BIOCHEMICAL EVENTS OCCURRING IN CHICK EMBRYO TISSUE CULTURE CELLS INFECTED WITH NEWCASTLE DISEASE VIRUS

by

MICHAEL PAUL LERNER

B. A., University of California, Los Angeles, 1963

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

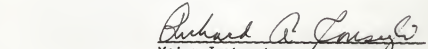
MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1967

Approved by:



Major Instructor

TABLE OF CONTENTS

INTRODUCTION

The infection of cells by animal viruses results in the formation and release of new virus particles and profound biochemical changes within the host cell. By using the tissue culture cell system, it is possible to study the interaction of the virus with the host cell *in vitro*. Since only two viral-specific enzymes have been shown to occur, the host cell must possess most of the enzymes required for viral production. Therefore, the viral nucleic acid, once separated from its protein coat, acts as a messenger by redirecting cellular enzymatic, protein, and nucleic acid synthesis, resulting in viral production and cellular disintegration and destruction. In most cases of infection by viruses containing ribonucleic acid (RNA) as the genetic material, the infectious cycle in the host cell is completed within 8-12 hours. Therefore, the specific biochemical events involved in the synthesis of new viral particles must take place rapidly and in an ordered manner.

The purpose of the work reported in this thesis was to investigate certain biochemical and enzymatic events which occurred in chick embryo tissue culture cells infected with Newcastle disease virus, an RNA virus. Three different types of biochemical events occurring in the course of infection were examined. First, studies of the effects of infection on certain cellular energy mechanisms were performed. Next, the aspects involved in cellular synthesis of viral RNA were studied with the specific aim of demonstrating and isolating a double-stranded, replicative form of NDV RNA. Finally, the fate of the viral protein coat during the course of infection was investigated.

REVIEW OF LITERATURE

Newcastle disease, a respiratory infection of chickens, was first recognized in 1926 in Newcastle-on-Tyne in Great Britain (Doyal, 1927) and in Java (Kranevels, 1926). By 1940, it had been reported in California (Beach, 1944) and throughout the United States (Beaudette and Black, 1945).

Characteristics of Newcastle Disease Virus

Newcastle disease virus (NDV) is a myxovirus. The myxoviruses, so named because they have an affinity for mucoproteins, were first named by Andrewes, Bang, and Burnette (1955). There are two different groups within the myxoviruses, based mainly on size and site of nucleic acid synthesis (Andrewes, 1965). Group I consists of the true influenza viruses, ranging in size from 80-120 mu in diameter and having viral nucleic acid produced in the nucleus of infected cells. Group II consists of Newcastle disease virus, mumps virus, and the parainfluenza viruses. This group is from 120-300 mu in diameter and viral nucleic acid is synthesized in the cytoplasm of infected cells.

The genetic material of all myxoviruses is ribonucleic acid (RNA). Treatment of this group of viruses with ether results in the complete loss of infectivity. The myxoviruses possess two specific antigenic components, an internal ribonucleoprotein and viral protein coat. Hirst (1942) reported that influenza virus could adsorb to and agglutinate red blood cells, and that this virus eluted spontaneously from these RBC's after incubation at 37C. He referred to the substance responsible for

RBC adsorbtion and elution as viral hemagglutinin. Neuriminidase, an enzyme which destroys cell receptor sites, and is specifically responsible for viral elution from RBC's, was isolated and identified by Gottschalk (1958). All myxoviruses possess a neuriminidase and hemagglutinin which are located on the viral protein coat.

Newcastle disease virus was first characterized by Bang (1946) as pleomorphic, but later work (Bang, 1948) indicated that the pleomorphic forms were due to different salt concentrations, and that the virus, when dried from distilled water, was spherical. Electron micrographs and chemical analysis showed that the particle consisted of a nucleoprotein core surrounded by a lipoprotein envelope, which had spiked projections (Horne et al., 1960).

By treatment of virus particles with ether, the inner core and envelope can be separated. The nucleoprotein part consists of 10% RNA (Waterson, 1964), which has a molecular weight of about 10^7 daltons (Duesberg and Robinson, 1965). Based on its size, Waterson (1964) has suggested that viral RNA is in an extended form within the core. The base composition indicated that the RNA was single-stranded (Kingsbury, 1966a).

The protein part of the viral coat has the hemagglutinating properties common to all myxoviruses and, in addition, the ability to lyse RBC's. Studies with influenza virus (Noll et al., 1962) and NDV (Rott et al., 1962) indicated that the hemagglutinin was located in the spikes on the protein coat, and the neuriminidase activity was stacked between these spikes. Three types of hemagglutinins were demonstrated in virus infected tissue culture cells; the complete virus, a small, noninfectious

component, and viromicrosomes or cell bound hemagglutinin (Granoff et al., 1950). The viromicrosomes have been obtained only from artificially disrupted cells and were devoid of spiked projections, although they did agglutinate RBC's. Schafer (1963) has suggested that the viromicrosomes represent the cellular site of hemagglutinin production. The small, noninfectious component resembled viral protein coat devoid of nucleoprotein (Rott, 1964).

Cunha et al. (1947) reported that NDV contained about 25% lipid, 65% protein, and 7% carbohydrate. The exact chemical nature of these components has not yet been reported.

Newcastle Disease Virus in Tissue Culture Systems

According to Chanock and Parrot (1965), NDV was able to grow in a wide variety of tissue culture cells; chick embryo, human heteroploid cells, such as Hela and L cells, primary simian and bovine epithelium, and bovine fibroblasts. The allantoic membrane cells of chick embryos produce about 1000 virus particles/cell while the yield from tissue culture cells ranges from 1-40 viruses/cell. Apparently, there is a defective mechanism in the tissue culture system.

The sequence of events which occur during the infection of animal cells by RNA viruses has been summarized by Waterson (1961). These events include adsorbtion, penetration, uncoating, synthesis of new viral RNA and protein, aggregation of viral components and release of progeny viruses. Levine and Sagik (1957) found that NDV attached to specific, complementary receptor sites on chick embryo cells by electrostatic and hydrogen bonding. The cell receptor site has been shown to

consist of N-acetylneuramic acid (Gottschalk, 1958). Cations, to reduce the force of repulsion, were needed for attachment, since both the virus and cell were negatively charged (Levine and Sagik, 1957). Also, for efficient viral adsorbtion, 0.15M NaCl and a pH of 6.8-7.7 were required. The reaction was independent of temperature between 2-30C. Although 100% of infecting virus eluted from RBC's within 2 hours at 37C (Sagik and Levine, 1954), no viral elution occurred in tissue culture cells under similar conditions.

Following adsorbtion, the virus particle enters the host cell. Electron micrographs by Silverstein and Marcus (1964) of Hela cells infected with NDV showed that the complete virus was engulfed by an invagination of the cell membrane, a process similar to pinocytosis in amoebae. Single virus particles were found to be enclosed in a cytoplasmic vacuole in the cell.

After penetration, the virus enters the eclipse phase. During this period the virus is truly intracellular and complete viral particles cannot be detected inside of the cell. Uncoating is the first step in the eclipse phase. Silverstein and Marcus (1964) reported that immediately following penetration, the cytoplasmic vacuole of the cell began to swell and the virus lost its outer membrane. Within 30 minutes after viral adsorbtion, intact virus particles were observed within the cell (Mussgay and Weibel, 1962). The enzymes responsible for removing the viral coat from nucleic acid have not yet been identified, and are probably normal cell enzymes. Holland and Hoyer (1962) favored the idea that the plasma membrane of the cell aids in the release of the virus.

Once the viral nucleic acid is inside the cell and is separated from

its protein coat, it begins the process of taking over the cell and producing viral progeny. All of the synthetic processes involved in the production of NDV have been shown to occur in the cytoplasm of infected cells (Wheelock and Tamm, 1959; Reda, Rott and Schäfer, 1964).

It has been reported that p-fluorophenylalanine, an amino acid analog, prevented the synthesis of fowl-plague (an influenza virus) virus (Scholtissek and Rott, 1961) and NDV (Scholtissek and Rott, 1965). These results, along with similar results on poliovirus infected cells (Darnell, 1962), indicated that protein synthesis was the first step in viral replication, Wilson and LoGerfo (1964) found that puromycin, a protein synthesis inhibitor, prevented the synthesis of NDV RNA for 4 hours after infection. They also showed that protein synthesis preceded viral RNA synthesis and that the protein was made even if RNA synthesis was prevented.

A new enzyme, RNA-dependent RNA polymerase, involved as a template in the synthesis of new viral RNA, has been shown to occur in cells infected with poliovirus, mengovirus and influenza virus (Baltimore and Franklin, 1963; Glasky et al., 1964). The appearance of this RNA polymerase paralleled the synthesis of new viral RNA. The incorporation of radioactive uridine and cytidine into NDV RNA was shown to begin about 4 hours post infection (Scholtissek and Rott, 1965; Granoff and Kingsbury, 1964). Since the incorporation of these compounds into viral RNA occurred at the same time as did new viral protein synthesis, the early protein might be NDV viral RNA polymerase. Therefore, as Wilson and LoGerfo (1964) suggested, the NDV viral genome could be acting directly as messenger RNA, since early protein synthesis occurred in the presence

of RNA inhibitors. Penman et al. (1964) found that poliovirus RNA was not degraded during infection and served as a direct template for early viral protein synthesis. Glasky and Holper (1963) reported the isolation of an RNA polymerase from chick embryo cells infected with NDV.

Following the discovery of RNA polymerase, Montagnier and Sanders (1963) discovered a double-stranded, replicative form of viral RNA. This type of RNA, being similar to DNA (deoxyribonucleic acid), would allow the efficient production of new single-stranded RNA molecules by the base-pair copying method proposed by Watson and Crick (1953). While a double-stranded RNA has not yet been found in NDV infected cells, Kingsbury (1966b) has reported finding a single-stranded NDV RNA which is complementary to parental RNA and which might be utilized as a template for new viral synthesis.

The synthesis of viral protein in infected cells has been shown to increase at 3 and between 5-12 hours after infection (Wheelock, 1963; Scholtissek and Rott, 1965). The increase at 3 hours could be related to the early viral proteins, while the increase beginning at 5 hours reflected new viral protein synthesis. By increasing the multiplicity of infection or treating cells with actinomycin D, which inhibits DNA-directed RNA synthesis, Barry (1965) noted that viral components were produced earlier than in cells without the antibiotic. These results indicated that the time of viral component synthesis could be changed by varying certain cellular growth conditions.

Biochemical changes occurred in cells infected with NDV, according to Wheelock (1962). He reported inhibition of cell protein synthesis was due to the synthesis of viral RNA. Bolognesi and Wilson (1966)

found that when cells were synthesizing as little as 35% of the maximum amount of viral RNA, the inhibition of cell protein synthesis occurred. They reported that by 9 hours after infection, 85% of the cell protein synthesis was inhibited. Bolognesi and Wilson postulated that the early virus specific proteins, synthesized from the viral genome, remained stable within the cell and were also responsible for inhibition of cell protein synthesis. They also stated that the inhibition of cell protein synthesis might lead to degradation of cell polysomes and the eventual use of these polysomes to synthesize viral components.

In the eclipse period, the final phases, which occur between 5-8 hours post infection, are aggregation and release of viral particles. The components of the virus were shown to be assembled into complete particles at the cell surface (Waterson, 1961). In certain unsuitable cell lines, such as Hela and L cells, excess viral proteins and RNA were produced (Wheelock, 1962). The lipid part of the virus came from the host cell, according to Marcus (1962). He demonstrated that infected Hela cells were covered by the viral antigen responsible for hemagglutination. Drake and Lay (1962) and Durand and Eisenstark (1962) also showed that the host cell contributed certain nongenetic, physical and chemical properties to the virus. Adams (1966) reported that extensive gradient purification of NDV RNA failed to remove small residual amounts of host cell RNA. The complete virus particles were shown to be released slowly from infected cells (Waterson, 1961) with the aid of neuriminidase, which has also been shown to be involved in the release of influenza virus particles (Padgett and Walker, 1964).

MATERIALS AND METHODS

Virus

The Roakin strain of Newcastle disease virus was used in all experiments. Stock virus was made by infecting the allantoic cavity of 10-12 day old embryonated hens' eggs with 10^4 plaque-forming-units (pfu) of NDV. The eggs were incubated in a humidified 38C incubator for 36-48 hours or until embryonic death was observed. The allantoic fluid was harvested and frozen and thawed to release ureates, which were removed by centrifugation for 30 minutes at 2500 rpm. All centrifugation was done in an International refrigerated centrifuge (Model PR2) unless otherwise noted. The clear viral supernatant was dialyzed for 16 hours at 4C against 200 volumes of phosphate (0.01M, pH 7.2, 0.15M NaCl) buffered saline (PBS). The suspension was then mixed with serum-free Eagle's (MEM) medium and stored at -20C in 50 ml aliquots until use. Stock virus prepared in the above manner had a titer of 1-2 x 10^8 pfu's/ml.

Cell Culture

Primary chick embryo tissue culture cells were made from 10-12 day old embryonated eggs. The embryo's, after being aseptically removed from the eggs, were minced and washed in PBS. After 10 minutes exposure to 0.25% trypsin (Difco 1:300), the cells were sedimented by centrifugation for 10 minutes at 1000 rpm. This process was repeated until all of the tissue was trypsinized. The cell suspension was counted in a hemocytometer, diluted to 1.0-1.2 x 10^6 cells/ml in Eagle's medium

supplemented with 10% horse serum, and seeded into glass or Falcon plastic Petri dishes. After incubation for 48-72 hours in a humidified 37C, 5% CO_2-air incubator, complete monolayers of cells were obtained and were used either for experiments or for growing radioactive virus.

Radioactive Virus

Virus containing H^3-uridine RNA was made by infecting complete monolayers of cells with 1 pfu/cell of stock virus. After a 30 minute adsorbtion period at 37C the infected cells were washed with PBS and overlayed with Eagle's medium containing 5% dialyzed (against 200 volumes PBS for 24 hours at 4C) horse serum and 0.5uc/ml of H^3-uridine (Schwartz BioResearch Inc., Orangeburg, N. Y.; specific activity 20.0c/mM). Infected cultures were incubated at 37C in a humidified CO_2 incubator for 36-48 hours or until extensive cellular degeneration was observed. The cells and growth medium were collected, frozen and thawed 3 times, and centrifuged for 30 minutes at 2000 rpm. The clear supernatant was saved, and the precipitate, consisting of cellular debris, was mixed with 10ml of the clear supernatant and treated with 1.0ml receptor destroying enzyme (Microbiological Associates, Bethesda, Md.) for 2 hours at 37C. This mixture was then centrifuged at 3000 rpm for 30 minutes and pooled with the original supernatant. The suspension containing radioactive virus was precipitated with 33% saturated $(NH_4)_2SO_4$ and sedimented at 8000 rpm for 3 hours in a Serval (SS-3) centrifuge at 4C. The supernatant was discarded and the viral pellet was resuspended in 5ml PBS and dialyzed against 3000 volumes PBS for 16 hours at 4C. The suspension was then treated with the following

enzymes: DNAse (10ugm/ml) and RNAse (10ugm/ml), in the presence of 10^{-3}M $MgCl_2$, and trypsin (0.1mg/ml) for 30 minutes at 37C. Following low speed centrifugation for 15 minutes at 3000 rpm in the International refrigerated centrifuge, the virus was pelleted in 2 hours at 35,000 rpm in the SW39 rotor of a Spinco Model L ultracentrifuge. The virus was resuspended in 5ml of tris (hydroxymethyl) amino methane (Tris) buffered saline, and stored frozen. H^3-valine-labeled NDV was prepared in the same manner as was H^3-uridine NDV with one exception; infected monolayers were overlayed with 50% valine deficient (0.24 gms/l) Eagle's medium containing 5% dialyzed horse serum and 0.5uc/ml of DL-valine-3,4-H^3 (New England Nuclear Corp., Boston, Mass.; specific activity 100uc/mM). Purified, nonradioactive virus was also prepared in the above manner.

Plaque Assay

All virus was titered before use by a modified plaque assay method for animal viruses (Delbecco, 1952). Primary monolayers of chick embryo cells were grown as previously described. The cells were washed once in PBS and 0.5ml of various log dilutions of virus were added. After a 30 minute adsorbtion period at 37C, infected monolayers were covered with 8 ml of an equal mixture of 2X Adeno medium (Consigli et al., 1966) and 2% purified agar (Difco). After 3 days incubation in a CO_2 atmosphere at 37C, 2.5ml of Adeno medium-agar mixture containing 0.01% neutral red (1/52,000 final concentration/ml) were added. Plaques were clearly visible 12 hours after the addition of the dye.

Hemagglutination

Hemagglutination (HA) titrations were performed when a rapid estimation of the number of virus particles was desired. Two-fold serial dilutions of NDV in 0.5ml of PBS were made in plastic HA trays, followed by the addition of 0.5ml of a 1% suspension of washed chicken red blood cells (final concentration of RBC's was 0.5%). Results were read after 30 minutes incubation at room temperature.

Enzyme Studies on NDV-Infected Chick Cells

The determinations of uridine kinase, carbamyl phosphates synthetase, adenosine triphosphatase (ATPase), and hexokinase were performed simultaneously on infected and control cells. Primary monolayers of chick tissue culture cells, grown in 100mm diameter Falcon plastic Petri dishes, were pretreated 4 hours prior to infection with lugm/ml of actinomycin D, an inhibitor of DNA-dependent RNA. One group of dishes was infected with 50 pfu's/cell of stock virus containing lugm/ml of actinomycin D. After an adsorbtion period of 35 minutes at 37C in a CO_2 incubator, the dishes were washed with PBS and overlayed with Eagle's medium containing 5% dialyzed horse serum and lugm/ml of actinomycin D. Control dishes were treated in the same manner. The cells were incubated in a 37C incubator and were harvested at 1, 3, 5 and 8 hours after infection. The following procedure to harvest the cells and to release cellular enzymes was followed in all experiments.

The growth medium was removed and 2ml of phosphate-free 0.15M NaCl were pipetted onto the monolayers. The cells were scraped off

of the dishes with a rubber policeman, washed once in phosphate-free 0.15M NaCl and pelleted by centrifugation for 10 minutes at 1000 rpm. The cells were resuspended in 4ml of phosphate-free 0.15M NaCl and 3ml of phosphate-free Tris-HCl (0.02M, pH 8.0) and were kept for 10 minutes at room temperature. The cells were broken by 15 strokes of a Ten Broek Pyrex tissue grinder (Corning Glass Works, Corning, New York). Cellular debris was then removed by centrifugation for 30 minutes at 3200 rpm. The resulting cell-free suspension was used as the source of enzymes for all assays. Protein determinations, indicating the number of cells involved at each assay period, were also taken from this cell-free suspension. Proteins were measured by the method of Lowry et al. (1951), using crystalline bovine albumin as a standard.

UMP Synthesis. Uridine kinase was assayed according to a modification of the method of Skold (1960). The reaction mixture consisted of 15uM of ATP, 10uM of $MgCl_2$, 0.25uM uridine and 2uc of H^3-uridine, all in 0.25ml of 0.1ml Tris-HCl, pH 8.0. After adding 0.5ml of enzyme, the mixture was incubated for 15 minutes at 37C. Enzymatic action was stopped by the addition of 5% trichloroacetic acid (TCA) followed by 40 minutes incubation in an ice bath. The acid soluble material was separated by centrifugation and frozen. Uridine monophosphate (UMP) was separated from all other products by two dimensional descending chromatography on Whatman number 3 MM paper. The first dimension solvent, which separated uridine from the uridine nucleotides, consisted of n-butanol, distilled water and 15N NH_4OH (785:150:11 v/v). The second dimension solvent, consisting of isobutyric acid, distilled water, 15N NH_4OH and 0.1M ethylenediaminetetraacetic acid (EDTA) (132:66:2:2 v/v),

separated UMP from other nucleotides. The area on the chromatogram containing UMP was located by ultraviolet light using a known UMP standard, cut out and hydrolyzed with 0.01N HCl, and counted for radioactivity in a Packard Tricarb liquid scintillation counter. The scintillation fluid, which was used in all radioactive determinations, contained: 360ml toluene, 360ml dioxane, 216ml absolute ethanol, 80 gms naphthelene, and 5 gms POP (2,5 diphenyloxazole). Calculations of uridine kinase activity were based on the total radioactive counts per minute of UMP formed per milligram of protein in each sample.

ATP Degradation. ATPase, which degrades ATP, was assayed according to a modification of the method of Lowry et al. (1954). The reaction mixture, which contained 5uM ATP and 2uM $MgCl_2$, in 0.5ml of phosphate-free Tris-HCl (0.03M., pH 8.0), along with 1.0ml of cell enzyme, was incubated for 30 minutes at 37C. The reaction was stopped with 5% TCA, and the inorganic phosphate, found in the TCA soluble fraction, was determined by the method of Bruemmer and O'Dell (1956), using Na_2HPO_4 as a standard. The results were based on the ugms of inorganic phosphate per mg of cell protein.

Enzymatic Phosphorylation: ATP and Glucose-6-Phosphate (G-6-P) Synthesis. The enzymatic substrate for the determination of carbamyl phosphate (CP) synthetase, involved in ATP formation, consisted of 10uM ADP (adenosine diphosphate), 16uM carbamyl phosphate, 1mgm yeast hexokinase, 1uM $MgCl_2$ and 50uM glucose, all dissolved in 1.0ml of phosphate-free Tris-HCl (0.03M, pH 8.0). The reaction mixture for the determination of hexokinase, involved in G-6-P synthesis, contained 10uM ATP, 50uM glucose, and 1uM of $MgCl_2$, all dissolved in 1.0ml of phosphate-free

Tris-HCl (0.03M, pH 8.0). After adding 1.0ml of enzyme to the hexokinase and CP synthetase substrates, they were incubated for 1 hour at 37C. The reactions were stopped by adding 5% TCA, and the TCA soluble fractions were assayed for ATP and glucose-6-phosphate. The yield of products formed from the phosphorylation of glucose to glucose-6-phosphate and ADP to ATP, trapping the inorganic phosphate cleaved from ATP as glucose-6-phosphate, was determined by the reduction of pyridine nucleotides (Horecker and Kornberg, 1948). Calculations were based on the published molar extinction coefficients of reduced triphosphopyridine nucleotides (NADPH) which were read at 340 mu on a Zeiss model PMQ II spectrophotometer.

Fate of the Viral Protein Coat

In order to follow the fate of the viral protein coat during an 8 hour infectious cycle, monolayers of cells, grown on 60mm diameter Falcon plastic Petri dishes, were infected with purified H^3-valine-labeled NDV. Also, the effects on the process of viral uncoating in the presence of actinomycin D and puromycin, a protein synthesis inhibitor, were studied. Three experimental conditions were used. One set of dishes, the control, was infected with 20 pfu's/cell of virus only, another set with 20 pfu's/cell of virus containing 50ugms/ml of puromycin hydrochloride, and a third set with the same virus concentration containing 5ugms/ml of actinomycin D. After a 30 minute adsorbtion period, all dishes were washed three times with PBS and overlayed with 50% valine-deficient Eagle's medium containing 5% dialyzed horse serum and the same concentrations of inhibitors as previously mentioned.

Infected dishes were incubated at 37C in a CO_2 incubator and harvested at 0.5, 1, 2, 3, 4, 5, 6 and 8 hours post infection. During harvesting, the media from each experimental set of dishes were removed and frozen. The cells were scraped off of the dishes, washed once in PBS, pelleted by centrifugation at 1000 rpm for 10 minutes in an International refrigerated centrifuge, resuspended in 0.5ml of 0.15M neutral saline, and frozen. The growth medium from the three sets of dishes was thawed and centrifuged for 10 minutes at 500 rpm to remove any cellular debris. A sample was removed and counted for radioactivity, which reflected degraded viral protein coat material. To another sample of media was added 10% TCA, and after 40 minutes in an ice bath and centrifugation at 2500 rpm for 30 minutes, the acid soluble fraction was counted. These counts were to determine if any of the original counts from the media were due to complete virus, which was then removed from the TCA soluble fraction. The cells, after being thawed, were precipitated with TCA (10% final concentration). The acid soluble fraction was removed and counted for radioactivity. The acid precipitate was washed once in 10% TCA, dissolved in 1N NaOH and counted for radioactivity. In order to determine if virus was being produced from these cells, samples of the three sets of dishes were kept in the incubator for 18 hours after infection. The media were removed and the amount of virus determined by plaque titrations.

Studies on RNA in Virus-Infected Cells

Three different experiments were undertaken to study RNA synthesis in NDV-infected cells. They were the incorporation of tritiated

uridine, the fate of radioactive viral RNA and its susceptibility to ribonuclease (RNAse) during an 8 hour growth period, and an attempt to isolate viral specific, RNAse-resistant RNA. The first experiment was the determination of H^3·uridine incorporation into infected and control cells. Monolayers of cells, grown in 60mm diameter Falcon plastic Petri dishes, were pretreated 4 hours prior to infection with 1 ugm/ml of actinomycin D. The cells were infected with 50 pfu's/cell of stock virus in the presence of 1 ugm/ml actinomycin D, and after a 30 minute adsorbtion period at 37C, Eagle's medium containing 5% dialyzed horse serum, 1 ugm/ml actinomycin D, and 0.25 uc/ml H^3·uridine was added. Control cells were treated in the same manner. All cells were incubated at 37C and harvested at 1, 2, 3, 4, 5, 6 and 8 hours post infection. The cells were washed in PBS and pelleted by centrifugation. A sample was removed for protein determination. RNA was isolated from the cells by the method of Schmidt and Thannhauser (1945), and counted for radioactivity.

The fate of radioactive viral nucleic acid was also studied. In this experiment, monolayers of cells were infected with 50 pfu's/cell of H^3·uridine-labeled NDV. After a 30 minute adsorbtion period, the cells were washed 3 times with PBS followed by the addition of Eagle's medium supplemented with 5% dialyzed horse serum. Groups of dishes were harvested at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 8 hours after infection. After washing and centrifuging the cells, they were resuspended in PBS and frozen and thawed three times. Clumps of cells were broken up by pipetting. From each sample, a fraction was removed for plaquing. This was called the pre-RNAse fraction. The remaining suspension was

then treated with 100 ugms of RNAse for 2 hours at 37C. A post-RNAse fraction was removed for plaquing. To the remaining suspension, kept at 4C, was added 1 mg of bovine albumin, a protein co-precipitate, followed by 5% TCA. After centrifugation for 0.5 hours at 3000 rpm, the acid soluble fraction was removed and counted for radioactivity.

In a similar experiment, cells were grown for 24 hours on Eagle's medium containing 7% horse serum and 0.2uc/ml of H^3-uridine, followed by 24 hours in the same medium without the radioactive compound. Three hours prior to infection, the cells were pretreated with 1 ugm/ml of actinomycin D. Half of the cultures were infected with 50 pfu's of stock nonradioactive virus while the other half served as the control uninfected group. After viral adsorbtion, the infected and control cells were both washed 3 times with PBS and Eagle's medium containing 5% horse serum was added to each dish. A set of dishes from both groups was harvested every hour from 1-6 and at 8 hours after infection. As previously mentioned, the cells were scraped, washed in PBS, centrifuged, resuspended in PBS, and frozen. After freezing and thawing all samples 3 times, they were treated with 100 ugms/ml of RNAse for 2 hours at 37C. After all samples were placed in an ice bath at 4C, 1 mg of bovine albumin and 5% TCA were added. After centrifugation, the acid soluble fraction was counted for radioactivity.

An attempt was also made to isolate an RNAse-resistant RNA from infected cells. Monolayers of cells were grown in 100mm diameter glass Petri dishes. One group was infected with 50 pfu's/cell of stock virus, which was adsorbed for 30 minutes at 37C. Another group of cells was not infected and served as the control group. After adsorbtion, both

control and infected cells were washed with PBS and overlayed with Eagle's medium containing 3% dialyzed horse serum and 0.25uc of H^3-uridine/ml of medium. Both groups were harvested at 3.5 hours post infection. The radioactive medium was removed and the cells were scraped and washed in PBS, and pelleted by centrifugation for 10 minutes at 1000 rpm. The cells were resuspended in 2.5ml of "diluted buffer" (Hausen, 1965), made of 0.01M Tris-HCl, pH 7.4, and containing 0.0015M $MgSO_4$ and 0.01M NaCl. The cells were allowed to swell for 10 minutes at room temperature, and were then broken by 15 strokes of a Ten Broeck Pyrex tissue grinder. Immediately, 0.3ml of bentonite, to inhibit RNAse, and 0.1mg pronase, to liberate any protein-bound RNA, were added to the disrupted cells. After a 10 minute incubation period at room temperature, the RNA was extracted two times with 5ml of (60C) redistilled liquified phenol (Fischer Scientific Company). After centrifugation for 15 minutes at 3,200 rpm, the RNA contained in the aqueous layer was precipitated with 3 volumes of cold ethanol. The RNA precipitate which formed within 6 hours was dissolved in 2ml of buffer A (Hausen, 1965) which contained 5mM/ml Tris-HCl, pH 7.4, 0.1M NaCl and 0.5% sodium dodecylsulfate (SDS). The RNA solution was then layered onto the top of a 30 ml preset 10-30% (w/v) sucrose-buffer A gradient and centrifuged at 15C for 16 hours at 22,000 rpm in the SW 25.1 rotor of a Spinco model L ultracentrifuge. Fractions of 1 ml were collected with the aid of a Buchler piercing unit (Buchler Instruments, Inc., Fort Lee, New Jersey). These pre-RNAse samples were counted for radioactivity. All samples were then diluted to 3.0 ml with "diluted buffer" and the optical densities were read at 260mu on a Zeiss spectrophotometer. To each sample

was added purified carrier yeast RNA, and all samples were then precipitated with 3 volumes of cold ethanol. The precipitate from each fraction was dissolved in 1 ml of PBS and treated with 20 ugms/ml of RNAse for 30 minutes at room temperature. After adding enough SDS to each sample to bring the final concentration to 0.5%, the post-RNAse fractions were counted for radioactivity.

RESULTS

Viral Nucleic Acid

As previously mentioned, other investigators, using different strains of NDV, have demonstrated that infected cells incorporated radioactive RNA precursors into viral RNA (Granoff and Kingsbury, 1964; Scholtissek and Rott, 1965). In order to correlate all of our experimental results, it was necessary to determine the time of uridine incorporation into viral RNA in chick cells infected with the Roakin strain of NDV. As shown in Fig. 1, infected cells, beginning at 3 hours and continuing until 8 hours post infection, incorporated more uridine than did control cells. The presence of actinomycin D in the growth medium greatly reduced the incorporation of uridine into cellular RNA as the radioactive counts from the control cells remained constant. The increase in radioactivity between 1-2 hours in both the control and infected cultures was due to the depletion of the cell's nucleic acid pool and the use of the exogenous H^3-uridine.

In order to follow the fate of viral RNA, and to determine the sensitivity of the RNA to RNAase, cells were infected with H^3-uridine-labeled NDV and analyzed as previously described (Materials and Methods). The results of this experiment are shown in Fig. 2. By one hour after infection, the biological activity, as measured by pfu's, of the pre-RNAse and post-RNAse samples had decreased, since uncoating had occurred. From 1.5 to 5 hours, the biological activity of both samples remained low. During this eclipse period, the viral particles were truly intracellular and few complete particles could be demonstrated. The

EXPLANATION OF FIG. 1

Uridine incorporation into RNA during
an 8 hour period.

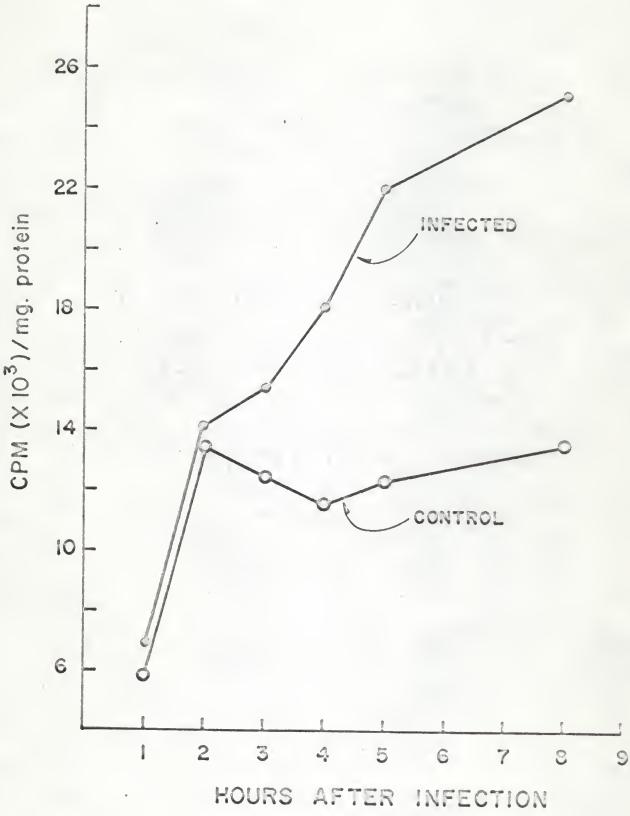

26
22
18
14
10
6
CPM (X 10³)/mg. protein
INFECTED
CONTROL
1
2
3
4
5
6
7
8
9
HOURS AFTER INFECTION

FIG. 1

EXPLANATION OF FIG. 2

The fate of the radioactive viral RNA
and a NDV growth curve.

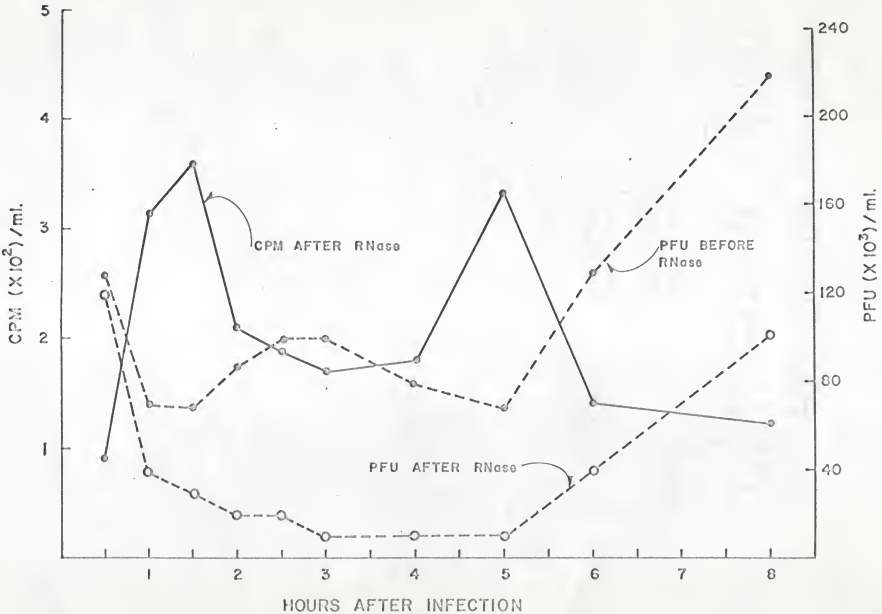

CPM. (X10²) / ml.
PFU (X10³) / ml.
CPM AFTER RNase
PFU BEFORE RNase
PFU AFTER RNase
HOURS AFTER INFECTION
1
2
3
4
5
6
7
8
40
80
120
160
200
240

FIG. 2

difference between the number of pfu's in the pre-RNAse and post-RNAse samples during the eclipse period was probably due to the effects of the uncoating enzymes, which weakened the viral protein coat and allowed the exogenous RNAse to inactivate the virus. The increase in pfu's beginning at 5 hours indicated that complete viral particles were being produced. In the RNAse treated sample, the addition of the enzyme resulted in the destruction of some progeny viral RNA and the production of fewer complete viral particles as compared to the untreated samples.

The amount of single-stranded, RNAse-susceptible, viral RNA increased within the cells during the first 2 hours after infection. This increase was due to uncoating and release of the viral nucleic acid from its protein coat. From 2-4 hours post infection, the amount of viral RNA that was degraded by RNAse declined. This might indicate the formation of a double-stranded, RNAse-resistant RNA. By 5 hours post infection, RNAse-susceptible, single-stranded RNA increased, since infected cells were producing new viral RNA. The aggregation and release of complete virus occurred from 5-8 hours post infection as shown by the increase in pfu's. During the same time period, the decrease in RNAse susceptibility was due to the packaging of new particles which were resistant to RNAse.

In order to determine if cellular RNA synthesis was involved in viral nucleic acid production, cells containing radioactive RNA were infected with stock virus. Actinomycin D was used to inhibit normal cellular RNA synthesis. The susceptibility of viral RNA to RNAse was determined as in the previous experiment. The results, as shown in Fig. 3, were similar to those in Fig. 2. In control cultures, the amount of

EXPLANATION OF FIG. 3

The fate of RNA from radioactive cells infected with NDV.

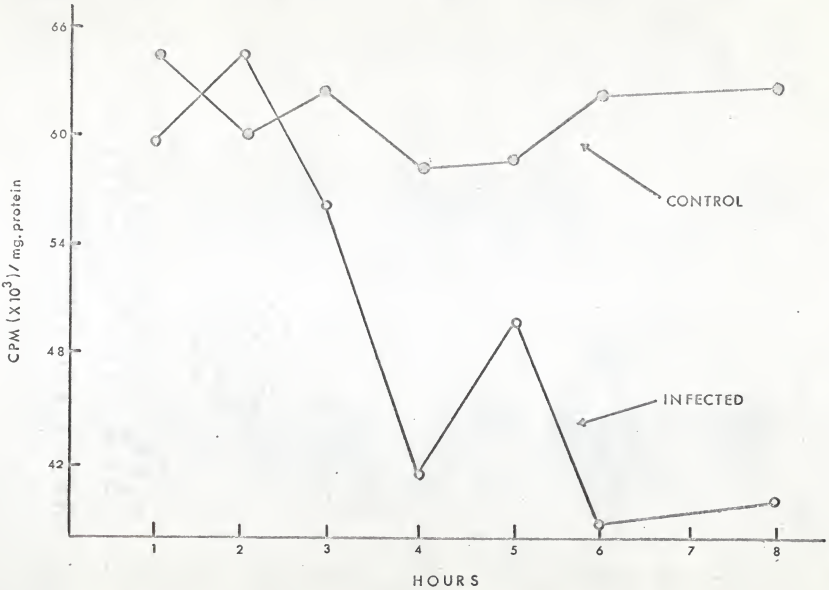

66
60
54
48
42
CPM (X 10³)/ mg. protein
CONTROL
INFECTED
1
2
3
4
5
6
7
8
HOURS

Fig. 3

RNAse-susceptible RNA remained constant. A decrease in RNAse susceptibility occurred from 3-4 hours post infection and was followed by an increase in susceptibility occurring at 5 hours. By the 6th hour, when complete viral particles were being produced, the amount of RNAse-susceptible RNA was much less than in control cells. By this time, complete virus particles were resistant to RNAse.

An attempt was also made to isolate a RNAse-resistant RNA from cells infected for 3.5 hours with NDV. For comparison RNA was also isolated from control noninfected cells. The results of these experiments are shown in Figs. 4 and 5. The optical densities from both control and infected cells indicated 3 peaks of RNA. However a band of radioactive RNA was found in infected cells which was not present in control cells (fractions 6-12). Although treatment of these fractions (6-12) with RNAse resulted in some loss of radioactivity, the profile of this RNA remained the same. The decrease in radioactivity after RNAse treatment might be due to destruction of some cellular RNA, but the appearance of the RNA profile after RNAse action might indicate an RNAse-resistant RNA.

Enzyme Studies

Uridine Kinase Activity. Tissue culture cells infected with NDV incorporated more radioactive uridine and cytidine than did noninfected cells (Granoff and Kingsbury, 1964; Scholtissek and Rott, 1965). They demonstrated that these precursors were incorporated specifically into viral RNA. Based on these results, we suspected that virus-infected cells would contain greater amounts of uridine kinase, an enzyme

EXPLANATION OF FIG. 4

Sucrose density gradient centrifugation of
RNA from control cells.

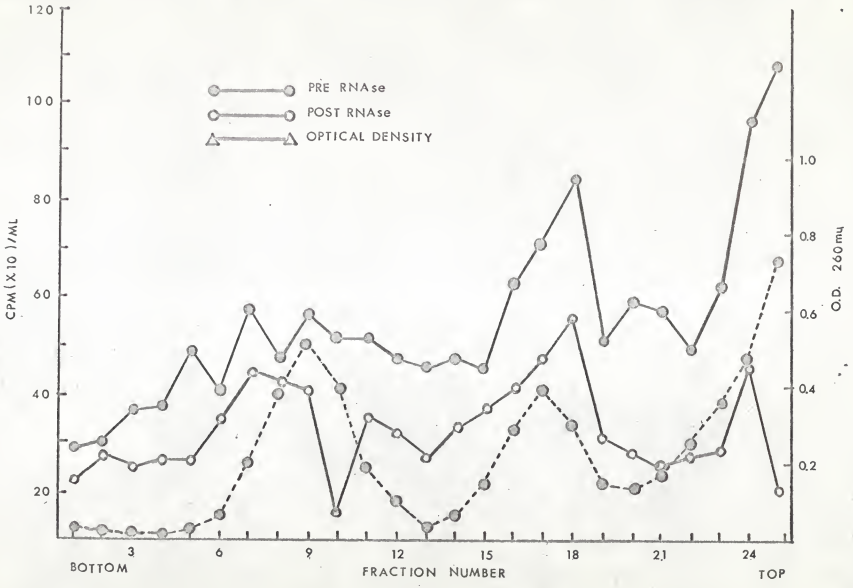

PRE RNAse
POST RNAse
OPTICAL DENSITY
CPM(X10)/ML
O.D. 260mμ
FRACTION NUMBER
BOTTOM
TOP
120
100
80
60
40
20
1.0
0.8
0.6
0.4
0.2
3
6
9
12
15
18
21
24

Fig. 4

EXPLANATION OF FIG. 5

Sucrose density gradient centrifugation of
RNA from NDV-infected cells.

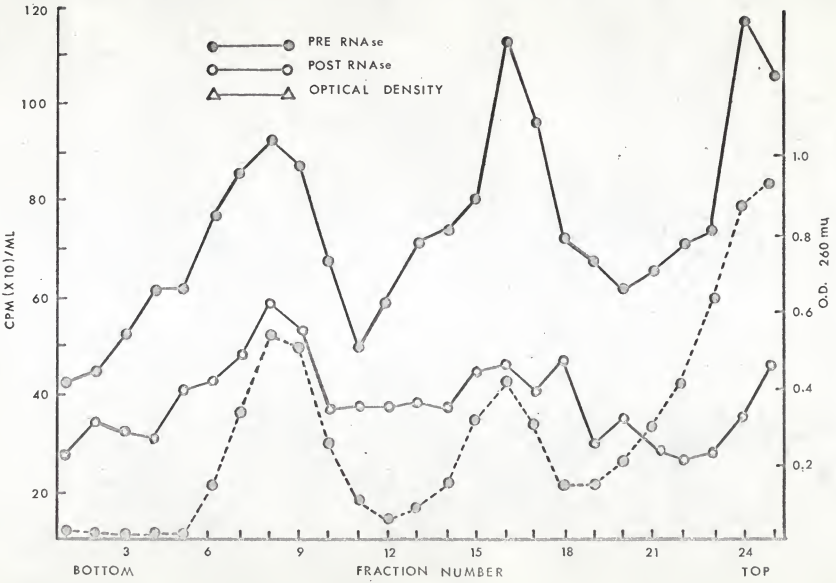

PRE RNAse
POST RNAse
OPTICAL DENSITY
CPM (X10)/ML
O.D. 260 mu
120
100
80
60
40
20
1.0
0.8
0.6
0.4
0.2
3
6
9
12
15
18
21
24
BOTTOM
FRACTION NUMBER
TOP

Fig. 5

responsible for converting uridine to UMP, than did uninfected cells. However, as shown in Fig. 6, the activity of uridine kinase, based on the chromatographic isolation of UMP, was the same in control and infected cells during an 8 hour period.

ATPase Activity. Since most of the energy for normal cellular synthesis comes from the high energy phosphate bonds present in ATP, studies were undertaken to determine if viral infection influenced certain cellular energy mechanisms. Figure 7 shows that ATPase, which breaks down ATP and releases energy, did not increase in infected cells. In both control and infected cells during an 8 hour period, the level of cell-released inorganic phosphate due to ATPase activity remained constant.

Hexokinase Activity. The phosphorylation of glucose to glucose-6-phosphate has been shown to occur early in the Embden-Myerhof reaction. The enzyme involved in this conversion, which results in the utilization of carbohydrate compounds, is hexokinase. Since control and infected cells contained the same amounts of G-6-P (Fig. 8), the amount of cellular hexokinase did not change during NDV infection.

Carbamyl Phosphate Synthetase Activity. Biological systems utilize ATP for the storage of high energy phosphate bonds and for the synthesis of nucleic acids. Carbamyl phosphate synthetase has been shown to synthesize ATP from ADP and carbamyl phosphate in vertebrate systems (Jones, 1964). It was interesting to determine if NDV infection would cause a change in the cellular requirement for ATP synthesis. Experimental results, as shown in Fig. 9, indicated that infected cells, especially at 5 hours, contained more ATP and thus more CP synthetase than did

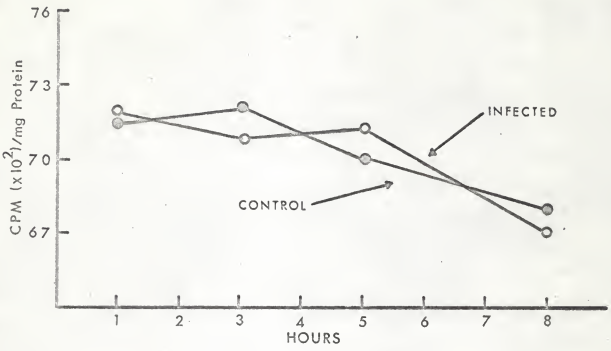


Fig 6 . URIDINE KINASE ACTIVITY

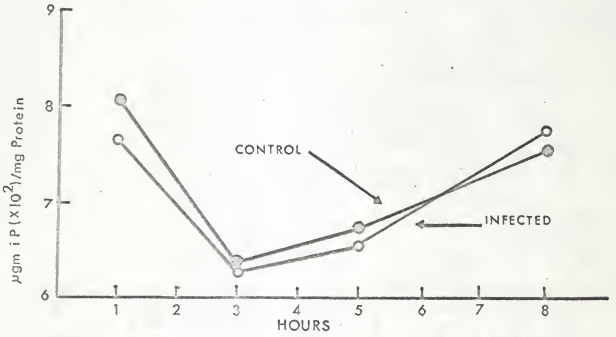


Fig. 7 ATPase ACTIVITY

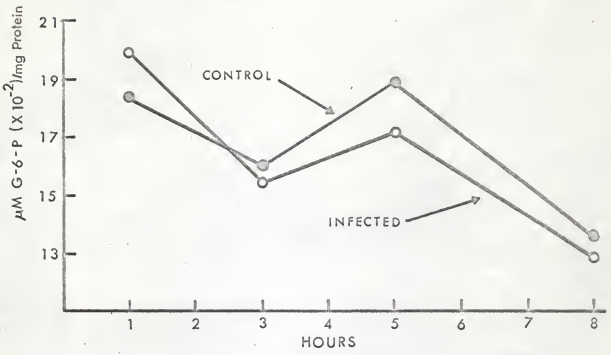


Fig. 8 HEXOKINASE ACTIVITY

Fig. 9 CARBAMYL PHOSPHATE SYNTHETASE ACTIVITY

control, noninfected cells.

Fate of the Viral Protein Coat

By infecting cells with purified virus which contained H^3-valine in part of its protein coat, it was possible to study the fate of the viral protein coat. In addition, known inhibitors of protein synthesis (puromycin) and of DNA-directed RNA synthesis (actinomycin D) were added to infected cells throughout an 8 hour growth cycle, in order to determine if these inhibitors affected the release or reutilization of the viral protein coat. Figure 10 shows the radioactive counts which were found in the cell growth medium in this experiment. These results indicated that as the infectious cycle progressed, greater amounts of H^3-valine were released into the medium from the cells. Since the above results could have been due to the release of complete, non-degraded virus particles, a sample of the growth medium was precipitated with 10% TCA, and the acid soluble fraction was again counted for radioactivity. As shown in Fig. 11, the results of this experiment were similar to the results in Fig. 10, indicating that most of the radioactivity in the growth medium was due to degraded viral protein coat material. Experimental evidence showed that actinomycin D and puromycin did not greatly interfere with the process of uncoating, and that the degraded material was not reutilized within 8 hours for the synthesis of progeny viruses.

In order to confirm the above results, the infected cells, after being thoroughly washed to remove traces of the growth medium, were subjected to treatment with 10% TCA. Both the acid soluble and

EXPLANATION OF FIG. 10

The fate of the viral protein coat in the growth medium of infected cells.

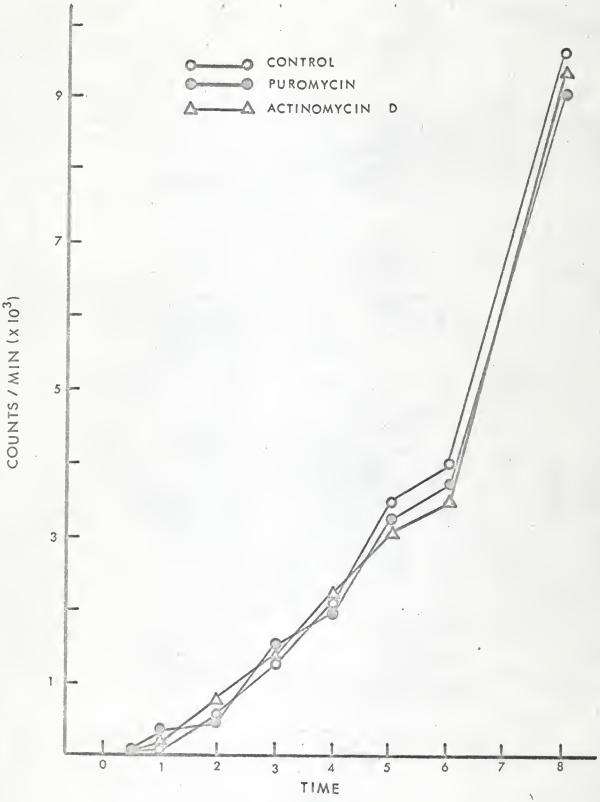

CONTROL
PUROMYCIN
ACTINOMYCIN D
COUNTS / MIN (x 10³)
9
7
5
3
1
0
1
2
3
4
5
6
7
8
TIME

Fig. 10

EXPLANATION OF FIG. 11

The fate of the viral protein coat in the growth medium of infected cells after TCA precipitation.

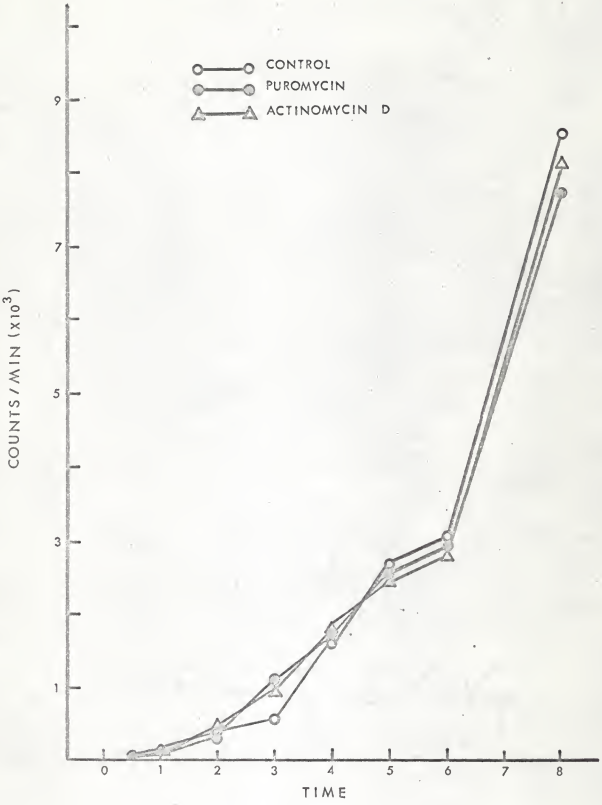

CONTROL
PUROMYCIN
ACTINOMYCIN D
COUNTS / MIN (x10³)
9
7
5
3
1
0
1
2
3
4
5
6
7
8
TIME

Fig. 11

insoluble fractions were counted for radioactivity. As expected, the radioactive counts from the TCA precipitate declined from 1 to 8 hours post infection (Fig. 12), indicating that degraded protein coat material was being released into the growth medium. From 0.5 to 1.0 hours post infection, the radioactivity in the acid precipitate increased, indicating that uncoating was occurring during this time period. Although the process of uncoating occurred in the presence of inhibitors, the results shown in Fig. 12 indicated that actinomycin D, and to a greater extent puromycin slightly reduced or slowed down the process of uncoating when compared to the untreated sample. Actinomycin D has been shown to decrease the amount of progeny virus production (Granoff and Kingsbury, 1964) while puromycin has been shown to prevent virus production when added during the first few hours after infection (Wilson and LoGerfo, 1964).

The radioactivity found in the acid soluble cellular material was never greater than 50 counts/minute and was probably due to light particles of the acid precipitate which were not completely sedimented, or to the small size of the amino acid pool of chick embryo cells.

Samples of infected cells, grown with and without the inhibitors, were kept in a 37C incubator for 18 hours and harvested. The growth medium was then assayed for infectious viral particles by the plaque assay method. The results, shown in Table I, indicated that puromycin completely prevented the growth of NDV while actinomycin reduced the amount of progeny viruses by about 40% as compared to control infected cultures.

Residual, unremoved virus particles which adsorbed to the cells

EXPLANATION OF FIG. 12

The fate of the viral protein coat in the TCA acid precipitate of infected cells.

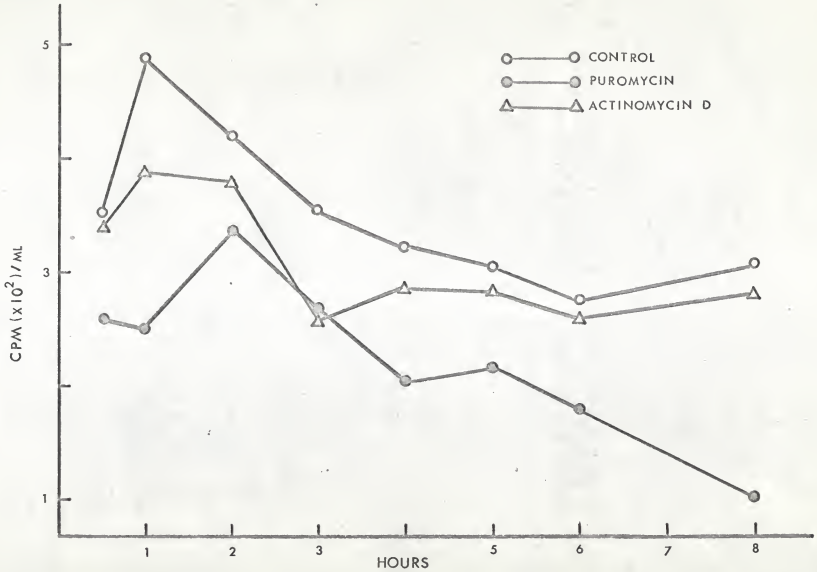

CONTROL
PUROMYCIN
ACTINOMYCIN D
CPM (x 10²) / ML
5
3
1
1
2
3
5
6
7
8
HOURS

FIG. 12

Table I. The effect of puromycin and actinomycin D on the amount of virus particles in the growth medium after 18 hours incubation at 37C.

Culture	Number of plaque-forming-units/ml
Control - no inhibitors	8.2×10^5
Actinomycin D - 5 ugms/ml	5.0×10^5
Puromycin - 50 ugms/ml	4.0×10^2

probably accounted for the 400 pfu's obtained in the puromycin sample, since the number of pfu's from the control sample was 2000 times greater than found in the puromycin sample.

DISCUSSION

Although NDV infection resulted in an increase in uridine incorporation, no increase in uridine kinase activity was observed. During the infection of chick embryo cells and chick chorioallantoic membrane with an RNA tumor virus, Rous sarcoma virus (RSV), an increase in uridine kinase activity was observed (Gelbard et al., 1966). However, enzyme activity in RSV-infected cells did not occur until 4 days after infection. Since cultivation of tissue culture cells resulted in a loss of certain enzymatic activities within several days (Kit et al., 1962), and tumor tissue cells had an altered metabolic pattern and a high level of uridine kinase activity (Kara et al., 1963), the infection of cells with RSV may be different than infection with NDV.

In the present experiment, complete virus was produced within 8 hours after infection, and uridine kinase activity was measured by the conversion of uridine to UMP only. Anderson and Brockman (1964) found that CTP (cytosine triphosphate) was the most potent feedback inhibitor of uridine kinase and that with a sufficient amount of CTP, no UMP was produced from uridine. Therefore if the chick tissue culture cells possessed high levels of CTP, increased amounts of UMP would not be formed. Also, chick cells could have a sufficient amount of uridine kinase to synthesize viral RNA without any change in enzymatic activity.

Energy for normal cellular metabolism comes mainly from ATP, which is formed primarily through oxidative processes, and to some extent by glycolytic processes. Experiments were performed to investigate the effects of viral multiplication on certain cellular energy mechanisms.

It was essential to study cellular ATP synthesis, since an increase in ATP would provide a means of storing and obtaining energy necessary for viral synthesis. Carbamyl phosphate synthetase was shown to increase in infected cells, especially at 5 hours post infection. At this same time, studies on the NDV growth curve indicated that the aggregation of viral components and subsequent release of progeny virus occurred.

Certain methods by which viral infection caused cells to utilize newly formed ATP were also investigated. Hexokinase, which phosphorylates glucose in the presence of ATP, did not increase during infection. Since this enzyme is involved in the Embden-Myerhoff glycolytic pathway, which results in the utilization of carbohydrates for energy and eventual protein and nucleic acid synthesis, the results indicated that this metabolic pathway was not involved during NDV infection, or that cells normally contain enough enzyme to utilize the newly formed ATP. Another mechanism by which cells utilize ATP for energy involves the enzyme ATPase, which cleaves the terminal phosphate group of ATP and liberates ADP, inorganic phosphate and free energy. The results of the present experiments on ATPase indicated that this enzyme did not increase in infected cells and implied that uninfected cells contained enough of the enzyme for viral synthesis or that ATPase was not involved in the utilization of ATP.

Since no increase in certain enzymes which utilize ATP for the production of energy were found, it is possible that the ATP was utilized for other purposes, such as the synthesis of viral RNA or in the activation of amino acids for viral protein synthesis. Both of these events would occur at about 5 hours post infection, the time when

increased ATP synthesis was observed.

During the period in which uridine incorporation occurred, infected cells were synthesizing viral nucleic acid and protein. A new viral-specific enzyme, RNA-dependent, RNA polymerase, which was involved in the synthesis of a replicative form of viral RNA, was shown in cells infected with NDV (Glasky and Holper, 1963) and polio and mengo viruses (Baltimore and Franklin, 1963). Also, in cells infected with polio and mengo viruses, a replicative form of RNA was demonstrated. However, there have been no reports of a RNAse-resistant, replicative form of RNA in NDV-infected cells.

The present investigation indicated that a replicative form of RNA was present in chick cells infected with NDV. By following the fate of radioactive viral RNA, it was demonstrated that at various times within the growth cycle, the RNA was either sensitive to or resistant to the action of RNAse. Sensitivity to RNAse, indicating single-stranded viral RNA, was shown to occur after viral uncoating and prior to aggregation of complete virus particles. Between these two events, viral RNA was found to be resistant to RNAse, a factor which implied the presence of a double-stranded NDV RNA.

In order to confirm the above results, an experiment using non-radioactive virus grown in radioactive cells was performed. The sensitivity of viral nucleic acid to RNAse was followed. The results again indicated the presence of a replicative form of RNAse-resistant RNA, and also showed that the host cell might participate in the formation of this RNA.

Experiments were then performed to isolate this type of RNA from

cells infected for 3.5 hours with NDV. The method of Hausen (1965) was followed for this experiment since he had successfully isolated double-stranded RNA from L cells infected with ME virus. The results of the NDV experiment showed a band of RNA which appeared at a sucrose concentration of about 25%, was resistant to RNAse, and was not found in control cells.

Therefore, these results indicated the appearance of a rapidly sedimenting, RNAse-resistant RNA, which was formed in infected cells with cellular RNA participation. Recently, Brown and Martin (1965) obtained similar results from cells infected with foot-and-mouth disease virus. They found that infected cells contained a rapidly sedimenting RNA which contained RNA molecules larger than those which were incorporated into purified virus. They postulated that the double-stranded RNA consisted partly of the infecting viral genome and a circular complementary strand. As RNA polymerase rotated around the circular strand of RNA, a long molecule of new viral RNA was produced and was eventually broken down into smaller, viral-sized units and into messenger RNA, which could code for viral protein synthesis.

In NDV, the rapidly sedimenting RNA might serve in the same manner as described by Brown and Martin for foot-and-mouth disease virus. If the new long-chained viral RNA were produced in association with cellular ribosomes, the participation of the host cell in NDV replication could be explained. Another possible method of viral replication would directly involve cellular ribosomes. The infecting viral genome might form a double-stranded RNA by pairing with a complementary region on the ribosome. This type of RNA could then function as a template for new

RNA synthesis by the asymmetric semiconservative method as described for ME virus by Hausen (1965). After the triplex, consisting of infecting viral RNA, complementary ribosomal RNA, and newly synthesized RNA, was formed, an enzyme might cleave this complex into smaller units such as new viral RNA and pieces of ribosomal material. Kingsbury (1966b) reported that large amounts of complementary viral RNA were present in cells infected with NDV. Further experimentation is needed to elucidate the exact method of NDV RNA replication.

By following the fate of the radioactive viral protein coat, it was shown the degraded coat material continually increased in the growth medium while it simultaneously decreased inside the cells. This indicated that the protein coat of progeny viral particles was not made from degraded coat material of infecting virus after 8 hours. Also, this showed that the amino acid pool of chick fibroblasts was small, since this cellular pool did not incorporate any degraded viral protein material. However, the cellular pool was sufficient for the synthesis of small amounts of complete virus after an 8 hour period. Results of other experiments performed in this laboratory indicated that after a 24 hour infectious cycle, the constituents for the viral protein coat came partly from the amino acid pool which was supplied by the cellular growth medium (R. C. Consigli and H. Minocha, personal communication). The latter results, in conjunction with the present studies, imply that by 24 hours after infection, progeny virus protein coat would contain material degraded from the original infecting viral particles.

By adding puromycin to infected cultures, it was possible to study certain events occurring during viral uncoating, which was shown to

occur within 1 hour after infection. Since this process was not prevented by puromycin, it indicated that uncoating occurred without new cellular protein synthesis. Therefore, the host cell contained an enzyme(s) which removed the viral protein coat. Joklik (1964) found that the first step in the uncoating process of vaccinia virus also occurred without new cellular protein synthesis. Although puromycin did not prevent viral uncoating, it did prevent the synthesis of new virus. Therefore, protein synthesis was required for viral replication. Also, cellular DNA was not required since the addition of actinomycin D did not prevent viral multiplication.

SUMMARY

Certain biochemical events occurring in chick embryo tissue culture cells infected with the Roakin strain of Newcastle disease virus have been studied. Although infection resulted in increased uridine incorporation, an increase in uridine kinase activity was not observed. A rapidly sedimenting, double-stranded, replicative form of viral RNA was isolated from cells after 3.5 hours infection. Although infected cells produced more ATP than control cells, hexokinase and ATPase did not increase during the course of infection. Viral uncoating was not prevented by puromycin or actinomycin D. The degraded viral protein coat material was released from the cells into the growth medium and was not utilized in the synthesis of new virus.

ACKNOWLEDGMENTS

I sincerely thank my major professor, Dr. Richard A. Consigli, for his continual encouragement and willing help.

I am very thankful for the help of my wife, Harriet Ann, who made many sacrifices throughout the period of this work.

LITERATURE CITED

Adams, W. R. 1966. Cellular origin of the slowly sedimenting ribonucleic acid (RNA) fraction associated with partially purified Newcastle disease virus (NDV). Fed. Proc. 25(1):422.

Anderson, E. P., and R. Brockman. 1964. Feedback inhibition of uridine kinase by cytidine triphosphate and uridine triphosphate. Biochim. Biophys. Acta 91:380-386.

Andrewes, C. H. 1965. Viruses and Noah's ark. Bacteriol. Revs. 29:1-8.

Andrewes, C. H., F. B. Bang, and F. M. Burnet. 1955. A short description of the myxovirus group (influenza and related viruses). Virology 1:176-184.

Baltimore, D., and R. M. Franklin. 1963. A new ribonucleic polymerase appearing after mengovirus infection of L-cells. J. Biol. Chem. 238:3395-3400.

Bang, F. B. 1948. Studies on Newcastle disease virus III. Characters of the virus itself with particular reference to electron microscopy. J. Exptl. Med. 88:251-266.

Bang, F. B. 1946. Filamentous forms of Newcastle virus. Proc. Soc. Exptl. Biol. Med. 63:5-7.

Barry, R. D. 1965. Factors which affect the release of Newcastle disease and Sendai viruses from infected allantoic cells. J. Gen. Micro. 39:229-238.

Beach, J. R. 1944. The neutralization in vitro of avain pneumoencephalitis virus by Newcastle disease immune serum. Science 100:361-362.

Beaudette, F. R., and J. J. Black. 1945. Newcastle disease in New Jersey, p. 2. Proc. 17th Ann. Meeting, Northeastern Conf. Lab. Workers Pullorum Disease Control, Kingston, R. I. Original not seen. Reference from Shope, 1964.

Bolognesi, D. P., and D. E. Wilson. 1966. Inhibitory proteins in the Newcastle disease virus-induced suppression of cell protein synthesis. J. Bacteriol. 91:1896-1901.

Brown, F., and S. J. Martin. 1965. A new model for virus ribonucleic acid replication. Nature 208:861-863.

Bruemmer, J. H., and B. L. O'Dell. 1956. Inhibition of color development in the Lowry-Lopez phosphorus method by sulfhydral compounds and its counteraction by copper. J. Biol. Chem. 219:283-294.

Chanock, R. M., and R. H. Parrot. 1965. Newcastle disease virus, p. 769-774. In F. L. Horsefall and I. Tamm (ed.), Viral and Rickettsial Infections of Man. J. B. Lippincott Co., Philadelphia and Montreal.

Consigli, R. A., H. C. Minocha, and H. Abo-Ahmed. 1966. Multiplication of polyoma virus II. The source of constituents for viral DNA and protein synthesis. J. Bacteriol. 92: In press.

Cunha, R., M. L. Weil, D. Beard, A. Taylor, D. Sharp, and J. W. Beard. 1947. Purification and characteristics of the Newcastle disease virus (California strain). J. Immunol. 55:69-89.

Darnell, J. E. 1962. Early events in poliovirus infection. Cold Spring Harbor Symp. Quant. Biol. 27:149-158.

Delbecco, R. 1952. Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. Natl. Acad. Sci., U.S. 38:747-752.

Doyal, T. M. 1927. A hitherto unrecorded disease of fowls due to a filter-passing virus. J. Comp. Pathol. Therap. 40:144-169. Original not seen. Reference from Shope, 1964.

Drake, J., and P. Lay. 1962. Host controlled variation in Newcastle disease virus. Virology 17:56-67.

Duesberg, P. H., and W. S. Robinson. 1965. Isolation of the nucleic acid of Newcastle disease virus (NDV). Proc. Natl. Acad. Sci., U.S. 54:794-800.

Durand, D. P., and A. Eisenstark. 1962. Influence of host cell type on certain properties of Newcastle disease virus in tissue culture. Am. J. Vet. Res. 23:338-342.

Gelbard, A. S., S. H. Kim, and M. L. Eidinoff. 1966. Nucleoside kinase activities in tissues infected with Rous sarcoma virus. Cancer Res. 26:748-751.

Glasky, A. J., and J. C. Holper. 1963. Inhibition of ribonucleic acid synthetase from influenza- and Newcastle disease virus-infected cells by various antiviral compounds, p. 688-692. In J. Sylvester (ed.), Antimicrobial Agents and Chemotherapy--1963. American Soc. for Microbiology, Ann Arbor.

Glasky, A. J., L. Simon, and J. C. Holper. 1964. Interferons: Selectivity and specificity of action in cell-free systems. Science 144:1581-1583.

Gottschalk, A. 1958. The influenza virus neuriminidase. Nature 181:377-378.

Granoff, A., and D. W. Kingsbury. 1964. Effect of Actinomycin D on the replication of Newcastle disease and influenza viruses, p. 96-115. In G. Wolstenholme and J. Knight (ed.), Ciba Foundation Symp. on Cellular Biol. Myxovirus Infections. J. Churchill, Ltd., London.

Granoff, A., O. C. Liu, and W. Henle. 1950. A small hemagglutinating component in preparations of Newcastle disease virus. Proc. Soc. Exptl. Biol. Med. 75:684-691.

Hausen, P. 1965. Studies on the occurance and function of virus-induced double-stranded RNA in the ME-virus L-cell system. Virology 25:523-531.

Hirst, G. K. 1942. Adsorbtion of influenza hemagglutinins and virus by red blood cells. J. Exptl. Med. 76:195-209.

Holland, J. J., and B. H. Hoyer. 1962. Early stages of enterovirus infection. Cold Spring Harbor Symp. Quant. Biol. 27:101-112.

Horecker, B. L., and A. Kornberg. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. 175: 385-390.

Horne, R. W., A. P. Waterson, P. Wildy, and A. E. Farnham. 1960. The structure and composition of the myxoviruses I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virology 11:79-98.

Joklik, W. K. 1964. The intracellular uncoating of poxvirus DNA II. The molecular basis of the uncoating process. J. Mol. Biol. 8:277-288.

Jones, M. E. 1963. Carbamyl phosphate. Science 140:1373-1379.

Kara, J., F. Sorm, and A. Winkler. 1963. Determination of uridine kinase activity in some human tumours and normal tissues, using C^{14} azauridine as substrate. Neoplasma 10:3-10.

Kingsbury, D. W. 1966a. Newcastle disease virus RNA I. Isolation and preliminary characterization of RNA from virus particles. J. Mol. Biol. 18:195-203.

Kingsbury, D. W. 1966b. Newcastle disease virus RNA II. Preferential synthesis of RNA complementary to parental viral RNA by chick embryo cells. J. Mol. Biol. 18:204-214.

Kit, S., Y. Valladares, and D. R. Dubbs. 1964. Effects of age of culture and vaccinia infection on uridine kinase activity of L-cells. Exptl. Cell Res. 34:257-265.

Kraneveld, F. C. 1926. Over een in Ned-Indie heerochende ziekte onder het pluimyes. Nederland Indisch Blad. Diergeneesk. 38: 448-450. Original not seen. Reference from Shope, 1964.

Levine, S., and B. P. Sagik. 1957. The interactions of Newcastle disease virus with chick embryo tissue culture cells: Attachment and growth. Virology 2:57-65.

Lowry, O. H., N. R. Roberts, M. Wu, W. S. Hixon, and E. J. Crawford. 1954. The quantitative histochemistry of brain II. Enzyme measurements. J. Biol. Chem. 207:

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Marcus, P. I. 1962. Dynamics of surface modification in myxovirus-infected cells. Cold Spring Harbor Symp. Quant. Biol. 27:351-365.

Montagnier, L., and F. Sanders. 1963. Replicative form of encephalomyocarditis virus ribonucleic acid. Nature 199:664-667.

Mussgay, M., and J. Weibel. 1962. Early stages of infection with Newcastle disease virus as revealed by electron microscopy. Virology 16:506-509.

Noll, H., T. Aoyagi, and J. Orlando. 1962. The structural relationship of sialidase to the influenza virus surface. Virology 18: 154-157.

Padgett, B. L., and D. L. Walker. 1964. Enzymatic variants of influenza virus III. Function of neuriminidase in the viral growth cycle. J. Bacteriol. 87:363-369.

Pennman, S., Y. Becker, and J. E. Darnell. 1964. A cytoplasmic structure involved in the synthesis and assembly of soliovirus components. J. Mol. Biol. 8:541-555.

Reda, I. M., R. Rott, and W. Schafer. 1964. Fluorescent antibody studies with NDV-infected cell systems. Virology 22:422-425.

Rott, R. 1964. Antigenicity of Newcastle disease virus, p. 133-146. In R. P. Hanson (ed.), Newcastle Disease Virus: An Evolving Pathogen. Univ. Wis. Press, Madison and Milwaukee.

Rott, R., I. M. Reda, and W. Schafer. 1962. Isolation and characterization of hemagglutinating noninfectious particles produced during multiplication of Newcastle disease virus (NDV). Virology 16: 206-209.

Sagik, B. P., and S. Levine. 1954. The interactions of Newcastle disease virus (NDV) with chick red cells, attachment, elution and hemolysis. Bacteriol. Proc. p. 71.

Schäfer, W. 1963. Structure of some animal viruses and significance of their components. Bacteriol. Rev. 27:1-17.

Schmidt, G., and S. J. Thannhauser. 1945. A method for determination of desoxyribonucleic acid, ribonucleic acid, and phospho-proteins in animal tissues. J. Biol. Chem. 161:83-89.

Scholtissek, C., and R. Rott. 1965. Metabolic changes in chick fibroblasts after infection with Newcastle disease virus. Nature 206:729-730.

Scholtissek, C., and R. Rott. 1961. Influence of p-fluorophenylalanine on the production of viral ribonucleic acid and on the utilizability of viral protein during multiplication of fowl plague virus. Nature 191:1023-1024.

Shope, R. E. 1964. The birth of a new disease, p. 3-22. In R. P. Hanson (ed.), Newcastle Disease Virus: An Evolving Pathogen. Univ. Wis. Press, Madison and Milwaukee.

Silverstein, S. C., and P. I. Marcus. 1964. Early stages of Newcastle disease virus--Hela cell interaction: An electron microscope study. Virology 23:370-380.

Skold, O. 1960. Uridine kinase from Ehrlich ascites tumor: Purification and properties. J. Biol. Chem. 235:3273-3279.

Waterson, A. P. 1964. The morphology and composition of Newcastle disease virus, p. 119-132. In R. P. Hanson (ed.), Newcastle Disease Virus: An Evolving Pathogen. Univ. Wis. Press, Madison and Milwaukee.

Waterson, A. P. 1961. Introduction to Animal Virology. Cambridge Univ. Press, London.

Watson, J. D., and F. H. Crick. 1953. The structure of DNA. Cold Spring Harbor Symp. Quant. Biol. 18:123-131.

Wheelock, E. 1963. Intracellular site of Newcastle disease virus nucleic acid synthesis. Proc. Soc. Exptl. Biol. Med. 114:56-60.

Wheelock, E. F. 1962. The role of protein synthesis in the eclipse period of Newcastle disease virus multiplication in Hela cells as studied with puromycin. Proc. Natl. Acad. Sci., U.S. 48:1358-1366.

Wheelock, E. F., and I. Tamm. 1959. Mitosis and division in Hela cells infected with influenza or Newcastle disease virus. Virology 8:532-536.

Wilson, D. E., and P. LoGerfo. 1964. Inhibition of ribonucleic acid synthesis in Newcastle disease virus-infected cells by puromycin and 6-azauridine. J. Bacteriol. 88:1550-1555.

BIOCHEMICAL EVENTS OCCURRING IN CHICK EMBRYO TISSUE CULTURE CELLS INFECTED WITH NEWCASTLE DISEASE VIRUS

by

MICHAEL PAUL LERNER

B. A., University of California, Los Angeles, 1963

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1967

Certain biochemical and enzymatic events occurring in chick embryo tissue culture cells infected with the Roakin strain of Newcastle disease virus (NDV) were studied.

During an 8 hour growth cycle, infected cells were shown to incorporate more radioactive uridine than control cells. In order to determine if the uridine was utilized in the synthesis of viral RNA (ribonucleic acid) precursors, the enzyme uridine kinase, which phosphorylates uridine to uridine monophosphate (UMP), was studied. Experimental results, based on the chromatographic isolation of UMP, indicated that both control and infected cells possessed equal amounts of uridine kinase.

By following the fate of radioactive single-stranded viral nucleic acid, it was shown that this RNA was either sensitive to or resistant to the action of RNAse during an 8 hour period. Sensitivity to RNAse occurred after viral uncoating (0.5-1.5 hours post infection) and during the aggregation and release of progeny virus (5-8 hours). Between 2-5 hours post infection, viral RNA was resistant to degradation by RNAse, a factor indicating the presence of a double-stranded RNA. In a similar experiment, radioactive cells were infected with nonradioactive virus. The results, as in the previous experiment, indicated the presence of an RNAse-resistant RNA and implied that the host cell participated in the formation of this RNA. The RNA from cells infected for 3.5 hours and from control cells was extracted and subjected to sucrose density gradient centrifugation. Analysis of the gradient showed that infected cells contained a rapidly sedimenting, RNAse-resistant, replicative RNA which was not present in control cells. Although the exact method of

synthesis of NDV RNA is unknown, several methods involving both host cell RNA and the rapidly sedimenting replicative RNA were postulated.

Experiments were performed on certain enzymatic mechanisms by which cells obtain energy. Carbamyl phosphate synthetase, which synthesizes ATP (adenosine triphosphate), increased in infected cells. ATPase, which degrades ATP with the liberation of energy, did not increase in infected cells. Hexokinase, an enzyme involved in carbohydrate metabolism and ATP utilization, did not increase during virus infection. These results suggested that the newly synthesized ATP was involved in other cellular reactions such as nucleic acid or protein synthesis.

By following the fate of NDV containing a radioactive protein coat, it was shown that the degraded coat material continually increased in the cellular growth medium while it decreased within the cell. This indicated that chick tissue culture cells have a fairly small amino acid pool, and that the degraded coat material was not reutilized for new viral synthesis within an 8 hour period. Puromycin, a protein synthesis inhibitor, did not prevent viral uncoating but did prevent viral multiplication. Therefore, although the host cells normally contained the uncoating enzyme(s), new protein synthesis was required for complete viral replication. Actinomycin D, an inhibitor of cellular DNA synthesis, did not prevent the formation of new viral particles.