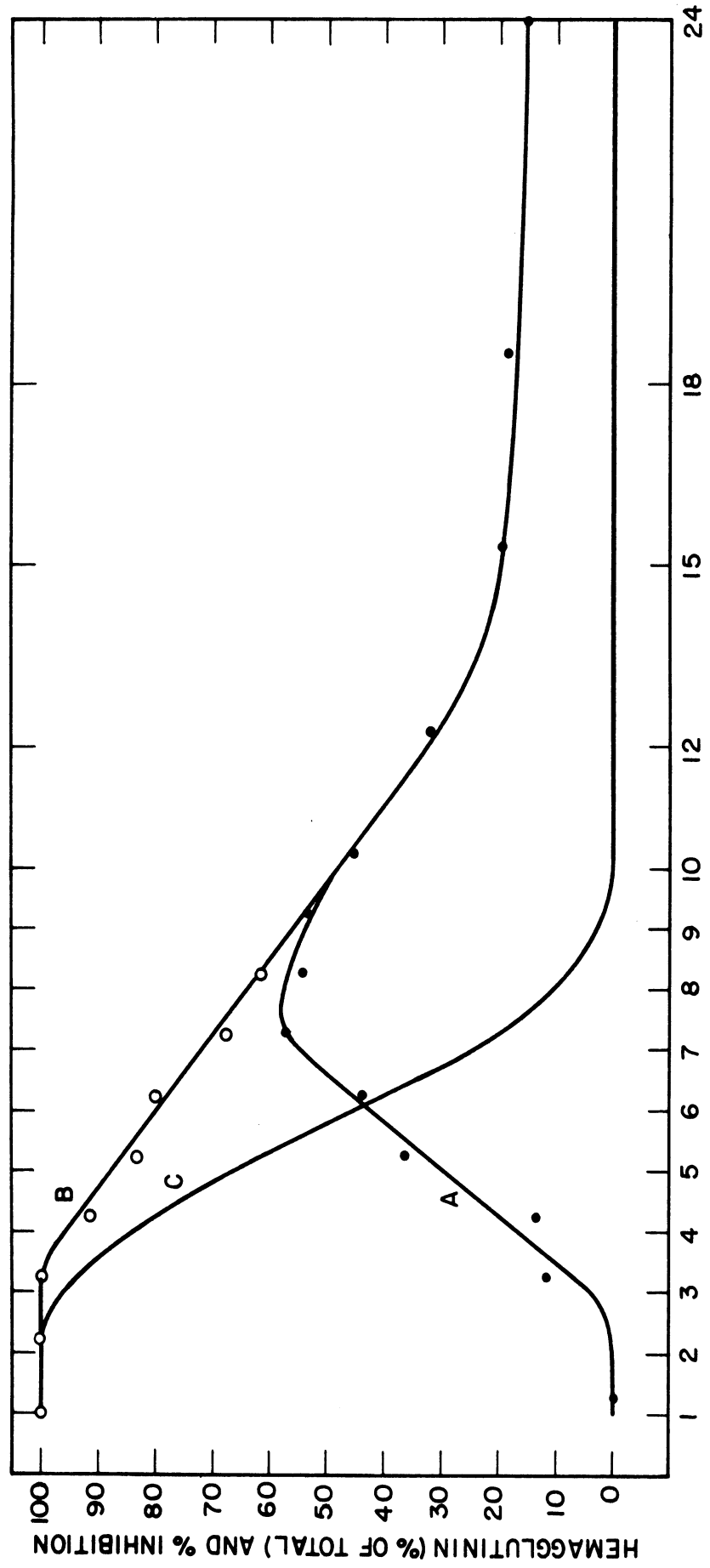


Fig. 5. The yield of virus subsequent to the addition of p-fluorophenylalanine at various times.—Curve A represents the yield of virus (that released plus that in the membrane at 24 hours) after addition of p-fluorophenylalanine at the times indicated on the abscissa. Curve B shows corresponding yields of virus from a control membrane. Curve C shows the percent inhibition produced.

the tenth hour, the amount of virus released is the same as that from the control (no inhibition). Between these two extremes the percent inhibition varies consistently with the time of addition of FPA. Thus it is apparent that the stage of viral synthesis for which phenylalanine is important, i.e., protein synthesis, begins during the second hour after initiation of infection and is not completed until the 10th hour.

It must be emphasized that the curves in Fig. 5 show the amount of virus released from the CAM after addition of FPA. A portion of this was present in the CAMs as pre-emergent virus (PEV) at the time the inhibitor was added. If the ordinates of the PEV curve (curve A, Fig. 2) are subtracted from the ordinates of the curves showing the amount of virus released from the inhibited CAM (curve A, Fig. 5) and the control CAM (curve B, Fig. 5), and the differences are plotted against the time of addition of FPA, the resulting curves represent the amount of new virus which is produced by the membranes. These curves are shown in Fig. 6, curves A and B representing the new virus produced after addition of FPA by the inhibited and control membranes, respectively.

While new virus is produced in the presence of maximal inhibitory levels of FPA, it is believed that the synthesis of viral protein is completely inhibited by FPA no matter when the inhibitor is added. It is also believed that the yield of new virus after complete inhibition of protein synthesis represents a stage in the synthesis of PR8 type A influenza virus in the cells of the chorio-allantoic membrane at which protein synthesis is complete and, therefore, not sensitive to FPA, a stage at which, however, the ability to agglutinate red blood cells has not yet been acquired. This nonhemagglutinating protein will be referred to as precursor protein. Further evidence for the existence of this stage will be presented in the following parts of this section.

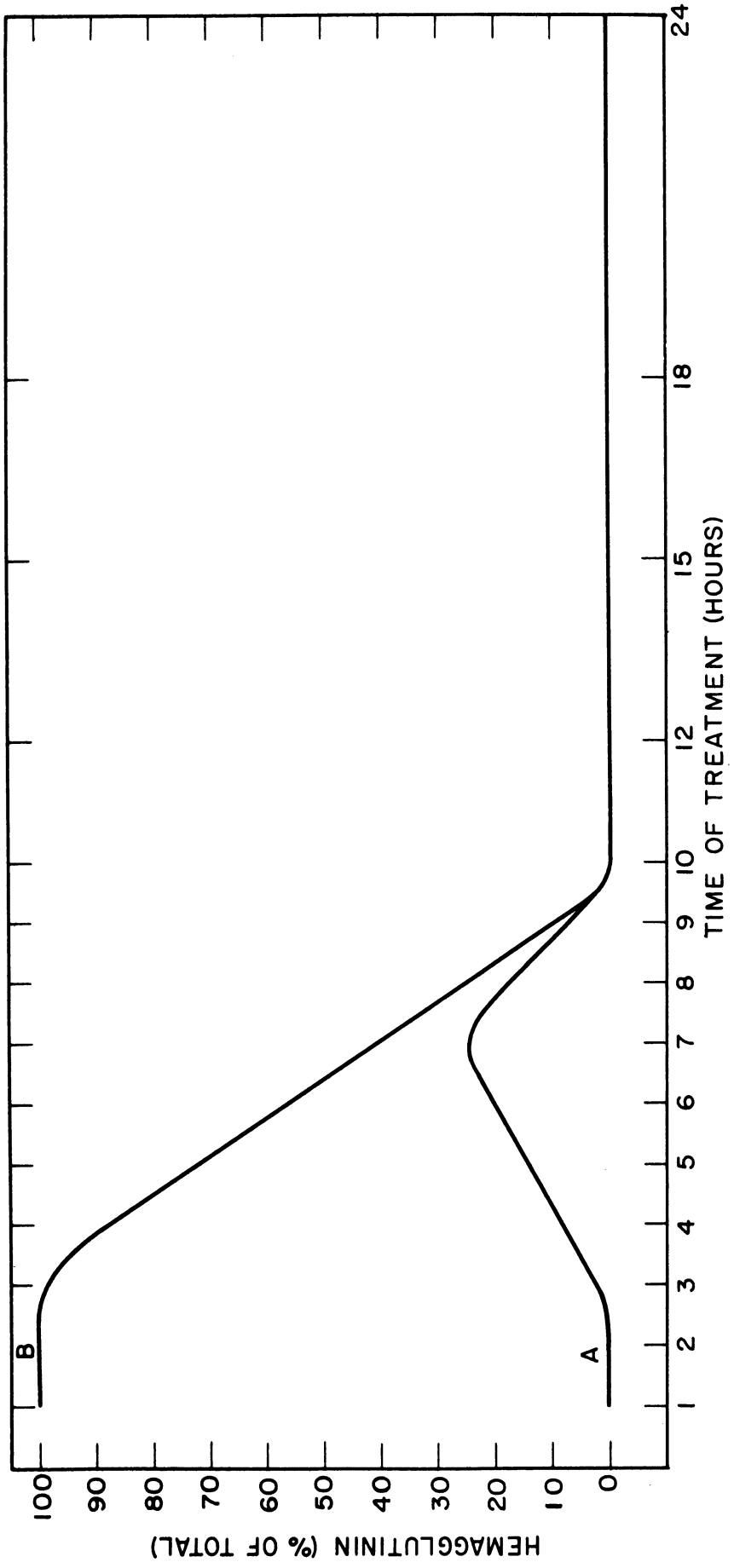


Fig. 6. The synthesis of virus subsequent to the addition of p-fluorophenylalanine.—Curves A and B represent the amount of new virus produced by the membrane receiving p-fluorophenylalanine and the control membrane, respectively (see text).

III. The effect of immune serum (IS) on the production of virus.

In the previous section evidence was given for the existence of an immature form of virus (precursor protein) in the chorio-allantoic membrane during the period of viral synthesis. This precursor protein was characterized as being complete with respect to its protein complement but incomplete with respect to its ability to produce hemagglutination.

It was assumed that the precursor protein was also antigenically complete or at least antigenically similar to hemagglutinating virus, and therefore, would react with antibodies present in specific antiviral antiserum. This assumption was adopted as a heuristic hypothesis, and the effect which treatment of the infected CAM with IS has on subsequent virus production was studied. For these experiments IS was added to the system at various times after initiation of infection and then removed by dilution. The effect of this treatment on the pre-emergent virus of the CAM at the time of treatment and the effect on the subsequent production of virus was determined. For the results to be meaningful it was necessary (1) to show that the effect of the IS was attributable entirely to its antiviral properties, (2) to find the optimal conditions for the action of IS, and (3) to show that there was no residual antiviral action due to incomplete removal of IS.

The immune serum was obtained from rabbits which had been injected with PR8-containing allantoic fluid. In addition to virus, infected allantoic fluid contains debris from the cells lining the allantois (Hoyle, 1950). It would be expected, therefore, that immunization of a rabbit with this fluid would stimulate the production of antibodies against host-cell protein as well as against viral protein. That this was the case and that the anti-host antibodies could be effectively removed from the IS is

shown below.

The removal of anti-host antibodies from immune serum.—Immune serum was adsorbed with powdered normal chorio-allantoic membrane (CAM-powder). (See Methods and Materials, page 26.) To do this CAM-powder and IS were mixed in the ratio of 20 mg of the powder to a milliliter of serum diluted to 20 percent in ESM, and the mixture was incubated at 37°C for 90 minutes and then at 4°C for 8 hours. It then was centrifuged at 18,000 rpm for 30 minutes in a refrigerated angle-head centrifuge. This procedure was repeated on a portion of the supernatant fluid, and then again on a portion of the supernatant fluid from the second adsorption. Normal serum (NS) was adsorbed twice in the same manner. A clear fluid resulted in each case. The unadsorbed IS produced a double ring of antigen-antibody precipitate when it was overlaid with a saline extract of CAM in a small-diameter tube, while the once-, twice-, and three-times-adsorbed IS and the unadsorbed and twice-adsorbed NS did not. There was no change in the hemagglutination-inhibition titer of the IS after repeated adsorption.

Six replicate composite CAMs infected for one hour with $10^{8.68}$ EID_{50} PR8 virus were incubated until 6 hours in ESM, and then incubated for 15 minutes at 37°C in unadsorbed, once-, twice-, and three-times-adsorbed IS,* in twice-adsorbed NS,* and in ESM. They were washed in two changes of 50 ml PBS at 20°C before treatment and in three changes afterward. The treated, washed membranes were incubated in ESM for the remainder of the 24-hour period, and then extracted into the 6-1/4- to 24-hour fluids. The fluids were titrated for HA activity. The data, shown in Table X, indicated that the unadsorbed IS suppressed the subsequent production of virus

*The sera were diluted to 2.5 percent in ESM for this treatment. This concentration was used on the basis of preliminary experiments.

TABLE X

THE YIELD OF VIRUS SUBSEQUENT TO TREATMENT AT SIX HOURS WITH UNADSORBED AND ADSORBED IMMUNE SERA

Treatment at 6 Hours[a]	Yield of Virus	
	1 to 6 hours[b]	6-1/4 to 24 hours[c]
ESM	400[d]	1280
Twice-adsorbed NS[e]	400	1280
Unadsorbed IS[f]	400	400
Once-adsorbed IS[g]	400	640
Twice-adsorbed IS[h]	400	640
3-times-adsorbed IS[i]	400	640

a. Sera diluted to 2.5 percent in ESM were present from 6 to 6-1/4 hours.
b. Yield of virus before treatment.
c. Yield of virus, including that in the membrane at 24 hours after treatment.
d. Hemagglutinin units per milliliter; total volume, 3 ml in all cases.
e. Normal serum adsorbed twice with powdered chorio-allantoic membrane.
f. Unadsorbed immune serum.
g. Immune serum adsorbed once with powdered chorio-allantoic membrane.
h. Immune serum adsorbed twice with powdered chorio-allantoic membrane.
i. Immune serum adsorbed three times with powdered chorio-allantoic membrane.

to a greater extent than did the adsorbed sera, and that adsorbing once with CAM-powder effectively removed this extra inhibitory action. It was anticipated that the twice-adsorbed NS might have produced an increase in the subsequent yield due to enrichment of the medium; it did not, however. A different lot of IS, having a lower HAI titer and therefore used at a higher concentration, was utilized in some preliminary experiments. This IS showed a much greater inhibition due to antibodies which could be removed by adsorption with CAM-powder. Unless otherwise specified, immune

serum which had been adsorbed twice with CAM-powder was used in the following experiments.

The minimal inhibitory level of immune serum.—An experiment was performed in order to determine the minimal level of IS necessary to neutralize completely the pre-emergent virus in the CAM at 6 hours. Membranes infected with $10^{8.68}$ EID_{50} virus were treated with 0.25 to 8 percent CAM-adsorbed IS and NS for 15 minutes at 6 hours. Immediately after treatment the CAMs were washed in three changes of 50 ml PBS at 20°C and extracted. Titrations for HA activity were performed on the extracts. The results, shown in Table XI, indicate that 0.25, 0.5, and 1.0 percent IS partially neutralized the PEV, and that 2 percent IS produced complete neutralization.

TABLE XI

THE NEUTRALIZATION OF PRE-EMERGENT VIRUS BY VARIOUS CONCENTRATIONS OF CAM-ADSORBED IMMUNE SERUM

CAM-adsorbed IS[a] (%)	CAM-adsorbed NS[a] (%)	Pre-emergent Virus[b]
8.0		0[c]
4.0		0
2.0		0
1.0		160
0.5		200
0.25		256
	8.0	400
	0.25	400

a. Membrane treated for 15 minutes at 37°C at 6 hours with immune or normal sera which were adsorbed twice with powdered chorio-allantoic membrane.
b. The virus content of the membrane immediately after treatment.
c. Hemagglutinin units per milliliter; total volume 3 ml; "0" implies no agglutination at 1:2 dilution.

The optimal duration of treatment with 2.5 percent immune serum.— Eight replicate composite CAMs infected with $10^{8.68}$ EID_{50} were treated at 37°C with 2.5 percent CAM-adsorbed NS and IS at 6 hours for 5, 10, 15, and 20 minutes, then washed in three changes of 50 ml PBS at 20°C, and incubated for the remainder of the 24-hour period in 3 ml ESM. The CAMs were extracted at the end of the experiment. Another 8 membranes were treated in the same manner, but were washed and extracted immediately after treatment. Titrations for HA activity were performed on the fluids and extracts. The results are shown in Table XII.

TABLE XII

THE EFFECT OF VARYING THE DURATION OF TREATMENT WITH 2.5 PERCENT IMMUNE SERUM

Treatment[a] with	Duration of Treatment (min)	PEV[b] at 6 Hours	Yield of Virus After Treatment		
			In Fluid	In CAM	Total
2.5% IS[d]	5	128[c]	640	320	960
	10	32	640	200	840
	15	0	400	200	600
	20	0	320	200	520
2.5% NS[e]	5	512	2048	400	2448
	10	512	2048	400	2448
	15	512	2048	400	2448
	20	512	2048	400	2448

a. The membranes were treated at 37°C for the indicated length of time beginning at 6 hours.
b. The pre-emergent virus remaining after treatment in a comparable set of membranes.
c. Hemagglutinin units per milliliter; total volume 3 ml.
d. Immune serum adsorbed twice with powdered chorio-allantoic membrane, and diluted to 2.5 percent in ESM.
e. Normal serum adsorbed twice with powdered chorio-allantoic membrane, and diluted to 2.5 percent in ESM.

The results show that incubation of the infected CAMs at 6 hours for 5 or 10 minutes was insufficient and that incubation for 15 minutes was sufficient for complete neutralization of the PEV in the membrane at that time. (See column 3.) It is also seen that the subsequent yield of virus from the immune-serum-treated membranes decreased as the duration of treatment was increased. (See column 6.)

The independence of inhibition due to anti-host antibodies and anti-viral antibodies.—For this experiment a low-titered IS pool diluted to 20 percent was used, and the membranes were not composite ones but were chosen on the basis of the hemagglutinin they produced from 1 to 5 hours. For these reasons the experiment is not strictly comparable to the other experiments described in this study; it is included here because it clearly shows the effect of both antibodies. Normal and immune sera were adsorbed with powdered chorio-allantoic membrane (CAM-powder), with formalized human type-O red blood cells coated with PR8 virus (PR8-antigen)—see Methods and Materials, page 26—and with both these antigens, in order to remove the corresponding antibodies. As a control measure, sera not adsorbed with the PR8-coated cells were adsorbed with uncoated cells. The sera were adsorbed in a manner similar to that given above for the adsorption with CAM-powder, except that only one cycle of adsorption was employed. When titrated for hemagglutination-inhibiting activity, it was found that only adsorption with PR8-antigen decreased the HAI titer. An additional serum was prepared by diluting IS, adsorbed with CAM-powder, with NS so that it had the same HAI titer as the IS adsorbed with CAM-powder and PR8-antigen. The HAI titers of the adsorbed sera are shown in column 4 of Table XIII.

Sixteen infected CAMs were treated in duplicate with these sera, the

time of treatment being from 6-3/4 to 7 hours. After treatment one of each was washed and stored at 4°C and the other was washed and then incubated in 3 ml ESM for the remainder of the 24-hour period. The membranes were transferred to fresh media at 8-1/2 hours. At the termination of the experiment all the CAMs were extracted. The HA titers of the fluids and extracts are shown in Table XIII.

By referring to column 5 it is seen that the IS which was not adsorbed with the PR8-coated cells (A, B, and C) completely neutralized the PEV, and that adsorption with CAM-powder (C) did not inhibit the neutralization; while the IS adsorbed with PR8-coated cells (D and E) as well as the diluted IS (H) neutralized about one half of the PEV. In the last column it is seen that the IS (E) adsorbed with both antigens produced the least amount of inhibition of subsequent yield while the IS (A and B) adsorbed with neither antigen produced the greatest amount of inhibition. The IS (C) adsorbed with the CAM-antigen and the IS (D) adsorbed with the PR8-antigen produced an amount of inhibition intermediate between these extremes. The inhibition produced by the IS (ϵ) adsorbed with both antigens was probably due to the incomplete removal of anti-PR8 antibody, for the effect of this serum is paralleled by that of the serum (H) which was diluted to have the same HAI titer. The results of this experiment show that: treatment of an infected CAM for 15 minutes at 6-3/4 hours with IS containing antibodies against protein of the host cell and against viral protein produced a large inhibitory effect on the subsequent yield of virus. Removal of either antibody reduced this inhibitory effect, and removal of both antibodies almost completely prevented the inhibitory effect, the small residual inhibition being due to the incomplete removal of the antiviral antibody.

TABLE XIII

THE EFFECT OF IMMUNE SERUM ADSORBED WITH VIRUS-COATED RED BLOOD CELLS AND WITH POWDERED CHORIO-ALLANTOIC MEMBRANE

Treatment[a] With			HAI Titer[c]	PEV at 6 hr[d]	Yield of Virus After Treatment			
Serum[b]		Adsorbed With			7-8.5 hr	8.5-24 hr	24 hr CAM	Total
IS	A	None	512	0[e]	0	80	160	240
	B	Uncoated red cells[f]	512	0	0	80	160	240
	C	Uncoated red cells + CAM-powder[g]	512	0	16	256	200	472
	D	PR8-coated red cells[h]	10	320	80	320	256	656
	E	PR8-coated red cells + CAM-powder	10	320	80	800	400	1280
NS	F	Uncoated red cells	---	640	320	800	400	1520
	G	Uncoated red cells + CAM-powder	---	640	320	800	400	1520
IS	H	"C" diluted 1 to 50 with "G"	10	256	80	800	400	1280

a. Membranes were incubated in the sera at 37°C from 6-3/4 to 7 hours.
b. The sera were diluted to 20 percent in ESM before adsorption.
c. Hemagglutination-inhibition titer of the adsorbed sera (2.5 percent) in units per milliliter.
d. Pre-emergent virus detectable in the membrane extract immediately after treatment.
e. All titers in hemagglutination units per milliliter; total volume, 3 ml; "0" indicates no agglutination.
f. Formalized human type-O red blood cells.
g. Powdered chorio-allantoic membrane.
h. PR8-coated formalized human type-O red blood cells.

Demonstration of the absence of residual antiviral action due to incomplete removal of immune serum by washing.—The sera used for the following series of experiments had undergone two cycles of adsorption with powdered chorio-allantoic membrane. For a series of four experiments extracts were prepared from normal and infected membranes which had been treated with NS or IS for 15 minutes at 6 hours and then washed in three changes of PBS at 20°C. These extracts were tested for hemagglutinin and hemagglutination-inhibition activities, and for ability to interfere with the production of virus by infected membranes. The latter was determined by an experiment performed according to the following schedule.

0 to 1 hr	Washed once	1 to 6 hr	Washed twice	6 to 6-1/4 hr	Washed 3 times	6-1/4 to 24 hr
Infected with $10^{8.68}$ EID_{50} PR8 in 3 ml		Incubated in 3 ml ESM		Incubated in 3 ml NS, IS, or ESM		Incubated in extracts of CAM

Some of the membranes were washed at 24 hours and extracted. All the washings were in 50 ml PBS at 20°C. The NS and IS were diluted to 2.5 percent in ESM. For Experiment 159 (see page 71), the CAMs were incubated from 6-1/4 to 24 hours in a homogenate of CAM obtained by the regular procedure of extraction but with no centrifugation. In all cases the extracts in which the membranes were incubated were prepared in ESM.

By referring to Table XIV it is seen that in Experiment 107 the CAM (B) incubated in an extract (N-IS) of a noninfected membrane which had been treated with IS and the CAM (D) incubated in an extract (I-IS) of an infected membrane which had been treated with IS produced as much virus (column 7) as did the control membrane (A) even though the (N-IS) extract had a titer of 12 hemagglutination-inhibition units (HAIU) per milliliter.

TABLE XIV

THE EFFECT OF INCUBATION IN EXTRACTS OF NONINFECTED OR INFECTED MEMBRANES TREATED WITH NORMAL OR IMMUNE SERUM

Exp. No.	CAM No.	Treatment		Titers of Extract[a]		Yield of Virus from Treated CAM (6-1/4 to 24 hr)		
		6 to 6-1/4 hr	6-1/4 to 24 hr	HA	HAI	Fluid	CAM (24 hr)	Total
107	A	NS[b]	N-NS extract[c]			1600[d]		
	B	NS	N-IS extract		12	1600		
	C	NS	I-NS extract	400		2048		
	D	NS	I-IS extract	0	0	1600		
111	E	NS	N-NS extract			2560	640	3200
	F	NS	I-IS extract	0	0	2560	640	3200
	G	IS	N-NS extract			1024	256	1280
	H	IS	I-IS extract	0	0	1024	256	1280
	I	NS	ESM			2560	640	3200
	J	IS	ESM			1024	256	1280
	K	NS	Extracted after 6 to 6-1/4-hr treatment for PEV - 1024					
	L	IS	Extracted after 6 to 6-1/4-hr treatment for PEV - 0					
115	M	NS	N-NS extract			1600	400	2000
	N	IS	N-NS extract			640	160	800
	O	IS	I-IS extract	0	0	640	160	800
	P	NS	Extracted after 6 to 6-1/4-hr treatment for PEV - 640					

a. Hemagglutinin and hemagglutination-inhibition titers of the extracts in which the membranes were incubated from 6-1/4 to 24 hours.

b. NS, 2.5 percent normal serum adsorbed twice by powdered chorio-allantoic membrane; IS, 2.5 percent immune serum adsorbed twice by powdered chorio-allantoic membrane.

c. Extracts of normal membrane (N-) or infected membrane (I-) which were treated for 15 minutes at 6 hours with normal serum (-NS) or immune serum (-IS).

d. Hemagglutinin units per milliliter; total volume, 3 ml.

The extract (I-NS) of an infected membrane which had been treated with NS contained 400 HAU per milliliter, and this is reflected in the greater apparent yield from the CAM (C) incubated in this extract. In Experiment 111, membrane F corresponds to membrane D, and, as above, it produced as much virus as did the control membrane (E). Membranes G and H treated with IS for 15 minutes prior to incubation in N-NS and I-IS extracts, respectively, produced the same amount of virus but less than did the control membrane E. Membranes I and J were incubated in ESM rather than in N-NS extract, and these produced the same amount as did the corresponding membranes E and G. It is seen that the 15-minute IS treatment completely neutralized the pre-emergent virus (membranes K and L). In Experiment 115 membranes M, N, and O were treated in the same manner as membranes E, G, and H of Experiment 111, and the results are comparable. In Experiment 159, Table XV, CAMs were incubated in treated-membrane extracts which were not centrifuged. This was done to rule out any antiviral activity which might have remained adsorbed to the membrane fragments. In this experiment membranes Q, R, S, and T are comparable to membranes A, B, C, and D of Experiment 111, and the results are the same, except that the N-IS extract produced a small amount of inhibition.

Since in no case was it possible to detect any antiviral activity in or from infected membranes which had been treated with IS for 15 minutes at 6 hours and then washed in 3 changes of 50 ml PBS, it was concluded that this washing was sufficient. Any effect which treatment of an infected CAM with IS had on the subsequent production of virus was entirely attributable to the neutralization of viral antigenic material in the CAM during treatment.

Attention is called to the fact that in several of the foregoing ex-

TABLE XV

THE EFFECT OF INCUBATION IN HOMOGENATES OF NONINFECTED OR INFECTED MEMBRANES TREATED WITH NORMAL OR IMMUNE SERUM

Exp. No.	CAM No.	Treatment		Yield of Virus[a]
		6 to 6-1/4 hr	6-1/4 to 24 hr	
159	Q	ESM	N-NS ext + sed[b]	1600[c]
	R	ESM	N-IS ext + sed	1280
	S	ESM	I-NS ext + sed	2048
	T	ESM	I-IS ext + sed	1600

a. Virus released from 6-1/4 to 24 hours.
b. Extracts of normal membrane (N-) or infected membrane (I-) which were treated for 15 minutes at 6 hours with normal serum (-NS) or immune serum (-IS). In this experiment, the whole homogenate was used.
c. Hemagglutinin units per milliliter of fluids; total volume, 3 ml.

periments treatment of infected membranes for 15 minutes with 2.5 percent IS, from which the anti-host antibodies had been removed, reduced the subsequent yield of virus to a greater extent than would be expected if the sole action of IS was that of neutralizing the PEV in the membrane at the time of treatment. In Table XIII it can be seen that the IS-treated CAM (C) produced 472 HAU, while the control membrane (P) produced 1520 HAU, a difference of 1048 HAU, but that only 640 HAU pre-emergent virus were neutralized. In Table XIV, Experiment 111, PEV in the amount of 1024 HAU was neutralized (K and L) while the treated (J) and control (I) membranes produced 1280 and 3200 HAU, respectively, a difference of 1920 HAU. In Experiment 115, 640 HAU pre-emergent virus was present in the membrane (P) while the treated membrane (N) produced 800 HAU and the control membrane (M) produced 2000 HAU, a difference of 1200 HAU. In each case the difference between the amount of virus produced by the IS-treated and control membranes was about twice as great as would have been predicted on

the basis of the neutralization of PEV only. This "discrepancy" supports the hypothesis which was stated at the beginning of this section; namely, that there was present in the membrane a precursor protein which is an immature form of virus and that this precursor protein reacts with antibodies present in specific antiviral antiserum. The effect of IS was further clarified by the following experiments.

The effect of varying the time of treatment with immune serum on the production of virus by the chorio-allantoic membrane.—For this study a series of experiments, each utilizing six replicate composite CAMs, was performed. The membranes were treated according to the schedule below, in which the values of t were 1, 2, ..., 9, 10, 12, 15, and 18 hours.

	0 to 1 hr		1 to t hr		t to t + 1/4 hr		t + 1/4 to 24 hr	
A	Infected with $10^{8.68}$ EID_{50} PR8 virus in 3 ml ESM	Washed once	Incubated in 3 ml ESM	Washed twice	IS + FPA	Washed 3 times	FPA	Washed twice; All membranes extracted
B					IS		ESM	
C					IS		(stored at 4°C)	
D					NS + FPA		FPA	
E					NS		ESM	
F					NS		(stored at 4°C)	

All washings were in 50-ml volumes of PBS at 20°C. The data from the control membranes (E) relating to the rate of release of virus from the CAM were reported on page 42; data from membranes F were discussed on page 45 in the study of variation of PEV with time; and data from membranes D were used with reference to the effect of time of addition of FPA on viral synthesis (page 55).

Data concerning the effect of IS on viral production were obtained from membranes A, B, and C. These three membranes were incubated at var-

ious times for 15 minutes in IS* (see schedule above) from which the anti-host antibodies had been removed by adsorption with powdered CAM. Membranes C were extracted after the IS treatment in order to determine whether the PEV was completely neutralized; it was in every case. The extract from this membrane was also tested for inhibitory activity by HAI titration; in no case was there any detectable HAI activity. Membranes A, which received both FPA and IS, were included as another test of the working hypothesis. If the FPA treatment completely inhibited further protein synthesis and if the IS neutralized the precursor protein along with the PEV, then a CAM treated with both FPA and IS should produce no virus after initiation of treatment, regardless of the time of treatment. It was found that the combined treatment did completely suppress subsequent production of virus in every case. The amount of virus produced by membranes B subsequent to treatment was determined by HA titration of the 6+1/4- to 24-hour fluid and the 24-hour CAM extract. The data for this series of experiments are tabulated in the Appendix on page 105. In Fig. 7 the yield of virus from membranes B subsequent to IS treatment and the corresponding yield from the control membranes E are plotted against the time of treatment (curves B and A, respectively). As before, the data were first converted to percentage of total virus produced by the control membrane in each experiment. In Fig. 8, curve A represents the difference between the amounts of virus produced by the control and the IS-treated membranes (obtained by subtracting the ordinates of curve A from those of curve B in Fig. 7). The curve showing the variation of PEV is reproduced in this figure (curve B).

*The concentration of IS was varied from 2.5 percent in accordance with the amount of PEV known, from results of preliminary experiments, to be present in the membrane at the time of treatment. From the preliminary experiments a "PEV-curve" essentially the same as that in Fig. 2 was obtained.

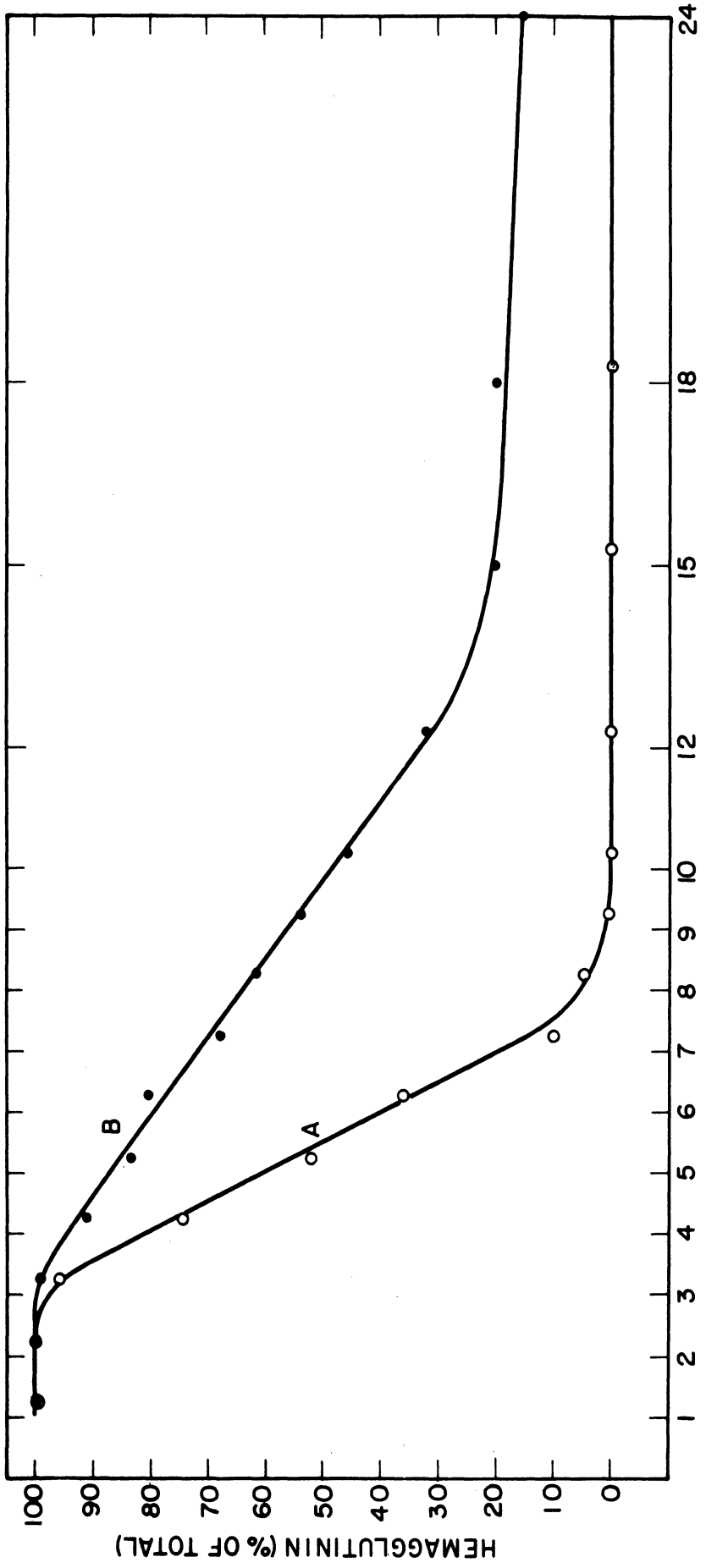


Fig. 7. The yield of virus subsequent to treatment with immune serum.— Curves A and B represent the yield of virus (that released plus that in the membranes at 24 hours) after treatment with immune serum at the times indicated in the abscissa, and from the control membranes, respectively (see text).

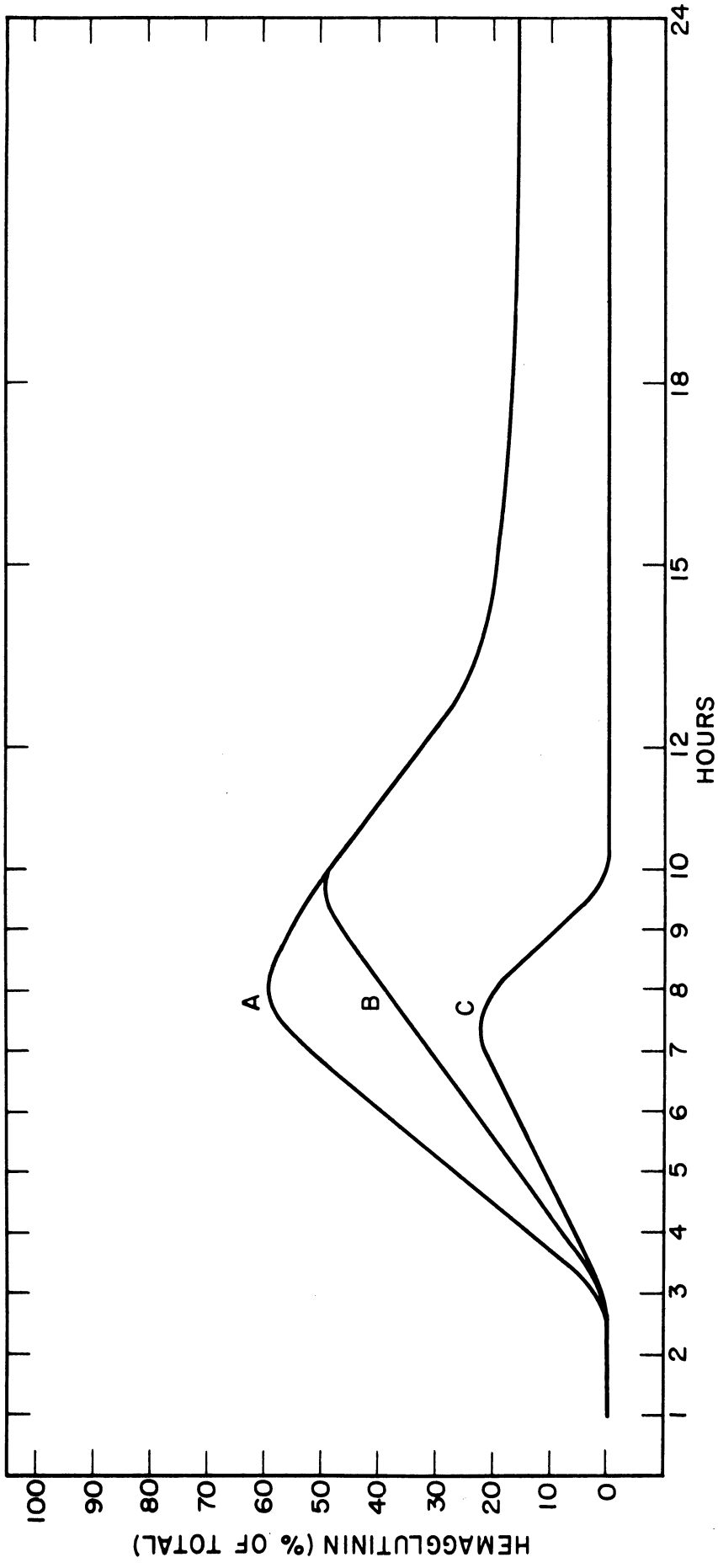


Fig. 8. The inhibition produced by treatment with immune serum.—Curve A shows the difference between the virus produced by the control membranes and the IS-treated ones. Curve B represents the amount of pre-emergent virus in the membrane. Curve C shows the difference between the inhibition produced by immune serum and the amount of pre-emergent virus in the membrane (see text).

Referring to curve B in Fig. 7 it is evident that specific antiviral IS had no inhibitory effect if it was added at one or two hours after initiation of infection. During this period there is no viral antigenic material in the host cells. This period is the "latent" or "eclipse" period during which the infecting virus loses its integrity and the host cell undergoes a reorganization preparatory to synthesis of new virus. It seems, however, that IS is capable of interfering with viral synthesis before the appearance of hemagglutinin, since virus is first detectable in the membrane shortly before 3 hours, yet IS begins to exert an inhibitory effect shortly after 2 hours (compare curve B, Fig. 7, or curve C, Fig. 2, with curve A, Fig. 7). It is seen from curve A, Fig. 7, that after 10 hours, IS treatment completely suppresses the subsequent yield of virus. This is consistent with the observation in part I of this section that viral synthesis is complete at this time, and that after 10 hours virus which appears in the fluid phase is merely "leaking" out of the membrane at the expense of the PEV. In Fig. 8, it can be seen that the magnitude of suppression of viral yield (curve A) is greater than the amount of PEV in the membrane (curve B) at the time of treatment until 10 hours, after which time they are equal. Curve C, which is a plot of the difference in amounts of suppression and PEV, thus represents the magnitude of the suppression which cannot be attributed to neutralization of PEV. This suppression is believed to be due to the "neutralization of viral antigenic material (precursor protein) which cannot be detected directly because it does not agglutinate red blood cells."

IV. The ultracentrifugal sedimentation of extracted and spontaneously released virus

The evidence for the existence of the protein pool was derived from two experimental facts: (1) that after addition of the maximal inhibitory

level of FPA more hemagglutinating virus was produced than was present in the membrane as PEV at the time of addition of the inhibitor, and (2) that after treatment with IS the suppression of viral yield was greater than could be accounted for by the neutralization of the PEV at the time of treatment with IS. In both cases, the discrepancy could be due to the measurement of PEV indicating less hemagglutinin than was actually in the membrane. It was suggested that the intracellular virus might exist in the form of aggregates which deaggregate as the virus is released spontaneously from the intact cell, but do not deaggregate in the extraction procedure or afterward.

When FPA was added at 6 hours, the inhibited membrane produced roughly twice as much virus as could be measured as PEV, and the treatment at this time with IS produced about twice as much suppression of yield as could be accounted for by neutralization of the PEV pool. For these results to be explained by the existence of aggregates of virus in the membrane at 6 hours, either a large fraction of the virus would have to be in the form of small aggregates (say dimers) or a small fraction would have to be in the form of large aggregates. As regards the second possibility, Hoyle (1950) found filamentous forms of virus—which Heinmets (1948) believed to be simple aggregates of elementary particles—in the allantoic fluid of infected eggs but none in extract of infected chorioallantoic membranes. Hoyle (1950) believes that the filaments are formed during the natural "excretion" of virus from the cells. The possibility that the majority of the intracellular particles are in the form of small aggregates was tested by the following experiment.

The method used was that of Pardee and Schwerdt (1952) for determining the size of viruses in impure preparations by sedimentation in a ser-

ies of sucrose concentrations in the angle-head centrifuge. These authors used the Spinco Model L ultracentrifuge with the No. 40.2 rotor, without refrigeration. In the present case the No. 40 rotor was used. A preliminary run was made using allantoic fluid virus. The virus was first partially purified by centrifugation at 2000 rpm for 15 minutes, followed by centrifugation of the supernatant fluid at 30,000 rpm for 30 minutes. This was sufficient to precipitate all the virus. The pellet was resuspended in distilled water and the partially purified virus was then centrifuged at 20,700 rpm for 40 minutes (time at full speed plus 1/3 the time required for acceleration and deceleration) in sucrose concentrations ranging from 3 to 36 percent by weight, in 3-percent steps. The temperature increased about one degree during the centrifugation, the average being about 30°C. The top 10 ml of the supernatant fluids were quickly and carefully removed by aspiration and titrated for HA activity. The percentage of the original activity remaining in the supernatant fluid was calculated and plotted against the sucrose concentration (Fig. 9, curve A). In the same manner an extract of 6-hour-infected CAM and the virus released into the fluid phase from 1 to 6 hours was partially purified and centrifuged in the graded sucrose concentrations. In this case, however, the fluids were centrifuged at 15,600 rpm at an average temperature of 25°C for 39.55 minutes, so that the density of sucrose in which complete sedimentation occurred would be small and hence the difference between the density of the suspending medium and the sedimenting particles, large. The top 10 ml was removed and assayed for HA activity.

When the hemagglutinin from the extract of the membranes was compared with that from the fluid phase of the culture, no essential difference was found in the percentage of virus which remained unsedimented in the vari-

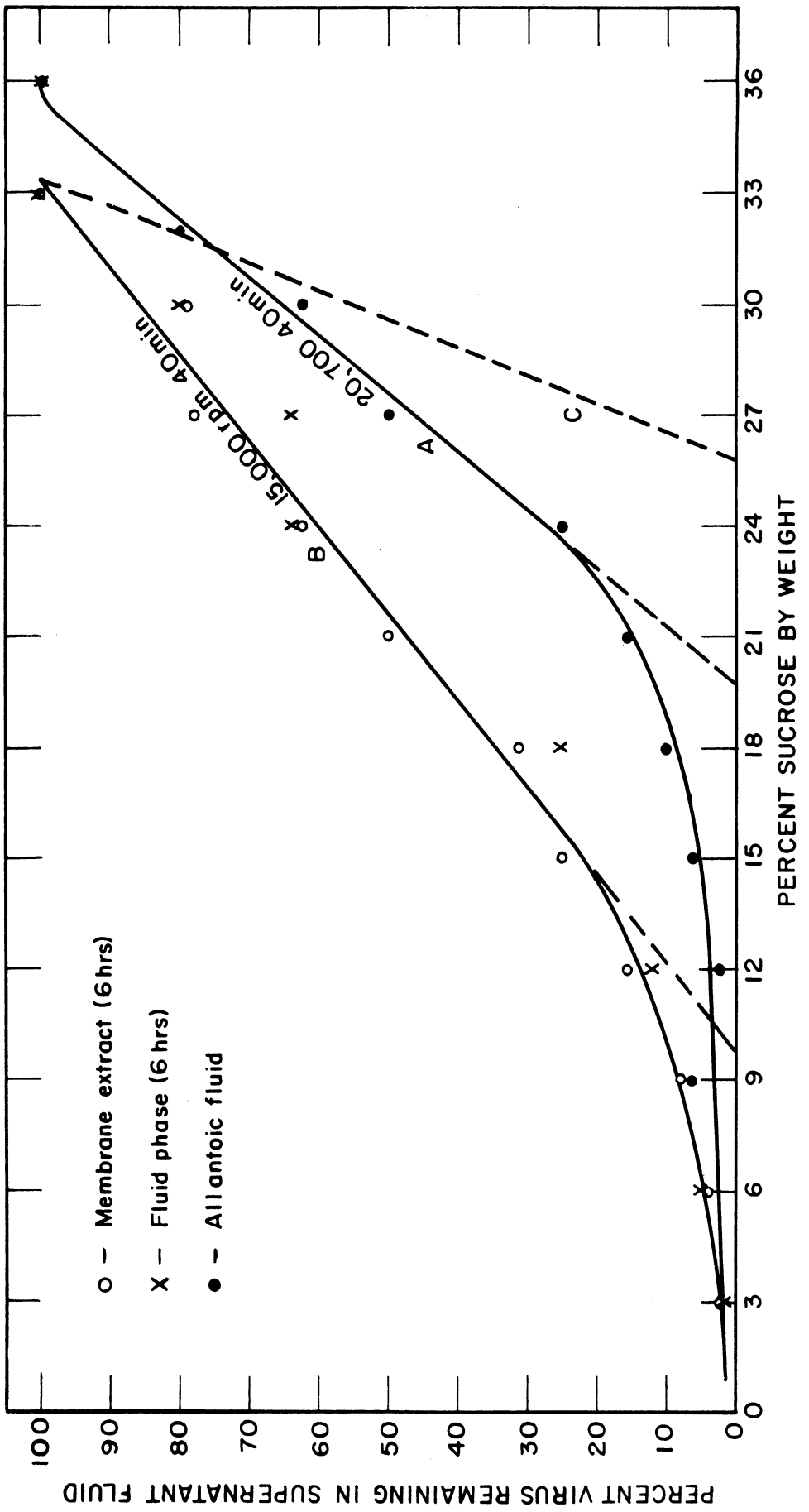


Fig. 9. The ultracentrifugal sedimentation of virus.—Curve A represents the sedimentation of allantoic fluid virus at 20,700 rpm for 40 minutes. Curve B shows the sedimentation of pre-emergent virus and released virus at 15,600 rpm for 39.55 minutes. Curve C is a theoretical curve for the sedimentation of a particle twice the diameter of the virus at 15,600 rpm for 39.55 minutes (see text).

ous sucrose solutions. This would not obtain if the intracellular virus were in small aggregates which were not broken up by the extraction procedure but that released from the cell by natural means was deaggregated. Thus the virus extracted from membranes and that released spontaneously appear not to differ in their degree of aggregation.

The percentage of the original activity remaining in the supernatant fluid was plotted against the sucrose concentrations as in the preliminary experiment. A single curve (curve B) was drawn through the two sets of points derived from the sedimentation of the extracted and naturally released virus. The HA titers of the fluids are shown in the Appendix, page 106.

The concentration of sucrose at which complete sedimentation occurred was obtained by extrapolation of the linear portion of the curve. The activity remaining in the supernatant fluid at the lower concentrations of sucrose probably represents virus which was washed off the pellet when the fluid was removed. The values of the viscosities of the sucrose solutions were obtained from Bates (1943) and those of the densities from the Handbook of Chemistry and Physics (1951-1952).

The density and diameter of the sedimenting particles were calculated by using formula (1) from Pardee's paper.

$$d = K\sqrt{\frac{\eta}{x^2 t\ (\sigma-\rho)}} \qquad (1)$$

where d is the hydrated diameter of the sedimenting particle in millimicrons, η is the viscosity of the suspending fluid in centipoises, x is revolutions per minute, t is time in minutes, ρ is the density of the suspending medium in grams per milliliter, σ is the density of the sedimenting particle in grams per milliliter, and K is a constant, the value of

which depends upon the physical parameters of the rotor. The value of K varies from one rotor to another according to the relation (2),

$$\frac{K}{K'} = \sqrt{\frac{\ell n\ R_{max} - \ell n\ R_{min}}{\ell n\ R'_{max} - \ell n\ R'_{min}}} \qquad (2)$$

where R_{max} and R_{min} are the maximum and minimum distance of the fluid from the axis of rotation, and the primed and unprimed constants refer to two rotors. Pardee, using polystyrene latex particles, found the value of K to be 3.4×10^6 for the No. 40.2 rotor. For the present experiments, the No. 40 rotor was used and a value of 3.25×10^6 was calculated by substitution of values for R_{max} and R_{min} which are given in the Spinco technical manual for the model L ultracentrifuge.

The allantoic fluid virus was completely sedimented at 20,700 rpm in 40 minutes in 19.5 percent sucrose which has a density of 1.0805 grams per milliliter and a viscosity of 1.470 centipoises at 30°C, and the virus derived from the suspended CAM cultures was completely sedimented at 15,600 rpm in 39.55 minutes in 9.8 percent sucrose whose density is 1.0378 and viscosity at 25°C is 1.232. Assuming that the virus has the same density and size in the two cases, its density was calculated by formula (3) which is derived from formula (1) by elimination of d and K.

$$\sigma = \rho_1 \frac{\eta_1 t_2 x_2^2 (\rho_2 - \rho_1)}{\eta_1 t_2 x_2^2 - \eta_2 t_1 x_1^2} \qquad (3)$$

The subscripts refer to the two centrifugations. From this formula the density of the hydrated virus was found to be 1.164 grams per milliliter. Using this value in equation (1) the diameter of 103 millimicrons was found. These values are in reasonable agreement with those found in the literature.

Lauffler and Stanley (1944) believe that the type of curve shown in

Fig. 9 should not be linear due to slight increases in the density of influenza virus because of loss of water in higher sucrose concentrations. These authors found that virus fails to sediment in a sucrose solution of 1.18 grams per milliliter and that in the absence of sucrose the hydrated density is about 1.1 grams per milliliter. They found the diameter to be about 100 millimicrons.

From equation (1) it is seen that if a particle of the same density but twice the diameter were centrifuged at the same speed and at the same temperature, the value of $\eta/(\sigma-\rho)$ would be four times greater. Assuming these conditions, a particle of diameter of 206 millimicrons (twice the diameter that was found) would precipitate in a sucrose solution of about 25.8 percent, which has a density of 1.1072 grams per milliliter and a viscosity of 2.216 centipoises at 25°C. On this basis a theoretical curve (curve C) is drawn for the sedimentation of a particle twice the diameter* as that found.

These sedimentation experiments indicate that the aggregation of the virus in extracts of the CAM cannot account for the results that were obtained from the experiments in which immune serum and p-fluorophenylalanine were used.

V. Summary and conclusions

The existence of a precursor protein was postulated on the basis of the incomplete inhibition of viral synthesis by p-fluorophenylalanine (FPA) and the known mode of action of this inhibitor. Since a part of the anti-

*Actually in the present case if the virus existed in the membrane extract as a dimer, the volume would be twice as large and would have an axial ratio of 2:1. This axial ratio, however, would make a small difference in the rate of sedimentation as compared with the difference between the sedimentation rates of two particles whose volumes were different by a factor of two.

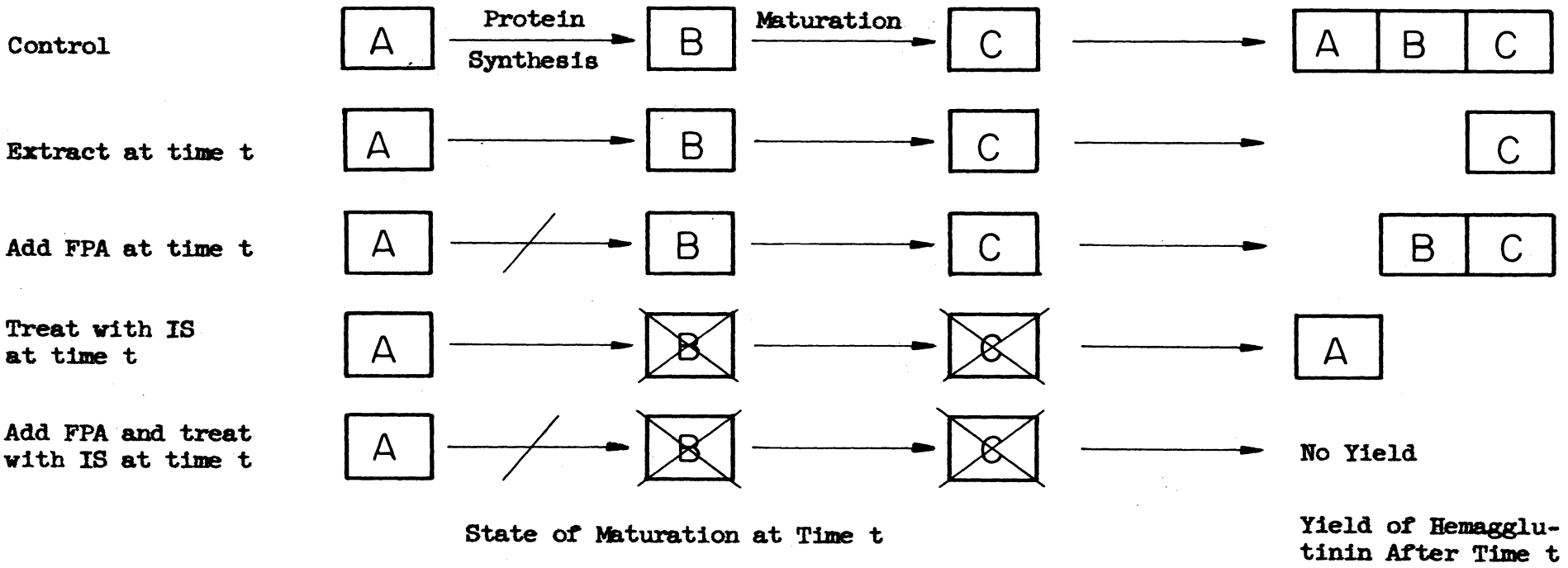
genicity of the mature virus should be due to this protein if it is a precursor, an attempt was made to further test the postulate by neutralization with antiviral serum. The concept of a precursor protein was found to be in keeping with the observation made both with the inhibition of protein synthesis by FPA and the neutralization of intact protein by antiviral immune serum (IS). Two independent estimates of the amount of precursor protein were made based upon these two types of observations and they were found to be essentially the same. The precursor protein was quantitated in terms of its capacity to give rise to hemagglutinin. This is shown diagrammatically in Fig. 10.

The synthesis of virus can be represented as a sequence of stages constructed upon the properties of the following intermediate forms:

$$\text{Pre-protein pool} \xrightarrow[\text{Synthesis}]{\text{Protein}} \text{Precursor Protein} \xrightarrow{\text{Maturation}} \text{PEV}$$

The pre-protein pool is composed of small molecular species destined to form virus and does not react with specific antiviral antibodies nor agglutinate red blood cells; the precursor protein possesses the former but not the latter property; and the PEV (pre-emergent virus) possesses both these properties. The intracellular biochemical process which converts the first stage to the second is protein synthesis. The nature of the maturation process which ultimately changes the precursor protein into hemagglutinating virus is not known. If the protein in question is a direct precursor of hemagglutinin, maturation may be the acquisition of the necessary structure or geometry for attachment to the combining sites on the red blood cells.

In the section dealing with the normal production of virus by the CAM, a curve was drawn which represents the appearance of hemagglutinin as a



A — Nonantigenic, nonhemagglutinating pre-protein pool

B — Antigenic, nonhemagglutinating precursor protein

C — Antigenic, hemagglutinating pre-emergent virus

Fig. 10. Summary of the use of p-fluorophenylalanine and immune serum for quantitation of the precursor protein.

function of time of incubation. This was called the normal growth curve (Fig. 2, curve C). The slope of this curve which represents the rate of production of hemagglutinin was shown in Fig. 3 (curve C). The latter curve can now be interpreted as showing the rate of the maturation process. The results of experiments in which p-fluorophenylalanine and immune serum were used suggested that the product of protein synthesis was a protein which is capable of reacting with antibodies in specific antiviral immune serum, i.e., a protein which is a specific viral antigen. A curve can be constructed which shows the manner in which this antigenic viral protein increases with time of incubation. The quantity of protein which has been synthesized by a certain time was calculated by summation of the amounts of hemagglutinin produced by that time and protein pool in the membrane at that time. The amount of hemagglutinin produced as a function of time of incubation is repeated in Fig. 11 (curve B). Curve C represents the amount of precursor protein in the membrane at any time. This is a plot of the averages of the ordinates of the precursor protein curves derived from the FPA and immune serum experiments. Curve A, which is the resultant of curves B and C, shows the total amount of protein synthesized as a function of time. The slopes of curves A and B of Fig. 11 are plotted in Fig. 12 (curves A and B, respectively). The latter curves show the manner in which the rates of protein synthesis and maturation vary with the time of incubation.

Protein synthesis and maturation are two intracellular stages in the production of type A influenza virus in the cells of the chorio-allantoic membrane. Figure 12 shows that the synthesis of viral protein begins at about 2-1/4 hours after initiation of infection and reaches its maximum rate of about 20 percent per hour at 3-1/4 hours. This high rate contin-

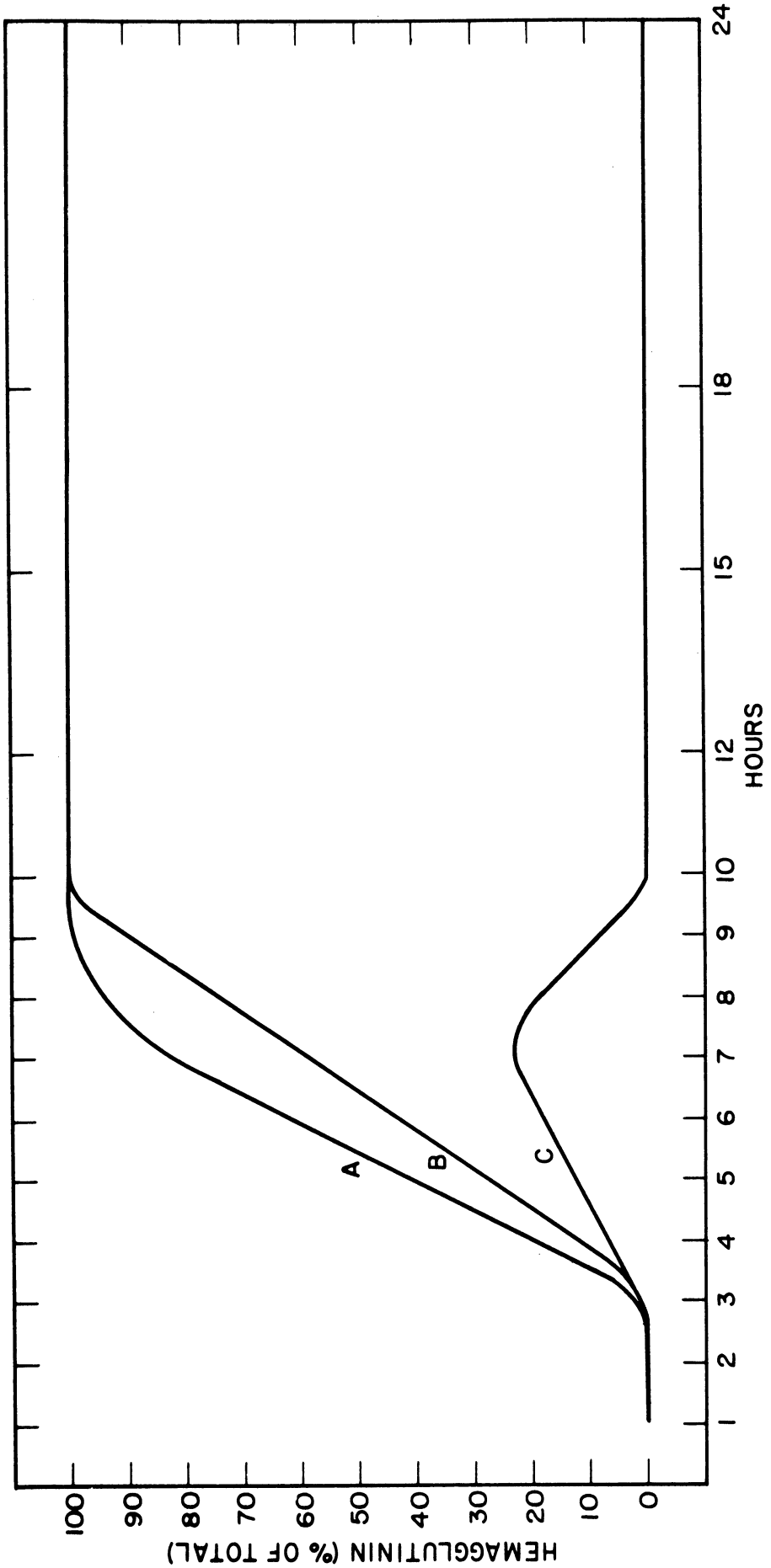


Fig. 11. Protein synthesis and the maturation of the precursor protein.— Curve C shows the amount of the protein pool in the chorio-allantoic membrane. Curves B and A show the total amount of virus and the amount of viral protein produced by the chorio-allantoic membrane, respectively.

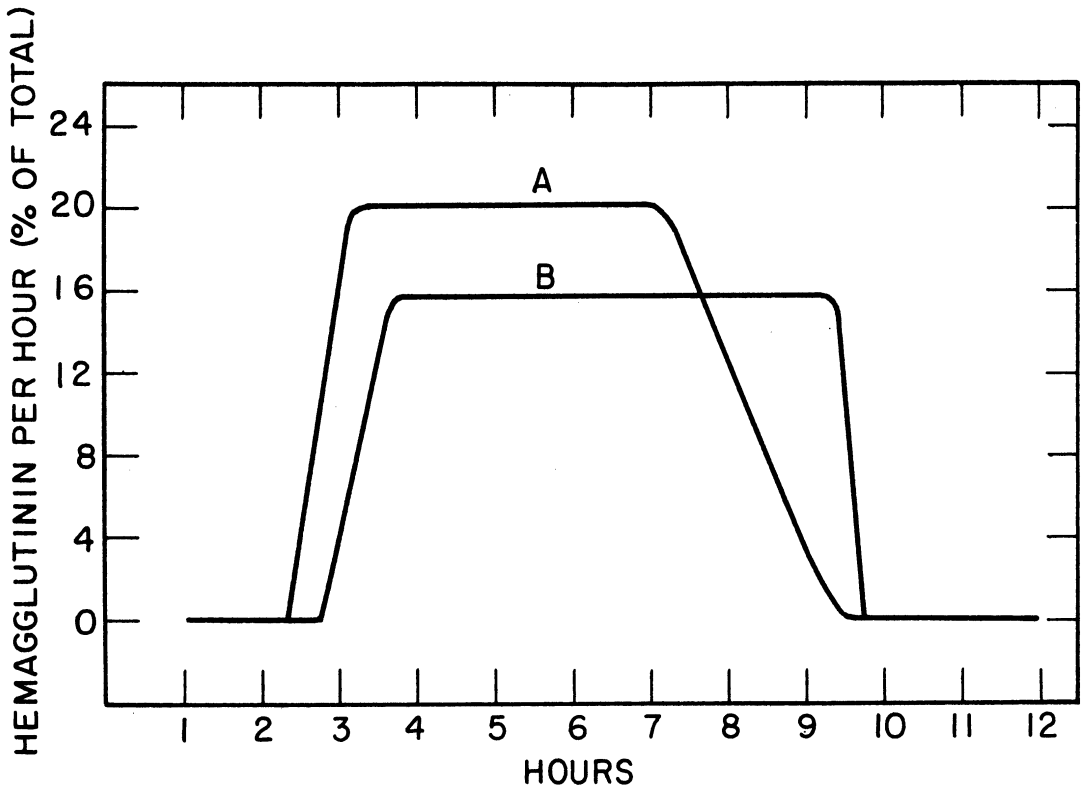


Fig. 12. The rates of protein synthesis and maturation of the precursor protein.—Curve A shows the rate of synthesis of viral protein. Curve B represents the rate of maturation of viral protein.

ues until about 7 hours and then slowly decreases to zero by 9-1/2 hours. The rate of maturation, i.e., the rate at which the protein becomes hemagglutinating, reaches its maximum rate of about 15.5 percent per hour at about 3-3/4 hours and continues at this rate until about 9-1/4 hours. From 7-1/4 to 9-1/4 hours, therefore, the pool of nonhemagglutinating precursor protein in the CAM decreases. The accumulation of precursor protein in the membrane is a consequence of the fact that the protein is synthesized at a faster rate than it matures. Maturation appears to be the rate determining step in the production of hemagglutinin.

GENERAL DISCUSSION

The following picture of the growth of PR8 influenza virus in the cells of the chorio-allantoic membrane has emerged from an interpretation of the results of these studies: (1) the virus is synthesized in the individual entodermal cells of the chorio-allantoic membrane in a continuous fashion over an extended period; (2) each virus particle proceeds through an intermediate form which is complete with respect to its protein composition and is antigenic but has not yet acquired the ability to agglutinate red blood cells; (3) the rates of synthesis of viral protein, of acquisition of the property of hemagglutination (maturation), and of release of mature virus from the cells are limited; and (4) the processes of protein synthesis and maturation reach their maximum rates quickly and maintain them for most of the viral productive period, after which time they quickly fall to zero. Release of virus also rapidly reaches a maximum rate, which is extended over a longer time but decreases more slowly.

Although the appearance of new virus over a period of time could result from the individual cells of the CAM having latent periods of varying lengths of time followed by a rather rapid production and release of virus, there are several lines of evidence to show that this is not the case. If the latent periods of the individual cells of the CAM were quite different, one would expect their lengths to fit a normal distribution about some mean. This would result in a sigmoid curve if the total amount of virus produced were plotted against the time of incubation. The curves obtained from the present investigation were not sigmoidal but linear.

Cairns (1952) has shown that release of virus from a single infected cell occurs over a period of several hours, i.e., that the virus is not released in a "burst," as is bacteriophage. Since Cairns measured only the released virus, his results could be interpreted as indicating the gradual release of virus synthesized during a short interval after initiation of infection. If this were true, however, the cells would contain a large amount of virus early in the productive phase, which would then decrease as the virus was released. The present study shows that this does not occur, but that the amount of virus in the cell increases during the entire period of production of new virus.

In the above discussion it was tacitly assumed that the protein-pool(s), as evidenced by the experiments in which p-fluorophenylalanine and immune serum were used, represented a precursor of mature virus. It was further assumed that the two types of experiments measured two properties of the same biologic entity. This interpretation seemed the simplest and most attractive of the several that presented themselves.

It is possible, first of all, that the protein pool(s) measured by the two experimental procedures are in reality two different stages in the multiplication of influenza virus. One stage, for example, might be a protein which is complete in its complement of amino acids and, therefore, beyond the stage of inhibition by p-fluorophenylalanine, but which requires further modification before it can combine with antibodies in antiviral serum.

By degradation of intact virus by ether (Hoyle, 1952; Frisch-Niggemeyer and Hoyle, 1956) and by repeated freezing and thawing (Tyrrell and Horsfall, 1954) a hemagglutinating particle much smaller than the intact virus has been demonstrated. The sedimentation experiments showed that

the product of the maturation of the "precursor protein" referred to in the present work was not a small particle, but one the size of intact virus.

There are two indications that the protein pool measured by the two experimental procedures is a precursor of mature virus. First, the protein pool has disappeared at the time when the production of new virus stops, and second, the product of protein synthesis is neutralized by specific antiviral serum. With respect to the second line of evidence it is also possible that the protein in question is not a precursor but that it and virus are synthesized *para passu*. If this were the case the nonprecursor protein would have to be capable of reacting with antibodies in immune serum prepared against mature virus.

The linear form of the curves representing protein synthesis and maturation is due to the fact that these processes are proceeding at their maximum rate. This probably means that the rates of these reactions are diffusion limited or are limited by the concentration of one or more enzymes mediating the reactions, and that these enzymes are saturated during most of the viral productive phase.

In several papers the constant or nearly constant rate of production of virus has been obscured by plotting the data on a semi-logarithmic coordinate system. In most cases (Hoyle, 1950; Cairns and Mason, 1953; Cairns, 1952; Finter *et al.*, 1954) the curves so plotted are not linear. In one case at least (Cairns and Mason, 1953) transformation to linear coordinates produced a straight line. Horsfall (1953, 1955), however, stated that the increase in hemagglutinin was logarithmic, and he suggested that reproduction is an autocatalytic reaction in which the rate is proportional to the amount of material produced. It may well be that

viral synthesis is essentially an autocatalytic process, but in many cases the kinetics do not show this because of some other factor, such as a saturated enzyme, limiting the rate.

There are two features of the "release curve" which must be reckoned with. The first of these is that the rate of release is constant during the first 9 hours of release, and the second is that it decreases rapidly after this period. One might expect that a certain intracellular concentration of virus would be needed for release to take place, and therefore, that once virus production has begun a certain quantity of virus would remain in the membrane. If this were the case the rate of release, after a delay, would be equal to the rate of synthesis. This is not the case; a constant fraction of the virus being produced remains in the tissue during the first 8 hours of viral increase. One might then suspect a state of equilibrium between the virus in the cells and that in the suspending medium. This also cannot be, since renewal of the medium does not affect the rate of release. If the mechanism of release of virus from the infected cell were an active biochemical process on the part of the cell, the constant-release period could be explained by some limiting process, perhaps an enzymatic reaction. There is some evidence for this. Schlesinger (1956b) showed that the release of virus is associated with the stepwise breakdown and restoration of the cellular mucoprotein substrate. If either the rate of breakdown (presumably by the viral enzyme) or the rate of restoration of the substrate were limited, the rate of release would also be limited and constant. Such a mechanism, however, does not account for the rapid decrease in the rate of release at about 12 hours, at which time about 35 percent of the total virus remains in the CAM. It is quite possible that after this time, there is no more restoration of

the mucoprotein—the synthesis of viral protein ceased at 10 hours—and the cell is damaged to the extent that viral release is no longer an active process but one associated with gradual disintegration of the cell.

It was seen (Fig. 4) that when p-fluorophenylalanine was added at six hours the subsequent rate of release decreased exponentially, suggesting that once protein synthesis was stopped the rate of release was proportional to the amount of viral protein remaining in the tissue at any time. It was also noted (page 50) that in the normal infection the rate of release followed these kinetics after 12 hours but only if it was assumed that a fraction of the virus was nonreleasable. These two observations are not necessarily contradictory because of the possible different state of the cells of the CAM at 6 hours and at 12 hours. The first observation, however, does appear to contradict the idea that release is an active process until about 12 hours and a passive one after this time, i.e., one would expect that the rate of release would continue at its maximum rate after addition of FPA at 6 hours. It is possible that FPA interferes with the active release of virus, and therefore after addition of the inhibitor at 6 hours the virus is released passively.

Hoyle (1950) found that filamentous forms are found in extracellular fluid but not in extracts of the chorio-allantoic membrane. It is, thus, probable that although the hemagglutinin titers of extracts gave a true indication of the number of virus particles, the titration of virus in the fluid phase indicated fewer elementary particles than were actually present. If this is true, the precursor protein is larger in amount than that indicated by the present studies.

The important problem concerning the action on virus-infected cells

of specific antibodies against the virus has been generally neglected. This may be due in part to the assumption that cells, normal or infected, are not permeable to gamma globulin. The work of Gitlin et al. (1953) strongly suggests, but does not conclusively prove, that gamma globulin does penetrate into living cells. Proteins comparable in size to gamma globulin have been shown to penetrate the membrane of intact cells. Yuile et al. (1951) working with C^{14}-labeled serum proteins demonstrated that plasma proteins are incorporated into protoplasm without previous breakdown into low molecular weight molecules. Bernhold (1956) showed that membranes of intact cells were permeable to dye-conjugated serum albumin and alpha globulin. Rosenfeld and Bascom (1956) presented evidence that excessive degradation of plasma proteins was not necessary for their penetration into hepatic cells. In the present investigation the results of experiments concerning the effect of antiviral immune serum indicate that immune gamma globulin does penetrate PR8-infected chorioallantoic cells. The unadsorbed immune serum which contained antibodies removable by adsorption with extracts of normal membrane definitely inhibited the synthesis of virus in cells treated with this serum. Inhibition was produced at a time when the cells were actively producing virus. This effect was probably attributable to an inhibition of some metabolic process in the host cell, perhaps due to the presence of antibodies against some enzyme necessary for the synthesis of viral protein. The possibility that this effect of the immune serum was due to antibodies against components on the surface of the host cell is possible though unlikely. The main line of evidence that gamma globulin does penetrate the infected cell is that an immature intracellular stage of virus, the precursor protein, is neutralized by exposure of the infected cell to immune serum. Pre-emergent forms of mature virus were also neutralized by immune

serum. These forms, however, are probably on or just inside the cell membrane (Eddy and Wyckoff, 1950).

As a consequence of the neutralization of an immature intracellular form of virus by exposure of the infected cell to specific antiviral immune serum, the cell synthesizes less virus than it would if the immune serum had not been present. This is the most significant finding that has emerged from these investigations. Rivers (1952) said:

> "In certain viral diseases, the administration of immune sera after exposure and before appearance of clinical signs and symtoms is efficacious, but, as a rule, once signs and symptoms of a viral or rickettsial disease have manifested themselves, the administration even of large quantities of immune sera is not very effective. Some workers disagree with this statement to a certain extent, but they have been unable to bring convincing evidence against the soundness of the view. In contesting the view, investigators admit that viruses or rickettsiae already in cells are not affected by immune substances but stress the fact that immune sera will prevent other cells in the body from becoming infected. The fallacy in the argument lies in the fact that, by the time patients have signs and symptoms sufficient to warrant the attendance of a physician, practically all cells in their bodies that are going to be infected have already been invaded by the viruses."

It is known, however, that the administration of concentrated convalescent gamma globulin may alter the course of mumps if given after the onset of symptoms (Rivers, 1952). A dose of unconcentrated adult serum can attenuate the severity of measles if the serum is given up to the 9th day after exposure (Janeway, 1944). The average incubation period of measles is 14 days, with a range of 12 to 14 days. The rash is preceded by 1 to 5 days of prodromal symptoms, and the incubation period for these symptoms may be as short as 7 days (Rivers, 1952). Thus it is apparent that immune serum can attenuate the clinical symptoms of measles when it is administered at a time when the virus is actively multiplying. In these cases the efficacy of administration of sera containing antibodies to the virus

may not be due entirely to the neutralization of extracellular virus and thus the prevention of infection of more cells, but may be due in part to the neutralization of intracellular viral precursors.

It is possible that in the future the combined use of an effective chemotherapeutic agent and specific antisera when administered after the onset of viral diseases may attenuate the severity of the disease.

SUMMARY

1. The suspended chorio-allantoic membrane culture system was improved to eliminate the differences between the membranes from individual embryonate eggs and found to be well adapted to the study of the multiplication of the PR8 strain of type A influenza virus.

2. Exposure of the chorio-allantoic membrane to $10^{8.68}$ EID_{50} virus for fifteen minutes or less is sufficient to infect all the entodermal cells of the membrane and produce a single cycle of multiplication.

3. The rate of release of virus from the cells of the chorio-allantoic membrane is not altered by repeatedly exchanging the suspending medium.

4. The rate of release of virus from the chorio-allantoic membrane is constant from 3-1/2 to 12 hours after initiation of infection.

5. The rate of production of virus in the cells of the chorio-allantoic membrane is constant from 3-1/2 to 9-1/2 hours and twice the rate of release of virus.

6. The production of virus is complete by 12 hours.

7. p-Fluorophenylalanine is capable of completely inhibiting the synthesis of viral protein regardless of when the inhibitor is added, and this inhibition is prevented in the presence of phenylalanine.

8. New virus is produced after the addition of p-fluorophenylalanine due to the maturation of a precursor protein.

9. Immune serum prepared by injecting rabbits with virus-infected allantoic fluid contains anti-chorio-allantoic membrane antibodies and these antibodies are cabable of inhibiting the multiplication of virus in

the chorio-allantoic membrane. These antibodies can be removed by adsorption with normal membranes.

10. Specific antiviral immune serum produces no inhibition of viral synthesis until approximately two hours and complete inhibition after 10 hours measured from the initiation of infection.

11. The precursor protein which exists in the cells of the chorio-allantoic membrane is neutralized by specific antiviral immune serum.

12. The rate of synthesis of viral protein is constant from 3 to 7 hours, is complete at 9-1/2 hours and is greater than the rate of maturation of the viral protein.

13. The two most significant results obtained from the study presented in this dissertation are: the finding of an intermediate stage of the synthesis of influenza virus in the cells of the chorio-allantoic membrane, namely, a precursor protein which is a specific viral antigen but does not have the property of hemagglutination; and the finding that specific antiviral immune serum is able to inhibit the production of virus in infected cells by virtue of its neutralizing a viral precursor.

APPENDIX

Release of Virus from the Chorio-Allantoic Membrane: Sampling Technique*

n	t_n	x_n	y_n	Y_n	$\frac{Y_n}{Y_{total}} \times 100$
2	2	0	0	0	0
3	3	32	32	32	0.92
4	4	200	179	211	6.07
5	5	400	267	478	13.75
6	6	512	245	723	20.81
7	7	640	299	1022	29.41
8	8	768	341	1363	39.22
9	9	768	256	1619	46.54
10	10	800	288	1907	54.86
11	12	1024	491	2398	69.00
12	15	1024	341	2739	78.82
13	18	800	117	2856	82.18
14	24	640	107	2963	85.26
24-hour CAM		512			(14.73)
Total				3475	99.99

*Figure 1

n = sample number

t_n = time sample was taken in hours after initiation of infection

x_n = HA titer of sample in HAU per milliliter

y_n = $(x_n - 2/3\ x_{n-1})$ = the HA released between times t_n and t_{n+1}

Y_n = $\sum^{n} y_n$ = the amount of HA released from time t_1 to time t_n

$\frac{Y_n}{Y_{total}} \times 100$ = the amount of HA released from time t_1 to time t_n as percentage of total virus produced (3475 HAU)

Release of Virus from the Chorio-Allantoic Membrane:
Complete Change of Medium*

n	t_n	x_n	Y_n	$\frac{Y_n}{Y_{total}} \times 100$
2	2	0	0	0
3	3	40	40	1.02
4	4	200	240	6.12
5	5	320	560	14.27
6	6	320	880	22.43
7	7	320	1200	30.58
8	8	320	1520	38.74
9	9	320	1840	46.89
10	10	320	2160	55.05
11	12	512	2672	68.09
12	15	512	3184	81.14
13	18	128	3312	84.40
14	24	100	3412	86.95
24-hour CAM		512		
Total			3924	100.00

*Figure 1

n = sample number

t_n = time sample was taken in hours after initiation of infection

x_n = HA titer of sample in HAU per milliliter

$Y_n = \sum^{n} x_n$ = the amount of HA released from time t_1 to time t_n

$\frac{Y_n}{Y_{total}} \times 100$ = the amount of HA released from time t_1 to time t_n as percentage of total virus produced (3924 HAU)

Time Study*

t	A	B	C (A+B)	D	E (C+D)	F	G	H (E+F+G+)
1	0	0	0	0	0	1600	200	1800
2	0	0	0	0	0	2048	400	2448
3	3	16	19	40	59	2048	400	2467
4	160	80	240	128	368	2048	400	2688
5	512	100	612	640	1252	2560	512	3684
6	800	160	960	1200	2160	3200	640	4800
7	640	160	800	800	1600	1280	400	2480
8	800	200	1000	1024	2048	1280	320	2600
9	1024	200	1224	1280	2504	1024	400	2648
10	1280	160	1440	1280	2720	800	400	2640
12	2048	128	2176	1024	3200	640	400	3216
15	2048	80	2128	512	2640	128	400	2648
18	1600	100	1700	400	2100	100	320	2120
24	2560	---	2560	---	2560	---	400	2960

	C'	D'	E'	H'
1	0	0	0	100
2	0	0	0	100
3	0.77	1.62	2.39	100
4	8.93	4.76	13.69	100
5	16.61	17.37	33.98	100
6	20.00	25.00	45.00	100
7	32.26	32.26	64.52	100
8	38.46	39.39	78.77	100
9	46.22	48.34	94.56	100
10	54.55	48.49	103.03	100
12	67.66	31.84	99.50	100
15	80.37	19.34	99.71	100
18	80.19	18.89	99.06	100
24	86.49			100

*Figure 1: Refer to Column C'.
Figure 2: Curve A; refer to Column D'.
Curve B; refer to Column C'.
Curve C; refer to Column E'.

At t hours after initiation of infection, a membrane was washed, incubated until t + 1/4 hours in fresh medium, washed and incubated for the remainder of the 24-hour period in fresh medium; another membrane was extracted after t + 1/4 hours.

A = amount of HA released from 1 to t hours
B = amount of HA released from t to t + 1/4 hours
C = A+B = amount of HA released from 1 to t + 1/4 hours
D = amount of HA in membrane at t + 1/4 hours
E = C+D = total amount of HA produced from 1 to t + 1/4 hours
F = amount of HA released from t + 1/4 hours to 24 hours
G = amount of HA in membrane at 24 hours
H = E+F+G = total amount of HA produced from 1 to 24 hours
C',D',E', and H' = C/H x 100, D/H x 100, E/H x 100, and H/H x 100, respectively

The Effect of p-Fluorophenylalanine
on the Production of Virus*

Hours After Initiation of Infection	Control		FPA (1.1 mg/ml) at 6 hours	
	Incremental Yield HA/ml	Cumulative Yield HA/ml	Incremental Yield HA/ml	Cumulative Yield HA/ml
1	0	0	0	0
2	0	0	0	0
3	50	50	40	40
4	512	562	400	440
5	512	1074	400	840
6	512	1586	400	1240
7	512	2098	320	1560
8	512	2610	256	1816
9	512	3122	256	2072
10	400	3522	160	2232
11	512	4034	128	2360
12	512	4546	100	2460
24	1280	5826	100	2560

*Figure 4

Time Study*
The Effect of p-Fluorophenylalanine

t	A	B	C (A+B)	D	E	F	G (E+F)
1	1600	200	1800	1800	0	0	0
2	2048	400	2448	2448	5	0	5
3	2048	400	2448	2467	256	32	288
4	2048	400	2448	2688	320	50	370
5	2560	512	3072	3684	1024	320	1344
6	3200	640	3840	4800	1600	512	2112
7	1280	400	1680	2480	1024	400	1424
8	1280	320	1600	2600	1024	400	1424
9	1024	400	1424	2648	1024	400	1424
10	800	400	1200	2640	800	400	1200
12	640	400	1040	3216	640	400	1040
15	128	400	528	2648	128	400	528
18	100	320	420	2120	100	320	420

	C'	D'	G'
1	100	100	0
2	100	100	0.20
3	99.23	100	11.07
4	91.07	100	13.76
5	83.39	100	36.48
6	80.00	100	44.00
7	67.74	100	57.42
8	61.54	100	54.77
9	53.78	100	53.78
10	45.46	100	45.46
12	32.34	100	32.34
15	19.94	100	19.94
18	19.81	100	19.81

*Figure 5

After time t_1 p-fluorophenylalanine was present in the concentration of 1.2 mg per ml, total volume 3 ml.

A = amount of HA released from t + 1/4 to 24 hours
B = amount of HA in membrane at 24 hours
C = A+B = amount of HA released from t + 1/4 to 24 hours plus that in the membrane at 24 hours
D = total amount of HA produced from 1 to 24 hours
E = amount of HA released from t + 1/4 to 24 hours in the presence of FPA
F = amount of HA in membrane at 24 hours in the presence of FPA
G = E+F = amount of HA released from t + 1/4 to 24 hours plus that in the membrane at 24 hours in the presence of FPA
C', D', and G' = C/D x 100, D/D x 100, and G/D x 100, respectively

Time Study*
The Effect of Immune Serum

t	E	F	G	G'
1	1600	200	1800	100
2	2048	400	2448	100
3	2048	320	2368	95.99
4	1600	400	2000	74.40
5	1600	320	1920	52.12
6	1600	128	1728	36.00
7	200	50	250	10.08
8	100	32	132	5.08
9	20	20	40	1.51
10	0	0	0	0
12	8	4	12	0.37
15	0	0	0	0
18	0	0	0	0

*Figure 7

At time t the membrane was treated with immune serum for 15 minutes.

See page 104 for columns A, B, C, and D.

E = the amount of HA released from t + 1/4 to 24 hours after treatment with immune serum
F = the amount of HA in the membrane at 24 hours after treatment with immune serum
G = the amount of HA released plus that in the membrane at 24 hours, after treatment with immune serum

The Ultracentrifugal Sedimentation of Virus*

Concentration of Sucrose % by Weight	HA Remaining in Supernatant Fluid					
	Allantoic Fluid		HA Released from 1-6 hr		HA in Extract at 6 hr	
	20,700 rpm 40 min		15,600 rpm 39.55 min		15,600 rpm 39.55 min	
	HA	%	HA	%	HA	%
3	3	1.88	4	2	6	2.3
6	5	3.12	10	5	10	3.9
9	10	6.25	16	8	20	7.8
12	4	2.50	25	12	32	15.6
15	10	6.25	50	25	64	25
18	16	10	50	25	80	31.2
21	25	15.6	100	50	128	50
24	40	25	128	64	160	62.5
27	80	50	128	64	200	78.1
30	100	62.5	160	80	200	78.1
33	128	80	200	100	256	100
36	160	100	200	100	256	100
Controls	160	100	200	100	256	100

*Figure 9

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