Studies on the factors involved during the maturation of Newcastle disease virus

John R. Burdick  
Iowa State University

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Studies on the factors involved during the maturation of Newcastle disease virus

by

John R. Burdick

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Bacteriology
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Iowa State University
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INTRODUCTION

The animal viruses are rather simple organisms yet they can take over the host cell synthesizing machinery, utilize host enzymes for their own reproduction and undergo self-assembly. Viruses, besides being clinically important, have provided scientists with vast amounts of genetic, metabolic and structural information from the cells they parasitize.

Virus maturation is a unique process that may be defined as the sequence of events which follows the synthesis of all viral components and which culminates in the production of an intact virion. The maturation process of viruses must be a very precise and structured event because viruses are efficient and specific during their infection processes.

The structural appearance of many of the viruses is fairly elaborate. Many of the virus groups can be identified from their electron micrographs because of their characteristic size and shape. Examples are the brick-shaped poxviruses, the characteristic spikes of the adenoviruses, the bullet-shaped rhabdoviruses and even the icosahedral symmetry of the T-phage head. The paramyxoviruses are an exception. They range in size from 70 to 400 nm and the shape of the virion varies from spherical to filamentous.

Over the years our laboratory has been involved with research concerning several aspects of virus-host interaction.
These studies dealt primarily with the early stages of virus infection. A study of the maturation process of NDV would lend a new and different viewpoint which would add further insight to our studies on virus-host interactions. The pleomorphism exhibited by NDV intrigued us. Perhaps by studying the product we could learn more about the process.
REVIEW OF LITERATURE

Virus Characteristics

Newcastle disease virus (NDV) has been classified as a paramyxovirus (Melnick, 1973). Electron micrographs show the particle to be very heterogeneous with respect to size and shape. The virus particles vary in size from 100 to 400 nm in diameter and the shape is spherical to ellipsoidal. Filamentous forms of NDV, thought at one time to be rare, are now considered to be quite common. These filamentous forms can be as much as 600 nm in length (Feller et al., 1969).

Chemically, NDV is composed of RNA, protein, lipid and carbohydrate. The percent dry weight of these components seems to vary with the host cell type. Klenk (1973) reported values of 0.9% RNA, 73% protein, 20% lipid and 6% carbohydrate. The molecular weight of the virion is \( 800 \times 10^6 \) (Rott, 1964).

NDV is an enveloped animal virus. The envelope meets all criteria for cellular membranes; morphologically it possesses a trilaminar or unit membrane structure and chemically it is composed of proteins, lipids and carbohydrates. The carbohydrates are covalently linked to the proteins or lipids (Klenk, 1974). The proteins of the virus envelope are virus-specific; however, since the virus makes extensive use of the host membrane during assembly, a small amount of host antigen can frequently be found in the particle (Lenard and Companes, 1974).
major glycoproteins found in the envelope are hemagglutinin and neuraminidase (Laver and Valentine, 1969). These glycoproteins function during attachment, penetration and release of the virion (Dales, 1973). The core protein (M-protein) is a non-glycosylated polypeptide which surrounds the nucleocapsid and extends into the lipid matrix of the envelope (Lenard et al., 1974). The M-protein may play a central role in the assembly and maturation processes.

Virus Assembly

The topics of virus assembly and structure have been covered in several recent reviews (Blough and Tiffany, 1973; Lenard and Compans, 1974; Rifkin and Quigley, 1974). There are basically two types of virus envelopment: 1) budding through the internal cellular membranes and 2) budding through the surface or plasma membrane.

The herpesviruses are DNA-containing viruses which bud through an internal cellular membrane, namely the nuclear membrane (Morgan et al., 1959). Radioactively-labeled viral proteins are first detected in the cytoplasm of infected cells; later they migrate to the nucleus (Fujiwara and Kaplan, 1967). The site for glycosylation of envelope proteins appears to be at the nuclear membrane where preformed cellular enzymes act in the glycosylation of viral proteins (Spear and Roizman, 1970). During maturation, proteins of the host-cell nuclear membrane neither become part of the viral envelope nor are they lost from the membrane. It is therefore assumed that the viral
proteins become integrated into newly-formed nuclear membrane after which they are glycosylated (Ben-Porat and Kaplan, 1972).

It is generally believed that the envelope of herpes-viruses is derived from the inner lamella of the nuclear membrane; however, it appears that some members of the group may bud through cytoplasmic membranes (Epstein and Holt, 1963). The sequence of events involving the envelopment of the herpes viruses are best dramatized by electron microscope studies (Morgan et al., 1959; Epstein, 1962). Mature virions can remain cell-associated for prolonged periods of time during which cellular integrity is maintained. Release of virions occurs by egestion from cytoplasmic vacuoles or by shedding from the cell surface (Fenner et al., 1974). Another group of enveloped DNA-containing viruses is the poxviruses. They are unique in that members of this group exhibit de novo synthesis of their envelope (Dales and Mosbach, 1968).

The RNA-containing arbovirus group is the largest and most diversified group of animal viruses and contains both enveloped and non-enveloped members. The enveloped arboviruses are divided into three groups: the togaviruses, the rhabdoviruses, and the Bunyamwera viruses. The togavirus family is currently divided into two genera; the alphaviruses, formerly the group A arboviruses and the flaviviruses, formerly the group B arboviruses (Fenner et al., 1974). The alphaviruses characteristically bud through
the plasma membrane of the host cell (Matsumura et al., 1971) whereas in the flavivirus genus, budding occurs on thickened membranes of the cytoplasmic vacuoles (Filshie and Rehacek, 1968).

Sindbis virus, type species of the alphavirus genus, is a small, spherical, enveloped virus. The virus has three structural proteins and two of them, the envelope proteins, are glycosylated (Grimes and Burge, 1971). The phospholipids of the viral envelope are cellular in origin, and it appears that the viral proteins do not select a special population of host cell phospholipids or glycolipids (Hirschberg and Robbins, 1974). There appears to be a higher cholesterol to phospholipid ratio in the mature virion in comparison to the host cell plasma membrane (Sefton and Gaffney, 1974). The authors suggest that this may be due to the tendency of the virus to assemble in cholesterol-rich regions of the membrane.

The flaviviruses and members of the Bunyamwera group are somewhat similar in their mode of maturation. Viruses of the Bunyamwera group bud from intracytoplasmic membranes into vacuoles or cisternae. Release occurs by cell disruption or migration of the vacuole to the cell surface. The budding process is not preceded by any morphological evidence of virus maturation (Murphy et al., 1968). Filshie and Rehacek (1968) first described the maturation process of two
flaviviruses, Murray Valley encephalitis and Japanese encephalitis viruses. When these agents were grown in mosquito cell culture, the budding of virus occurred on cytoplasmic organelles with accumulation of mature particles in the lumina of the endoplasmic reticulum. The budding or maturation of Dengue virus (a flavivirus) shows a similar method of virus envelopment (Matsumura et al., 1971).

Other viruses which acquire their envelope by budding through intracytoplasmic membranes are the Coronaviruses (Melnick, 1973) and, under certain conditions, members of the Rhabdovirus group (Zee et al., 1970).

Those viruses which acquire their envelope by budding through the host cell surface membrane include the para­myxoviruses, the orthomyxoviruses, rhabdoviruses, the leuk­oviruses and the arenaviruses. Similarities between these virus groups include sensitivity to ether treatment, site of capsid assembly and nucleocapsid envelopment and finally, four of the five groups exhibit helical capsid symmetry (Fenner et al., 1974; Melnick, 1973). The capsid symmetry of the arenaviruses is unknown. The envelope of these viruses contains lipid, viral-specific proteins, including at least one glycoprotein and sometimes glycolipids (Eiserling and Dickson, 1972).

The modification of the host cell plasma membrane by enveloped animal viruses is similar in that viral struc-
tural proteins associate as patches within the lipid bilayer of the host surface membrane. In a recent review by Lenard and Compans (1974), alternative modes for the generation of maturation sites on the surface membrane were presented as five possibilities: 1) viral proteins may be inserted into the membrane and act as a nucleation site for further insertion, leading to progressive growth of the patch; 2) viral polypeptides may be added gradually to a defined domain of the cell surface. Host cell proteins could be absent or progressively displaced from such regions; 3) insertion of viral proteins may be random, followed by lateral diffusion and aggregation into patches; 4) a segment of membrane containing a preexisting patch of viral proteins may fuse with the surface membrane; 5) a segment containing interspersed viral proteins may be inserted into the membrane followed by lateral diffusion and aggregation into patches. Although many feel that host cell polypeptides are replaced at maturation sites or that maturation occurs at areas of the membrane that are relatively free of host cell proteins, the presence of host antigen has been reported in enveloped viruses (Rifkin and Quigley, 1974; Rott et al., 1975).

Newcastle disease virus (NDV) replicates solely in the cytoplasm of the host cell (Bratt and Robinson, 1967). Membrane polypeptides are first detected about two hours post-infection (Hightower and Bratt, 1974) and become associated with the host membrane within two minutes after their syn-
thesis (Kaplan and Bratt, 1973). It is not known whether viral envelope proteins are synthesized on membrane-bound or membrane-free polysomes; however, because the host cell transferases necessary for glycosylation are membrane-bound one might suspect the synthesis of the viral membrane proteins occurs on membrane-bound polysomes (Fenner et al., 1974). Post-translational cleavage of membrane proteins is suspected for some of the paramyxoviruses. Scheid and Choppin (1974) have shown activation of fusion, hemolytic activity and infectivity resulting from proteolytic cleavage of a precursor protein of Sendai virus.

In the last two years there has been a considerable amount of research done with glycoproteins in biological systems. This interest is in part due to the belief that glycoproteins act or can act as communication links between cells and a modification of these glycoproteins may play a significant role in cell transformation (Sharon, 1974). All of the enveloped animal viruses contain at least one glycoprotein which forms surface projections on the viral envelope (Choppin et al., 1972). In the case of the paramyxoviruses, certain functions have been assigned to some of the glycoproteins; i.e. attachment for the hemagglutinin, virus release for the neuraminidase, and cell fusion may lie in the presence of a specific glycoprotein (Dales, 1973; Scheid and Choppin, 1974).
Although all of the information for the amino acid sequence of the glycoprotein is known to originate in the viral genome, the origin of the information for the carbohydrate moiety is unknown (Lenard and Compans, 1974). The enzymes for glycosylation are located in the membranes of the Golgi apparatus and the endoplasmic reticulum and are responsible for addition of oligosaccharides to the nascent polypeptide chains and ceramides (Hagopian et al., 1968). These transferases although being host-specific (Grimes and Burge, 1971) are probably altered as a result of virus infection (Defrene and Louisot, 1973). The utilization of host transferases by the smaller viruses is probably true for all; however, the larger viruses, such as herpes simplex virus may indeed code for one or more transferases (Ray and Blough, 1974).

The attachment of saccharide residues to viral-specific proteins appears to be a separate process by which individual monosaccharides are added in a stepwise fashion by transferases specific for that sugar (Kaplan and Bratt, 1973). Extensive work with influenza virus has shown that the addition of certain monosaccharides occurs on either the rough or smooth endoplasmic reticulum, depending on the sugar (Compans, 1973a; Compans 1973b). The specificity of the glycosylation of viral polypeptides is exemplified by work showing inhibition of virus maturation by the addition
of analog sugars to the system (Kaluza et al., 1972; Klenk et al., 1972; Ray and Blough, 1974). Although evidence has shown that host cell transferases act in the glycosylation of viral proteins one cannot overlook the possibility that this process is either directly or indirectly influenced by the virus. The membrane glycoproteins of Sindbis and Vesicular stomatitis viruses grown in the same host showed a difference in the sialic acid residues per glycoprotein (Burge and Huang, 1970).

Lipids

Enveloped animal viruses such as NDV possess a lipid-containing membrane which is acquired by budding through the host cell surface membrane. The lipid composition of the mature virion for the most part reflects the lipid composition of the host cell (Choppin et al., 1972). Reasons for this generally accepted hypothesis include: 1) Enveloped viruses contain most or all of the lipid classes present in the membranes of the host cell. Only in a few instances have the lipids found in the viruses not been detectable in the uninfected host cell; 2) Viruses grown in different host cells show differences in their lipid pattern; 3) Radioactively-labeled cellular lipids and in many cases, most significantly those that have been labeled before infection, are incorporated into virions; 4) The lipid pattern of viruses is as complex as that of the host cells. The information capacity of the genome of many
viruses appears too small to code for virus-specific lipid synthesizing enzymes (Klenk, 1973). For practically every reason listed above which supports the belief that viral lipids are host-derived there exists contradictory evidence. Current work has suggested that modification of the host cell membrane by many of the enveloped animal viruses involves not only the insertion of virus-specific proteins but also a redirection of host cell phospholipid synthesis as well as virus directed redisposition of pre-existing lipids within the membrane (Israel et al., 1975). Rifkin and Quigley (1974) have recently published an extensive review dealing with virus-membrane modification.

Biological membranes exist in a fluid or dynamic state rather than a static state (Capaldi, 1974). The degree of fluidity is influenced by both internal (lipid classes and membrane proteins) and external (pH, temperature, nutrients) factors (Wisnieski et al., 1974; Bach and Miller, 1976; Fukushima et al., 1976). Work has shown that the activation and/or deactivation of enzyme transport systems in the membrane are controlled by the lipid composition of the membrane (Farias et al., 1972).

Animal cells in general contain three types of lipids: phospholipids, glycolipids and sterols, usually cholesterol. There are two types of phospholipids, glycerol phospholipids and sphingophospholipids (Rouser et al., 1968).
Both quantitative and qualitative similarities among lipids have been noted between virus and host cell. The phospholipids found in the membrane of enveloped animal viruses invariably consist of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (Sph). Sph, PC, and PE are the predominant phospholipids, while PS and PI are found in smaller quantities (Klenk, 1974).

Hirschberg and Robbins (1974) reported that the proteins of Sindbis virus incorporate a non-specific population of host cell membrane phospholipids and glycolipids during viral morphogenesis, while other investigators have reported differences between Sindbis virus and the host cell plasma membranes (David, 1971; Pfefferkorn and Hunter, 1963). When fowlpox virus was grown in chorioallantoic membrane and chicken embryo cell culture very little difference exists between virus and host-cell lipid composition; however, when the virus was grown in chicken scalp cells extensive alterations in the fatty acid composition were noted (Lyles et al., 1976). These authors suggest that the changes in the fatty acid composition observed in the virus infected chicken scalp epithelium may be a host response to virus infection rather than a virus-directed effect. One cannot exclude the possibility that the observed results are virus-directed.
Experiments involving other enveloped animal viruses including Rhabodoviruses (McSharry and Wagner, 1971), RNA tumor viruses (Quigley et al., 1972) and orthomyxoviruses (Kates et al., 1962) show that quantitative differences exist between the virus particle and the host cell membrane lipids. Of particular interest is the constant finding of a high sphingomyelin content in these viruses. Herpes viruses also are enveloped, however, they derive their envelope from the nuclear membrane which already possesses high levels of sphingomyelin. This could explain why no differences between the virion and nuclear membrane lipids were seen (Ben-Porat and Kaplan, 1972).

Landsberger et al. (1973) have recently shown that the rigidity of the viral envelope depends on the lipids and is independent of the type of proteins present. These data were obtained by electron spin resonance (ESR) methods. In this technique a fatty acid or phospholipid derivation containing a paramagnetic nitroxide ring is intercalated into the viral membrane. The ESR spectra from different probes provide information concerning the fluidity, orientation, polarity and translational motions of lipid molecules. Although this method is frequently used to investigate lipid mobility there are significant drawbacks when applied to intact virus (Blough and Tiffany, 1975).

Much work has been directed toward learning the origin of viral lipids. Earlier experiments with orthomyxoviruses
(Kates et al., 1961) and paramyxoviruses (Klenk and Choppin, 1969) found that viral lipids were similar to the uninfected host lipid in composition. Blough and Lawson (1968) however, observed significant differences between the lipids of NDV and Sendai virus which had been grown in ovo. The results of later work suggested a possible selective rearrangement of membrane lipids (Klenk and Choppin, 1970b) or even a redirection of host cell lipid synthesis (Shibuta and Matumoto, 1972).

In a more recent report workers found that NDV modifies phospholipid synthesis in the host and that the lipids of the mature virion were slightly different than those of the modified host membrane (Israel et al., 1975). The physical state of the host cell phospholipids has been shown to be a significant determinant in the infection process of the paramyxoviruses (Haywood, 1975; Li et al., 1975). Quigley et al. (1971) have reported similarities between the phospholipid content of Rous sarcoma, NDV, Sindbis and Sendai viruses which were different than those of common host systems in which they were grown. These similarities may reflect requirements for the budding process or a similar requirement for subsequent adsorption to the host cell. It is apparent that the membrane lipids play a role in virus maturation; how much of a role is as yet undetermined.

Membrane Modification

The method by which the glycosylated membrane proteins
are inserted through the lipid region of the host cell membrane is not known. Several models for assembly of virus-specific areas of the membrane in the orthomyxoviruses and paramyxoviruses have been reported (Tiffany and Blough, 1970; Choppin et al., 1972). These models suggest that the glycoproteins are inserted in a random fashion, floating in a sea of membrane lipids. In order for the maturation patches to form there has to be some form of recognition across the lipid bilayer between the external proteins and the internal proteins. The surface glycoproteins have a hydrophobic end which extends only into the lipid matrix of the outer leaflet of the bilayer (Laver and Valentine, 1969). Gahmberg et al. (1972) identified the hydrophobic segment of the glycoprotein of Semliki forest virus which may anchor the protein to the lipid bilayer. A possible method for recognition involves the specific interaction between the inner leaflet and the internal membrane (M-protein) (Lenard et al., 1974).

The M-protein of NDV comprises about 3% of the total virion protein, is non-glycosylated and has a molecular weight of 26,500 (Iinuma and Simpson, 1974). Although the M-protein does not appear to be a part of the nucleocapsid it is believed that it extends into the lipid bilayer of the viral envelope (Lenard et al., 1974). Nermut (1972) suggested that a space exists between the lipid bilayer and the internal
structure of the virion. This suggestion was based on electron microscopic observations. Just prior to budding, the M-protein, visible as an electron-dense layer appears on the inside of the modified plasma membrane. The nucleocapsid aligns itself under the thickened portion of the membrane and budding begins (Lenard et al., 1974; Choppin et al., 1972). The M-protein could act as a target for the nucleocapsid and its intimate association with the lipid bilayer may cause the final redisposition of membrane lipids which are found in the mature virion. Evidence for this stems from the fact that the emerging virion is covered with viral spikes down to the base of the budding particle, but they are not seen on the adjacent area of the modified membrane (Choppin et al., 1972).

The term self-assembly has been applied within a wide range of biological systems from simple poly-enzymes to the more complex bacterial and animal virus systems. Considering all available data a plausible and compatible hypothesis has been put forth which states: "the formation of hydrophobic bonds between nonpolar regions of viral proteins and the hydrocarbon moieties of lipids could provide the major driving forces, free energy, for this part of virus membrane assembly" (Eiserling and Dickson, 1972).
MATERIALS AND METHODS

Solutions and Media

All water used for solutions and preparation of media was obtained by passing distilled water through a Super-Q Ultra Pure Water System (Millipore Corp., Bedford, Mass.). All salts were of analytical grade.

Alsever’s Solution

The anticoagulant was used in the preparation of chicken red blood cells for hemagglutination assay. The solution was prepared by adding to 800 ml of water, 0.8 gm of sodium citrate, 2.05 gm of glucose, 0.055 gm of citric acid, and 0.42 gm NaCl. The solution was brought to 1 liter with water, sterilized by autoclaving and stored at 4 C. It was used in equal amounts with chicken blood.

Balanced Salt Solution Without Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (BSS) 10X

To 500 ml of water were added 80.0 gm of NaCl, 4.0 gm of KCl, 3.5 gm of NaHCO\textsubscript{3}, 10.0 gm of glucose, 100 ml of 0.2% aqueous phenol red; the total volume was brought to 1000 ml with water. One-ml of chloroform was added as a preservative and the 10X BSS was stored at room temperature. A working 1X solution was obtained by diluting the stock solution 1:10 with water and sterilizing the 1X solution in the autoclave at 121 C for 15 min.

Growth Medium (BME)

BME-Hanks base with L-glutamine and without sodium
bicarbonate (Microbiological Associates, Bethesda, Maryland) was the base used for growth media. To the rehydrated base were added 5% v/v calf serum, 5% w/v tryptose phosphate broth (Difco), 100 units/ml K Penicillin G, 100 µg/ml streptomycin sulfate and 0.3M HEPES (N-2-hydroxy ethylpiperazine-N'-2-ethanesulfonic acid) buffer. The medium was adjusted to pH 7.2, filter-sterilized and stored at -20 C.

Hank's Balanced Salt Solution (HBSS) 10X

Solution A was prepared by dissolving 80.0 gm of NaCl, 4.0 gm of KCl, and 2.0 gm of MgSO$_4$·7H$_2$O in 300 ml of H$_2$O. Solution B was prepared by dissolving 1.85 gm of CaCl$_2$·2H$_2$O in 100 ml of H$_2$O. Solution C was prepared by dissolving 1.13 gm of Na$_2$HPO$_4$·7H$_2$O, 0.6 gm of KH$_2$PO$_4$, and 10.0 gm of glucose in 300 ml of H$_2$O. To solution C was added 100 ml of 0.2% phenol red.

Solution B was added to solution A with constant mixing, then solution C was added. The solution was brought to 1 liter with water, filter-sterilized and stored at 4 C.

Phosphate Buffered Saline (PBS) 10X

This buffer was prepared by adding 105.7 gm of Na$_2$HPO$_4$·7H$_2$O, 27.0 gm of KH$_2$PO$_4$, 41.0 gm NaCl and brought to 1 liter with water. A 1X solution was obtained by diluting the stock 1:10 and adjusting the solution to pH 7.2 with 1N NaOH.

Trypsin-EDTA Solution

To 1000 ml of sterile BSS were added 0.6 gm of 1:250
trypsin (Difco Laboratories, Detroit, Michigan) and 0.2 gm EDTA (ethylene-diamine-tetraacetic acid). To this solution were added 100,000 units of K penicillin G and 100,000 micrograms of streptomycin sulfate. The mixture was sterilized by filtration. The final solution was stored at -20 C until used.

Scintillation Counts of Radioisotopes

The scintillation fluid (Cocktail D, Beckman) was prepared by adding 100.0 gm of naphthalene to 5.0 gm of PPO (2,5 diphenyloxazole). The mixture was dissolved in the balance of a liter of dioxane. The solution was stored in an amber-colored bottle in the dark. For determinations of radioactivity, 10 ml of scintillation fluid were added to each sample to be counted. The loaded vials were allowed to equilibrate for 12 to 18 hr and then were counted in a Packard Tri-Carb liquid scintillation counter. Suitable blanks and standards were also counted. Each vial was counted for a period of 10 min in triplicate. The average of the counts minus the blank or background was recorded. The counting efficiency for 14C was 90%.

Overlay Medium 2X

This medium was prepared as double-strength BME. The medium was stored at -20 C. When needed, it was thawed to 46 C and mixed in equal proportions with a molten Purified Agar solution (13 gm/liter H2O) (Baltimore Biological
Laboratories, Inc., Baltimore, Maryland). The mixture was maintained at 46°C until added to monolayers.

**Staining Agar**

Thirteen gm of purified agar and 0.4 gm of neutral red were brought to 1 liter with water. This mixture was then sterilized by autoclaving for 15 min at 121°C. The mixture was used for staining monolayers to make plaques more visible. For use, the mixture was first melted, then cooled to 46°C before being added to the plates at a volume of 1.0 ml per plate.

**Virus Stock**

The virus used in this study was Newcastle disease virus, (NDV) strain Cal. RO. It had originally been obtained from the Newcastle disease repository of the University of Wisconsin and was supplied by Professor D. P. Durand.

Virus stocks were prepared by inoculation of the allantoic cavity of ten-day-old embryonated chicken eggs. A small hole was aseptically punched in the shell above the air sack. An injection of 0.2 ml of virus stock was delivered into the allantoic cavity using a 1.0-ml syringe with a 6.08-cm, 23-gauge needle. After 48 hr of incubation at 39°C, the allantoic fluid was harvested aseptically, pooled and stored at -56°C until sufficient volume was obtained to warrant purification of the virus. Partial purification was accomplished by centrifugation
in a Sorvall RC-2B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) with a GSA rotor at 11,700 xg for 20 min. The pellet containing cellular debris was discarded. The virus, contained in the supernatant, was collected by centrifugation at 54,000 xg for 2.5 hr using a Beckman L265-B ultracentrifuge. The resulting pellet was suspended in PBS overnight at 4 C. This material was then purified by centrifugation to equilibrium (90,000 xg for 2.5 hr) in a preformed 10-4.5% (w/w) potassium tartrate gradient. The visible band was collected and dialyzed against several volumes of PBS for 24 hr at 4 C. The dialyzate was titered for plaque-forming units (PFU). This suspension was stored in three-ml volumes at -56 C.

**Hemagglutination Titration**

Chicken red blood cells were secured by cardiac puncture using a 10-ml syringe and a 6.35 cm, 18-gauge needle previously moistened with Alsever's solution. The red blood cells were washed three times in PBS. Following the final wash, the cells were resuspended in PBS to a 0.5% cell suspension.

Plastic titration wells (Linbro Chemical Co., New Haven, Conn.) were used, into each of which was placed 0.2 ml PBS; 0.2 ml of virus sample was diluted in a two-fold series. To each well was added 0.2 ml of the red blood cell suspension. After an hour of incubation at room temperature the test was read. The hemagglutinating units (HAU) are reported as the reciprocal of the highest dilution showing
partial agglutination x 10^7. The factor of 10^7 was computed by the method as described by Friedenald and Pickels (1944).

Preparation of Chicken Embryo Cells (CEC)

Ten-day-old embryonated chicken eggs were candled for viability and swabbed on the blunt end with 95% ethyl alcohol. Embryos were aseptically removed and placed in a petri plate containing BSS. Following decapitation and evisceration, the embryos were washed in another portion of fresh BSS. The embryos were then expressed through a 20-ml syringe into about 50 ml of fresh BSS and the large fragments were allowed to settle for about 5 min. The supernatant was decanted and a second wash of BSS was added to suspend the fragments. When the larger fragments had settled, the supernatant was again decanted and about 5 ml of cold trypsin-EDTA solution per embryo were added to the cells. A sterile magnetic stirring bar was added to the flask and the mixture was agitated on a Magnestir (Labline, Inc., Chicago, Illinois) for 20 min. The resulting solution was then filtered through two layers of sterile cheesecloth into about 20 ml of cold BME. The cells were then centrifuged at 287 xg for 5 min. The cell pellet was suspended in a volume of fresh BME and filtered through three layers of sterile cheesecloth. The cells in the resulting suspension were adjusted to a cell density of about 1.5 x 10^6 cells per ml with fresh BME. Samples of the cell suspension
were placed in culture vessels and then incubated at 39 C for 48 hr or until a confluent monolayer of cells was obtained.

**Plaque Titration**

Plaque assays for the virus were done by using chicken embryo cell monolayers. The growth medium was removed by aspiration and appropriate dilutions of the virus, made in BME, were applied in a 0.5-ml volumes. The virus was allowed to adsorb to the cells for 1 hr at 39 C. The inoculum was removed at this time and the plates were overlayed with 6 ml of the overlay medium-agar mixture. Following solidification of the overlay the plates were incubated at 39 C for 72 hr. Plaques were made visible by overlaying the medium-agar mixture with staining agar and then the incubation period was continued for an additional 3 to 4 hr. At this time the plaques were discernible as unstained areas of the monolayer. Plaque enumeration was done with the aid of a 10X dissecting microscope.

**Electron Microscopy**

All electron microscopy was done with a Hitachi HU-11C transmission electron microscope at an accelerating voltage of 75 KV. Negative-stained preparations of NDV were made by placing a drop of the virus suspension on a formvar-coated grid for 1 min. Excess sample was drawn off with a piece of filter paper and a drop of 3% phosphotungstic acid, pH 7.0, was placed on the grid and removed after a
few seconds.

Plasma membrane samples to be embedded for thin sectioning were isolated and purified as described later in this section. The membranes were fixed in 4% aqueous KMnO₄ for 30 min at room temperature. The sample was washed once with water and embedded in 3% agar. The agar merely made handling of the sample easier. The membranes were then post-fixed for 30 min with 2% OsO₄ in .01M phosphate buffer. Dehydration of the sample was accomplished by an acetone series of 30%, 60%, 90%, and 100% acetone. Following dehydration the agar plug was embedded in plastic (Spurrs resin) using the following series of resin:acetone changes: 1 to 3 for 20 min, 1 to 1 for 30 min, 3 to 1 for 1 hr, pure resin for 24 hr. The plastic was polymerized in the following manner: 37 C for 24 hr, 45 C for 24 hr, and 60 C for 72 hr. Sections were stained with 0.5% uranyl acetate in methanol for 30 min.

Polyacrylamide Gel Electrophoresis (PAGE)

Buffer: 1.38 gm of barbital; 8.7 gm of sodium barbital and 0.38 gm of calcium lactate were brought to 1 liter with water. The pH was adjusted to 7.2 with 1N NaOH.

Acrylamide (Eastman Kodak Co., Rochester, N.Y.) was re-crystalized by dissolving 50 gm acrylamide in 1 liter of chloroform at 60 C. The residue was removed by filtration and the solution was cooled slowly to -20 C. The resulting crystals were collected and dried.
$N,N'$-methylene-bisacrylamide (Eastman Kodak Co.,) Rochester, N.Y) was recrystallized by dissolving 10 g bisacrylamide in 1 liter of acetone at 55 C. The solution was filtered and cooled slowly to -20 C and the crystals were collected and dried.

The acrylamide-bisacrylamide solution was made in water at a ratio of 30.0:0.08 respectively.

**Running Gel:** The following reagents composed the 7.25% acrylamide gel: 6.25 ml acrylamide-bisacrylamide solution; 18.0 ml buffer; 0.045 ml $N,N,N'$-tetramethylethylene-diamine; 0.025 ml 10% ammonium persulfate (freshly prepared). Gels were cast in glass tubes having an inside diameter of 5mm to a length (height) of 10 cm.

**Procedure:** The PAGE apparatus was a product of Buchler Instruments Inc., Fort Lee, N.J. Both the radioactive whole virus samples and the isolated M-protein samples were prepared for electrophoresis as described by Scheid and Choppin (1973).

Electrophoresis was started at 1.5mA/gel until the sample had entered the gel. The amperage was then increased to 4.0mA/ gel and was continued until the dye front moved approximately 5.0 cm. The gels were removed from the tubes very carefully so as not to fragment the polyacrylamide. This was accomplished by expression of the gel into a dish containing buffer solution.
For the determination of radioactivity of samples the gels were sliced into 1-mm thick sections using a razor blade. Radioactivity was eluted from the slices using 0.5 ml of PBS. The sections were prepared for radioactive determination as previously described.

Isolation of the M-protein of NDV

The non-glycosylated membrane protein (M-protein) of NDV was isolated by the procedure of Scheid and Choppin (1973). Purity of the M-protein preparation was determined by PAGE.

Isolation of Lipids

Lipids of CEC and CEC plasma membranes were isolated by a modification of the method of Folch et al. (1957). The material for lipid extraction was placed in acid-cleaned 16 mm screw-capped tubes and washed twice with physiological saline and suspended in 3.0 ml of anhydrous methanol which was deoxygenated by bubbling the solvent with nitrogen gas. The tubes were then gassed with nitrogen for air displacement, sealed and heated for 30 min at 55°C. After cooling, chloroform, which had also been deoxygenated, was added at a volume of 6 ml. The tubes were regassed, sealed and allowed to stand overnight at room temperature. The chloroform-methanol insoluble material was removed by filtration and the filter was washed with 1 ml of CHCl₃-M₃OH (2:1). The filtrate was extracted twice with 0.2 volume of 0.02M KC1.
Following each extraction the aqueous layer was removed by aspiration being careful not to disturb the interface. After the second aspiration the interface was washed twice with 0.5 ml of chloroform: methanol: water (3:48:47). Residual water was removed from the material by passing it through a column made from 9-inch Pasteur pipettes stopped with glass wool and packed with hexane-washed Na$_2$SO$_4$. One ml of chloroform was passed through the column after the sample collecting all in a 16-mm screw-capped tube. The tubes were placed in a 55°C water bath and the solvent was evaporated with a continuous stream of nitrogen flowing over it. The last 0.5 ml was evaporated at room temperature to prevent oxidation.

The amount of phospholipid in the sample was determined using the method of Vaskovsky et al. (1975). Cholesterol content was measured by the procedure of Zlatkis et al. (1953).

Isolation of CEC Surface Membranes

Plasma membranes were isolated by the FMA (fluorescein mercuric acetate method of Scher and Barland (1972) as modified by Carlsen and Till (1975). The FMA was synthesized by the procedure of Karush et al. (1964). The absorption spectrum of the prepared FMA was identical to that reported by Karush et al.; absorption maxima were obtained at 248, 298, and 502 nm.
Gas Chromatography

**Sample Preparation:** Methyl esters of isolated fatty acids were prepared by adding 4.5 ml of anhydrous methanol-2% \( \text{H}_2\text{SO}_4 \) to the screw-capped tube containing the lipid sample. The tube was gassed for air displacement with nitrogen, resealed and allowed to stand overnight at room temperature. Four ml of water were then added to the tube and this material was extracted three times with 3.0-ml volumes of hexane placing the hexane layer in a separate tube. The combined hexane layers were then washed with 3.0 ml of water. The material was then passed through a \( \text{Na}_2\text{SO}_4 \) column followed by 1 ml of hexane. The material was collected and dried at 55°C under a continuous stream of nitrogen. The methylated fatty acids were stored at -20°C.

**Column Preparation:** Stainless steel columns (6' x 1/8") were washed with acetone and dried with a steam of nitrogen gas before packing. Equal amounts of packing (Liquid phase: 15% DEGS, Support 60/80 Gas Chrom P. Applied Science) was introduced into a matched pair of columns. Ends of the columns were plugged with dimethyldichlorosilane-treated glass wool (Applied Science, College Park, Pa.). Columns were conditioned in the gas chromatograph for 36-48 hr at a temperature which was ten degrees higher than the maximum operating temperature for that column. The detector ends
of the column were left disconnected during conditioning to prevent excess contamination of the flame detectors.

**Equipment:** Gas chromatography was performed on a Beckman GC-4 gas chromatograph. Chromatograms were recorded on an Esterline Angus 1101E recorder equipped with a model 247 Disc integrator. The gas chromatographic conditions were as follows:

- **Sample size:** 1.0-5.0 ul
- **Carrier flow:** 50 ml/min
- **Hydrogen flow:** 48 ml/min
- **Air flow:** 275 ml/min
- **Carrier make-up:** 70 ml/min
- **Inlet temperature:** 230 F
- **Detector line temp.:** 230 F
- **Detector temperature:** 260 F

The fatty acid peaks were tentatively identified by comparison to relative retention times of internal and external standards. The standards used in this study are listed in Table 5. The statistical test done on the fatty acid data was a three-way factorial analysis of variance.

Standard errors were computed by using the formula: 

\[ \sqrt{\frac{\sum(x_i - \bar{x})^2}{n(n-1)}} \]
RESULTS

NDV Morphology

The negative-stained preparation shown in Figure 1 is representative of the virus, NDV strain Cal. RO, which was used in this study. The virus surface is studded with spike-like projections. Helical nucleocapsid symmetry can be clearly seen in the micrograph. The diameter of the helix was 17 nm. The negative-stained preparations of NDV shown in Figure 2 are presented to exemplify the heterogeneity which was seen in populations of the virus. The forms range in shape from spherical to filamentous and in size from 90 to 315 nm.

A purified preparation of CEC-grown NDV was centrifuged through a linear sucrose gradient (4% to 54%). The virus sample was layered on the top of the gradient and centrifuged at 17,000 rpm in a Beckman model L 265-B ultracentrifuge by using an SW-27 rotor. The material was spun for 3 hr at a temperature of 10°C. The material in the tubes was fractionated in 2.0-ml volumes using a gradient fractionator (Instrumentation Specialities Co., Lincoln, Nebraska), and each fraction was assayed for HAU (Table 1). Fractions 3-7, 8-13 and 14-18 were each combined and the virus contained in these fractions was assayed for HAU and PFU and the HAU to PFU ratio (HAU/PFU) was determined. The sucrose densities of the three combined fractions were also determined. The results showed that the distribution of HAU represents a bell-shaped curve.
Figure 1. Negative stained preparation of NDV showing surface topography and nucleocapsid, 176,000 X.
Figure 2. Negative stained preparations of NDV showing heterogeneity among virus population, 95,000 X.
Table 1. The HAU, computed HAU/PFU and densities of fractions obtained from density gradient centrifugation of NDV grown in CEC culture at 39 C.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>HAU</th>
<th>HAU/PFU of Combined Fractions</th>
<th>Sucrose Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$1.4 \times 10^{11}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>$3.3 \times 10^8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$2.2 \times 10^8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>$1.4 \times 10^8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>$6.4 \times 10^8$</td>
<td>$2300$ (fractions 3-7)</td>
<td>1.06 g/cc</td>
</tr>
<tr>
<td>6</td>
<td>$5.5 \times 10^9$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>$1.1 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>$2.9 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>$6.6 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>$9.4 \times 10^{10}$</td>
<td>$370$ (fractions 8-13)</td>
<td>1.14 g/cc</td>
</tr>
<tr>
<td>11</td>
<td>$5.4 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>$4.7 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>$3.1 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>$1.0 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>$1.9 \times 10^{10}$</td>
<td>$808$ (fractions 14-18)</td>
<td>1.23 g/cc</td>
</tr>
<tr>
<td>16</td>
<td>$1.2 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>$8.3 \times 10^9$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>$3.2 \times 10^8$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The computed HAU/PPU represents the total number of virus to infectious virus. The population forming in the middle of the gradient contain a greater number of infectious particles when compared to the total population than either of the virus populations found in the other fractions.

Samples from each of the combined fractions were prepared for observation by electron microscopy (Figure 3). Those particles found in the top portion of the gradient (fractions 3-7) were small; particle diameters ranged from 70 to 100 nm. I observed that many of these virus-like particles appeared to be without nucleocapsid. The middle portion (fractions 8-13) contained mostly spherical particles ranging from 100 to 170 nm in diameter those virions found in the bottom fractions were irregularly shaped and ranged upwards to 300 nm in diameter. The presence of ruptured particles among those found in the bottom fractions also can be seen.

Cholesterol to Phospholipid Ratio in CEC

Primary cultures of CEC were infected with NDV at a MOI = 8 for 1 hr at 39 C. At various times post infection the culture fluid was assayed for HAU and PFU. The lipids were extracted from the cells at those times and the molar ratio of cholesterol to phospholipid (C/P) was determined as previously described (Table 2). The results show that the HAU/PFU increases during the 24-hr period of infection.
Figure 3. Negative stained preparations of NDV obtained from the top (a), middle (b) and bottom (c) fractions of a sucrose density gradient, 80,000 X.
The C/P of mock-infected cell monolayers at 0 time post infection was determined to be 0.26. The C/P of infected CEC increased during the first four hours. At 4 hr post infection, a time when progeny virus were first being released, the C/P reached its maximum and began to decline during the next eight hours. During the remaining 12 hr the C/P gradually increased.

Table 2. The effects of time on the HAU, PFU, HAU/PFU, and C/P of whole CEC which were infected with NDV.

<table>
<thead>
<tr>
<th>Time Post Infection</th>
<th>HAU</th>
<th>PFU</th>
<th>HAU/PFU</th>
<th>C/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.5 x 10^7</td>
<td>8.0 x 10^4</td>
<td>187</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>4.0 x 10^7</td>
<td>1.6 x 10^5</td>
<td>250</td>
<td>0.64</td>
</tr>
<tr>
<td>6</td>
<td>1.1 x 10^8</td>
<td>2.8 x 10^5</td>
<td>392</td>
<td>0.45</td>
</tr>
<tr>
<td>8</td>
<td>2.1 x 10^8</td>
<td>5.0 x 10^5</td>
<td>420</td>
<td>0.41</td>
</tr>
<tr>
<td>12</td>
<td>1.3 x 10^8</td>
<td>2.1 x 10^6</td>
<td>619</td>
<td>0.49</td>
</tr>
<tr>
<td>21</td>
<td>2.1 x 10^9</td>
<td>2.9 x 10^7</td>
<td>724</td>
<td>0.56</td>
</tr>
<tr>
<td>24</td>
<td>2.4 x 10^9</td>
<td>3.2 x 10^7</td>
<td>750</td>
<td>0.59</td>
</tr>
</tbody>
</table>

The results of the previous experiments indicated that there may be a relationship between virus phenotype and the potential of the virion to act as an infectious entity. It also was felt that the lipids may play a role in that relationship.
Temperature Studies

The following experiments were designed to examine the effects of a low (3\(^{\circ}\) C) versus a high (39) temperature on some parameters of virus production (maturation).

Since virus maturation and release occur at the cell surface information derived from isolated plasma membranes might be beneficial to the study. CEC plasma membranes were isolated from CEC monolayers and then prepared for observation by electron microscopy as previously described. Figure 4a is an electron micrograph of a negative-stained preparation of the isolated plasma membrane of CEC and shows the characteristic sheet-like appearance of isolated surface membranes. Figure 4b shows the thin-sectioned preparation. Present in this micrograph are numerous membrane vesicles and some ribosome-like particles; however, the membranes appear free of other cellular debris.

Effect of Temperature on C/P

The C/P of CEC, their membranes and NDV, all grown at 34\(^{\circ}\) C or 39\(^{\circ}\) C were determined. The results, presented in Table 3, show that in all cases the C/P of the 34\(^{\circ}\) C grown material was less than that of the 39\(^{\circ}\) C grown material. The results represent the averages from three independent experiments, and in every instance the values for the 34\(^{\circ}\) C material was less than the 39\(^{\circ}\) C material.
Figure 4. Electron micrographs of isolated plasma membranes of CEC.
Table 3. The effect of growth temperature on the C/P of CEC, CEC plasma membranes, and purified NDV.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>CEC</th>
<th>Plasma Membranes</th>
<th>NDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 C</td>
<td>0.17 (0.03)*</td>
<td>0.46 (0.5)</td>
<td>0.78 (0.10)</td>
</tr>
<tr>
<td>39 C</td>
<td>0.26 (0.05)</td>
<td>0.54 (0.06)</td>
<td>0.99 (0.04)</td>
</tr>
</tbody>
</table>

*± standard error, based on data from 3 experiments.

CEC grown at either 34 C or 39 C were each infected with NDV as described earlier. At various times post infection the plasma membranes were isolated and the C/P determined (Figure 5). At both temperatures the C/P increased following infection and continued to increase up to the point where new virus were beginning to be released (4 hr post infection). The ratios then decreased substantially over the next two hours and then leveled off. It was noted that the C/P of the plasma membranes grown at 34 C were usually lower than those grown at the high temperature.

Effect of Temperature on Virus Production

Results of experiments (data not shown) indicated that over a 24-hr period of infection there were fewer virus produced at 34 C than at 39 C; however, the HAU/PFU of 39 C grown virus was greater than that of the 34 C grown virus.

Growth curves of NDV produced at 34 C and 39 C were
Figure 5. The C/P of CEC-plasma membranes isolated at various times post infection. 34 °C○○○; 39 °C●●●.
constructed (Figure 6). At 23.5 hr post infection there was a log difference between the two virus populations in terms of PFU/ml. The low incubation temperature did not substantially effect the onset of virus maturation which occurred for both between 3 and 4 hr post infection. Following the initial increase, the rate at which virus was released in the two cultures changed. It is apparent from the data presented that the rate of virus release from the 39 C cultures was greater than that of the 374 C cultures, from 4 hr onward. Incubation was continued to 28 hr post infection and the number of infectious virus seen in the 374 C culture was less than the total number of infectious virus found in the 39 C culture fluid at 23.5 hr post infection. At 28 hr post infection, cultures were treated with 5 units/ml of neuraminidase for 30 min to release attached virus and the fluid was assayed for PFUs. PFUs present in the 39 C culture equaled 4.7 x 10^4 PFU and 7.9 x 10^4 PFU at 374 C.

Inactivation of Free Virus Particles

Known concentrations of NDV suspended in BME were incubated at either 374 C or 39 C for a total of 24 hr. At various times during the incubation period a sample was removed to determine virus numbers (Figure 7). The results indicate that there was a significant decrease in the population of infectious virus at both incubation temperatures; however, inactivation of virus at the higher
Figure 6. Growth curve comparison of NDV produced at 34°C and 39°C.
Figure 7. Inactivation of free-NDV particles incubated at 34°C or 39°C.
temperature was greater.

Early vs Late Progeny

Roller bottle cultures of CEC grown at 34°C or 39°C were each infected with NDV as described earlier. At 5 hr post infection the culture medium was removed and 25 ml of fresh BME containing 5 units/ml neuraminidase (Nase) were added to each and incubation was continued for 1 hr. At that time the medium was removed and the cells were washed twice with HBSS. Fresh growth medium was added to the cultures and incubation was continued to 11 hr post infection at which time the same procedure for Nase treatment was repeated. Virus contained in the culture media plus the wash solutions for each temperature at 6 and 12 hr post infection was isolated by polyethylene glycol precipitation (Scheid and Choppin, 1973), layered onto a linear sucrose gradient and centrifuged according to the procedure described earlier in this section. The tube contents were fractionated into 2.0-ml volumes and assayed for HAU. The results of the 39°C grown virus are presented in Figure 8. The majority of the virus population produced during the first 6 hr post infection was detected in the middle of the gradient while that produced from 6 to 12 hr post infection was detected in the lower portion of the gradient. Data from the 34°C experiment are presented in Figure 9. It was apparent that there was little difference between the virus formed early in infection as
Figure 8. Distribution of HAU in sucrose gradient containing virus produced early and late in infection at an incubation temperature of 39°C.
Figure 9. Distribution of HAU in a sucrose gradient containing virus produced early and late in infection at an incubation temperature of 34°C.
compared to those formed late in infection in terms of HAU. At both times a large majority of the virus population was detected in the lower portion of the gradient. Comparison of HAU/PPU and C/P for early and late progeny were made at each temperature (Table 4). Results were similar to earlier findings concerning virus which was found in middle and lower portions of sucrose gradients.

Table 4. Determination of HAU/PPU and C/P of early (6 hr) and late (12 hr) progeny produced at 34 C or 39 C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>HAU/PPU</th>
<th>C/P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hr</td>
<td>12 hr</td>
</tr>
<tr>
<td>39 C</td>
<td>702</td>
<td>917</td>
</tr>
<tr>
<td>34 C</td>
<td>440</td>
<td>702</td>
</tr>
</tbody>
</table>

Fatty Acid Studies

Table 5 shows the relative retention times for the fatty acid standards. The retention times were calculated as a function of the retention time of palmitic acid which was arbitrarily set at 1.00. Each day when unknown samples were to be passed through the gas chromatograph for analysis, the relative retention times for the fatty acid standards were determined. This procedure was followed to
adjust for day-to-day variations that might be encountered. The presence of unsaturated fatty acids in the samples was verified by microhydrogenation of unsaturated bonds; alterations in the mobilities of fatty acids by hydrogenation were noted.

Fatty acid preparations made from infected (4 hr) and non-infected cells or plasma membranes of CEC grown at 34 C or 39 C were methylated as described and assayed by gas chromatography. The data presented in Table 6 are averages from three independent experiments, and the standard error for each value is also presented. Significant differences between the fatty acids of the cells and cell membranes were noted. The effect of virus infection on the fatty acid composition of CEC and CEC plasma membranes was independent of temperature except for C_{14:0}, C_{16:0}, C_{20:0} and C_{20:4}. Saturated fatty acids were slightly higher in the 34 C than in the 39 C cultures. Also, a substantial difference existed in the ratios of C_{18:0} to C_{18:1} in plasma membranes of cells grown at the two temperatures.

Fatty acids of purified NDV were also analyzed by gas chromatography. Samples included virus grown at either 34 C or 39 C and obtained from the middle and lower portions of linear sucrose gradients. The relative percentages of the fatty acids are presented in Table 7.
Table 5. Relative retention times of fatty acid standards.

<table>
<thead>
<tr>
<th>Code</th>
<th>Fatty Acid</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₀:₀</td>
<td>Capric</td>
<td>0.10</td>
</tr>
<tr>
<td>C₁₂:₀</td>
<td>Lauric</td>
<td>0.34</td>
</tr>
<tr>
<td>C₁₄:₀</td>
<td>Myristic</td>
<td>0.55</td>
</tr>
<tr>
<td>C₁₅:₀</td>
<td>Pentadecanoic</td>
<td>0.77</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>Palmitic</td>
<td>1.00</td>
</tr>
<tr>
<td>C₁₆:₁</td>
<td>Palmitoleic</td>
<td>1.18</td>
</tr>
<tr>
<td>C₁₇:₀</td>
<td>Margaric</td>
<td>1.26</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>Stearic</td>
<td>1.54</td>
</tr>
<tr>
<td>C₁₈:₁</td>
<td>Oleic</td>
<td>1.64</td>
</tr>
<tr>
<td>C₁₉:₀</td>
<td>Nonadecanoic</td>
<td>1.82</td>
</tr>
<tr>
<td>C₁₈:₂</td>
<td>Linoleic</td>
<td>2.12</td>
</tr>
<tr>
<td>C₂₀:₀</td>
<td>Arachidic</td>
<td>2.20</td>
</tr>
<tr>
<td>C₂₂:₀</td>
<td>Behenic</td>
<td>2.61</td>
</tr>
<tr>
<td>C₂₄:₀</td>
<td>Lignoceric</td>
<td>3.04</td>
</tr>
</tbody>
</table>

The data show that there were no substantial differences in the fatty acid composition among the samples assayed.
A single virus preparation was made of the particles produced in three different cell culture experiments so that a detectable amount of lipid material was present.
Table 6. Relative percentages by weight and standard errors (in parentheses) of fatty acids detected in infected and non-infected CEC and CEC plasma membranes (CEC-PM) of cultures grown at 34 C or 39 C.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>39 C Infected</th>
<th>39 C Non-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEC</td>
<td>CEC-PM</td>
</tr>
<tr>
<td>C(_{12:0})</td>
<td>-</td>
<td>2.1(0.3)</td>
</tr>
<tr>
<td>C(_{14:0})</td>
<td>4.4(0.5)</td>
<td>4.2(0.4)</td>
</tr>
<tr>
<td>C(_{15:0})</td>
<td>3.4(0.3)</td>
<td>4.0(0.2)</td>
</tr>
<tr>
<td>C(_{16:0})</td>
<td>12.7(0.1)</td>
<td>10.4(0.8)</td>
</tr>
<tr>
<td>C(_{16:1})</td>
<td>2.2(0.2)</td>
<td>2.7(0.4)</td>
</tr>
<tr>
<td>C(_{17:0})</td>
<td>3.4(0.3)</td>
<td>3.1(0.2)</td>
</tr>
<tr>
<td>C(_{18:0})</td>
<td>19.2(1.1)</td>
<td>12.5(1.4)</td>
</tr>
<tr>
<td>C(_{18:1})</td>
<td>11.9(1.0)</td>
<td>20.3(1.4)</td>
</tr>
<tr>
<td>C(_{18:2})</td>
<td>5.1(0.5)</td>
<td>7.8(0.4)</td>
</tr>
<tr>
<td>C(_{19:0})</td>
<td>5.5(0.3)</td>
<td>6.2(0.5)</td>
</tr>
<tr>
<td>C(_{20:0})</td>
<td>9.2(0.6)</td>
<td>8.9(0.6)</td>
</tr>
<tr>
<td>C(_{22:0})</td>
<td>8.3(0.2)</td>
<td>9.4(0.9)</td>
</tr>
<tr>
<td>C(_{24:0})</td>
<td>5.1(0.5)</td>
<td>-</td>
</tr>
<tr>
<td>C(_{20:4})</td>
<td>9.6(0.2)</td>
<td>8.4(0.3)</td>
</tr>
<tr>
<td>Saturated</td>
<td>73.9</td>
<td>72.7</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>11.4</td>
<td>11.6</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>14.7</td>
<td>16.2</td>
</tr>
</tbody>
</table>

\(^*\)S indicates that there is a significant difference between the percentage of fatty acid in CEC and CEC-PM: C, infected and non-infected; T, 34 C and 39 C.
<table>
<thead>
<tr>
<th></th>
<th>Infected</th>
<th>Non-infected</th>
<th>Significance^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEO</td>
<td>CBC-PM</td>
<td>GEO</td>
<td>CEG-PM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>1.8(0.1)</td>
<td>-</td>
<td>4.5(0.5)</td>
</tr>
<tr>
<td>CE-PM</td>
<td>2.4(0.1)</td>
<td>2.9(0.3)</td>
<td>4.7(0.2)</td>
</tr>
<tr>
<td>CE</td>
<td>1.8(0.2)</td>
<td>1.1(0.4)</td>
<td>5.8(0.3)</td>
</tr>
<tr>
<td>CE-PM</td>
<td>5.3(0.6)</td>
<td>12.7(0.7)</td>
<td>9.7(0.4)</td>
</tr>
<tr>
<td>CE</td>
<td>1.2(0.5)</td>
<td>2.8(0.2)</td>
<td>3.8(0.4)</td>
</tr>
<tr>
<td>CE-PM</td>
<td>1.3(0.1)</td>
<td>5.9(0.1)</td>
<td>4.5(0.5)</td>
</tr>
<tr>
<td>CE</td>
<td>3.8(0.4)</td>
<td>22.5(0.9)</td>
<td>10.0(0.3)</td>
</tr>
<tr>
<td>CE-PM</td>
<td>13.4(0.6)</td>
<td>16.3(0.5)</td>
<td>21.0(1.0)</td>
</tr>
<tr>
<td>CE</td>
<td>5.7(0.6)</td>
<td>6.9(0.4)</td>
<td>6.4(0.3)</td>
</tr>
<tr>
<td>CE-PM</td>
<td>5.9(0.3)</td>
<td>6.5(0.5)</td>
<td>5.2(0.2)</td>
</tr>
<tr>
<td>CE</td>
<td>9.5(0.6)</td>
<td>6.7(0.1)</td>
<td>8.7(0.6)</td>
</tr>
<tr>
<td>CE-PM</td>
<td>8.5(0.1)</td>
<td>7.8(0.7)</td>
<td>8.1(0.7)</td>
</tr>
<tr>
<td>CE</td>
<td>9.9(0.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CE-PM</td>
<td>7.0(0.2)</td>
<td>7.9(0.4)</td>
<td>7.9(0.4)</td>
</tr>
<tr>
<td>CE</td>
<td>9.4(0.1)</td>
<td>5.3(0.3)</td>
<td>9.6</td>
</tr>
<tr>
<td>CE-PM</td>
<td>75.3</td>
<td>76.0</td>
<td>76.5</td>
</tr>
<tr>
<td>CE</td>
<td>15.1</td>
<td>11.2</td>
<td>14.5</td>
</tr>
</tbody>
</table>
Table 7. Relative percentages by weight of fatty acids found in purified NDV.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>39 C</th>
<th></th>
<th>34 C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>C_{12:0}</td>
<td>3.2</td>
<td>3.9</td>
<td>3.7</td>
<td>3.1</td>
</tr>
<tr>
<td>C_{14:0}</td>
<td>4.1</td>
<td>4.5</td>
<td>4.4</td>
<td>4.0</td>
</tr>
<tr>
<td>C_{15:0}</td>
<td>4.5</td>
<td>4.2</td>
<td>4.9</td>
<td>2.1</td>
</tr>
<tr>
<td>C_{16:0}</td>
<td>12.9</td>
<td>11.9</td>
<td>11.8</td>
<td>11.8</td>
</tr>
<tr>
<td>C_{16:1}</td>
<td>3.5</td>
<td>3.0</td>
<td>2.1</td>
<td>2.8</td>
</tr>
<tr>
<td>C_{17:0}</td>
<td>2.9</td>
<td>3.1</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>12.4</td>
<td>11.2</td>
<td>13.2</td>
<td>12.2</td>
</tr>
<tr>
<td>C_{18:1}</td>
<td>17.0</td>
<td>17.4</td>
<td>19.1</td>
<td>22.0</td>
</tr>
<tr>
<td>C_{18:2}</td>
<td>5.9</td>
<td>6.4</td>
<td>5.1</td>
<td>6.1</td>
</tr>
<tr>
<td>C_{19:0}</td>
<td>4.3</td>
<td>4.5</td>
<td>5.6</td>
<td>5.2</td>
</tr>
<tr>
<td>C_{20:0}</td>
<td>7.4</td>
<td>6.4</td>
<td>8.0</td>
<td>7.1</td>
</tr>
<tr>
<td>C_{22:0}</td>
<td>6.9</td>
<td>7.4</td>
<td>7.0</td>
<td>6.8</td>
</tr>
<tr>
<td>C_{24:0}</td>
<td>5.0</td>
<td>6.6</td>
<td>3.6</td>
<td>4.5</td>
</tr>
<tr>
<td>C_{20:4}</td>
<td>10.0</td>
<td>9.5</td>
<td>8.4</td>
<td>8.8</td>
</tr>
</tbody>
</table>

*a* Virus isolated from the middle portion of a sucrose gradient

*b* Virus isolated from the lower portion of a sucrose gradient
Studies on the M-protein of NDV

Roller bottle cultures of GEO grown at 39 C were starved for amino acids by incubating the cell in HBSS for 8 hr. The cells were then infected with NDV as described before. Following infection the cultures were incubated in BME supplemented with amino acids in the form of an algal profile of a ^14C-reconstituted protein hydrolysate (0.5 uCi/ml) for 24 hr. The virus produced from this infection was purified and the M-protein was isolated by using the procedure described by Scheid and Choppin, 1973. Radioactively-labeled virus and M-protein were prepared for PAGE in the manner described in Materials and Methods. The M-protein preparation was free of other viral proteins and non-viral contaminating radioactivity (Figure 10).

An experiment was designed to determine if the M-protein would bind to non-infected and/or infected GEO plasma membranes. Since M-protein is insoluble in 0.1M phosphate buffer but soluble in phosphate buffer containing 1.0M KCl, binding studies were carried out by using preparations of M-protein suspended in both buffers. Bovine serum albumin (BSA), at a concentration of 1.0 mg/ml, was added to certain reaction mixtures to serve as a nonspecific binding control. Unlabeled soluble M-protein was added as a possible specific binding control.

Reaction mixtures were incubated for 1 hr at 39 C. After the incubation period, plasma membranes were isolated
Figure 10. Distribution of radioactivity in polyacrylamide gels containing whole virus particles (A) or isolated M-protein preparation (B).
from the reaction mixtures, the mixtures were first subjected to centrifugation at 800 xg for 10 min. The supernatant (sample A) was saved for detection of radioactivity and the pellet was suspended in a small volume of buffer and centrifuge in a two-phase polymer system (Carlsen and Till, 1975) which allowed the plasma membranes to form at the interface. Samples from the top (sample B) and the bottom (sample C) of the gradient were saved to determine radioactive content. The plasma membranes were placed in 5.0 ml of phosphate buffer (pH 7.3) and centrifuge at 800 xg for 10 min. The supernatant (sample D) and the pellet (sample E) were assayed for radioactive content. The results of this experiment are presented in Table 8. BSA had little effect on the results obtained. The soluble-unlabeled M-protein control seemed to decrease the binding of the labeled M-protein. The data showed, however, that radioactivity was associated with only the infected CEC plasma membranes and not with the non-infected plasma membranes (sample E).
Table 8. The binding of radioactive M-protein to infected and non-infected CEC plasma membranes.

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infected CEC Plasma Membrane</th>
<th>Non-infected CEC Plasma Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td></td>
<td>BSA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SM&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>327&lt;sup&gt;e&lt;/sup&gt;</td>
<td>344</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>A= first supernatant; B= top of gradient; C= bottom of gradient; D= second supernatant; E= plasma membranes.
<sup>b</sup>BSA control.
<sup>c</sup>Soluble M-protein control.
<sup>d</sup>Labeled M-protein only
<sup>e</sup>Counts/min.
DISCUSSION

Some aspects of NDV maturation and release were studied. The heterogeneity in NDV morphology (Figure 2) is well known (Melnick, 1973; Blough and Tiffany, 1975; Roman and Simon, 1976). Filamentous forms of NDV have been reported (Feller et al., 1969). Blough (1963) reported that artificial filamentous forms could be produced by adding vitamin A to infected cells. Vitamin A is a fat-soluble vitamin, and the effect which was observed was probably due to alteration of the membrane lipids (Blough et al., 1967). A possible labilizing effect could increase membrane fluidity and enhance the production of filamentous virus forms.

The intention was to determine if any relationship existed between virus morphology and virus infectivity. Another objective was to discover if cellular factors might be involved in the actual packaging and/or release of progeny. I believe that the results in Table 1 indicate that there may be a relationship between virus size and virus infectivity. The HAU/PFU of virus in fractions 3-7 was extremely high; however, this is not too surprising. Pieces of modified host cell membrane, present as a consequence of cell destruction, tend to round-up and form hemagglutinating particles; thus, the HAU/PFU would be high. For this reason, I decided to eliminate the material found in the upper fraction from subsequent experiments. The
particles contained in the middle fractions (8-13) had the lowest HAU/PPU. Electron micrographs of virus contained in these fractions (Figure 3b) were mostly spherical, and ruptured particles were rarely seen. This would lead one to believe that the particles in fractions 8-13 were more stable than those in fractions 14-18 which might directly effect the HAU/PPU. Virus present in the lower portion of the gradient (fractions 14-18) had a relatively high HAU/PPU of 808. As can be seen in the electron micrograph showing virus particles found in these fractions (Figure 3c), there were several ruptured particles. Again one might hypothesize that these large, irregularly-shaped particles are unstable and therefore tend to rupture easily.

The C/P has been an important determinant for several lipid studies involving viruses (Blair and Brennen, 1972; Choppin et al., 1972; Klenk, 1974). The action of cholesterol in plasma membranes has been thought to be a stabilizing or condensing agent (Finean, 1953; Ashworth and Green, 1966; Papahadjopoulos et al., 1973). Results of recent work (Yeagle et al., 1975) suggested that the action of cholesterol may be dependent upon the polar head groups of membrane lipids. The reported stabilizing effect of cholesterol allowed me to speculate that the stability of NDV was linked to its cholesterol content. Animal cells reportedly have a molar C/P between 0.2 and 0.3 and NDV has a molar C/P of about 1.0 (Klenk, 1974).
The effect of virus infection on the C/P of CEC was determined and the HAU/PFU was also computed for various times post infection (Table 2). The HAU/PFU steadily increased during the course of infection. Both the inactivation of infectious virus during the 24 hr infection period plus the accumulation of hemagglutinating membrane fragments could explain these results. The C/P of mock-infected cells was 0.26; however, in virus-infected cells at 0 time post infection, the C/P was 0.31. The association of cholesterol-rich virus particles with the cells might explain the observed difference. During infection the C/P increased, reaching a maximum at 4 hr post infection. This was followed by a decrease during the next two readings taken over a 14 hr period. It seemed that virus infection was indeed modifying the C/P of the cells. At 4 hr post infection, virus progeny are being released from the cells. If the virus bud through cholesterol-rich regions of the membrane, the release of cell-associated virus would result in a decrease in the C/P of the cell. I was puzzled by the gradual increase in the C/P during the next 16 hr. Possibly this increase resulted from reassociation of released virus with available cell receptors.

At this point in the study I believed that there may be a relationship between virus phenotype and infectivity
and that cellular lipids could play a role in that relationship.

The lipid composition of cells in culture is ultimately controlled by the cell's genetic information, however, as with any organism the expression of cellular genes can be affected by external factors (e.g. available nutrients). Cellular lipid composition varies with temperature, pH, cation concentration, and nutrients (McElhaney, 1974; Jacobson and Papahadjopoulos, 1975; Fukushima et al., 1976). I chose to vary the temperature at which the cells were incubated and determine the effect on virus maturation.

Cells were grown at 39°C, the normal temperature, and at 34°C, and the effects of temperature on virus production over a 24 hr period were determined (Figure 6). Few differences were seen in virus production during the first 4 hr of infection, but virus maturation seemed to occur at both incubation temperatures at about 3.5 hr post infection. Following the initial increase of virus titer, the production of PFU in the 34°C culture was less than that of the 39°C. This effect could be caused by an overall slower metabolic rate (i.e., protein synthesis) in the 34°C culture than in the 39°C culture. However, when incubation was continued to 28 hr post infection the total PFUs in the 34°C culture had not increased significantly over the total PFUs at 23.5 hr post infection. At 28 hr post infection,
both cultures were treated with neuraminidase to release cell-associated virus. More virus was associated with the 34 C cells than the 39 C cells; however, the difference was not sufficient to explain the overall differences that I observed. The greater number of cell-associated virus in the 34 C than in the 39 C culture may have been caused by the presence of more available receptors in the 34 C culture as a result of less cytopathic effect because of reduced virus production.

I was interested in the high HAU/PFU in the 39 C incubated culture. Two questions came to mind: 1) was this a consequence of a less stable particle being produced as a result of increased virus production, or 2) was there greater inactivation of virus at the higher temperature? The data presented in the inactivation study (Figure 7) showed that the high HAU/PFU found in the 39 C culture was probably a result of a greater rate of inactivation caused by the elevated temperature.

Because NDV maturation and release occur at the host cell plasma membrane, and this membrane undergoes substantial modification as a result of infection (Lenard and Compans, 1974), I decided to look more specifically at membrane effects. The literature abounds with various methods of membrane isolation; however, I desired a rapid technique which would allow me to obtain several samples
in a relatively short period of time. The method of Scher and Barland (1972) as modified by Carlsen and Till (1975) was selected. This method employs fluorescein mercuric acetate (FMA) to stabilize the membranes and strip them directly from the cell monolayer. Standard procedures for determining membrane purity by utilizing enzyme assays were not possible with FMA isolated material because FMA inhibits enzyme activity. FMA has been reported to have no effect on membrane lipids (Klenk and Choppin, 1969). Electron micrographs of thin-sectioned and negative-stained membranes are shown in Figure 4. It is apparent that the preparation consisted mainly of plasma membrane. No nuclei or mitochondria were observed and relatively little endoplasmic reticulum was present. Although it can not be stated that a completely pure preparation of plasma membranes was obtained, the membranes were largely separated from other cellular structures, and they resembled the preparations obtained by Klenk and Choppin (1969).

I continued temperature studies by using the isolated membrane preparations. Table 3 shows the C/P of CEC, CEC-plasma membranes and purified NDV grown at two temperatures. As mentioned earlier, lipid composition varies somewhat with external and internal factors (e.g., cell cycle) and I tried to maintain identical conditions during each experiment. I cannot state that there was a
statistically significant difference between the C/Ps at the two incubation temperatures, but there was a tendency for the C/P to be lowest at the lower temperature. In Figure 5 results are presented that show the effects of NDV infection on the C/P of isolated plasma membranes from cells grown at 34°C and 39°C. The response was strikingly similar to the results obtained for whole cells grown at 39°C (Table 2) and could be explained in a similar manner. The C/P of the plasma membranes is comparable to other published values (Klenk and Choppin, 1970a; Klenk and Choppin, 1970b; Quigley et al., 1971; Quigley et al., 1972). Since the C/P of CEC-plasma membrane was about one-half that of the virus, I propose that the virus buds through cholesterol-rich regions of the membrane. This preferential budding has been suggested for a member of the arbovirus group (Sefton and Gaffney, 1974).

I considered the possibility that certain morphological types may be formed at certain times post infection. Could there be some limiting factor(s) which leads to the production of aberrant viral forms? Figure 8 shows a comparison of the distribution of HAU in a sucrose gradient containing virus present after the first 6 hr of infection to those virus produced from 6 to 12 hr post infection. A difference was observed at an incubation temperature of 39°C. The majority of early progeny were located in
the middle of the gradient. These virus particles had a low HAU/PPU and a high C/P, in comparison to those virus formed late in infection which were found primarily in the lower portion of the gradient and had a higher HAU/PPU and a lower C/P (Table 4). When I repeated this experiment at an incubation temperature of 34°C, there was no difference between early and late progeny in terms of HAU distribution (Figure 9). Very little difference between the HAU/PPU and C/P was observed for early and late progeny at 34°C (Table 4). In light of the results from these two experiments I concluded that the amount of cholesterol in the cell membrane may be a limiting factor, and therefore early progeny may bud through cholesterol-rich regions of the membrane.

If cholesterol acts to stabilize the membrane lipids (Papahadjopoulos et al., 1973) then the higher C/P found in NDV may be required to form a stable, and thus, infectious particle. I consistently found that the larger NDV particles contained a lower C/P and contend that this was caused by the depletion of cholesterol-rich regions of the membrane. This hypothesis is based on the observation that the large particles appear primarily late in infection (Figure 8). Blough and Merlie (1970) reported a lower C/P in the Von Magnus forms of influenza virus; these particles also were very large and non-infectious.
The lower amount of cholesterol would tend to expand the total area occupied by the lipids and permit greater mobility of the acyl chains. Blough and Merlie also reported that these incomplete particles had areas of their envelopes devoid of surface projections. I am unable to state if this was the case for the large virus particles. The instability of these particles, coupled with the possibility of fewer surface projections, could lead to a reduced ability of the virus to attach properly to the cell receptors. The increased fluidity of the viral envelope as a result of less cholesterol could promote fusion (Poste and Allison, 1973). This lipid variation may also affect the virus phenotype (Simpson and Hauser, 1966) Other studies conducted in my laboratory showed that larger virus particles have a greater tendency to cause fusion-from-without (Janet Beneke, Department of Microbiology, University of Minnesota, Rochester, Minnesota, personal communication, 1973).

In the case of the virus grown at $34 \, ^\circ \mathrm{C}$, the cholesterol level of the membrane of the cells is low (Table 3); thus, the production of the larger-type virus would be seen early as well as late in infection (Figure 9). Areas of the membrane with a low C/P may act as secondary sites for virus maturation. The slight difference in the membrane composition could lead to a reduced binding of the M-protein giving rise to unstable as well as non-infectious
virus. The lower temperature may not be affecting macromolecular synthesis because comparable levels of virus-specific proteins are found in cells infected with influenza virus at 34°C and 39°C (A. Kendal, Center for Disease Control, Atlanta, Georgia, personal communication, 1976). The production of infectious virus, as measured by plaque formation, has been related to different cell types and thus to variation in lipid composition (Kato et al., 1972).

I next turned my efforts to analyzing the fatty acids of both infected and non-infected host cell membranes. Variation in temperature affects cellular lipid composition (Jacobson and Papahadjopoulos, 1975), as well as virus infection (Li et al., 1975). Data presented in Table 6 show few statistical differences between the amounts of fatty acids found at 34°C as compared to those at 39°C. Variation between infected and non-infected cultures were independent of temperature. This is understandable because enveloped animal viruses have been shown to modify host cell lipid synthesis (Blair and Brennan, 1972; Shibuta and Matumoto, 1972; Israel et al., 1975).

The results show that at the 34°C incubation temperature there was a tendency for the material to have a higher percentage of saturated fatty acids and a lower amount of monounsaturated fatty acids. One would expect the levels of monounsaturated fatty acids to increase at the low temperature in order to maintain membrane
fluidity (Fox, 1972). Because the temperature difference was small, perhaps the cells compensated for the lower temperature by decreasing the amount of cholesterol in the membrane. An encouraging observation was that the ratio of \( C_{18:0} \) to \( C_{18:1} \) was lower in the \( 34 \text{C} \) grown material. \( C_{18:1} \) is formed from \( C_{18:0} \) (D.C. Beitz, Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, personal communication, 1976) therefore, the increase in the unsaturated form may be a direct result of the lower temperature.

The results (Table 6) of the fatty acid studies were not conclusive. There were several values which were significantly different from other values obtained for the same fatty acid; however, I could not see any pattern or trend which could help explain some of the other data. A greater spread in the incubation temperature could have produced more differences in the overall fatty acid compositions. Also, if I had separated the neutral and polar lipids and then assayed the fatty acids of each, the results may have been more meaningful.

The fatty acid contents from NDV produced in cells grown at different temperatures and then isolated from the middle and lower portions of sucrose gradient, were determined (Table 7). Although the differences were not significant they may be rather important to the virus because only 20% of the fatty acids are hydrophobically bonded
to the viral structural proteins (Tiffany and Blough, 1970). If I had analyzed the neutral and polar lipids separately substantial differences may have been found, particularly between the viruses of different densities. The particles in the middle of the gradient may have shown a higher amount of glycerides in comparison to those found in the lower portion of the gradient (Joseph Tsang, Department of Chemistry, Illinois State University, Normal, Illinois, personal communication, 1976). The enveloped animal viruses are known to contain a higher level of sphingomyelin than their respective host (Kates et al., 1962; McSharry and Wagner, 1971; Quigley et al., 1972). The fatty acids of sphingomyelin are primarily long-chain fatty acids. There was no indication of a decreased level of these acyl chains among the virus samples analyzed. Such variation may have explained the differences between the virus characteristics. Indeed, there may be no differences between the virus samples analyzed. Tiffany and Blough (1969b) have shown differences in the fatty acid contents of three strains of NDV grown in a common host. This suggests that the viral membrane proteins select a certain population of membrane lipids and virion fatty acids are strain specific.

The inner side of the envelope of paramyxoviruses is coated by the non-glycosylated M-protein (Scheid and
Previous information on the assembly of paramyxoviruses has primarily been obtained from electron microscopic studies of virus infected cells (Compans et al., 1966). Nagai et al. (1976) recently presented data which suggests that the M-protein of NDV is incorporated into membranes that already contain viral glycoproteins and that this process is one of the last steps in envelope assembly. The interest in the M-protein that has arisen in the last few years is based on the general belief that this polypeptide may be the "maturation protein" for several enveloped animal viruses. New procedures for the isolation of the M-protein have provided investigators with further methods for determining the actual role of the M-protein during virus assembly (Scheid and Choppin, 1973; McSharry et al., 1975).

A question which came to mind during these studies involved the self-assembly process of NDV. Will the M-protein bind to the host cell plasma membrane in vitro? More specifically, will the M-protein bind to non-infected plasma membrane or does this event require the presence of viral glycoproteins?

NDV was grown in CEC in the presence of $^{14}C$-labeled amino acids and I obtained a preparation of radioactively-labeled M-protein (Figure 10). Table 8 shows the results of the binding experiment, and the results are inconclusive.
BSA, added to reaction mixtures as a non-specific binding control, did not have a substantial effect on the binding of insoluble M-protein to infected plasma membranes. Reduced binding of M-protein was observed however, with soluble M-protein in the presence of BSA. When unlabeled-soluble M-protein was added to reaction mixtures to serve as a specific binding control a decrease in labeled M-protein binding to infected cell membranes was observed.

There are several possible reasons for the radioactivity which was associated with the infected plasma membranes. The infected membranes were isolated at 2 hr post infection. This was done in an attempt to permit insertion of viral glycoproteins into the membrane and yet limit any M-protein association. The cells were not synchronized and this would lead to variability among the cell population which could result in at least three different stages of infection at 2 hr post infection. First, there could be a population of cells in which there was no membrane modification; second, there would be cells which may have a modified membrane and third, there would be cells which had a modified membrane and M-protein already associated with them. Consequently at 2 hr post infection there may exist only a small percentage of the cell membranes which could bind M-protein. The argument that the radioactivity found associated with infected plasma membranes and insoluble M-protein was a result of
some sort of entrapment is invalid because if this were true I would have also detected radioactivity in the mixtures containing non-infected plasma membranes and insoluble M-protein.

In light of the findings some important conclusions can be drawn. Viral glycoproteins are required for the binding of M-protein. Since the viral glycoproteins are believed not to transverse the cell membrane (Tiffany and Blough, 1970), then how does the M-protein recognize these modified areas? It has been reported that the insertion of glycoproteins into the cell membrane during virus infection causes a redistribution of membrane lipids (Israel et al., 1975). The specific lipid composition of these areas could then provide the recognition site for the M-protein binding. To a limited extent the M-protein is embedded in the lipid matrix (Lenard et al., 1974) therefore, the lipid composition could be very important for proper binding. The association may result in a final redistribution of the membrane lipids (Bach and Miller, 1976), as evidenced by the fact that viral glycoproteins are chemically detectable at the cell surface but morphologically, the spikes are not seen prior to budding and the emerging virion is only covered with spikes down to the base of the budding particle and not on adjacent areas of the modified membrane (Choppin et al., 1972). The final redistribution of lipids would
activate the glycoproteins (Orly and Schramm, 1975), and the M-protein could now act as a target for nucleocapsid binding (Shimizu and Ishida, 1975). Artificial assembly of active envelope particles of Sendai virus has been reported (Hosaka, 1975).
SUMMARY

The maturation process of NDV was studied. Experiments were undertaken to examine the heterogeneity of the virus particles and relate this to the lipid content of the host membrane. I also presented preliminary evidence that the binding of the M-protein to the cell membrane, which is considered to be one of the final stages in virus maturation, involves the specific recognition of areas of the membrane which have been modified by the insertion of viral-specific glycoproteins.

I propose a model for NDV maturation in which the viral-specific glycoproteins recognize and become associated with a cholesterol-rich region of the membrane. The association results in a limited redistribution of the membrane lipids, the composition of which is recognized by the internal membrane protein of the virus. The binding of M-protein to these maturation sites results in a final redistribution of membrane lipids; thus activating the envelope proteins, evident now as spikes on the membrane. The nucleocapsid associates with the M-protein and the budding process begins.

In the model I have described any variation in the lipid composition of the membrane would lead to the formation of an unstable, pleomorphic, non-infectious particle. Therefore, I propose that the membrane lipids
are the primary controlling factor for the maturation of NDV.

I feel that this work has provided a basis for further studies in the area of animal virus maturation. As with any form of scientific research however, the work is never complete. The accumulation and analysis of data provides new insight and these in turn, lead to further investigation with hopes of providing pertinent information to the scientific community.
LITERATURE CITED


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