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IMMUNOLOGICAL AND BIOCHEMICAL STUDIES ON  
INCLUSIONS PRODUCED IN CANINE ADENOVIRUS INFECTED CELLS

by

 MAHMOOD SHAMSI SHAHRABADI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH  
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DEPARTMENT OF MICROBIOLOGY

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THE UNIVERSITY OF ALBERTA  
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled, "Immunological and Biochemical Studies on the Inclusions Produced in Canine Adenovirus Infected Cells" submitted by Mahmood Shamsi Shahrabadi, D.V.M., M.Sc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The nature of the inclusions induced by the virus of canine laryngotracheitis in a canine cell line was determined by means of biochemical and immunological studies. In addition, the synthesis of macromolecules in infected cells was determined and compared with that in noninfected cells. The early detectable changes in infected cells were the increased synthesis of DNA, RNA, and protein. This increased amount of these macromolecules was detected at 8 hours after infection and continued until 20-28 hours.

To determine the antigenic content of the inclusions a method was developed by means of which the intracellular antigens could be detected at the level of the electron microscope by staining thin sections with ferritin conjugated antibody. Using this method combined with the fluorescent staining technique, the appearance and distribution of ICL virus capsid antigens in infected cells was determined. Fiber antigen was the first structural antigen which could be detected in the early and ring form inclusions at 9-10 hours after infection. Hexon and penton base antigens were detected 2 hours after the appearance of fiber antigen and were associated with the newly formed virus particles. At the later stages of infection light staining and dark staining inclusions were demonstrated in infected cells. The light inclusions which were inside the nucleus contained all three hexon, penton base and fiber antigens, whereas the dark inclusions which were observed both in the nucleus and cytoplasm contained only hexon antigen.

The early and ring form inclusions were purified from the in-

fecting cells and their chemical composition was determined. It was found that these inclusions contained DNA, RNA, and protein. Further analysis of the DNA by CsCl centrifugation revealed that the DNA content of the inclusions was viral. Electron microscope autoradiography showed that the early and ring form inclusions were the sites of viral DNA synthesis. In addition, association of a DNA polymerase with the inclusions was detected which in the presence of the four nucleoside triphosphates could incorporate  $^3\text{H}$ -TTP into the DNA product. The product had a density equal to that of viral DNA indicating its similarity to the viral DNA. The level of DNA polymerase in exponentially growing infected and uninfected whole cells was equal but when the enzyme activity in the nuclei purified from infected and noninfected cells was determined, a four fold increase in the polymerase level was found in infected nuclei over that in noninfected nuclei.

The presence of an RNA polymerase in the early and ring form inclusions was also detected although further characteristics of the enzyme were not determined. Polyacrylamide gel electrophoresis suggested the presence of several virus proteins in the early and ring form inclusions.

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LIST OF ABBREVIATIONS

c	- curie
$\mu$ c	- microcurie
ICL	- infectious canine laryngotracheitis
MDCK	- Madin-Darby canine kidney
SDS	- sodium dodecyl sulfate
TCA	- trichloroacetic acid
GMA	- glycol methacrylate
MEM	- minimal essential medium
HBSS	- Hank's balanced salt solution
P.F.U.	- plaque forming unit
CF	- complement fixation
HA	- hemagglutination
HI	- hemagglutination inhibition
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease
RNA	- ribonucleic acid
mRNA	- messenger RNA
c.p.m.	- counts per minute
DEAE	- diethylaminoethyl
OD	- optical density
cm	- centimeter
mm	- millimeter
$\mu$	- micron
$\text{\AA}$	- Angstrom
ml	- milliliter

$\mu$ l	- microliter
x g	- gravity (centrifugal force)
g	- gram
mg	- milligram
$\mu$ g	- microgram
M	- molar
mM	- millimolar
$\mu$ M	- micromolar
mmole	- millimole
moi	- multiplicity of infection
RSB	- reticulocyte standard buffer
r.p.m.	- revolutions per minute
EDTA	- ethylenediamine tetraacetate
SSC	- sodium chloride sodium citrate
TTP	- thymidine triphosphate
dATP	- deoxyadenosine triphosphate
dCTP	- deoxycytidine triphosphate
dGTP	- deoxyguanosine triphosphate
TC	- toluene diisocyanate
XC	- xylylene diisocyanate



# INTRODUCTION

Of the 55 different types of adenoviruses, some cause disease in man and animals, some are oncogenic in vivo and cause transformation of cells in vitro, and some infect tissue culture cells productively. This latter productive infection results in the sequential formation of various inclusions in the host cell. This provides an interesting natural system to study, the aim being to achieve an understanding of virus assembly and multiplication through analysis of content, structure, function, and time of appearance of the different inclusions. Such a study is important both medically and biologically and may be extended eventually to other kinds of virus as well, many of which share this same ability to produce inclusions.

The infectious canine laryngotracheitis (ICL) virus designated as A26/61 by Ditchfield et al. (1962) has been shown to be serologically and morphologically related to the adenovirus group (Ditchfield et al., 1962; Yamamoto, 1963, 1966, 1967; Yamamoto and Marusyk, 1968). It multiplies inside the nucleus of the infected cell and has similar growth characteristics to other adenoviruses.

Adenoviruses are icosahedral particles composed of DNA and protein (Green and Pina, 1963). The DNA is double stranded and in association with some internal proteins forms a nucleoprotein complex or virus core. The core is enclosed in a capsid composed of several morphological units called capsomers (Valentine and Pereira, 1965). On the basis of morphological features these capsomers are designated as hexons, penton bases, and fibers (Ginsberg et al., 1966). The total

number of capsomers composing the capsid is 252 and is constant in all types of adenoviruses. Two hundred and forty capsomers on the capsid are hexons, having 6 adjacent capsomers. The other 12 capsomers on the vertices of the icosahedron are the penton bases which have 5 adjacent capsomers. Each penton base is attached to a fiber with a knob on the end. Hexons, penton bases, and fibers are antigenically different and can be separated and purified by column chromatography (Valentine and Pereira, 1965; Marusyk and Norrby, 1970). Specific antisera can be prepared against these purified antigens to study the development of antigens in virus-infected cells (Russell et al., 1967). The structural diversity of adenovirus capsid proteins has been reviewed by Norrby (1969).

Polyacrylamide gel electrophoresis of particles of human adenoviruses disrupted by sodium dodecyl sulfate shows at least 9 distinct polypeptide bands (Maizel et al., 1968a,b). The location of 8 of the 9 polypeptides in the virion has been determined (Maizel et al., 1968a,b). Three of these 8 polypeptides are present in the capsid: the "hexon" polypeptide of molecular weight 120,000, the "penton base" polypeptide of molecular weight 70,000, and the "fiber" polypeptide of molecular weight 62,000. Two minor peptides of molecular weight 13,000 were also found to be associated with hexons (Maizel et al., 1968b). The remaining 3 polypeptides have been identified as viral core protein(s) (Russell et al., 1968; Prage et al., 1968). The core protein(s) comprises about 20% of the total viral protein (Laver et al., 1968).

The 3 coat proteins are made in considerable excess in infected cells and are designated as soluble antigens. These antigens can be isolated from the infected cells and examined in detail (Valentine and Pereira, 1965; Wilcox and Ginsberg, 1963; Marusyk and Norrby, 1970).

#### Intracellular Multiplication

##### A. Morphological Studies

Adenoviruses are known to multiply inside the nucleus of infected cells (Kjellen *et al.*, 1955; Morgan *et al.*, 1956). During multiplication they produce distinct morphological changes which are confined to the nucleus. These alterations were first studied by Boyer *et al.* (1957), who differentiated the various types of human adenoviruses into 2 groups. This classification was on the basis of the inclusions produced in the nuclei of infected HeLa or human amnion cells. The types were differentiated thus:

I. A group of adenoviruses including types 1, 2, 5, and 6 which produced eosinophilic Feulgen-negative inclusions at 14 to 16 hours after infection. These inclusions developed granular basophilic cores which were Feulgen-positive and eventually formed a dense basophilic mass. In addition to the above alteration, in type 5 adenovirus infected cells the appearance of bar-shaped eosinophilic crystals inside the nucleus was reported (Morgan *et al.*, 1957). These Feulgen-negative crystals were not found in other types of adenovirus infected cells.

II. A group of adenoviruses which caused the formation of irregular, granular, eosinophilic masses and the development of a rarified zone beneath the nuclear membrane which appeared 14 hours after infection. Later on, the central area of the nucleus became

more intensely basophilic and consisted of either densely packed granules or a strikingly regular honeycomb. These network masses were associated with the appearance of sharp-edged crystal-like structures which were basophilic and Feulgen-positive. In some cells there were ovoid or wedge-shaped compartments radiating out from a central mass, producing a flower-like nuclear form. These alterations were produced by adenovirus types 3, 4, and 7. However, there was no detectable change in the cytoplasm of host cells during the replication cycle of these two groups of viruses. The nuclear alterations of adenovirus infected cells have been reviewed by Ginsberg and Dingle (1965).

In ICL adenovirus infected cells the sequential cellular changes were found to be slightly different from those described for adenovirus types 1, 5, and 6 (Yamamoto, 1969b). The earliest morphological change which appeared twelve hours after infection was the appearance of basophilic inclusions in the nuclei of infected cells. These early inclusions which were Feulgen-positive increased in size to form rings which appeared 16 hours after infection. The outer part of the ring was basophilic and Feulgen-positive, surrounding the eosinophilic Feulgen-negative central part. Later on, the inclusions increased in size and the interior eosinophilic region became filled with DNA containing material which was sensitive to deoxyribonuclease digestion. The size of the nucleus markedly increased and reached a maximum at 28 hours after infection. As the infection proceeded, most of the nuclear space became occupied with an eosinophilic mass containing basophilic granules and finally the whole cell became pycnotic and the nucleus was indistinguishable from the cytoplasm.

With the aid of the electron microscope the morphological alterations in adenovirus infected cells were studied in more detail. Morgan et al. (1957, 1960) showed the presence of large protein crystals in the nuclei of cells infected with type 5 adenovirus. Similar crystalline structures were found in a strain of type 2 adenovirus infected cells by Weber and Stich (1969). The formation of the crystals was found to be a property related to the type and strain of adenovirus rather than to the host cell line (Boyer et al., 1959). Adenovirus type 12 induces the formation of different types of inclusions inside the nucleus of the host cell. These inclusions, studied by Martinez-Palomo et al. (1967) appeared about 20 hours after infection and were found to be of 4 different types consisting of either protein or nucleoprotein.

The sequential changes in primary dog kidney cells infected with ICL virus was studied with the electron microscope (Yamamoto, 1969a) and was correlated with the alterations observed with the light microscope (Yamamoto, 1969b). The penetration of virus particles into the cell was by a process similar to phagocytosis. The virus particles could be seen bound within a membrane in the cytoplasm 3 to 4 hours after infection. This process was similar to the entry of adenovirus types 2 and 4 into HeLa cells (Dales, 1962). The earliest alteration detectable in ICL virus infected cells was the granularity of the nucleus which appeared 8 hours after infection. These granules increased in size and formed irregular inclusions by 12 hours post infection. These early and ring form inclusions corresponded to the early basophilic granules and spherical basophilic structures

observed in infected cells by the light microscope (Yamamoto, 1969b). As the infection proceeded, the ring form inclusions joined together and the dark staining region expanded into the central core to form a uniformly dense inclusion inside which a few virus particles could be observed.

At 20 hours after infection, 2 kinds of distinct intranuclear inclusions could be observed:

I. Circular or oval dark staining inclusions with a very dense granular structure which were found usually close to the nuclear membrane.

II. Light staining inclusions of irregular shape with a homogeneous structure surrounded by virus particles and located near the nuclear membrane.

At this time, virus particles were seen to aggregate and later could be found as crystalline arrays. The release of virus from the nucleus into the cytoplasm was by formation of protrusions from the nuclear membrane containing numbers of virus particles. The membrane-bound virus was pinched off into the cytoplasm where the membrane was destroyed and the virus particles released.

The alterations produced by ICL adenovirus in the MDCK cell line were found to be similar to those produced in primary dog kidney cells (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971). In addition, it was shown that the early and ring form inclusions were susceptible to digestion by DNase, whereas the dark and light staining inclusions were readily digested by proteolytic enzymes. Furthermore, autoradiography studies showed the incorporation of thymidine, uridine, and

leucine into the early and ring form inclusions, indicating that they were composed of DNA, RNA, and protein. The DNA content of these inclusions was found to be incorporated into the virus particles, and since the host DNA was margined toward the nuclear membrane, it was concluded that these bodies contained only viral DNA.

The dark and light staining inclusions which did not contain nucleic acids were found to be protein. It was also shown that the protein of the dark inclusions was synthesized at the time of appearance of these bodies, whereas the light inclusions were formed from the accumulation of some protein(s) which was synthesized during the early period of infection.

#### B. Biochemical Studies

Events associated with the multiplication of adenoviruses can be outlined as follows:

- a) Attachment and penetration of virus into the host cell
- b) DNA synthesis
- c) RNA synthesis
- d) Protein synthesis

These studies have been done on human adenoviruses and mostly on adenovirus types 2, 4, and 5. It is assumed that all adenoviruses have similar patterns of multiplication, although the exact time of the events may vary.

##### a) Attachment and Penetration of Virus into the Host Cell

Studies by Lawrence et al. (1967) showed that within sixty minutes after adsorption of adenovirus type 5 to KB cells, 97% of the cell associated virus was still TCA insoluble but DNase sensitive. They

found that sensitivity of virus particles to DNase which occurred after penetration of the virus into the cells was due to the removal of some viral protein coat. The uncoated virus had a sedimentation coefficient value of 400S, whereas viral DNA and purified virus had values of 30S and 800S respectively. They concluded that the uncoated viral DNA was associated with some structural proteins and further alterations must occur either in the cytoplasm or in the nuclei before viral DNA replication commences. Burlingham and Doerfler (1971) reported that after penetration of adenovirus types 2 and 12 into the host cell, 3 size classes of parental viral DNA with sedimentation coefficients of 18S, 31S, and 45S could be extracted from the infected cells. Since they found that native viral DNA had a sedimentation coefficient of 31-33S, it was suggested that some of the native viral DNA (31S) was cleaved to 18S by the action of some specific endonucleases. Hybridization experiments showed that the 45S segments contained both viral and host DNA. This finding led the authors to assume that some of the 18S segments resulting from the cleavage of native viral DNA were incorporated into the host DNA to form 45S DNA.

b) DNA Synthesis

In 1959 Ginsberg and Dixon, studying multiplication of type 4 adenovirus in HeLa cells, found a two fold increase in the total amount of DNA over that measured in uninfected cells. A similar observation was made by Flanagan and Ginsberg (1962) who indicated that the increase in the amount of DNA in infected cells was due to the synthesis of viral DNA. They also reported that synthesis of DNA



essential for virus production began 10 hours after infection and was completed by 16 hours after infection.

Consider the DNA synthesis of the host cell after it is infected with adenovirus. Ginsberg et al. (1967) reported that host DNA synthesis was inhibited at the time when viral DNA synthesis began. In 1969 Hodge and Scharff showed that synthesis of host DNA in adenovirus type 2 infected cells was dependent on the stage of the life cycle at which the replication of viral DNA was initiated. They used synchronized HeLa cells and found that when viral DNA was synthesized during the G1 phase, the replication of cellular DNA stopped completely. When viral DNA synthesis began after the onset of the S phase, cellular DNA replication was partially but not completely inhibited.

In general it appears that the synthesis of adenovirus DNA in the host cell does not start until a relatively long period after infection. Once it begins, reinitiation of host DNA synthesis is inhibited.

#### c) RNA Synthesis

In 1964 Flanagan and Ginsberg showed an increased synthesis of RNA in HeLa cells infected with type 5 adenovirus. This RNA which was essential for production of infectious particles was found to be complementary to viral DNA. With different systems, synthesis of virus specific RNA started at different times, between 6 and 12 hours after infection in KB cells infected with adenovirus type 2 (Thomas and Green, 1966) and between 8 and 14 hours in HeLa cells infected with type 5 adenovirus.

Hybridization studies by Rose et al. (1965) showed that the increase of RNA synthesis in KB cells infected with type 2 adenovirus was for viral specific mRNA. This RNA in infected HeLa cells was found to be relatively stable with a functional half life of about 6 hours (White et al., 1969). Lucas and Ginsberg (1971), studying RNA synthesis in KB cells infected with adenovirus type 2, reported that there were 3 classes of viral specific RNA synthesized during infection:

I. Early virus RNA Class I whose synthesis began prior to viral DNA replication; it comprised about 70% of the early RNA species and was apparently degraded by 18 hours after infection.

II. Early RNA Class II whose synthesis began prior to virus replication and continued at an enhanced rate late in infection.

III. Late RNA whose synthesis began after the initiation of viral DNA synthesis.

However, any change in synthesis of host ribosomal RNA has not been reported. In a previous study (Yamamoto and Shahrabadi, 1971) it was shown that nucleoli in infected cells were active in RNA synthesis even at the late stage of infection. Recently Raska et al. (1971) using BHK-21 cells infected with adenovirus type 12 showed induction of a 3 to 5 fold increase in the rate of  $^3\text{H}$ -uridine incorporation into RNA. In addition to the synthesis of viral specific mRNA, they found that 45S, 32S, 28S, 18S, and 4S cellular RNA were all synthesized at an increased rate. Their results suggested that the synthesis of ribosomal RNA in infected cells continued at a rate greater than that in non-infected cells.

To summarize, it seems that the synthesis of both host RNA and

viral mRNA in adenovirus infected cells continues over a prolonged period. The continued synthesis of host ribosomal RNA may be required for viral protein synthesis. This is in contrast to cells infected with herpes virus in which the synthesis of host RNA is inhibited soon after infection (Roizman et al., 1965). As in other DNA containing viruses such as polyoma and SV 40 (Green, 1970), transcription of the adenovirus genome occurs sequentially. About one fifth to one half of the parental viral genome is transcribed before the initiation of viral DNA replication (early mRNA) and nearly all the virus genome is transcribed after the onset of viral DNA synthesis.

d) Protein Synthesis

The earliest detectable change occurring in KB cells infected with type 2 adenovirus was reported to be the synthesis of an early protein which started 4 hours after infection (Polasa and Green, 1965). Although the nature of this protein(s) was not known, its synthesis was found to be essential for replication. Therefore, the role of the early protein as an enzyme in DNA synthesis in infected cells was suspected. In 1964a, Green et al., studying the DNA synthesizing system in KB cells infected with adenovirus type 2, reported that there was no increase in thymidine kinase nor DNA polymerase, whereas in cells infected with vaccinia virus, a marked increase in these enzymes was found at an early stage of infection. Similar observations were made by McAuslan et al. (1965) in HeLa cells infected with adenovirus type 2. Using different types of adenovirus and host cell systems, Kit et al. (1966) observed somewhat different results. They demonstrated a pronounced increase in thymidine kinase activity in primary monkey kidney

cells infected with a simian adenovirus. This increase in enzyme activity started about 10 to 11 hours after infection and could not be correlated with the time of synthesis of the early protein which is initiated 4 hours after infection. However, the only protein so far detected at the early stage of infection (4 hours) in some adenovirus infected cells is "T antigen" (tumor antigen, Huebner et al., 1963). Whether there is a functional correlation between the early protein and T antigen is still obscure. Since T antigen does not have any role in DNA synthesis (Green, 1970), it seems more likely that early protein and T antigen are 2 different proteins synthesized during the early stage of infection. The time of synthesis of viral structural protein in adenovirus infected cells was first reported by Wilcox and Ginsberg (1963). These authors used HeLa cells infected with type 5 adenovirus and found that the synthesis of viral structural protein commenced at about 11 hours after infection which was about 2 to 3 hours before virus maturation. Further studies by Thomas and Green (1966) indicated that adenovirus type 2 coded proteins were made in the cytoplasm of KB cells. A similar result was obtained by Velicer and Ginsberg (1968) in KB cells infected with adenovirus type 5. They showed that the synthesis of viral capsid proteins occurred in the cytoplasm of infected cells, and then the proteins were transferred to the nucleus for assembly into virus particles. By pulse-chase experiments, it was found that peptides associated with the polyribosomes were the precursors of virus capsid protein and were incorporated into hexons and fibers in the nucleus 6 minutes after synthesis (Velicer and Ginsberg, 1970).

It was reported (Green, 1970) that only 6% of the viral protein synthesized during infection by adenoviruses is incorporated into infectious particles; some of the rest is present as soluble antigens and some is incorporated into non-infective particles.

The effect of adenovirus infection on the synthesis of host cell protein was studied by Bello and Ginsberg (1967). They indicated that protein synthesis of KB cells infected with adenovirus was inhibited about 16 hours after infection. They suggested that the eventual decline in host protein synthesis may be due to the accumulation of fiber antigen.

#### Immunological Studies

Immunological studies on the nature of adenovirus antigens have been reported by many investigators and reviewed by Ginsberg (1965). Following is a brief description of production and localization of antigens in infected cells.

In 1959 Boyer et al. studying the multiplication of adenovirus types 5 and 7 by means of fluorescent staining techniques, reported that the development and distribution of adenovirus antigen was confined to the nucleus of infected cells. The antigen appeared as discrete granules or rings in the nuclei and increased until the nuclei appeared as brightly fluorescent masses. Since that time many workers used the technique of fluorescent staining to localize the antigens of many different types of adenovirus in infected cells (Pereira et al., 1959; Pope and Rowe, 1964; Shimojo et al., 1967). When the method of separation of adenovirus capsid antigens was improved, different types of adenovirus capsid protein could be purified and antibody specific

against each antigen could be obtained. Using specific antisera against penton, hexon, and fiber, Hayashi and Russell (1968) were able to study the sequential appearance of various capsid antigens of adenovirus types 5 and 12 in HEK cells. They found that hexon and fiber antigens appeared at 10 hours after infection and penton base antigen was detectable two hours later.

However, the extent of localization of viral antigens in infected cells by fluorescent staining techniques is limited by the low resolution of the light microscope. Investigation of a detailed distribution of viral antigens in infected cells and determination of a definite correlation between the fluorescent antigens and the fine structure of infected cells can only be accomplished by immunoelectron microscopy.

#### Immunoelectron Microscopy

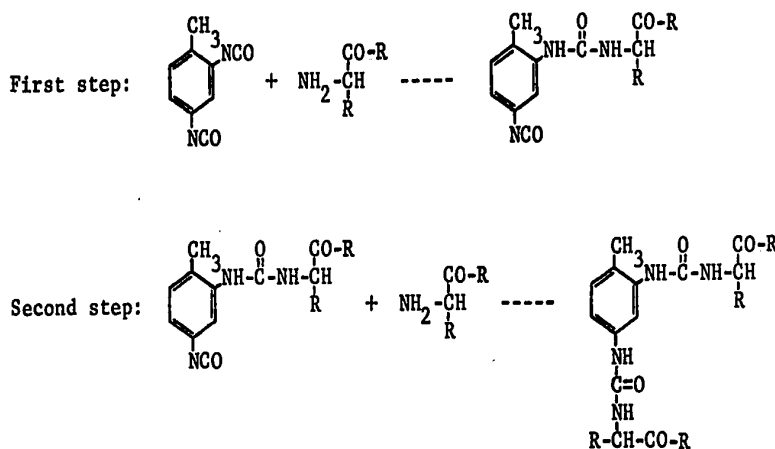
The development of techniques associated with electron microscopy has made it possible to visualize in fine detail the macromolecular content of thin sections in intact cells and cell organelles. More particularly, what is visualized is a map of the distribution of the electron scattering power of the fixed ultrathin sections, and methods are required for identifying and localizing individual macromolecular components on this map. Specific stains are required which scatter electrons in some characteristic manner.

In order to develop a technique of specific staining of antigens, similar to fluorescent staining techniques but to function at the resolution available with the electron microscope, antibody conjugate must be prepared which by virtue of its high electron scattering power

can be distinguished at the level of the electron microscope. To prepare a suitable antibody conjugate, groups of large atomic number must be chemically coupled to the antibody molecule. This has been accomplished by coupling ferritin to antibody.

Ferritin is an easily prepared crystalline protein which can be obtained in large quantity from horse spleen (Granick, 1946). Farrant (1954) characterized ferritin as a protein molecule with a molecular weight of 650,000 and an iron content of 23%. Because of the high electron scattering power conferred by the large iron content, individual ferritin molecules can readily be seen in the electron microscope. The protein part of the molecule when freed of the iron is called apoferritin. Both ferritin and apoferritin are nearly spherical molecules with a diameter of about 110 Å (Singer and Schick, 1961).

One problem encountered is destruction of antibody upon extensive reaction during conjugation (Sternberger, 1967). Suitable methods should be applied to prevent this destructive effect on specific combining sites. In 1961, Singer and Schick succeeded in conjugating ferritin with  $\gamma$ -globulin using toluene di-isocyanate as the cross-linking agent. Under certain conditions, in the first step of the reaction, one of the isocyanate groups forms ureido bonds with the  $\text{NH}_3$ -terminal and epsilon-lysine amino groups in the ferritin. In the second step, the other unreacted isocyanate group on ferritin-di-isocyanate conjugate becomes available for cross-linking onto immunoglobulin (Singer and Schick 1961; Sternberger, 1967). The reactions and the chemical configuration of the reactants are as follows:



One of the advantages of ferritin as a label is the fact that a specifically localized ferritin granule usually corresponds to a single antibody molecule. No such quantitation has been possible with other immunoelectron microscopic techniques (Sternberger, 1967).

Singer and Schick (1961) were the first to use ferritin conjugated antibody for localization of antigens on the surface of tobacco mosaic virus. In the intervening years this method has been used successfully to stain extracellular antigens of a variety of microorganisms such as the encapsulated strain of *Staphylococcus aureus* (Smith *et al.*, 1960) and *Klebsiella rhinoscleromatis* (Metzger and Smith, 1962). Other instances of specific localization of ferritin antibody on the cell surface include Kreb's ascites cells (Easton *et al.*, 1962) and influenza infected chorioallantoic membranes (Morgan *et al.*, 1961a). In all these studies the cells were exposed to conjugated antibody prior to fixation and embedding.

Ferritin labeled antibody, because of its large size, does not readily penetrate intact mammalian cells, bacteria or viruses (Easton



et al., 1962). For this reason it has been necessary to disrupt the cells by mechanical means, allowing the access of ferritin conjugate to intracellular antigens. Andres et al. (1962) used manual dissection of tissue with a razor blade to facilitate penetration of ferritin antibody into the kidney tissue. Other methods such as freezing and thawing of the formalin fixed tissue prior to the application of the ferritin conjugate were used to localize intracellular viral antigens in infected cells (Morgan et al., 1961). In these methods considerable damage to ultrastructure resulted from freezing and thawing. Since the accessibility of intracellular antigens to conjugated antibody depends upon damaging cellular membranes, small damage and incomplete breakage of cellular membrane would cause sluggish and incomplete penetration of labeled globulin into a cell (Vogt and Kopp, 1964). This also implies incomplete removal of nonspecific ferritin on washing, often observed as nonspecific staining in the interior of cells and tissue (Vogt and Kopp, 1964; Douglas et al., 1966). Considering these problems, it would be ideal to apply ferritin-immunoglobulin conjugate to the thin sections in which all intracellular antigens are at least in part exposed to the surface of the section and hence available to contact with antibody. However, staining of thin sections after embedding creates new problems. First, embedding media should be to some extent water soluble to allow the access of antibody to antigen. While epoxy resins are excellent for preservation of structural morphology, they are not water soluble and cannot be used for immunoferritin staining of thin sections. Second, the antibody combining site of antigens should be preserved during fixation, dehydration,

and embedding. Third, the nonspecific attachment of ferritin to the embedding media should be eliminated. It seems that the use of proper fixative and embedding media are essential. Osmium tetroxide is known to alter severely the protein configuration of most biological specimens (Lenard and Singer, 1968). Other fixatives such as glutaraldehyde and formalin have been reported to preserve at least some antigenic determinants of the fixed tissue (McLean and Singer, 1964, 1970). The problem of nonspecific attachment of ferritin to the embedding polymer has been overcome partly by McLean and Singer (1964) who used a hydrophilic cross-linked polyampholyte as the embedding medium. Since the use of this embedding medium was not routinely successful, other embedding media such as cross-linked bovine serum albumin was used (McLean and Singer, 1970). It was found that the antibody binding capacity of red blood cells and E. coli infected with T4 phage was preserved in this embedding polymer. This method seems to be tedious and the sections tend to fall apart when floated on distilled water.

In previous studies, using the techniques of enzyme cytochemistry and autoradiography, the chemical composition of inclusions produced in ICL virus infected cells was determined. The purpose of the present study was first, to develop a technique by which the appearance and distribution of specific intracellular antigens could be determined at the resolution of the electron microscope, second, using this technique to investigate the antigenic relationship of the inclusions with respect to viral capsid protein, and third, to determine the role of some of the inclusions in the process of virus multiplication.

## MATERIAL AND METHODS

### Virus

The canine adenovirus obtained from Dr. J. Ditchfield, University of Toronto, was provided by Dr. T. Yamamoto. The virus has been propagated on Madin-Darby canine kidney cell line (MDCK) and stored at -20°C.

### Cell Culture

Madin-Darby canine kidney cell line (MDCK) obtained from the American Type Culture Collection, Rockville, Md., was used throughout this study.

### Tissue Culture Media

Commercial Eagle's (1959) powdered minimal essential medium (MEM), supplied by General Biochemical, Grand Island, New York, was used in all of the experiments. The medium was supplemented with 5% calf serum and 100 I.U./ml of penicillin G and 100 µg/ml of streptomycin sulfate. The pH was adjusted to 7.4 with 7.5% sodium bicarbonate. Hank's (1949) balanced salt solution (HBSS) containing 0.5% lactalbumin hydrolysate (Difco) was used for the preparation of overlay medium for virus assay.

### Stock Virus Production

Each Roux bottle containing a complete monolayer of MDCK cells was inoculated with 4 ml of virus suspension containing  $2 \times 10^8$  P.F.U. per ml. The virus was allowed to adsorb for 1.5 hours at 37°C with frequent agitation at 15 minute intervals. After the adsorption period 85 ml of fresh medium was added and incubated at 37°C until the cytopathic effect was complete (2 days). The cells were harvested and

centrifuged at 1,500 r.p.m. in a clinical centrifuge for 10 minutes. The pellet was resuspended in 20 ml of growth medium and disrupted by freezing and thawing 5 times. The cell debris was sedimented by low speed centrifugation and the supernatant was stored as virus stock at -20°C until used.

#### Virus Assay

The plaque assay method was used for virus titration. The details of the procedures were described previously (Shahrabadi, 1969).

#### Virus Purification

##### A. ICL Virus

In each experiment 10 Roux bottles of MDCK cell monolayer infected with ICL virus were used. The infected cells were harvested 48 hours after infection and centrifuged as above. The pellet was suspended in 10 ml of 0.15 M NaCl and disrupted by freezing and thawing 5 times. The cell debris was separated by centrifugation at 6,000 r.p.m. in an RC-2B centrifuge. The supernatant was layered onto a preformed non-linear gradient of CsCl consisting of 1 ml at density 1.4, 1 ml at density 1.32 and 1 ml at density 1.2. When a large amount of virus was required, 21 ml of CsCl solution with the above densities (7 ml of each) were layered in a 50 ml centrifuge tube and the top of the tube was filled up gently with crude virus suspension. The gradient was centrifuged at 41,000 x g for 90 minutes in a Beckman Model L ultracentrifuge using either an SW-39 rotor (for 5 ml tubes) or an SW-25.2 rotor (for 50 ml tubes). The virus band was collected dropwise from the bottom of the tube. The region above the virus band was also collected and used as soluble antigens. Both virus and soluble antigens

were dialysed against 0.1 M phosphate buffer at pH 7.2 overnight and stored at -20°C.

#### B. Vaccinia Virus

Vaccinia virus was purified and used for antibody production. A crude suspension of vaccinia virus was provided by Dr. T. Yamamoto. Two ml of virus suspension was layered over 6 ml of 40-20% linear sucrose gradient in 0.01 M Tris buffer at pH 7.5 and centrifuged at 23,000 x g for 1 hour. The virus band was collected from the top of the tube by a syringe and layered over 4 ml of a linear CsCl gradient with a density ranging from 1.4 to 1.2. The gradient was centrifuged in a Beckman Model L ultracentrifuge at 41,000 x g for 3 hours. The virus band was collected with a syringe from the top of the tube, dialysed against 0.01 M Tris buffer, pH 7.0, overnight and stored at -20°C.

#### Preparation of Antisera Against Adenovirus and Vaccinia Virus

One ml of purified adenovirus containing 500 µg protein/ml was mixed with an equal amount of Freund's complete adjuvant (Difco) and injected into rabbits subcutaneously (1 ml each side). The injection was repeated 3 times at 7 day intervals. One month after the final injection, an intravenous boost was given with 1 ml of virus suspension (without adjuvant). The rabbits were bled 10 days later. The antisera prepared with this method against adenovirus had a hemagglutination inhibition titre of about 20,000 units/0.25 ml and a CF titre of about 2,000. Antisera against vaccinia virus was prepared in rabbits similarly to that of adenovirus. The activity of the antiserum was checked by immunogel diffusion test in which a 1/8 dilution of anti-

serum produced distinct precipitin lines.

Antisera against rabbit  $\gamma$ -globulin was prepared in sheep and goats. The animals were injected subcutaneously with 1 ml of purified rabbit  $\gamma$ -globulin (8 mg protein/ml) mixed with an equal volume of complete Freund's adjuvant. Three injections were given at weekly intervals. The final booster was given 4 weeks later and the animals were bled 12 days after the final injection. The activity of the antisera was checked by immunogel diffusion test.

Antisera against purified hexons, penton bases, and fibers of ICL virus was obtained from Drs. E. Norrby and R.G. Marusyk, Department of Virology, Karolinska Institute, Stockholm. These antisera had been adsorbed to the nonspecific ICL virus capsid antigens, i.e. hexon antiserum had been adsorbed to purified penton base and fiber antigens, penton base antiserum had been adsorbed to hexon and fiber antigens, etc. The purity and specificity of these antisera were confirmed by gel diffusion tests using ICL virus soluble antigens.

#### Purification of $\gamma$ -Globulin

The  $\gamma$ -globulin fraction of the sera was purified according to the method of Dedmon et al. (1965). Briefly, a 20 x 4 cm column of DEAE Sephadex (A 50) was prepared. The column was washed with 3 volumes of 0.02 M phosphate buffer at pH 7.6. Fifteen to 20 ml of serum was layered gently over the top of the gel and eluted with 0.02 M phosphate buffer at pH 7.6. The  $\gamma$ -globulin fraction passed through the column and was collected in a container. The remaining serum protein was eluted with 2 M NaCl. The  $\gamma$ -globulin fraction was then concentrated to the desired volume in an Amicon ultrafiltration system (Amicon Corp.,

21 Hartwell Ave., Lexington, Mass., U.S.A.) using a UM 10 filter.

#### Hemagglutination and Hemagglutination Inhibition

Serial 2-fold dilutions of ICL virus or infected cell extract were prepared in 0.5 ml volumes in physiological saline in 85 x 10 mm tubes. To each tube was added 0.25 ml of a 0.5% suspension of human type A red blood cells in saline. Tubes were shaken and incubated at room temperature for 2 hours. The hemagglutinin titre was taken as the highest dilution of virus which produced complete agglutination of the red blood cells (Rosen, 1960).

For the hemagglutination inhibition (HI) tests 0.25 ml of virus suspension, so diluted as to contain 4 hemagglutination units, was added to 0.25 ml of serial 2-fold dilutions of serum. The mixtures were shaken briefly and then allowed to stand at room temperature for one hour. Then 0.25 ml of 0.5% erythrocyte suspension was added and incubated at room temperature for 2 hours. The titre of serum was taken as that dilution which completely inhibited agglutination and the tube containing this dilution was considered to contain 1 unit of HI antibody (Rosen, 1960).

#### Agar Gel Double Diffusion Test

Agar gel double diffusion plates were prepared by adding 7 ml of melted 1% Noble agar in PBS to 6 cm diameter disposable tissue culture dishes which had been precoated with 2% agar. Sodium azide (0.2%) was added as a preservative. After the agar had solidified, circular wells 5 mm in diameter (5 mm interwell distance) were cut with a brass cork borer. After addition of antigens and antisera, the plates were sealed and kept at room temperature. In some cases clean glass slides were

used, which were similarly covered with 2 ml of solidified agar. The slides were kept in a humid chamber at room temperature. The plates and slides were examined daily and results were recorded usually after 3 to 4 days.

#### Immunofluorescent Staining

MDCK cells were grown as monolayers in Leighton tubes containing cover slips. The cells were infected with ICL virus at a multiplicity of infection of 100 P.F.U./cell. The virus was allowed to adsorb at 37°C for 1.5 hours. After the adsorption period, the unattached virus was removed and the monolayers were washed 3 times with prewarmed medium, then incubated with 2 ml of growth medium at 37°C. At 1 hour intervals, from 4 to 16 hours after infection, duplicate cover slips were removed, washed in 0.02 M phosphate buffer at pH 7.5 and fixed in acetone at 4°C for 10 minutes (Casals, 1967), then air dried.

To eliminate the nonspecific staining, antisera were adsorbed to MDCK cells for one hour at room temperature then filtered through a 0.45  $\mu$  millipore filter. Fluorescein conjugated to antirabbit globulin  $\gamma$ -globulin (Difco Laboratories, Detroit, Michigan) was first similarly adsorbed to MDCK cells then adsorbed to rabbit liver powder (Difco) and finally filtered through the above type of filter.

For staining, the indirect method was used. The cover slips were rinsed in phosphate buffered saline then covered with antiserum diluted 1/4 to 1/8 and incubated in a moist chamber at 37°C for 1 hour. They were washed in phosphate buffered saline for 10 minutes (3 changes), covered with fluorescein labeled antirabbit globulin  $\gamma$ -globulin and incubated at 37°C for 30 minutes. They were washed as above and counter



stained with rhodamine diluted 1/20 (Difco) at room temperature for 30 minutes, then washed in phosphate buffered saline and mounted in buffered glycerol at pH 7.5 (90% glycerol in 0.02 M phosphate buffer). The slides were examined immediately with an American Optical fluorescent microscope at 200 x magnification. Pictures were taken on 35 mm Kodachrome (ASA 64) with an exposure time of 2 to 3 minutes. In the quantitative experiments at least 10 different fields, each containing about 120 cells, were examined. The total number of cells and the number of cells showing fluorescence were counted and the percentage of the latter was calculated.

#### Immunoferritin Method

##### A. Purification of Ferritin

Purification of ferritin was performed according to the method of Nicolson (personal communication).

Ferritin, 6 times crystallized, Cd free (obtained from Poly-sciences, Inc., Rydal, Pa.) was diluted to 10 mg/ml with 2%  $(\text{NH}_4)_2\text{SO}_4$ . To 100 ml of the diluted ferritin was added 34 ml of 20%  $\text{CdSO}_4$  and the solution was stored in the cold room overnight then centrifuged for 1 hour at 4,500 x g in an RC-2B centrifuge. The supernatant was discarded and the pellet was dissolved in 2%  $(\text{NH}_4)_2\text{SO}_4$  to bring the volume to 100 ml. This solution was again centrifuged as before. To the 100 ml of supernatant from the second centrifugation was added 34 ml of 20%  $\text{CdSO}_4$  and this solution was again stored overnight in the cold room, then centrifuged a third time. The pellet was dissolved in 75 ml of 2%  $(\text{NH}_4)_2\text{SO}_4$ , then 75 ml of saturated  $(\text{NH}_4)_2\text{SO}_4$  was added. This solution was kept at 4°C for 1 hour then centrifuged at 12,000 x g for

15 minutes. The supernatant was discarded and the precipitate was dissolved in 75 ml of distilled water. Seventy-five ml of saturated  $(\text{NH}_4)_2\text{SO}_4$  was added and the preceding cold storage and centrifugation were repeated. The pellet was saved for dialysis.

The pellet was dissolved in a small amount of distilled water and dialysed overnight against cold running water (16°C). (The dialysis tube was originally half empty to allow a 100% increase in solution volume.) The solution was further dialysed overnight against 0.05 M phosphate buffer at pH 7.5 at 4°C. To precipitate the small aggregates, the solution was centrifuged at 12,000 x g for 5 minutes. The supernatant was saved and sterilized by passing it through a 0.45  $\mu$  millipore filter.

#### B. Conjugation of Ferritin with $\gamma$ -Globulin

The original method of Singer and Schick (1961) was used.

The solution of ferritin was removed from the bottle. The volume was made up to 10 ml with 0.05 M phosphate buffer at pH 7.5 and centrifuged in a Beckman Model L ultracentrifuge at 35,000 r.p.m. with an SW-39 rotor for 2 hours. The top solution (3/4 of the supernatant) was discarded and the pellet was allowed to dissolve overnight in the remaining solution at 4°C.

To 2 ml of a 15 mg/ml solution of ferritin vigorously stirring in an ice bath was added 0.1 ml of toluene-2,4-diisocyanate and this was allowed to react for 25 minutes. After the reaction time, the ferritin solution was transferred to an ice cold centrifuge tube with an ice cold Pasteur pipette and centrifuged at 4°C for 10 minutes at 12,000 x g. The supernatant was carefully removed with an ice cold Pasteur pipette

and allowed to stand for 1 hour at 0°C. It was then mixed with an equal volume of 15 mg/ml  $\gamma$ -globulin solution in borate buffer at pH 9.5 (the final pH was 9.2 to 9.3). The mixture was incubated at 37°C for one hour, dialysed in 0.1 M  $(\text{NH}_4)_2\text{CO}_3$  at pH 8.8 for 5 to 6 hours, then overnight in 0.05 M phosphate buffer at pH 7.5. To remove the precipitate and large aggregates, the conjugated solution was centrifuged for 15 minutes at 12,000 x g and the supernatant was collected.

#### C. Purification of Ferritin Conjugated $\gamma$ -Globulin

The crude conjugate obtained as above contained some unreacted  $\gamma$ -globulin and some free ferritin. To remove the unconjugated  $\gamma$ -globulin, the conjugate was layered over 5 ml of a 70-40% linear sucrose gradient in 0.05 M phosphate buffer at pH 7.5 and centrifuged at 24,000 r.p.m. for 6 hours in an ultracentrifuge using the SW-39 rotor. Ten drop fractions were collected from the bottom of the tube. The optical density of each fraction was measured at 440 m $\mu$  (maximum absorption for iron) in a Gilford 240 spectrophotometer. Antibody activity of the fractions was determined by the hemagglutination inhibition test.

To remove free ferritin, fractions which contained both ferritin and  $\gamma$ -globulin ferritin conjugate were pooled, dialysed against 0.05 M phosphate buffer at pH 7.5, and concentrated to 1 ml in an Amicon ultrafiltration system using a UM 10 filter. A 1 x 30 cm column of agarose A-1.5 (Biorad 200-400 mesh) was prepared and washed with 3 volumes of 0.05 M phosphate buffer at pH 7.5. One ml of the conjugate solution was carefully layered on the top of the gel. The column was eluted with the above buffer and 0.5 ml fractions were collected.

Hemagglutination inhibition activity and optical density at 440 m $\mu$  were determined. Fractions containing both ferritin and antibody activity were pooled, concentrated, and after passage through a 0.45  $\mu$  millipore filter, stored at 4°C until used.

D. Electron Microscopy

Monolayers of MDCK cells prepared in 3 oz bottles were inoculated with 2 ml of ICL virus with a multiplicity of infection (moi) of 100 P.F.U./cell. The virus was allowed to adsorb for 1.5 hours at 37°C. After the adsorption period, the unattached virus was removed and the monolayers were washed with 3 ml of the prewarmed medium and incubated at 37°C with 10 ml of fresh medium. At varying time intervals, the medium was removed and the cell monolayers were washed once with 3 ml of chilled medium then fixed. For immunoelectron microscopy the following methods of fixation were tried:

I. Fixation in 0.5% osmium tetroxide in 0.1 M phosphate buffer at pH 7.2 for 30 minutes.

II. Fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 at 4°C for 30 minutes. In some cases a low concentration (0.5%) of glutaraldehyde was used for a short time (10 minutes).

III. Fixation in 4% methanol-free formaldehyde (Pease, 1962; Robertson et al., 1963) in 0.1 M phosphate buffer at pH 7.2 containing 7.5% sucrose. The time of formaldehyde fixation was usually 30 minutes at 4°C.

Since, among the above fixations, formaldehyde fixation was found to preserve the antibody combining site of antigens, it was used in the subsequent immunoferritin experiments.

The fixed cells were scraped off the culture bottles with a rubber policeman and centrifuged at 1,500 r.p.m. for 10 minutes. The pellet was washed in 0.1 M phosphate buffer at pH 7.2 with 4 changes of buffer for a period of 16 to 24 hours. The pellet obtained by centrifugation was dehydrated and embedded in glycol methacrylate (Polysciences, Rydal, Pa.) according to the method of Leduc and Bernhard (1967). Polymerization was induced by ultraviolet radiation at 4°C with a lamp containing a fluorescent tube (Burton Manufacturing Co., Santa Monica, California). After 2 to 3 days, the blocks were hard enough to be sectioned. The blocks were kept at 4°C until used. Micrococcus sodenensis and the purified cell walls of this organism, obtained from Dr. J.N. Campbell, Dept. of Microbiology, University of Alberta, were similarly fixed in formaldehyde and embedded in glycol methacrylate.

Thin sections were cut with a diamond knife (E.I. Dupont de Nemours and Company, Wilmington, Delaware) on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.).

For morphological studies, cells were treated with glutaraldehyde followed by osmium tetroxide fixation, then embedded in epoxy resins according to the standard methods (Yamamoto, 1969).

#### E. Staining of Thin Sections with Ferritin Conjugated Antibody

Preliminary attempts at staining thin sections with ferritin conjugated antibody revealed the nonspecific attachment of ferritin to cellular components. This nonspecific staining was eliminated by the adsorption of the conjugate to cell homogenates using the following method:

MDCK cells were suspended in 0.05 M phosphate buffer, pH 7.5, homogenized in a Stir-R homogenizer (Tri-R Instruments, Rockville Center, N.Y.) at setting 3 for 5 minutes and centrifuged at 1,500 r.p.m. in an International centrifuge for 10 minutes. The supernatant was discarded and the pellet was resuspended in a solution of ferritin conjugated antibody (0.4 ml of cell pellet in 1 ml of conjugate). The mixture was incubated at room temperature for 1 hour, then centrifuged as above and the supernatant was sterilized by passing it through a millipore filter (0.45  $\mu$  pore size).

Sections floated on distilled water were picked up with a wire loop and floated on a solution of ferritin conjugated antibody in a porcelain spotting dish. The concentration of conjugate was 0.5 to 1 mg protein/ml in 0.05 M phosphate buffer, pH 7.5. The reaction was allowed to proceed at room temperature for 30 minutes. Excess conjugate was removed by floating the sections on three 3-minutes changes of phosphate buffer. The sections were then picked up on 200 mesh copper grids supported by a carbon-coated formvar film, rinsed in distilled water, dried, and stained with saturated uranyl acetate in water. For controls, sections were similarly treated with ferritin conjugated to heterologous antibody (vaccinia virus antibody). In some cases ferritin conjugated to  $\gamma$ -globulin obtained from unimmunized rabbits was used as a control.

#### F. Indirect Method of Ferritin Staining

The procedures for the indirect staining of thin sections with ferritin conjugated antibody were similar to those used in the indirect method of fluorescent staining. Thin sections were floated on a solution

of  $\gamma$ -globulin containing antibody specific for ICL virus capsid antigens. The concentration of  $\gamma$ -globulin was 8 mg protein/ml in 0.02 M phosphate buffer, pH 7.6. The reaction was allowed to proceed at room temperature for 30 minutes. The sections were washed in 0.05 M phosphate buffer (3 successive changes of buffer each for 3 minutes), then floated on a solution of anti-rabbit  $\gamma$ -globulin conjugate at a concentration of 0.5 mg protein/ml in phosphate buffer. They were allowed to react at room temperature for 30 minutes, then washed and electron stained similarly to the direct method.

#### Purification of Early and Ring Form Inclusions

MDCK cells were grown as monolayers in Roux bottles. At least 15 Roux bottles containing about  $6 \times 10^8$  cells were used in each experiment. The monolayers were infected with ICL virus with a multiplicity of infection of 100 P.F.U./cell. The virus was allowed to adsorb at 37°C for 1.5 hours (with frequent agitation at 15 minute intervals), then incubated with 100 ml of growth medium at 37°C. At 10 hours after infection, the cells were scraped off the bottles with a rubber policeman and centrifuged at 400 x g for 10 minutes. The pellet was resuspended in 5 ml of hypotonic buffer, RSB (reticulocyte standard buffer containing 0.01 M NaCl, 0.01 M Tris, 0.0015 M  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH 7.4). The cells were allowed to swell in an ice water bath for 15 minutes then homogenized in a Stir-R homogenizer (Tri-R Instruments, Rockville Center, N.Y.) at setting 3 for 6 minutes. The homogenate was centrifuged at 400 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in 5 ml of RSB and centrifuged as above. At this stage, 5 ml of RSB was added to the pellet and pipetted vigorously. Then a sample

was removed and stained with 1/1000 methylene blue and examined in the light microscope. It was found that the nuclei were relatively free of observable cytoplasmic tabs. There was contamination of 3 to 5% unbroken cells which were removed with the following procedure (Penman et al., 1966):

A mixture of one part of 10% solution of Tween-40 and 2 parts of 10% solution of sodium deoxycholate was prepared. Three tenths ml of this mixture was added to the suspension of nuclei, shaken on a vortex for 3 seconds, and centrifuged for 5 minutes at 400 x g. The pellet obtained was once more washed in 5 ml of RSB and sonicated as follows:

The nuclei preparation was resuspended in 4 ml of 0.25 M sucrose in Tris buffer, pH 7.2, containing 3 mM  $MgCl_2$ , and sonicated with a Brownwill Biosonic (Brownwill Scientific, Rochester, N.Y.) at setting 40 for a total period of 10-15 seconds. (The extent of breakage of the nuclei was determined by phase contrast microscopy after each 5 second period of sonication so that the inclusions would be subjected only to the minimum amount of trauma necessary to liberate them.) This period of sonication was usually sufficient to break over 99% of the nuclei.

The sonicate so obtained was homogenized with a Tri-R homogenizer for 2 minutes. The sucrose concentration in the sonicate was increased to 1.3 M (the sonicate was mixed with an equal volume of 2.35 M sucrose) then rehomogenized and centrifuged (using a swinging bucket) at 650 x g for a period of 20 minutes. The pellet which was mainly unbroken nuclei was discarded and the supernatant was again centrifuged at 2,500 x g for 20 minutes. The obtained pellet was resuspended in 6 ml of 0.88 M



sucrose in Tris buffer at pH 7.2 containing 3 mM  $\text{CaCl}_2$ , homogenized as above and centrifuged at 500 x g for 10 minutes.

At this stage, electron and light microscope examination of the pellet showed the inclusion preparation to be relatively pure. Occasionally there was 1 to 2% contamination of nucleoli and nuclear membrane. The following procedure removed the remaining contamination:

The inclusions were resuspended in 2 ml of 1 M sucrose and homogenized for 4 minutes to disperse the aggregates. One ml of the homogenate was layered onto 4 ml of linear CsCl gradient of density 1.6 to 1.2 and centrifuged at 30,000 r.p.m. for 3 hours using an S-39 rotor. The inclusions, located in a region with a density of 1.38 were collected with a syringe from the top of the tube. After dialysis in 0.01 M Tris buffer, pH 7.2, the inclusions were stored at  $-20^\circ\text{C}$  until used.

#### Electron Microscope Autoradiography

MDCK cells grown as monolayers in 3 oz bottles were infected with ICL virus with a multiplicity of infection of 100 P.F.U./cell. After the adsorption period (1.5 hours), they were incubated in 10 ml of medium at  $37^\circ\text{C}$ . Nine hours after infection the cells were exposed to a medium containing 50  $\mu\text{c}/\text{ml}$  of tritiated thymidine (specific activity 20 c/mmole, Schwarz BioResearch, Inc., Orangeburg, New York) and incubated at  $37^\circ\text{C}$  for 1 hour. In some cases the infected cells were labeled with 100  $\mu\text{c}$  of  $^3\text{H}$ -thymidine/ml of medium for 4 minutes. After pulsing, the cells were washed 5 times in 5 ml of cold medium, fixed in glutaraldehyde and osmium tetroxide, then embedded in Epon 812 as described.

For quantitative autoradiography, the membrane method (Salpeter

and Bachman, 1965) was used for the application of emulsion. Sections with the same color interference (same thickness) were picked up with a wire loop and placed on a clean glass slide coated with 1% collodion in amyl acetate. The location of sections was 2 cm from the lower end of the slide. The slides were dipped in Ilford L-4 emulsion (Ilford, Essex, England) diluted 1:6 according to the method described earlier (Shahrabadi, 1969). The slides were air dried for 30 minutes and kept in a light proof box at 4°C. The exposure time was 2 weeks for the 1 hour labeled cells and 8 weeks for the 4 minute labeled cells. After the exposure time, the slides were developed in D 19 (Kodak) at 18°C for 5 minutes, fixed in 25% sodium thiosulfate, and washed in distilled water for 5 minutes. Before the slides were dried, the collodion membrane was stripped onto the surface of distilled water. The sections were picked up on copper grids, stained with uranyl acetate and lead citrate according to the previous method (Shahrabadi, 1969).

At least 100 cells were examined under the electron microscope and 50 photographs were taken. The number of grains over different areas of the cells were counted directly on the electron microscope screen. The average surface of the cellular area was estimated from the photographs and the number of grains located outside the cellular area was counted and considered as background.

#### Extraction and Purification of MDCK Cell DNA

DNA from infected and noninfected MDCK cells was extracted according to the method of Pardue and Gall (1969). Cells were lysed in a solution of 1% sodium dodecyl sarcosinate in 0.05 M Tris buffer, pH 8.4, containing 0.1 M EDTA and 100 µg/ml of self digested pronase (B grade,

Calbiochem, Los Angeles, California). The mixture was incubated at 37°C for 3 hours. Residual protein was removed by 5 times extraction with water saturated phenol. The aqueous solution from the final extraction was brought to 0.1 M NaCl and the DNA was precipitated with 2 volumes of 95% ethanol. The precipitate was washed with 70% ethanol and dissolved in 1 x SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and dialysed against 2 liters of 1 x SSC for 24 hours (the dialysate was changed 2 to 3 times during this interval). The concentration of DNA was estimated from its absorption of light at a wavelength of 260 mμ. An absorbancy of 20 optical density units per mg was assumed for DNA.

#### Preparation of DNA from ICL Virus

The modified original method of Green and Pina (1964b) described by Rose et al. (1965) was used for extraction of viral DNA. Purified virus suspension in 0.01 M Tris buffer, pH 7.5, was treated with 0.2 mg per ml of papain (Worthington Biochemical Corporation) and 0.3 M mercaptoethanol. The mixture was incubated at 37°C for 5 hours. Then SDS was added to a final concentration of 1.25% and the preparation was incubated at 50°C for 30 minutes. The concentration of NaCl was raised to 0.1 M and the residual protein was removed by extraction with water saturated phenol 5 times. To remove the remaining phenol, the aqueous solution was dialysed against 3 liters of 1 x SSC buffer at 4°C for 30 hours (the dialysate was changed 5 times during this period) and the DNA concentration was determined as above.

#### DNA Determination

DNA was determined by the diphenylamine reaction described by

Ashwell (1957) using calf thymus DNA (Calbiochem, Los Angeles, California) for a standard.

#### RNA Determination

The orcinol method described by Ashwell (1957) was used for determination of RNA. Yeast RNA (Calbiochem, Los Angeles, California) was used for a standard.

#### Protein Determination

Protein was determined by Folin phenol reagent according to the procedure of Lowry et al. (1951). Bovine serum albumin was used for a standard.

#### DNA Polymerase Assay

DNA polymerase activity was determined by an assay similar to that of Richardson et al. (1964). Tritiated TTP, specific activity 18.5 c/mmole, was obtained from New England Nuclear, Boston, Mass. Other deoxynucleoside triphosphates were purchased from Calbiochem, Los Angeles, California.

The reaction mixture was prepared as follows:

dATP ..... 0.05 mM

dGTP ..... 0.05 mM

dCTP ..... 0.05 mM

<sup>3</sup>H-TTP ..... 0.3  $\mu$ M

Freshly purified inclusions ..... 100-400  $\mu$ g protein

The mixture was made in a total volume of 1 ml in 50 mM Tris buffer, pH 8.3, containing 10 mM MgCl<sub>2</sub>, 50 mM KCl, and 2 mM dithiothreitol. In some cases 200  $\mu$ g of activated calf thymus DNA (Aposhian

and Kornberg, 1962) was added as exogenous DNA. The reaction mixture was incubated at 37°C. At intervals, 100 µl samples were removed and added to 2 ml of cold 5% TCA then filtered through 25 mm millipore filters (pore size 0.45 µ). The filter papers were washed 3 times with 5% TCA and twice with 70% ethanol. The papers were dried and counted in 5 ml of Bray's (1960) solution in a liquid scintillation counter (Nuclear Chicago).

#### Treatment of Inclusions with Protamine Sulfate and Ammonium Sulfate

A sample of 0.5 ml of inclusions (800 µg protein in 0.05 M Tris buffer, pH 8) was mixed with 0.5 ml of 4% protamine sulfate in the above buffer. The mixture was shaken in ice for 10 minutes then centrifuged at 1,500 r.p.m. for 10 minutes. The supernatant was saved and the pellet was resuspended in 0.5 M  $(\text{NH}_4)_2\text{SO}_4$  in 0.05 M Tris buffer and centrifuged as above. Samples from both the supernatant and the pellet were assayed for DNA polymerase activity.

For ammonium sulfate treatment, to 1 ml of inclusions (1000 µg protein) in 0.05 M Tris buffer, pH 8, containing 5 mM dithiothreitol, was added ammonium sulfate to a final concentration of 3 M. The mixture was sonicated for 15 seconds then stirred in ice for 10 minutes and centrifuged at 5,000 r.p.m. for 10 minutes. The supernatant was discarded and the pellet was dissolved in 0.05 M Tris buffer, pH 8, and centrifuged as above. A sample of the pellet and supernatant were assayed for DNA polymerase activity.

#### Equilibrium Sedimentation Analysis of DNA

A solution of DNA was diluted in 1 x SSC to give approximately 50 µg DNA/ml. To 2.3 ml of this solution was added 2.9 gm of CsCl

(Fisher). The resulting solution with a density of about 1.7 gm/ml was centrifuged at 38,000 r.p.m. for 50 hours in an SW-50.1 rotor at 25°C. Three drop fractions were collected by piercing the bottom of the centrifuge tube. For radioactivity determination, 2 ml of 5% TCA was added to each fraction and the acid insoluble DNA was collected on millipore filters (0.45  $\mu$  pore size). After being washed with 70% ethanol, the filters were air dried and the radioactivity was counted in 5 ml of Bray's solution (Bray, 1960) in a liquid scintillation counter (Nuclear Chicago). In some cases the optical density at 260 m $\mu$  of each fraction was determined in a Gilford spectrophotometer, model 240. For analytical ultracentrifugation, approximately 3 to 4  $\mu$ g DNA were dissolved in a 1 ml solution of CsCl in 0.01 M Tris buffer, pH 7.00. The density of the CsCl solution was found to be 1.724 gm/ml. The mixture was centrifuged at 25°C in a Beckman Model E analytical ultracentrifuge at 44,000 r.p.m. for 24 hours. Photographs of the UV absorbing bands in the centrifuge cell were traced with a Beckman densitometer and their density was calculated according to the method described by Chervenka (A Manual of Methods for the Analytical Ultracentrifuge, Spinco Beckman)

#### Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed similarly to the method of Maizel et al. (1968a). ICL virus and inclusions were dissociated by treatment with 1% SDS in 0.02 M phosphate buffer, pH 7.2, containing 1% 2-mercaptoethanol. The time of dissociation was 10 hours at 37°C. Gels (10 cm x 5 mm) were prepared containing 5% acrylamide (Matheson Coleman and Bell, Norwood, Ohio) and 0.27% bisacrylamide

(Eastman Kodak). Polymerization was induced by 0.07% ammonium persulfate and 0.05% tetramethylethylenediamine. Electrophoresis was performed in 0.1 M phosphate buffer, pH 7.2, containing 0.1% SDS. The gels were run for 6 hours at 8 milliamperes per gel. At the termination of the electrophoresis, the gels were taken out and fixed overnight in 20% sulfosalicylic acid. They were stained in 0.25% Coomassie brilliant blue in water for 4 hours and the excess stain was removed by washing the gels in 3 changes of 7% acetic acid for 24 hours.

## RESULTS

The growth of ICL virus in a dog kidney cell line (DKL) has been studied by Yamamoto (1965). Since a different cell line (MDCK) was used throughout this study, experiments were designed to determine the one step growth of the virus in MDCK cells using plaque assay for virus titration. In addition, the synthesis of RNA, DNA, and protein in infected cells was investigated and compared with that in noninfected cells.

### One Step Growth of ICL Virus

Monolayers of MDCK cells in 3 oz bottles were infected with 2 ml of ICL virus (moi of 100 P.F.U./cell). The virus was allowed to adsorb for 1.5 hours at 37°C. The unadsorbed virus was removed by washing the monolayers 3 times with 5 ml of prewarmed medium and the cells were incubated in growth medium at 37°C. From 0 to 32 hours after adsorption samples were taken at 4 hour intervals and the supernatant separated from the monolayer. The cells were scraped off the bottles by a rubber policeman and suspended in 10 ml of growth medium. The total number of cells was determined by counting with a hemacytometer. The cells were disrupted by freezing and thawing 5 times. Samples from the supernatant and the cell suspension were assayed for infectivity and hemagglutinin.

Fig. 1 shows the results of growth of ICL virus in MDCK cells. At time 0 a small number of infectious particles (15 P.F.U./10<sup>3</sup> cells) was recovered from the infected cells. The number of cell associated viruses decreased until 4 hours. From 4 to 8 hours after infection, no



detectable infectious particle was found in infected cells. By 12 hours there was a marked increase in infectivity of intracellular virus which continued until 24 hours and reached levels of about 100 P.F.U. per cell at 32 hours.

The release of extracellular virus from the infected cells into the medium was first detected at 20 hours. From this time on, the extracellular virus continuously increased until 32 hours. Both intracellular and extracellular virus hemagglutinins were detected just at the time of appearance of infectious particles and increased simultaneously up to 32 hours (Fig. 2). Hemagglutinating capacity was concluded to be due to the formation of mature virus particles.

Fig 1

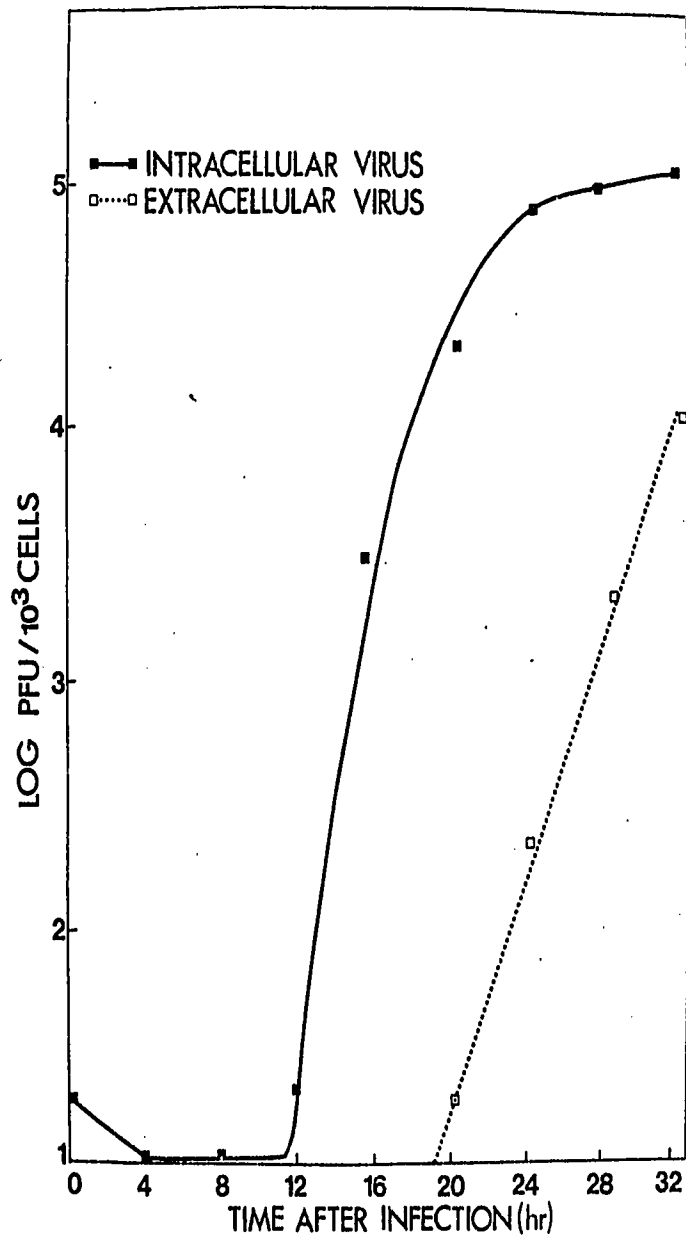


FIGURE 1

ONE STEP GROWTH OF ICL VIRUS IN MDCK CELLS

Monolayers of MDCK cells in 3 oz. bottles were infected with ICL virus. The unadsorbed virus was removed and the infected cells were incubated at 37° C. At intervals shown, samples were removed. The cells were separated from the supernatant and assayed for virus infectivity as described. The virus titer was calculated per 10<sup>3</sup> cells.

Fig 2

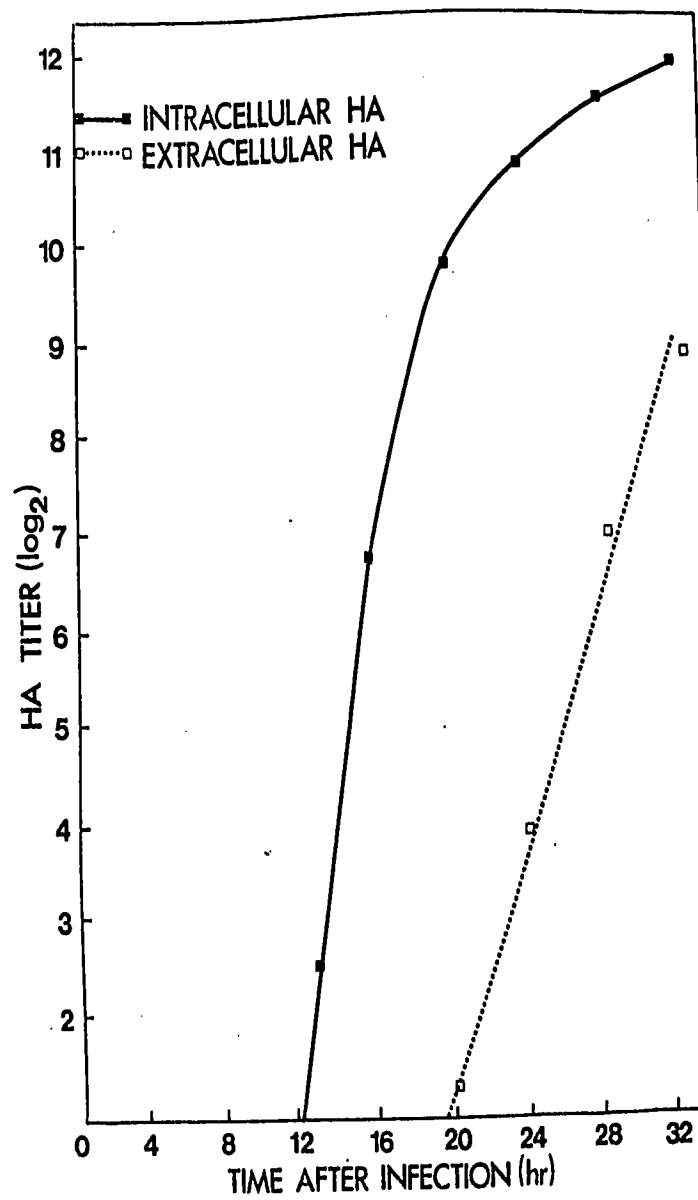


FIGURE 2

APPEARANCE OF ICL VIRUS HEMAGGLUTININ IN MDCK CELLS

A portion of each sample taken in Fig. 1 was removed and ICL virus hemagglutinin was determined according to "Material and Methods". The hemagglutinin (HA) titer was calculated per  $10^4$  cells.

#### Synthesis of Macromolecules in ICL Virus Infected Cells

Once the growth of ICL virus in infected cells was determined, it was decided to study the synthesis of macromolecules in infected cells in comparison to that in noninfected cells.

Monolayers of MDCK cells were infected with ICL virus as described above. From 0 to 28 hours after infection, equal numbers of both infected and noninfected cells were taken at 4 hour intervals. The cells were separated from the medium by low speed centrifugation. RNA was separated from DNA according to the method of Schneider (1957) and their amounts were determined as described in "Material and Methods". At similar intervals separate samples were taken and their protein content was estimated. An increase in the amount of DNA and RNA in infected cells was detected at 8 hours after infection and continued until 28 and 20 hours respectively (Fig. 3). The DNA content of infected cells nearly doubled over that of noninfected cells within 28 hours after infection whereas the RNA content increased to a level of about 48% over that of noninfected cells. The increase of protein in infected cells was detected at 8 hours after infection (Fig. 4). While the amount of protein per noninfected cell remained constant over a 32 hour period, the amount per infected cell increased by nearly 23% at 32 hours after infection.

Fig 3

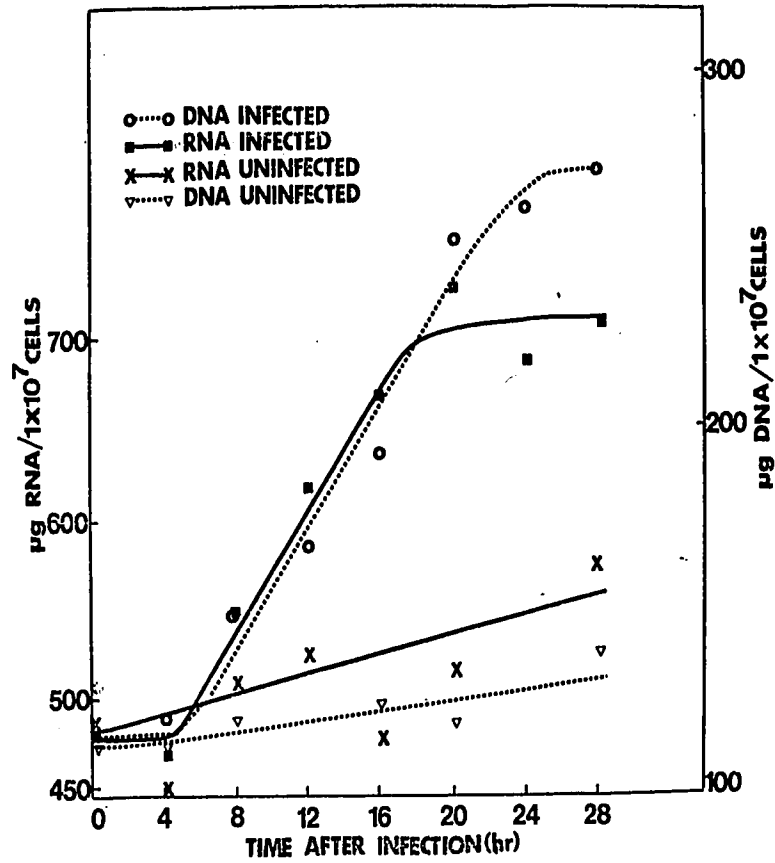


FIGURE 3

SYNTHESIS OF RNA AND DNA IN INFECTED AND UNINFECTED MDCK CELLS

Monolayers of MDCK cells were grown in 3 oz. bottles. The cells were divided into two groups. One group of cells was infected with ICL virus (100 P.F.U./cell). The other group of cells was uninfected (control). Both groups were incubated in 10 ml. MEM at 37° C. At the intervals shown, equal numbers of both infected and uninfected cells were removed and their RNA and DNA content was determined.



Fig 4

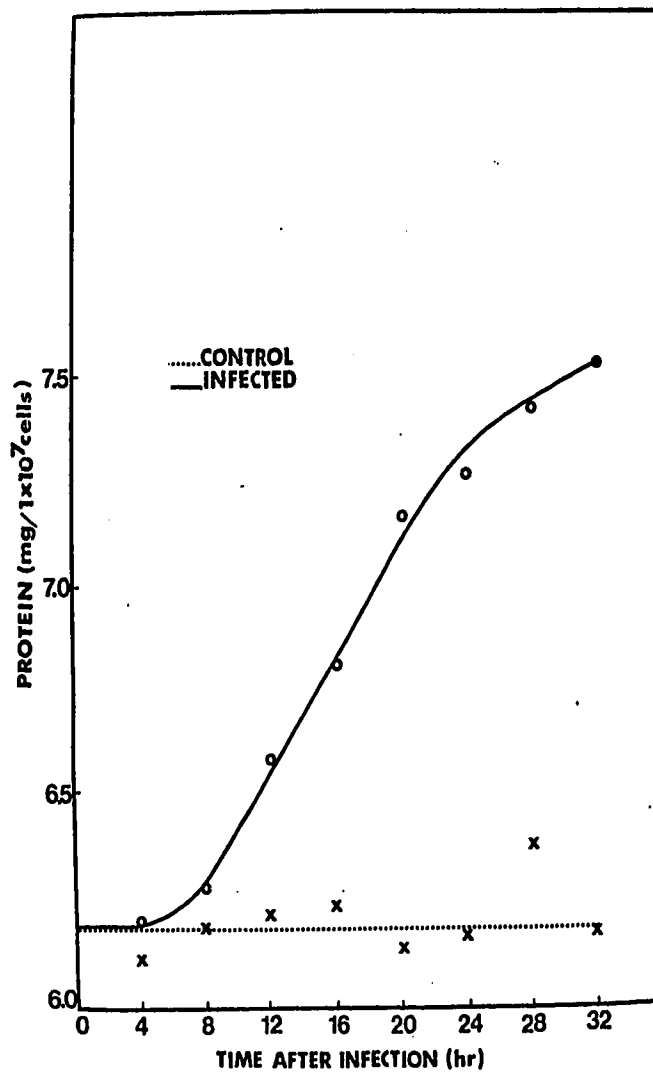


FIGURE 4

PROTEIN SYNTHESIS IN UNINFECTED AND ICL VIRUS INFECTED MDCK CELLS

Cells were grown under the same conditions as in Fig. 3. Equal numbers of uninfected (control) and infected cells were removed at intervals and their total protein content was measured.

#### Purification of ICL Virus

Since a pure preparation of ICL virus was required for the immunological and biochemical studies, a rapid and efficient method by which a large quantity of pure virus could be obtained was applied.

ICL virus was purified from the infected cells by CsCl centrifugation as described in "Material and Methods". To identify the virus band, 15 drop fractions were collected by puncturing the centrifuge tube with a Buchler drop collecting unit (Buchler Instruments, Fort Lee, N.J.). The fractions were diluted to the desired volume with 0.01 M Tris buffer, pH 7.2. The optical density of each fraction was measured at 260 m $\mu$  in a Gilford spectrophotometer 240 and infectivity was determined by plaque assay.

Fig. 5 demonstrates the virus bands obtained after centrifugation. Two separate bands of virus particles were obtained. The first band (from the bottom) was found to be infectious virus particles and the second band, which had a lower density, was not infectious. Electron microscope examination of the virus particles from each band revealed that the first band was composed of intact virus particles. The second band contained particles which appeared as empty capsids. In the subsequent experiments only the first band was collected and stored as pure ICL virus particles.

Fig 5

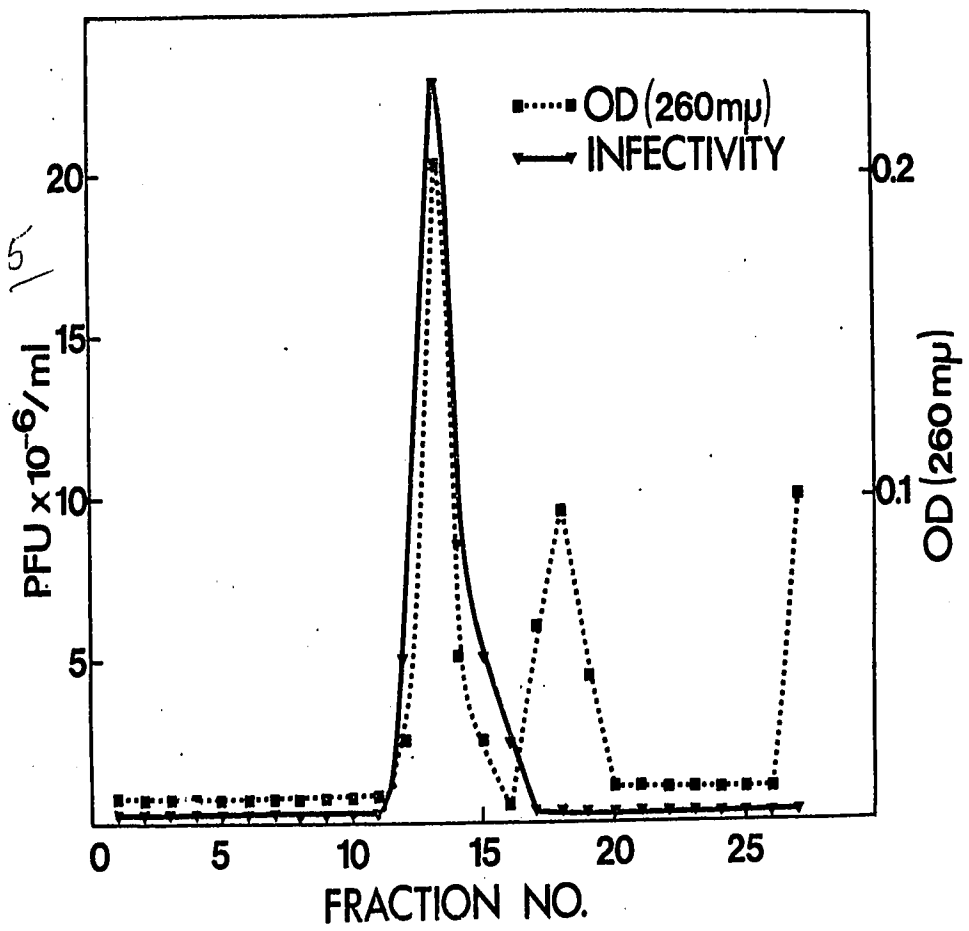


FIGURE 5

PURIFICATION OF ICL VIRUS IN CsCl

Crude virus suspension was layered on a pre-made non-linear gradient of CsCl (density 1.4, 1.32, 1.2 gm/ml) and centrifuged at 40,000 x g for 90 minutes in a Spinco SW<sub>39</sub> rotor. The infectious virus was separated as a single sharp band.

#### Conjugation of Ferritin with Immunoglobulin

Conjugation of ferritin with antibody was necessary in order to visualize the antibody-antigen reaction at the level of the electron microscope.

Purification and recrystallization of ferritin was described in "Material and Methods". For conjugation of ferritin with immunoglobulin both toluene-2,4-diisocyanate (TC) and meta-xylylene diisocyanate (XC) were tried as cross linking agents. Preliminary experiments indicated that the use of TC resulted in a higher yield of conjugate than the use of XC. Therefore, in subsequent experiments only TC was used for conjugation. The bulk of conjugate so prepared was a mixture of unreacted  $\gamma$ -globulin, unreacted ferritin, and ferritin conjugated  $\gamma$ -globulin.

The presence of unconjugated antibody interferes with localization of conjugated antibody by competing for antigen sites. For this reason, the unconjugated antibody was separated from the conjugated material by sucrose gradient centrifugation. The results of such separation are shown in Fig. 6. The unreacted antibody remained at the top of the tube and the bulk of conjugate containing both free ferritin and ferritin conjugated to antibody was obtained as a single band.

Although the unconjugated ferritin does not interfere with the antigen-antibody reaction, its presence will slightly increase the background of nonspecific staining. To remove the unreacted ferritin from the conjugate the bulk of conjugate obtained after separation from unconjugated immunoglobulin was passed through a column of

agarose A 1.5. Ferritin containing material came through the column as 2 separate peaks (Fig. 7). The first peak which contained both ferritin and antibody activity was considered to be the pure conjugate. The second peak in which antibody activity was not detectable was considered to be unconjugated ferritin. The preparation of conjugate obtained as above had an antibody activity of 20-25% of that of the original immunoglobulin.

To test the specificity of the ferritin conjugate, a mixture of ICL virus and vaccinia virus was sprayed on an electron microscope grid. A drop of ferritin conjugate containing ICL virus antibody was applied to the grid. After 5 minutes incubation at room temperature, the grid was washed in 0.05 M phosphate buffer, pH 7.5, rinsed in distilled water and examined in the electron microscope. It was found that ferritin conjugated antibody was heavily and specifically attached to the ICL virus particles (Fig. 8). In contrast, vaccinia virus particles treated with ferritin conjugated to ICL virus antibody were not stained with such heterologous antibody conjugate.

Fig 6

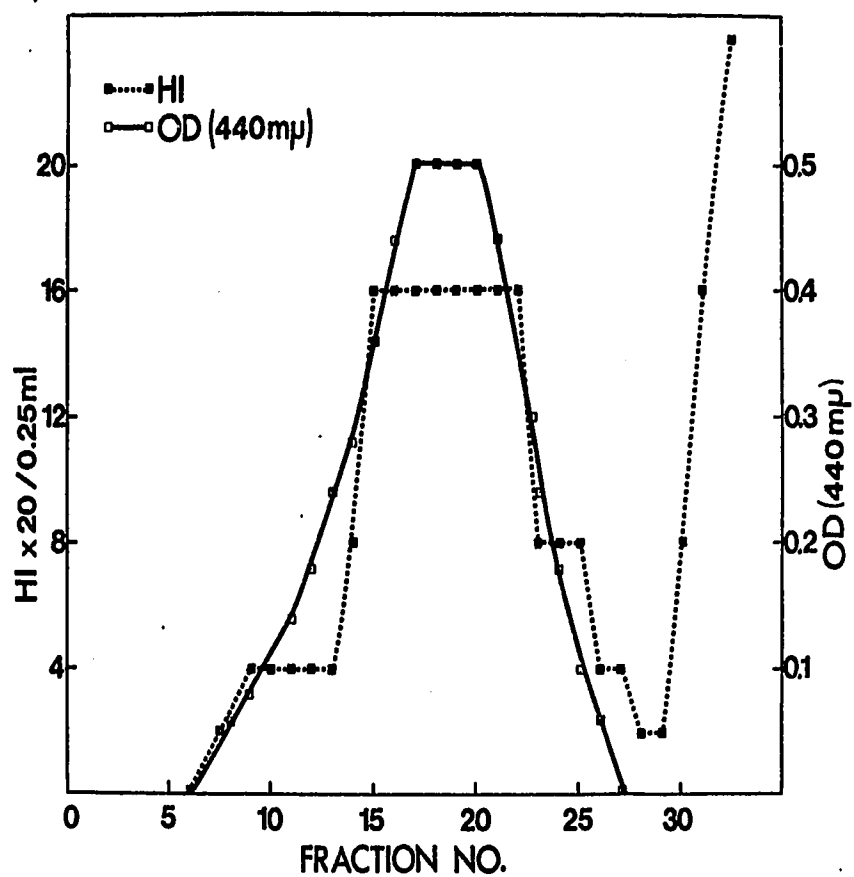




FIGURE 6

SEPARATION OF UNCONJUGATED

$\gamma$ -GLOBULIN FROM FERRITIN CONJUGATED  $\gamma$ -GLOBULIN

The crude ferritin conjugated anti-ICL virus  $\gamma$ -globulin was layered on a linear sucrose gradient (30-70%) and centrifuged as described in "Material and Methods". Fractions were collected from the bottom of the tube. Their ferritin content was determined by measuring the OD at 440 m $\mu$  and their antibody activity was measured by the hemagglutination inhibition (HI) test.

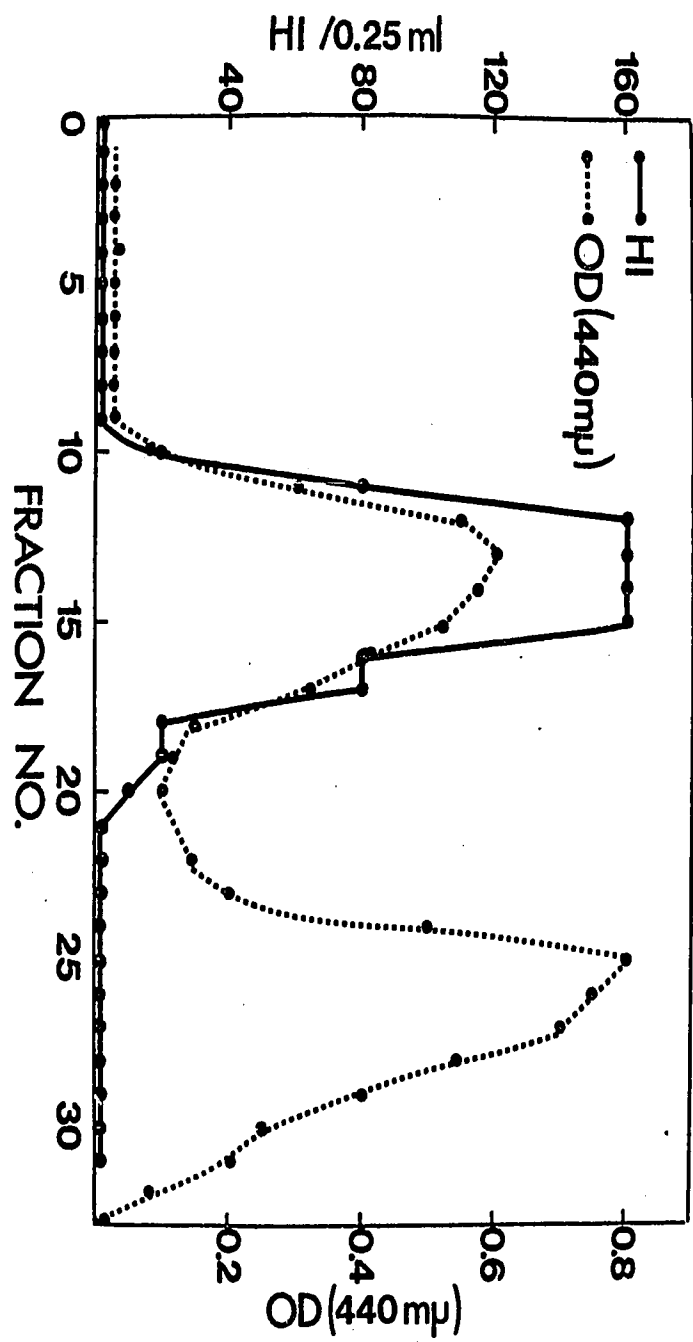


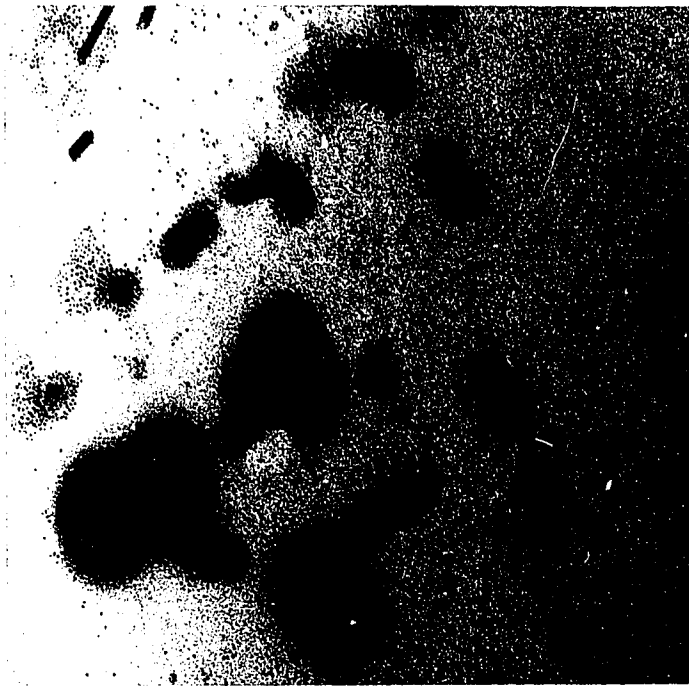
Fig 7

FIGURE 7

SEPARATION OF FREE FERRITIN FROM THE FERRITIN CONJUGATED  $\gamma$ -GLOBULIN

The crude conjugate after removal of free  $\gamma$ -globulin was passed through a column of Agarose A 1.5 according to "Material and Methods". Ferritin concentration and antibody activity of each fraction was measured as in Fig. 6. Free ferritin with no antibody activity had separated from the conjugate.

Fig 8



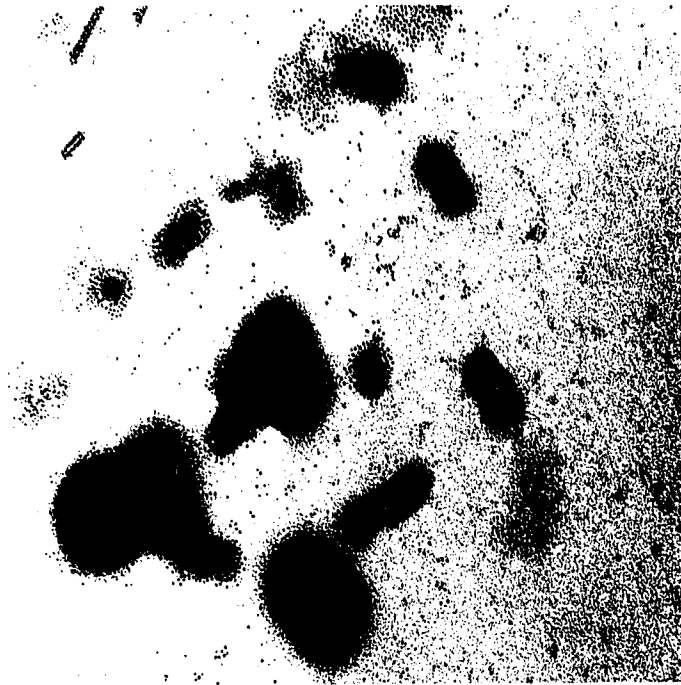


FIGURE 8

SPECIFICITY OF THE FERRITIN CONJUGATED ANTI-ICL VIRUS ANTIBODY

A mixture of purified ICL virus and vaccinia virus on a carbon coated grid was treated with ferritin conjugate specific for ICL virus. After being washed it was examined unstained in the electron microscope. Ferritin conjugate is specifically attached to ICL virus particles.

#### Staining of Thin Sections with Ferritin Conjugated Antibody

For localization of ICL virus antigens in infected cells, it was essential to develop a method whereby the intracellular antigens could become accessible to react with ferritin conjugated antibody. Since the existing methods of staining intracellular antigens with ferritin conjugate were inadequate, experiments were designed to use a proper fixative and embedding medium which would preserve the antigenicity of the embedded material and would allow the antigen-antibody reaction directly on thin sections. The first attempt was to stain thin sections of ICL virus infected cells with ferritin conjugated to ICL virus antibody.

Infected cells were fixed in formaldehyde and embedded in glycol methacrylate as described in "Material and Methods". In preliminary experiments sections were picked up on copper grids supported by a carbon coated formvar film and stained with the conjugate. It was found that the antigens in sections were stained with ferritin conjugate but there was a large amount of nonspecific attachment of ferritin to the supporting film. To eliminate this nonspecific attachment it was decided to carry out the antibody-antigen reaction on thin sections by floating them on a solution of ferritin conjugate. The procedures employed are schematically shown in Fig. 9. Using such a method, ICL virus particles in infected cells could be heavily and specifically stained with the ICL virus antibody conjugate (Fig. 10). There was almost no attachment of ferritin to the cell area surrounding the virus particles. To test the specificity of the ferritin staining, sections of ICL virus infected cells were similarly treated with

ferritin conjugated to vaccinia virus antibody. There was no reaction between the ICL virus and the antibody conjugate of vaccinia virus. Fig. 11 shows such a lack of attachment of ferritin to the virus particles. Similar lack of staining was observed when the sections were treated with ferritin conjugated to unimmunized  $\gamma$ -globulin.

In order to test the application of this technique to another antigen-antibody reaction, Micrococcus sodonensis cells and a purified cell wall suspension of this microorganism were similarly fixed in formaldehyde and embedded in glycol methacrylate (GMA). Thin sections were treated with ferritin conjugated antibody specific for M. sodonensis cell wall. Fig. 12 shows a section of a purified cell wall preparation stained with the ferritin conjugate. Ferritin can be seen to be attached to the cell wall. When the sections of whole cells were similarly stained with the conjugate, only the cell wall region was stained with ferritin (Fig. 13) thus showing its specificity. The control sections which were treated with heterologous antibody conjugate (ICL virus antibody) did not show any attachment of ferritin to the cell wall (Fig. 14). These results showed that the intracellular antigens in both eucaryotic and procaryotic cells could be localized by using this technique of staining thin sections with ferritin conjugated antibody.



Fig 9

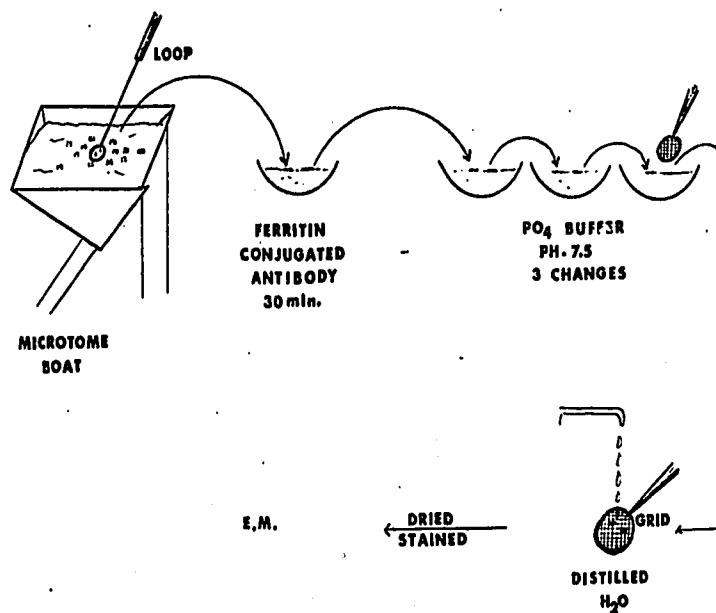


FIGURE 9

SCHEMATIC DIAGRAM OF THE PROCEDURES EMPLOYED FOR  
STAINING OF THIN SECTIONS WITH FERRITIN CONJUGATED ANTIBODY

Fig 10

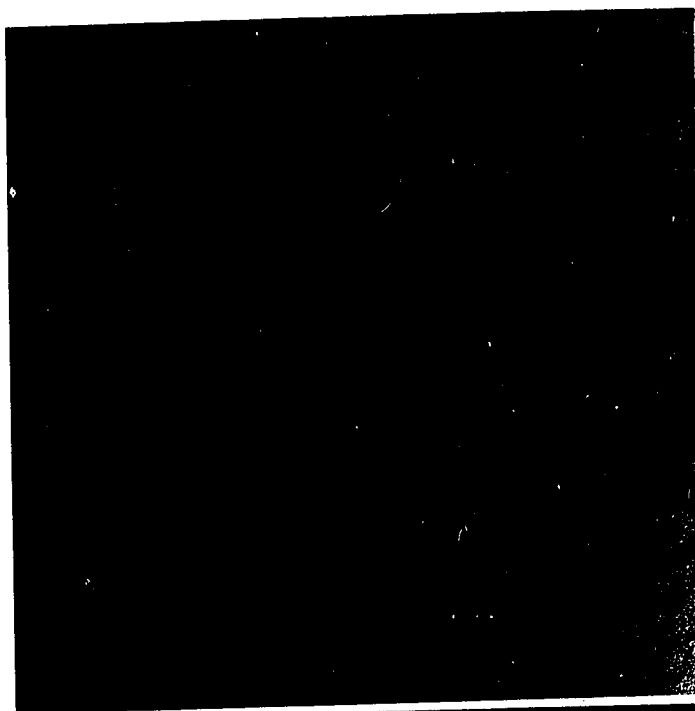
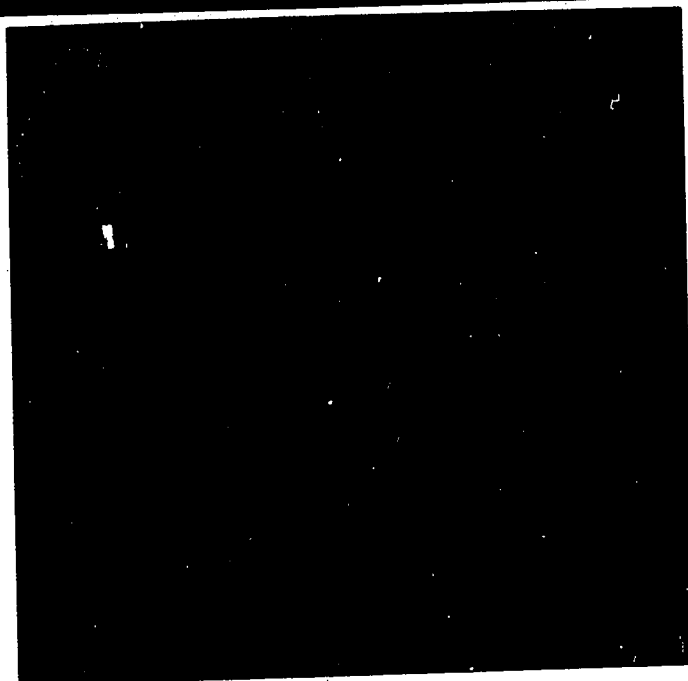


Fig 11



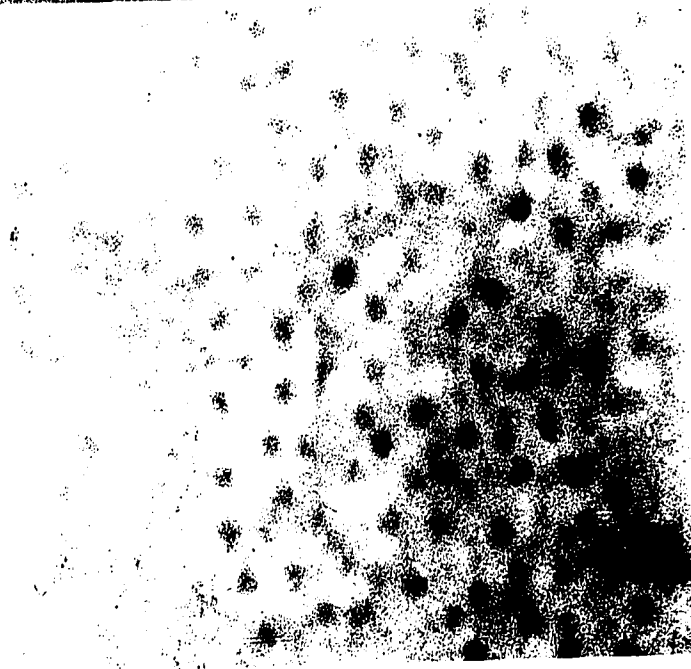
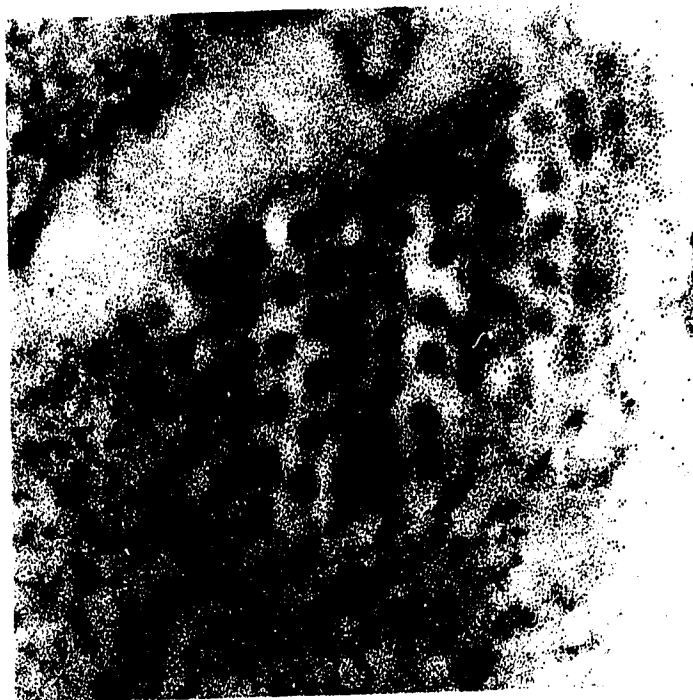


FIGURE 10

SPECIFIC STAINING OF INTRACELLULAR  
ANTIGENS WITH FERRITIN CONJUGATED ANTIBODY

MDCK cells were infected with ICL virus. The infected cells were fixed in formaldehyde and embedded in GMA. Thin sections were stained with ferritin conjugated anti-ICL virus antibody. The conjugate is attached specifically to virus particles.

FIGURE 11

LACK OF STAINING OF INTRACELLULAR ANTIGENS  
WITH HETEROLOGOUS FERRITIN ANTIBODY CONJUGATE

MDCK cells were infected as in Fig. 10. The sections were stained with ferritin conjugated anti-vaccinia virus antibody. ICL virus particles are not stained with the heterologous conjugate.

Fig 13



Fig 12

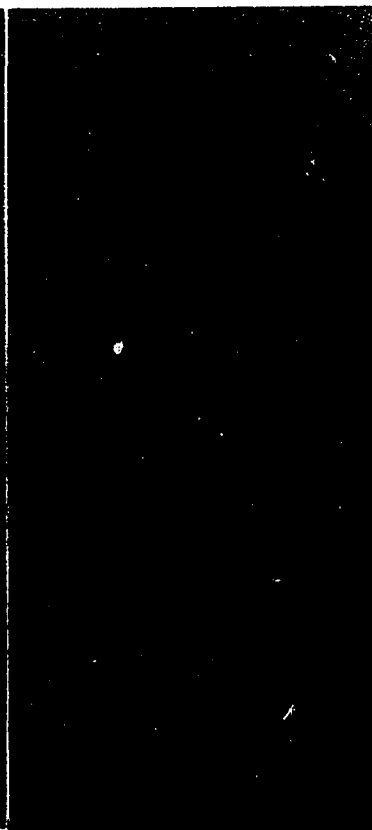


Fig 14





FIGURE 12 (top right)

STAINING OF PURIFIED CELL WALL

OF M. SODONENSIS WITH FERRITIN CONJUGATED ANTIBODY

Purified M. sodonensis cell wall was fixed in formaldehyde and embedded in GMA. Sections were treated with ferritin conjugated to anti-M. sodonensis cell wall antibody. Ferritin is attached to the cell wall.

FIGURE 13 (top left)

SPECIFIC STAINING OF CELL WALL

OF M. SODONENSIS WITH FERRITIN CONJUGATED ANTIBODY

Sections of M. sodonensis were prepared and stained with ferritin conjugate as in Fig. 12. Conjugate is specifically attached to the cell wall.

FIGURE 14 (bottom)

M. SODONENSIS CELL WALL TREATED WITH HETEROLOGOUS CONJUGATE

Sections similar to that in Fig. 12 were treated with ferritin conjugated to anti-ICL virus antibody and show no attachment of ferritin to the cell wall.



#### Localization of ICL Virus Capsid Antigens in Infected Cells

Following the development of the technique of staining antigens with ferritin conjugate, an attempt was made to apply the technique for localization and distribution of ICL virus capsid antigens in infected cells. Specific antisera against hexon, penton base, and fiber of ICL virus were used for ferritin staining. These antisera were tested for purity by gel diffusion tests. A single band was obtained from the reaction between the specific capsid antiserum and ICL virus soluble antigens, whereas antiserum against the whole virus particles formed at least 3 bands. Fig. 15 shows an example of the reaction between hexon antiserum and ICL virus soluble antigens. Since the amounts of capsid antisera were quite limited, it was not possible to use them for ferritin conjugation. For this reason, it was decided to develop an indirect immunoferritin method in which only small amounts of specific antisera would be required. Sections were first treated with rabbit  $\gamma$ -globulin containing antibody specific for ICL virus capsid proteins. After washing, they were stained with ferritin conjugated to anti-rabbit  $\gamma$ -globulin. The detail of the procedure was described in "Material and Methods". Using this method it was found that the intracellular antigens in thin sections could be stained with ferritin conjugate and the degree of specificity was equal to that of the direct method and staining appeared to be more intense.

The sequential appearance of inclusions in MDCK cells was similar to that described previously (Shahrabadi, 1969). In the present study, it was found that the time of appearance of inclusions was related to the multiplicity of infection. At a high multiplicity of infection (100 P.F.U./cell), the early granular inclusions could be observed by

light microscopy at 9 hours after infection. These inclusions rapidly increased in size so that by 10 hours after infection a number of cells contained the ring form inclusions. At 15 hours dark staining inclusions and light staining inclusions could be observed in some of the infected cells. The number of cells containing these inclusions increased with time after infection so that at 18 hours they could be seen in a majority of infected cells.

#### Fine Structure of Infected Cells Fixed in Formaldehyde

Although preservation of cellular organelles fixed in formaldehyde was not satisfactory, the use of this fixative was unsurpassed for immunoelectron microscopy (glutaraldehyde and osmium tetroxide were found to destroy the antibody combining sites of the antigens). The cytoplasm of the formaldehyde fixed cell appeared to be extracted and the mitochondria had lost their fine structure (Fig. 16). Marginated chromatin in the nuclei of infected cells appeared unstained but the inclusions and virus particles were adequately preserved.

#### Ferritin Staining of the Early and Ring Form Inclusions

At 9 hours after infection, staining of thin sections with ferritin conjugated antibody using fiber antiserum showed specific attachment of ferritin to the early inclusions (Fig. 17). Although the early inclusions were lightly labeled with ferritin, the number of ferritin molecules per unit surface area of inclusions was much higher than the number in the surrounding cell area. (In 10 photographs the number of ferritin molecules over the inclusion area and over the equivalent area of nucleoplasm and cytoplasm was counted. It was found that about 85% of the ferritin molecules were attached to the inclusions.) In contrast,

when penton base and hexon antisera were used, there was no attachment of ferritin to the inclusions (Fig. 18). At 10 hours after infection, sections similarly treated with fiber antiserum showed specific staining of the dark region of ring form inclusions (Figs. 19, 20), but there were no detectable penton base nor hexon antigens in these bodies (Fig. 21). These results indicated that the early inclusions and ring form bodies contained fiber antigen. Hexon antigen was detected at 11 hours in some of the infected cells. This antigen was only observed in those nuclei in which the infection had progressed to form virus particles (Fig. 22). Staining of similar sections using vaccinia virus antiserum was negative and no attachment of ferritin to the sections of adenovirus infected cells was observed.

#### Ferritin Staining of Dark and Light Inclusions

Although the dark and light inclusions appeared in the nuclei of some cells by 15 hours after infection, samples for immunoelectron microscopy were taken at 18 hours after infection by which time a large number of infected cells contained these inclusions. Sections were indirectly stained with ferritin conjugate using various specific capsid antisera. With fiber antiserum, the light staining inclusions were labeled with ferritin (Figs. 23, 24) and the virus particles surrounding the inclusions were also lightly labeled. However, the dark inclusions which could frequently be seen in the same infected cell were not labeled with ferritin (Fig. 25). When penton base antiserum was used in the staining procedures, ferritin conjugate was similarly attached to the light inclusions whereas the dark inclusions remained unstained (Fig. 26). For a control, similar sections were stained in-

directly with ferritin conjugate using vaccinia virus antiserum. The result of such staining was negative and no ferritin attachment was observed to the light inclusions (Fig. 27). When the sections were treated with hexon antiserum, ferritin staining resulted in heavy attachment of the conjugate to the light inclusions and virus particles (Figs. 28, 29). In contrast to the sections treated with penton base and fiber antisera, the dark inclusions treated with hexon antiserum were also labeled with ferritin (Fig. 30). These results indicated that the light staining inclusions contained all three (hexon, penton base, and fiber) antigens of the viral capsid, whereas the dark inclusions contained only hexon antigen. The presence of ICL virus capsid antigens in the inclusions as determined by the immunoferritin method are summarized below.

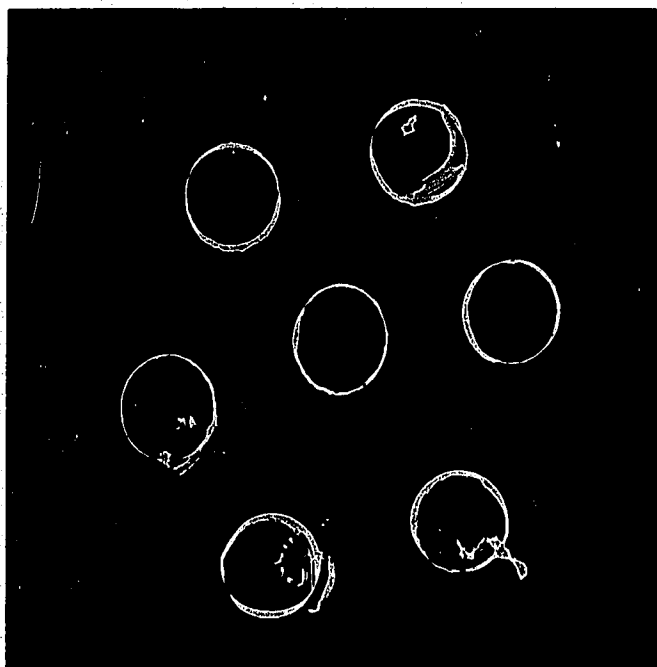
<u>ICL VIRUS CAPSID ANTIGENS</u>			
<u>INCLUSIONS</u>	<u>FIBER</u>	<u>PENTON BASE</u>	<u>HEXON</u>
Early and ring form	+	-	-
Dark staining	-	-	+
Light staining	+	+	+

+ = presence of antigen

- = absence of antigen

Early and ring form inclusions were examined in 10 hour infected cells. Dark staining and light staining inclusions were examined in 18 hour infected cells.

Fig 15  
—



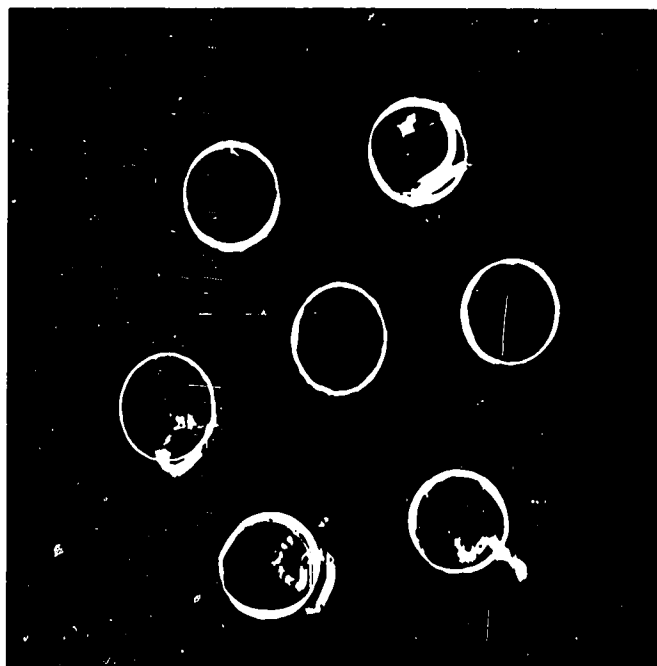


FIGURE 15

IMMUNOGEL DIFFUSION PATTERN OF ICL VIRUS HEXON ANTISERUM

Wells were cut in 1% solidified agar. The antigen-antibody reaction was allowed to proceed at room temperature for 3 days.

Center .....ICL virus soluble antigens

1 .....Undiluted ICL virus antiserum

2 ..... $\frac{1}{2}$  diluted ICL virus antiserum

3 and 4 .....ICL virus hexon antiserum diluted  $\frac{1}{4}$

5 .....ICL virus hexon antiserum diluted  $\frac{1}{2}$

6 .....Undiluted ICL virus hexon antiserum

Fig 16

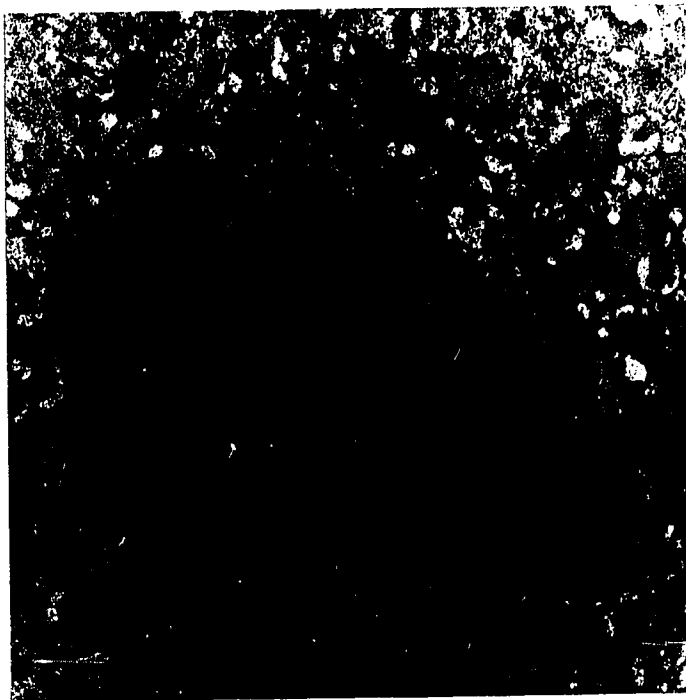
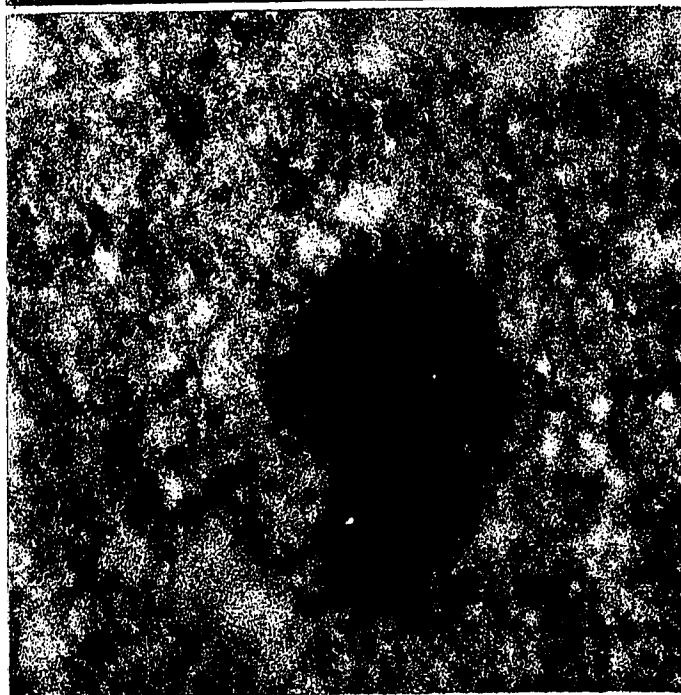


Fig 17





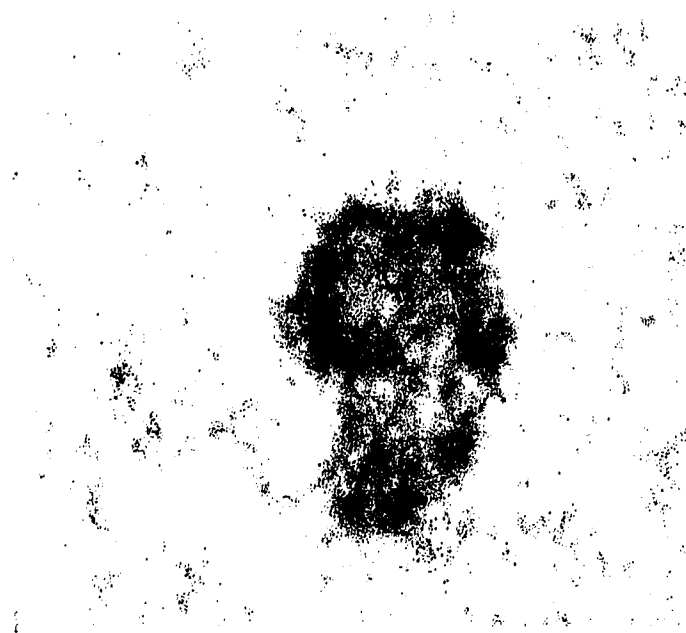


FIGURE 16

MDCK CELL NINE HOURS AFTER INFECTION WITH ICL VIRUS

Monolayers of MDCK cells in 3 oz. bottles were infected with ICL virus. Nine hours after infection, they were fixed in formaldehyde and embedded in GMA. The section was stained indirectly with ferritin conjugated antibody using fiber antiserum. Single arrows indicate the degree of preservation of mitochondria. Marginated chromatin close to the nuclear membrane is lightly stained. The early granular inclusions are distinct inside the nucleus. X 16,000

FIGURE 17

STAINING OF EARLY INCLUSION WITH FERRITIN CONJUGATE

A high magnification of an early inclusion shown in Fig. 16 by a double arrow. The inclusion is labeled with ferritin conjugate indicating the presence of fiber antigen in this body. X 60,000

Fig 18

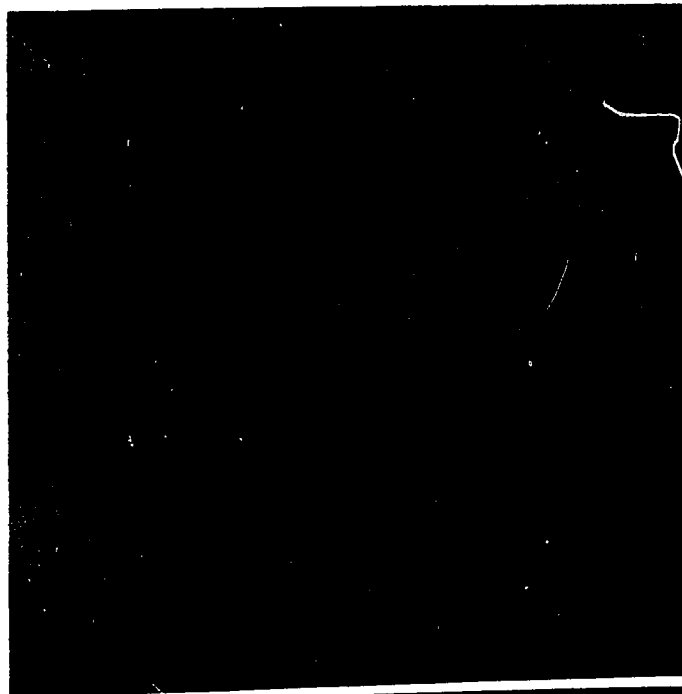


Fig 19

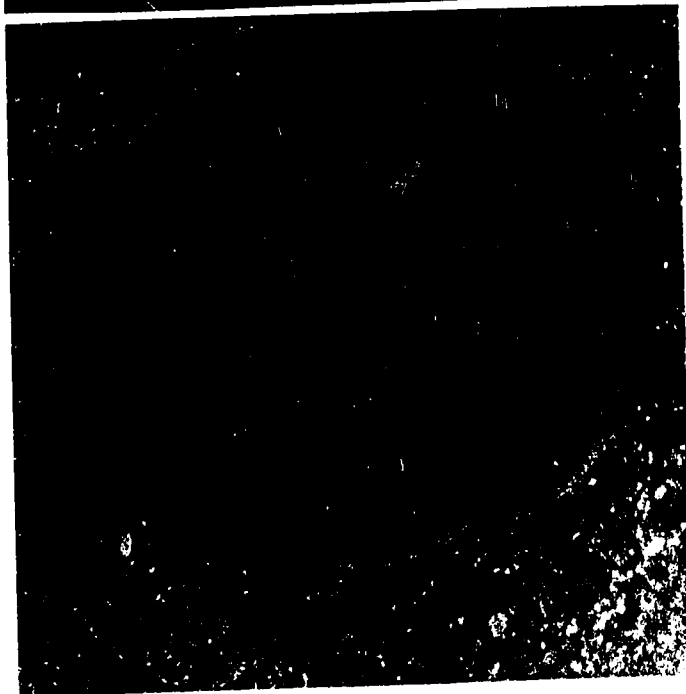




FIGURE 18

AN EARLY INCLUSION TREATED WITH ICL VIRUS HEXON ANTISERUM

A section of 9 hour infected cells was stained indirectly with ferritin conjugated antibody using hexon antiserum. The early inclusion is not labeled with ferritin. X 45,000

FIGURE 19

MDCK CELL TEN HOURS AFTER INFECTION

A section of a 10 hour infected cell showing numerous ring form inclusions inside the nucleus. The section was stained indirectly with ferritin conjugated antibody using ICL virus fiber antiserum. X 12,000

Fig 20

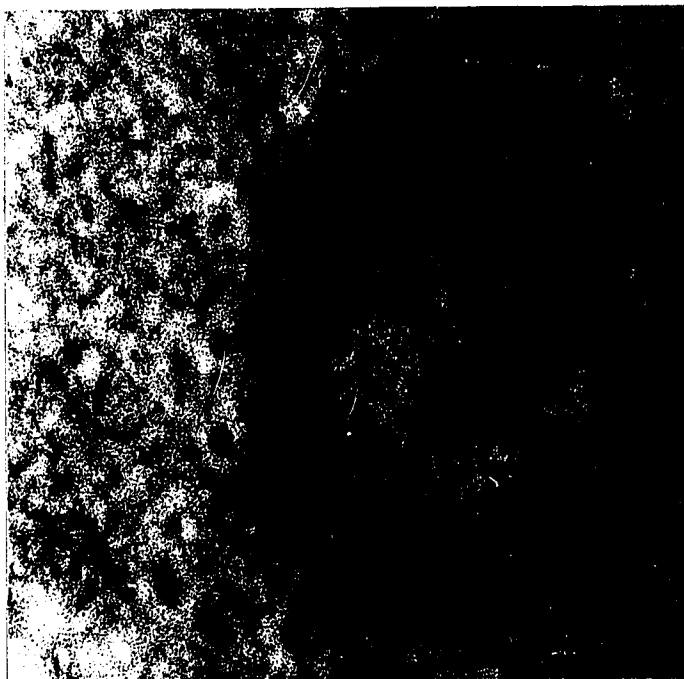
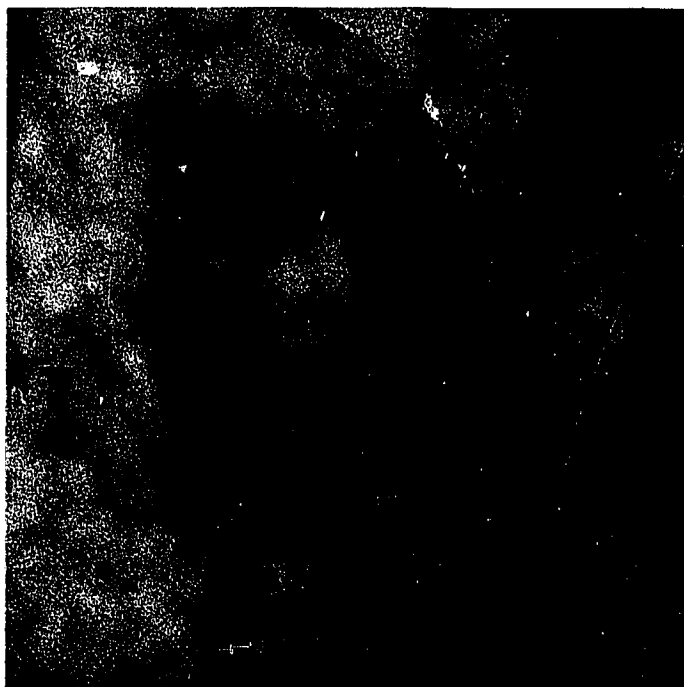


Fig 21



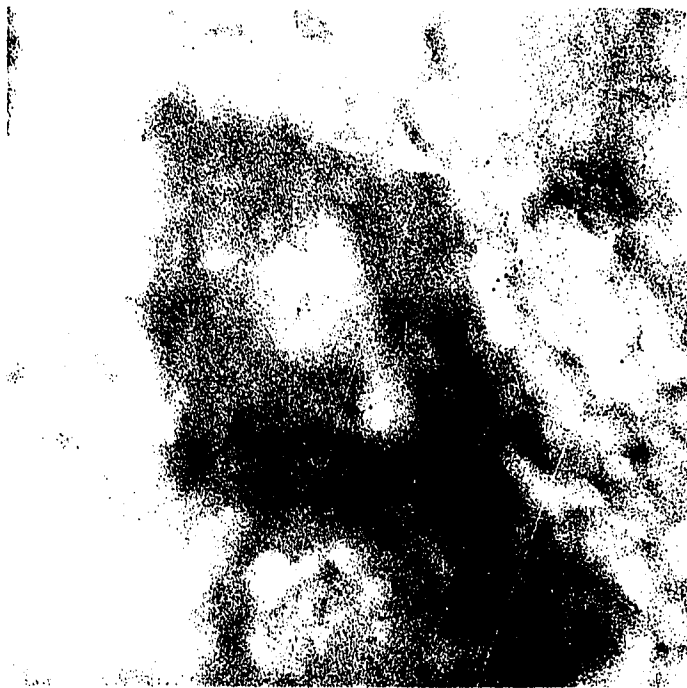
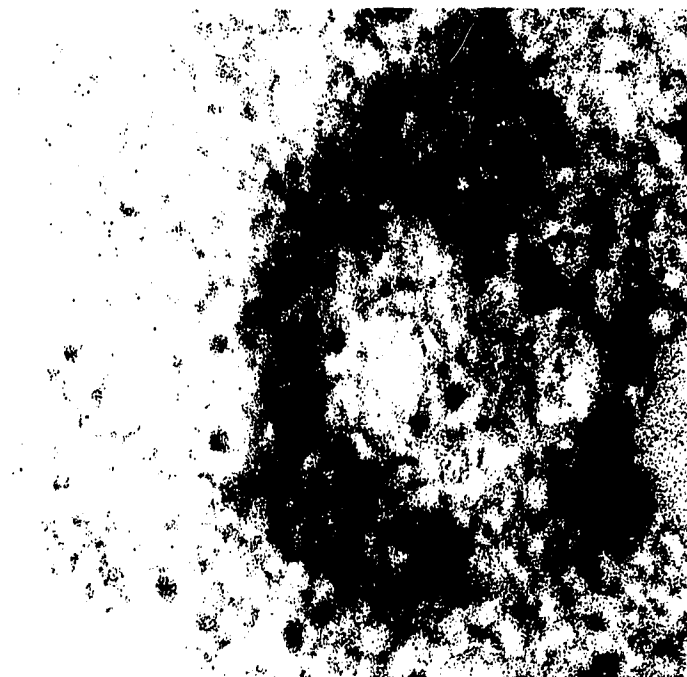


FIGURE 20

A RING FORM INCLUSION TREATED WITH FIBER ANTISERUM

A high magnification of the ring form inclusion shown in Fig. 19 by an arrow. Ferritin is specifically attached to the dark region of the inclusion. X 60,000

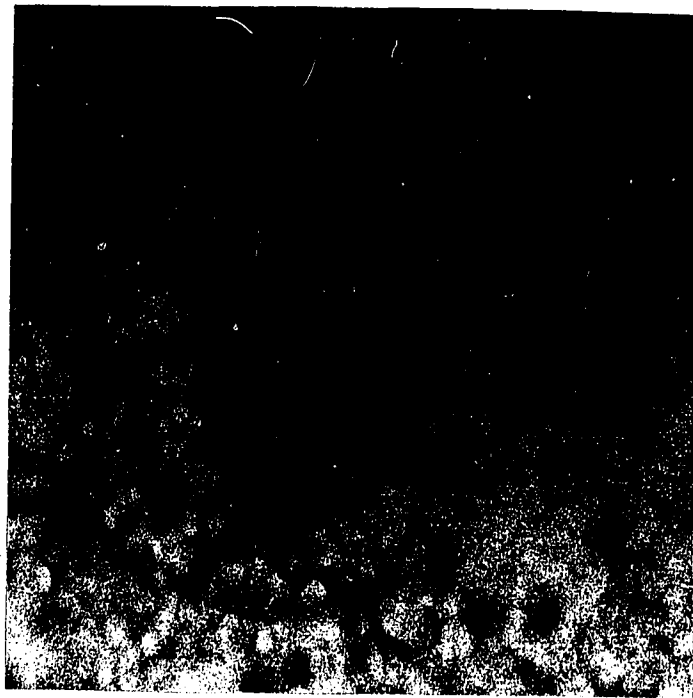
FIGURE 21

A RING FORM INCLUSION TREATED WITH HEXON ANTISERUM

A section of a 10 hour infected cell was stained indirectly with ferritin conjugated antibody using ICL virus hexon antiserum. Ferritin is not attached to the ring form inclusion. X 90,000



Fig 22



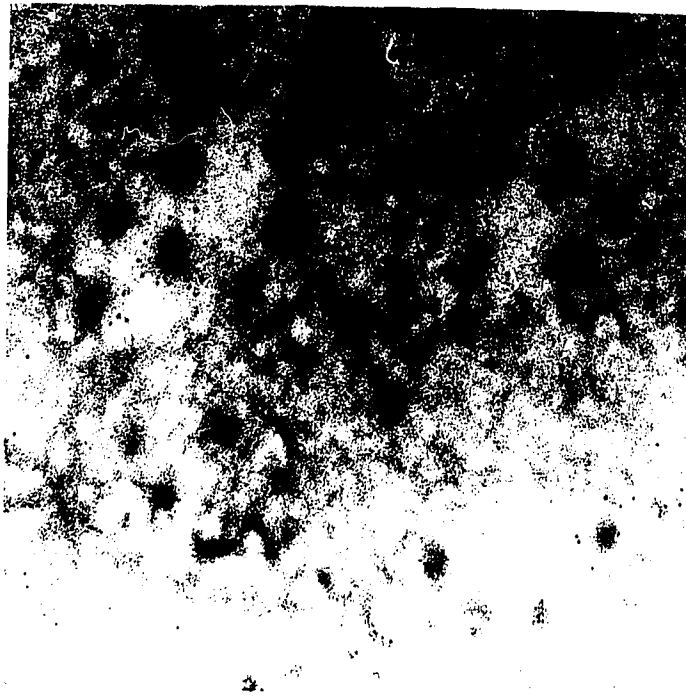


FIGURE 22

MDCK CELL ELEVEN HOURS AFTER INFECTION WITH ICL VIRUS

Section of an MDCK cell 11 hours after infection treated with hexon antiserum and stained with ferritin conjugate. Scattered virus particles inside the nucleus are labeled with ferritin. X 80,000

Fig 23

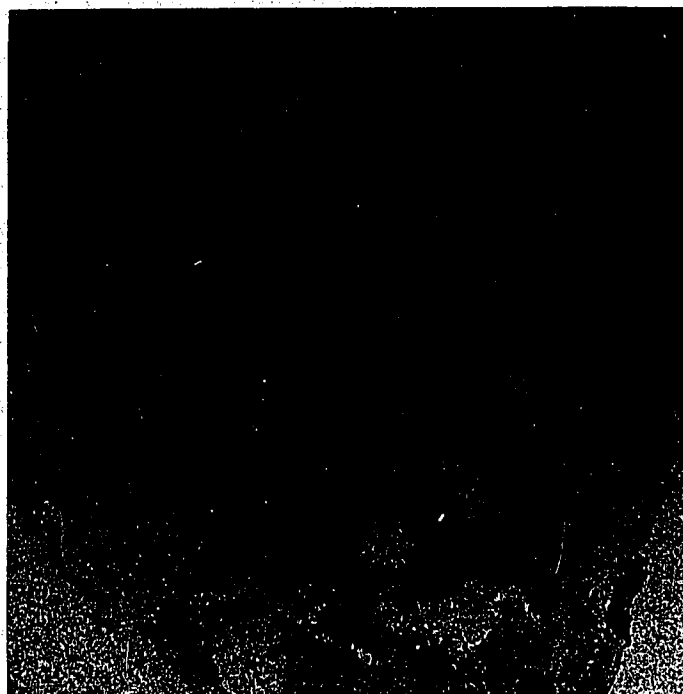
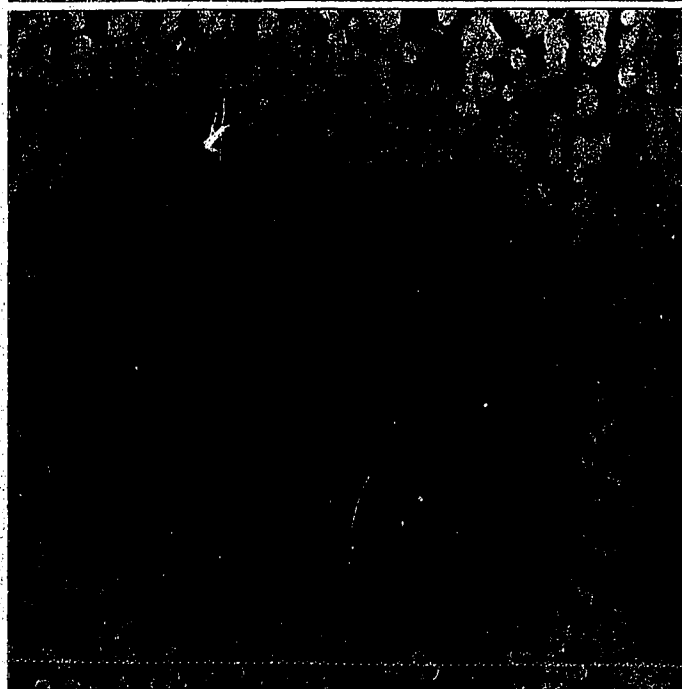


Fig 24



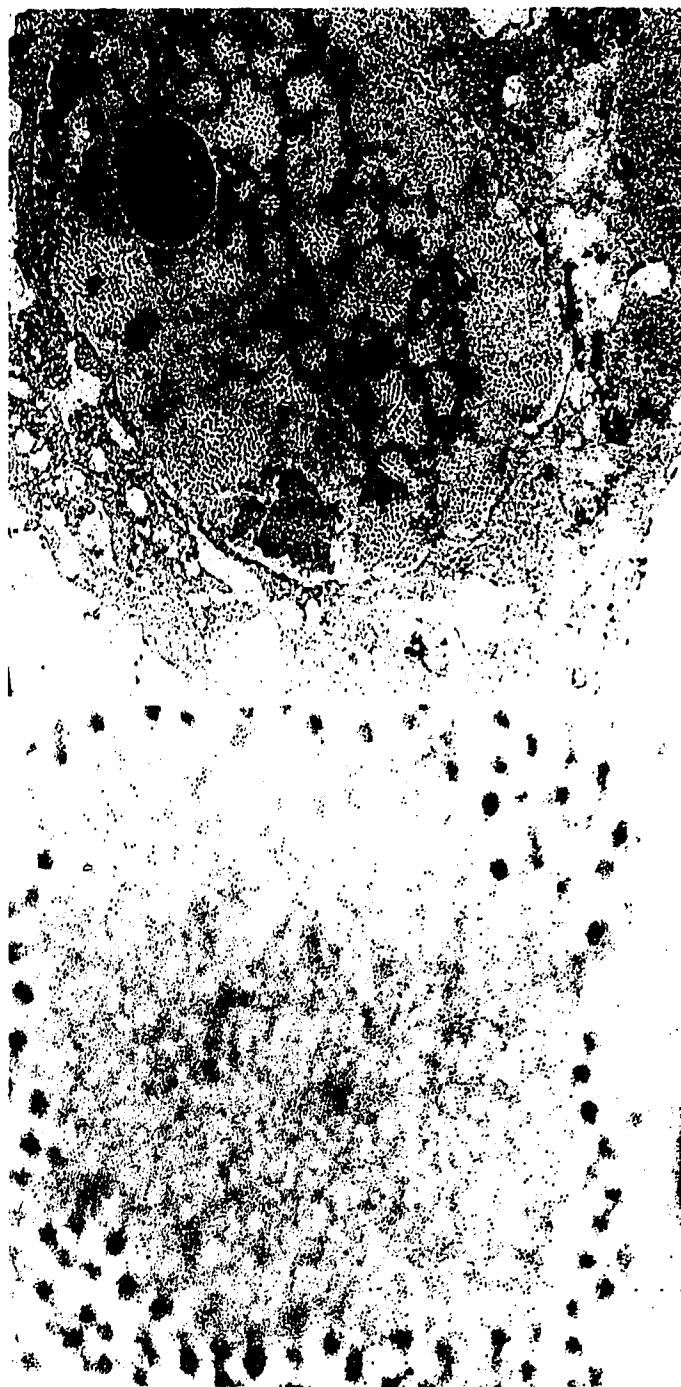


FIGURE 23

MDCK CELL EIGHTEEN HOURS AFTER INFECTION WITH ICL VIRUS

Cells were infected with ICL virus. Eighteen hours after infection they were fixed in formaldehyde and embedded in GMA. Dark staining and light staining inclusions are present inside the nucleus. The section was stained indirectly with ferritin conjugated antibody using ICL virus fiber antiserum. X 6,000

FIGURE 24

ICL VIRUS FIBER ANTIGEN IN LIGHT STAINING INCLUSION

A high magnification of the light inclusion shown by double arrows in Fig. 23. Ferritin is attached to the light inclusion. Virus particles are lightly labeled with ferritin. X 40,000

Fig 25

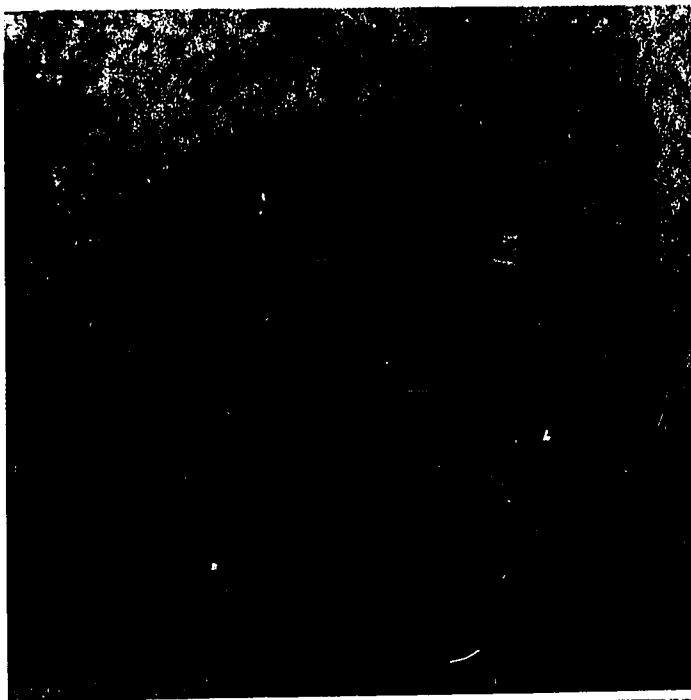
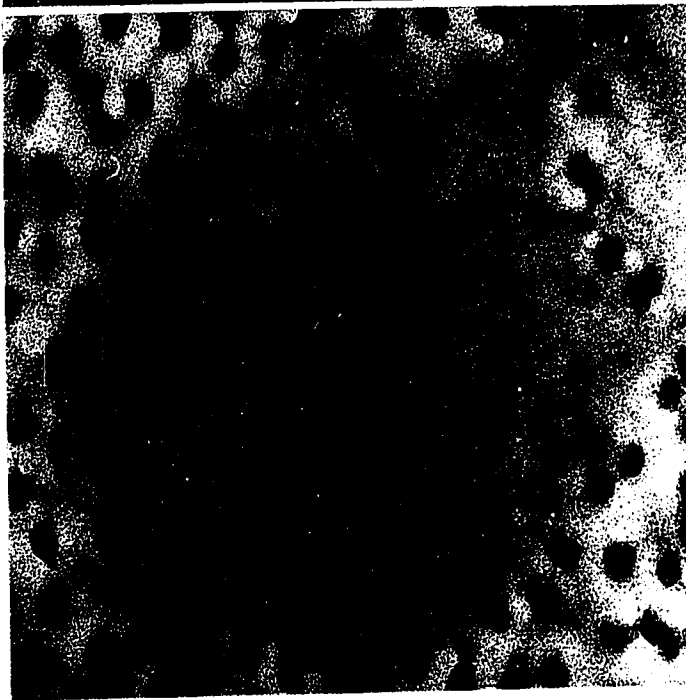


Fig 26



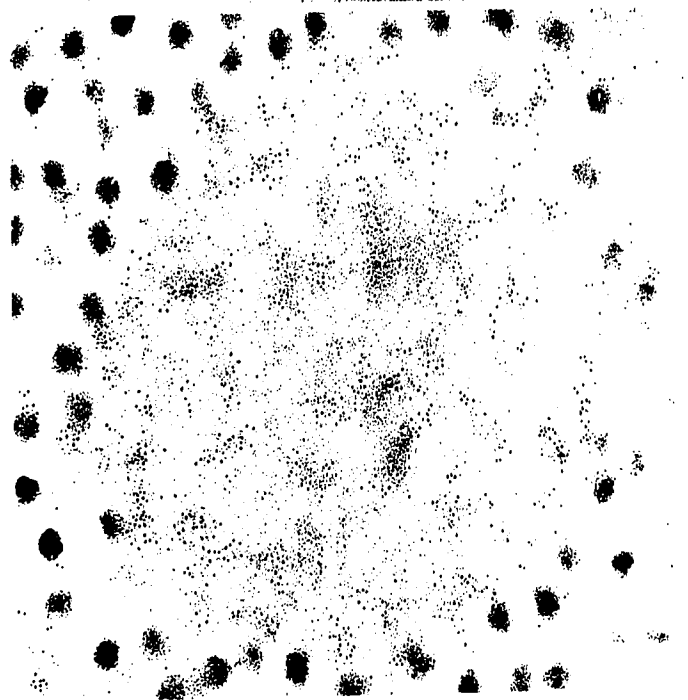
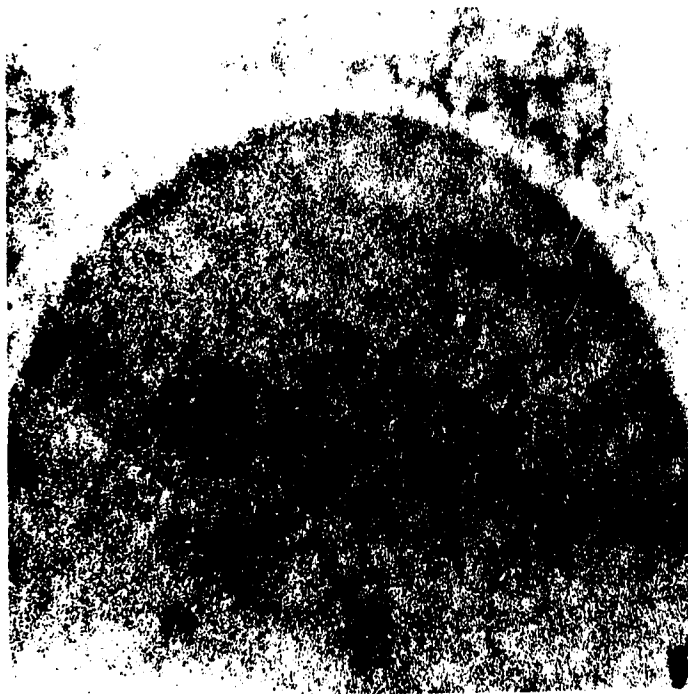




FIGURE 25

DARK STAINING INCLUSION TREATED WITH ICL VIRUS FIBER ANTISERUM

A high magnification of the dark inclusion in Fig. 23 shown by a single arrow. The inclusion is not labeled with ferritin conjugate.  
X 40,000

FIGURE 26

LIGHT STAINING INCLUSION TREATED WITH ICL VIRUS PENTON BASE ANTISERUM

Light inclusion in a section of an 18 hour infected cell stained indirectly with ferritin conjugated antibody using ICL virus penton base antiserum. The inclusion is labeled with ferritin. Some virus particles are also lightly labeled with ferritin. X 60,000

Fig 27



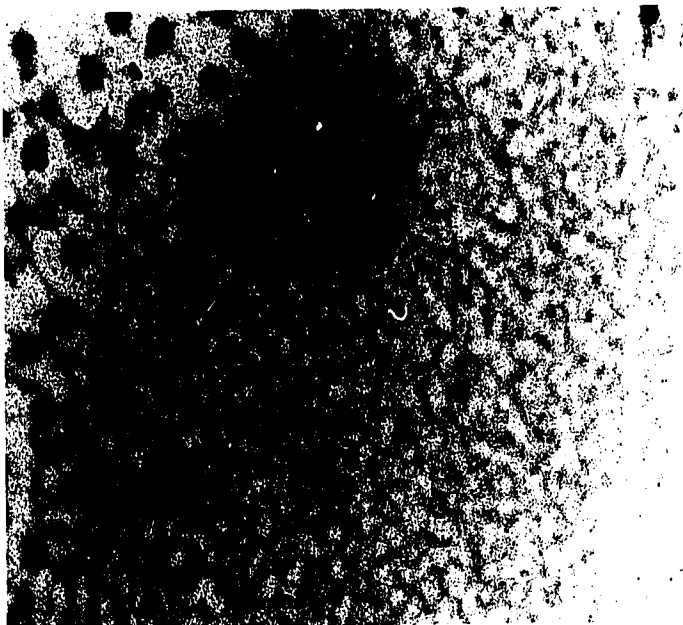


FIGURE 27

LIGHT STAINING INCLUSION TREATED WITH HETEROLOGOUS ANTISERUM

A light staining inclusion in a section of an 18 hour infected cell stained indirectly with ferritin conjugated antibody using vaccinia virus antiserum. The inclusion is not labeled with ferritin.

X 50,000

Fig 28

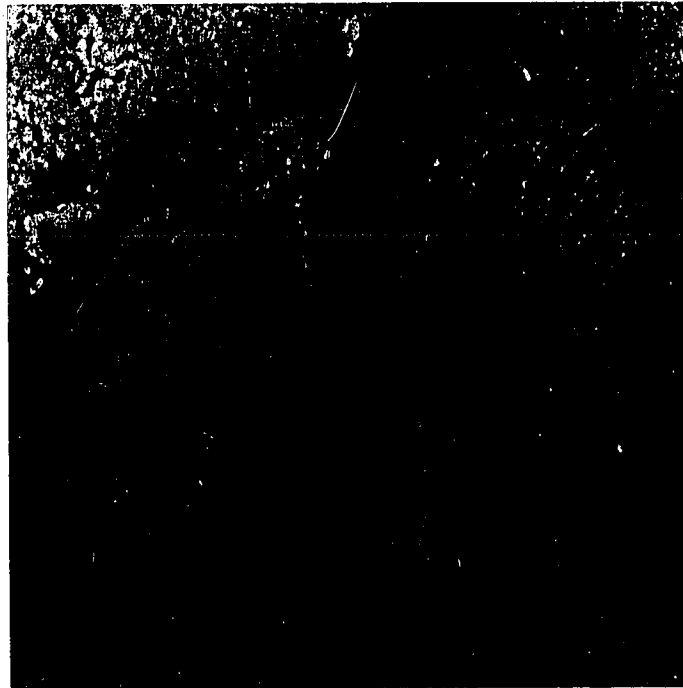
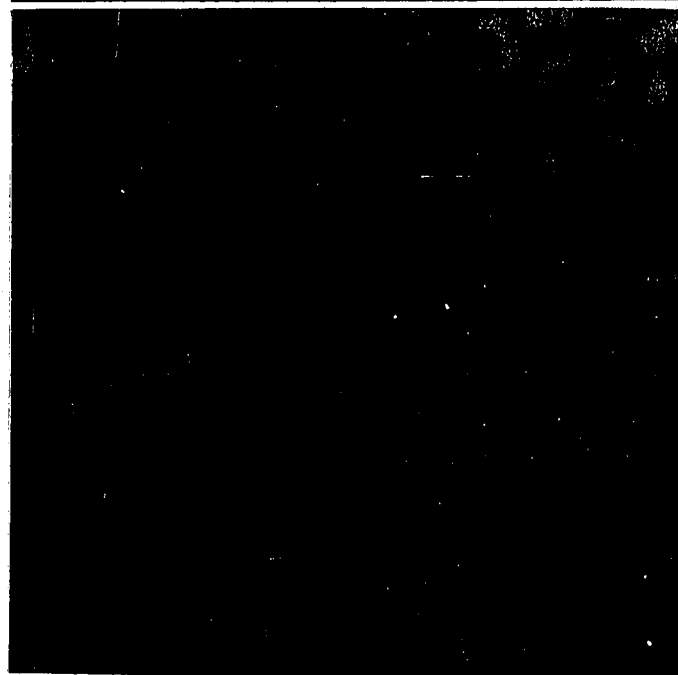


Fig 29



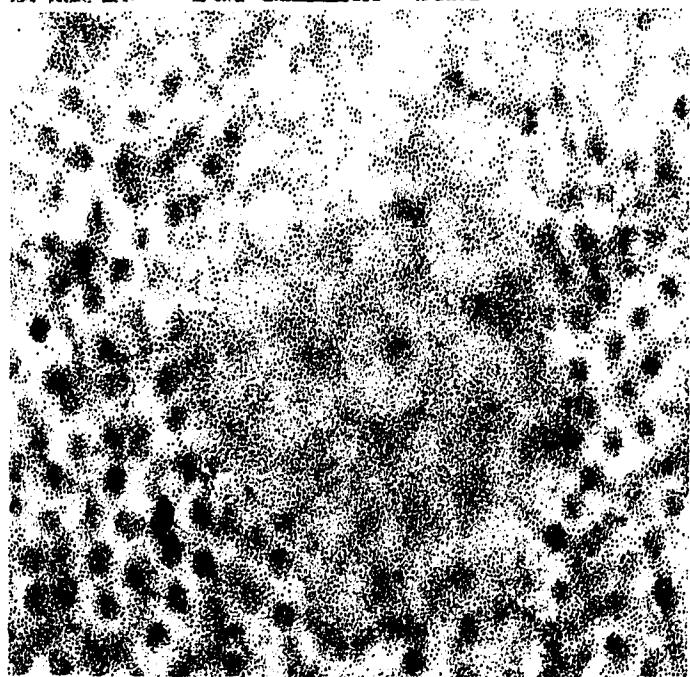


FIGURE 28

MDCK CELLS EIGHTEEN HOURS AFTER INFECTION WITH ICL VIRUS

A section of an infected cell similar to that in Fig. 23 showing dark staining and light staining inclusions inside the nucleus. The section was stained indirectly with ferritin conjugated antibody using ICL virus hexon antiserum. X 12,000

FIGURE 29

LIGHT STAINING INCLUSION TREATED WITH ICL VIRUS HEXON ANTISERUM

A high magnification of the light inclusion shown in Fig. 28 by a single arrow. Ferritin is attached to the inclusion and surrounding virus particles. X 70,000

Fig 30







FIGURE 30

DARK INCLUSION TREATED WITH ICL VIRUS HEXON ANTISERUM

A high magnification of the dark inclusion shown in Fig. 28 by double arrows. The inclusion is labeled with ferritin. X 80,000

#### Development of Fluorescent Antigens in ICL Virus Infected Cells

The appearance and the development of the viral fluorescent antigens in human adenovirus infected cells has been reported by many investigators (Boyer et al., 1959; Pope and Rowe, 1964; Hayashi and Russell, 1968). However, the fluorescent antigens detected with the light microscope have not been correlated with the inclusions observed at the level of the electron microscope. In the present study the antigenic content of the inclusions as determined by the immunoferritin method was compared with the distribution of fluorescent ICL virus capsid antigens.

MDCK cells grown in monolayers on cover slips were infected with ICL virus. Duplicate samples were taken at 1 hour intervals and stained for fluorescent antigens using specific antisera against the hexon, penton base, and fiber capsid antigens. The detail of the procedures was described in "Material and Methods".

The time of appearance of antigens and the number of cells demonstrating fluorescent stain is shown in Fig. 31. The first detectable fluorescent antigen in infected cells was fiber antigen. This antigen appeared in the form of fine flecks inside the nucleus at 8 hours after infection (Fig. 32). At this time only a small number of infected cells showed the presence of fiber antigen. Control infected cells treated with heterologous antiserum (vaccinia virus antiserum) did not show any fluorescence (Fig. 33). At 9 hours, the flecks increased in size and number (Fig. 34) and by 10 hours after infection they appeared as rings (Fig. 35). As the infection proceeded, the number of cells showing fiber antigen increased. The rings increased in size

and number and finally filled the entire nucleus (Fig. 36).

In contrast to the fiber antigen, hexon and penton base fluorescent antigens were not detectable in infected cells until 10 hours after infection. At this time, both antigens appeared as diffuse faint staining fluorescence with small intensely staining dots (Fig. 37). By 11 hours after infection, a large number of intensely fluorescent balls appeared in the nucleus (Fig. 38). At 12 hours, some of the intense fluorescence became marginated near the nuclear membrane (Fig. 39) which at a later stage (13 hours) joined together and formed a continuous concentric layer adjacent to the interior of the nuclear membrane (Fig. 40). By 16 hours after infection the whole nuclear area was filled with both hexon and penton base fluorescent antigens (Fig. 41).

Fig 31

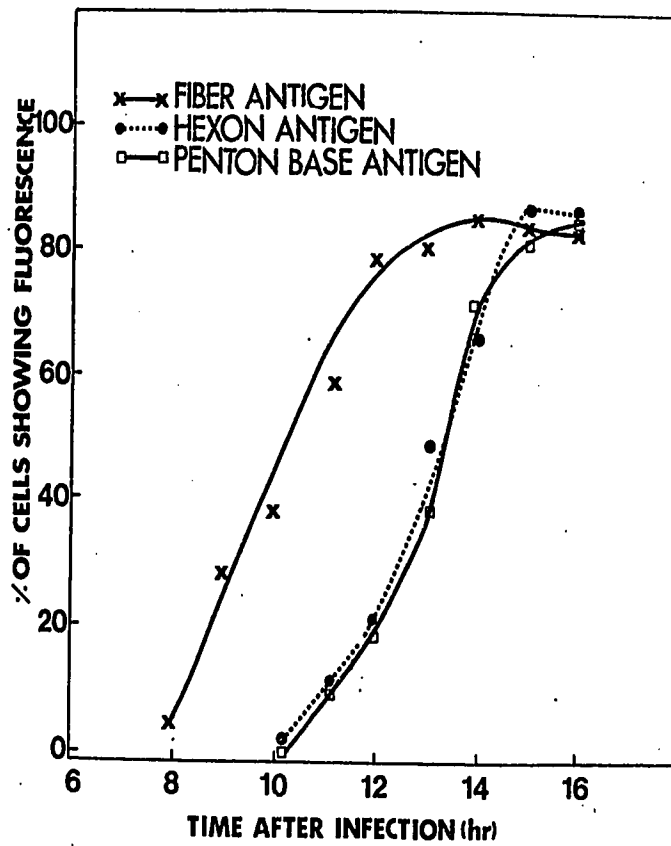


FIGURE 31

APPEARANCE OF ICL VIRUS CAPSID ANTIGENS IN MDCK CELLS

MDCK cells were infected with ICL virus with a multiplicity of infection of 100 P.F.U./cell. At one hour intervals duplicate samples were removed and stained indirectly with fluorescein conjugated  $\gamma$ -globulin. Approximately one thousand cells were counted from the samples of each time and the percent of cells showing fluorescence was calculated.

Fig 32

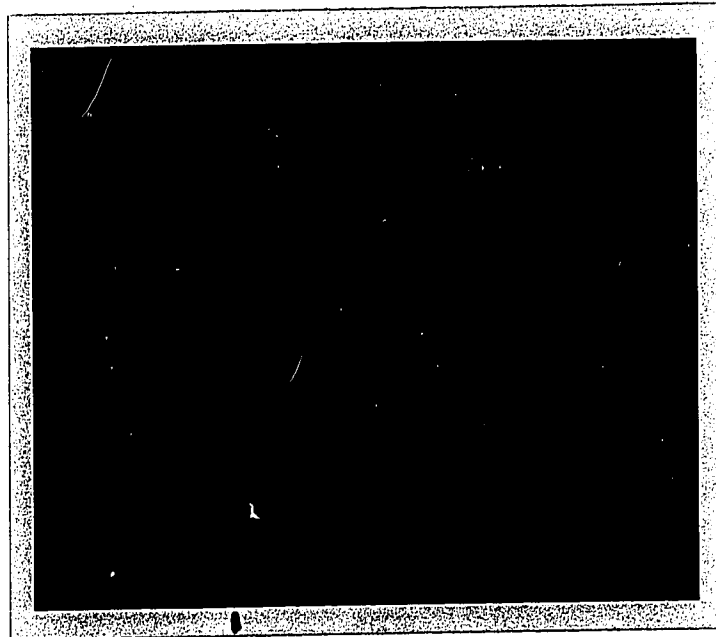
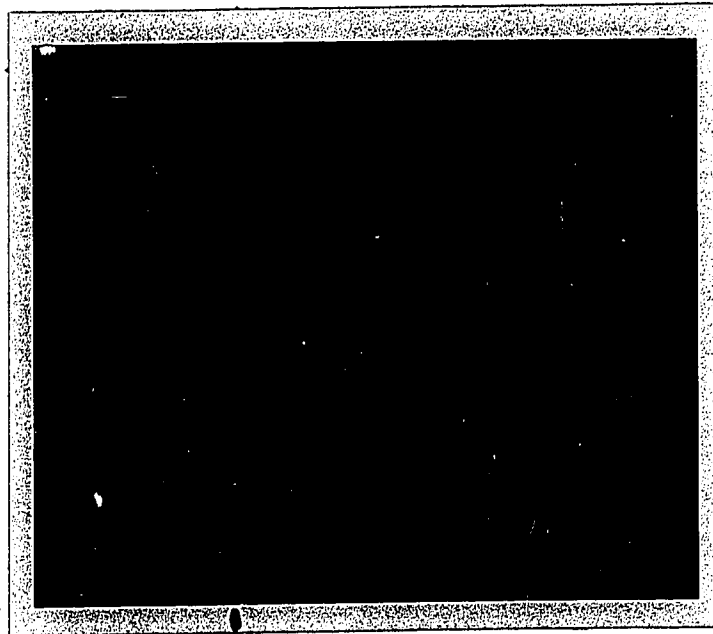


Fig. 33





1



1



FIGURE 32

FLUORESCENT FIBER ANTIGEN IN EIGHT HOUR INFECTED CELLS

MDCK cells grown as monolayers on coverslips were infected with ICL virus. Eight hours after infection the cells were stained indirectly with fluorescein labeled antibody using ICL virus fiber antiserum. Fluorescent fiber antigen is present in the form of fine flecks and dots inside the nuclei.

FIGURE 33

FLUORESCENT STAINING OF UNINFECTED MDCK CELLS

Uninfected MDCK cells were similarly stained as in Fig. 32 and show no fluorescence.

Fig 34

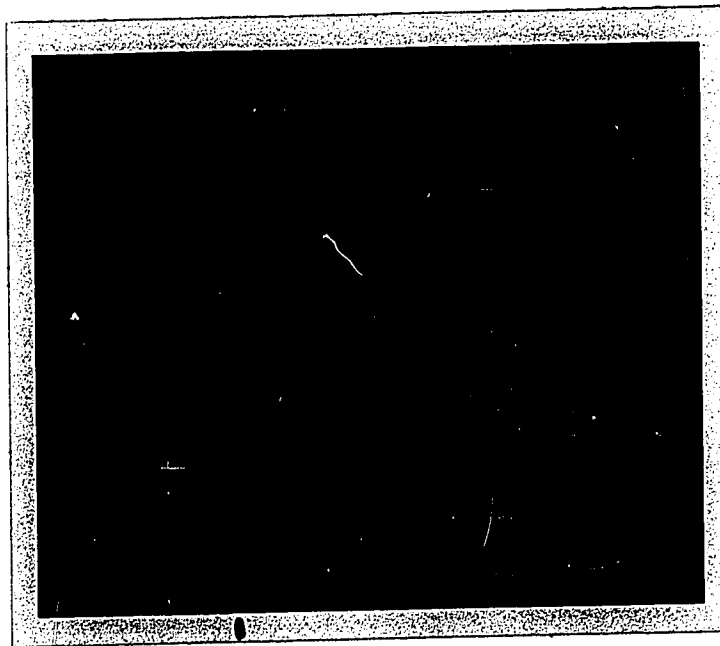
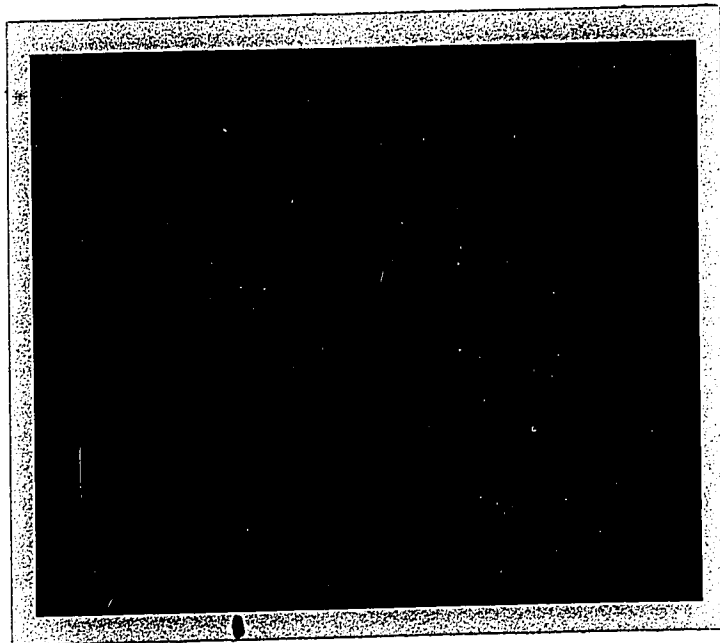
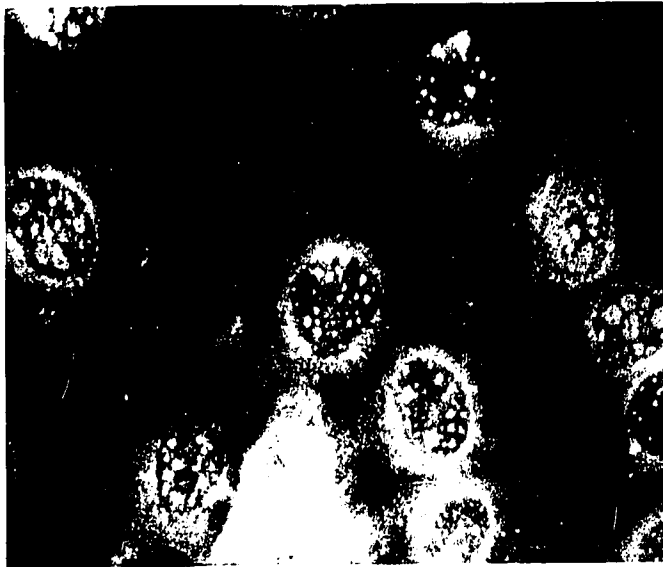


Fig 35





1



2

FIGURE 34

FLUORESCENT FIBER ANTIGEN IN NINE HOUR INFECTED CELLS

Nine hours after infection cells were stained for fluorescent fiber antigen as in Fig. 31. Numerous enlarged fluorescent flecks are present inside the nuclei. Some have developed to ring form patterns.

FIGURE 35

FLUORESCENT FIBER ANTIGEN IN TEN HOUR INFECTED CELLS

Ten hours after infection cells were stained for fluorescent fiber antigen as in Fig. 31. Fiber antigen shows as ring form fluorescence inside the nuclei.

Fig 36

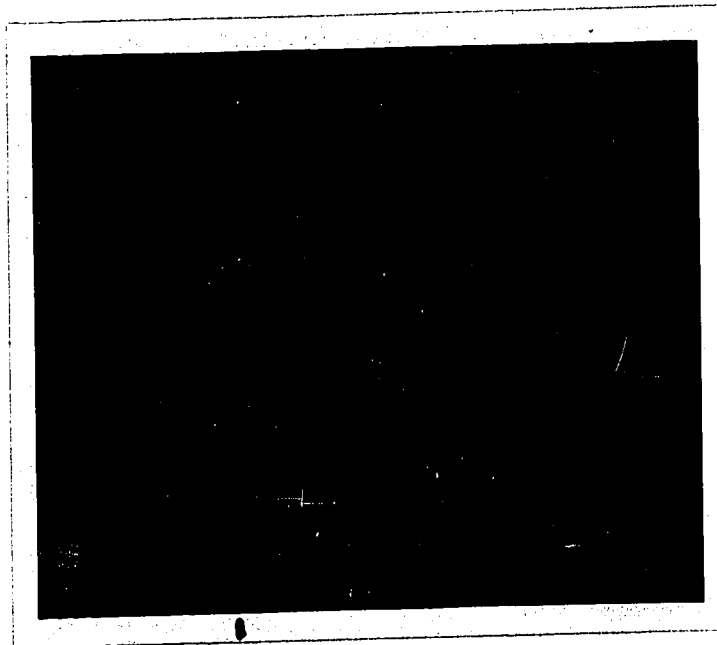
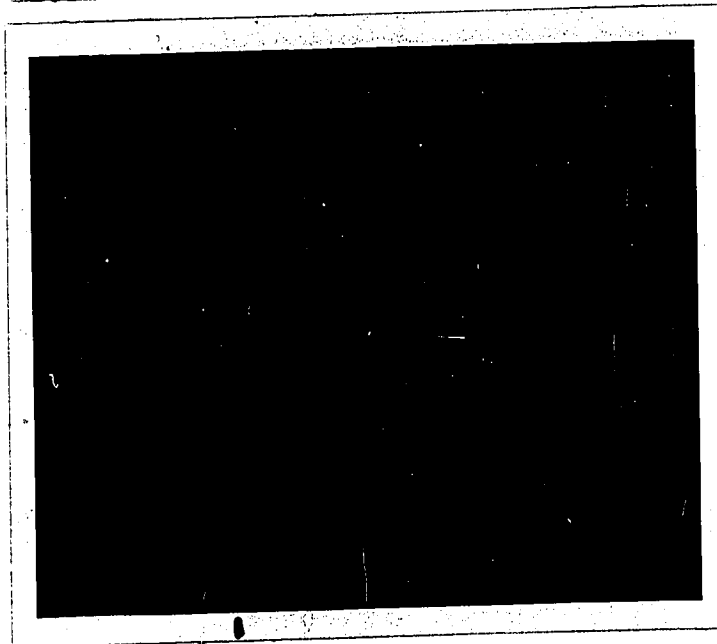


Fig 37





1



1

FIGURE 36

FLUORESCENT FIBER ANTIGEN IN FOURTEEN HOUR INFECTED CELLS

Fourteen hours after infection cells were stained for ICL virus fluorescent fiber antigen. The nuclei of infected cells are filled with fluorescent fiber antigen.

FIGURE 37

FLUORESCENT HEXON ANTIGEN IN TEN HOUR INFECTED CELLS

Ten hours after infection cells were stained indirectly with fluorescein labeled antibody using ICL virus hexon antiserum. Fluorescent hexon antigen is in the form of small dots inside the nuclei.

Fig 38

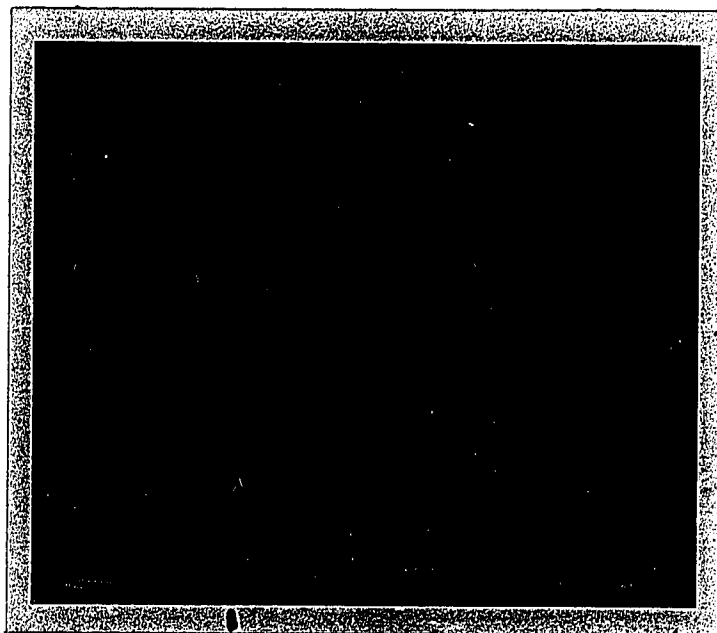
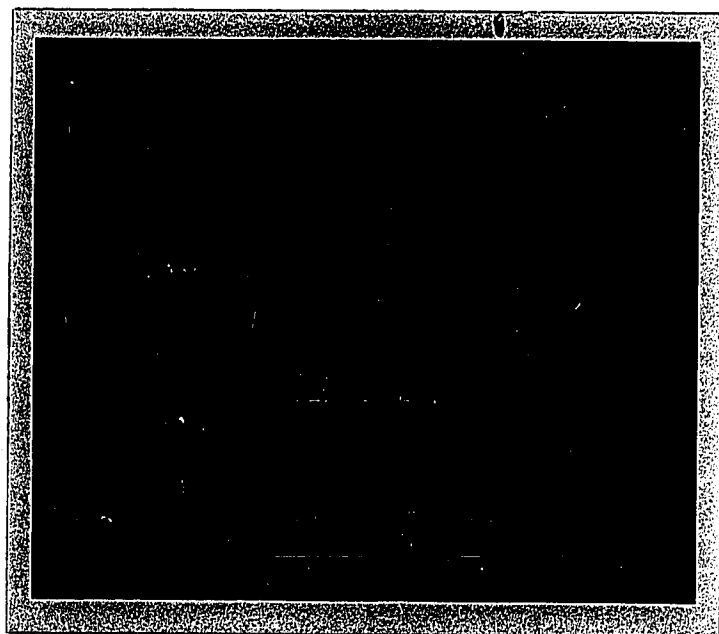


Fig 39





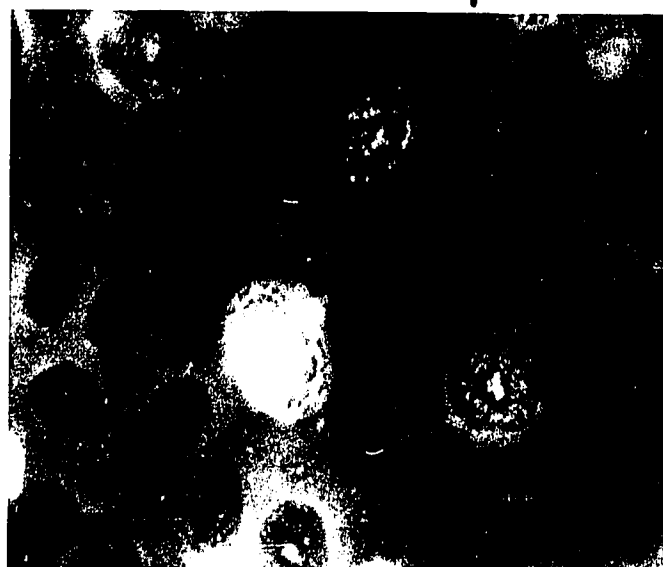


FIGURE 38

FLUORESCENT HEXON ANTIGEN IN ELEVEN HOUR INFECTED CELLS

Eleven hours after infection cells were stained for fluorescent hexon antigen. The antigen is in the form of numerous large fluorescent balls in a diffuse, faintly staining background.

FIGURE 39

FLUORESCENT HEXON ANTIGEN IN TWELVE HOUR INFECTED CELLS

Twelve hours after infection cells were stained for fluorescent hexon antigen. Intensely fluorescent balls are marginated toward the nuclear membrane.

Fig 40

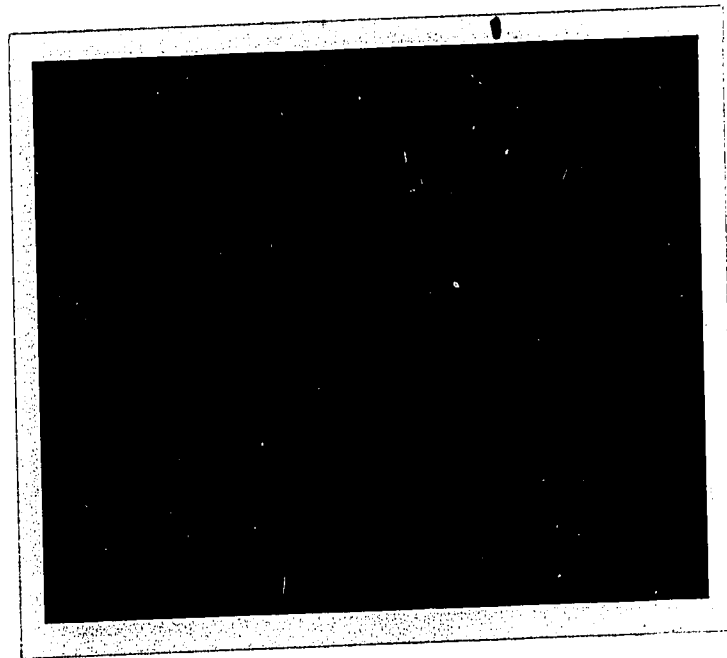


Fig 41

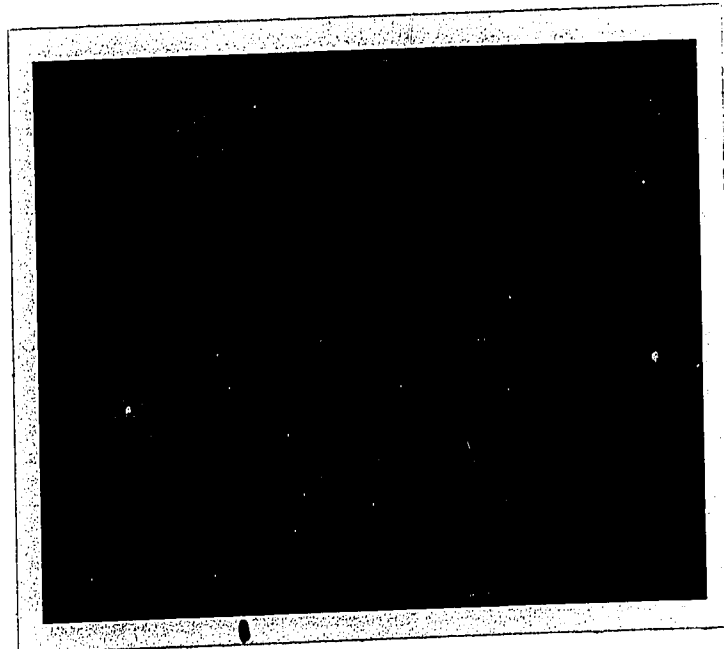




FIGURE 40

PENTON BASE FLUORESCENT ANTIGEN IN THIRTEEN HOUR INFECTED CELLS

Thirteen hours after infection cells were stained for fluorescent penton base antigen. The antigen is concentrated adjacent to the nuclear membrane.

FIGURE 41

PENTON BASE FLUORESCENT ANTIGEN IN SIXTEEN HOUR INFECTED CELLS

Sixteen hours after infection cells were stained for fluorescent penton base antigen. The nuclei of infected cells are filled with the fluorescent antigen.

#### Cytoplasmic Dark Inclusions in ICL Virus Infected Cells

Although inclusions in adenovirus infected cells are known to be confined to the nucleus, in electron microscopic examination of ICL virus infected cells some inclusions were also found in the cytoplasm (Fig. 42). These cytoplasmic inclusions were similar to the dark staining inclusions found in the nucleus. They were composed of protein and were labeled with ferritin conjugate using ICL virus hexon antiserum (Fig. 43). These unusual cytoplasmic inclusions were often observed near the nuclear membrane in such a configuration as to suggest imminent engulfment of the inclusions into the nucleus. For this reason it was thought that these inclusions were first formed in the cytoplasm then entered the nucleus. Since such an observation was not made with the electron microscope, it was decided to determine the number of these inclusions both in the nucleus and cytoplasm at different times after infection.

MDCK cells were infected with ICL virus (moi of 100 P.F.U./cell). From 10 to 20 hours after infection, samples were taken at 1 hour intervals, fixed and embedded for electron microscopy. A large number of infected cells were examined and the number of dark inclusions in both the nuclei and cytoplasm were counted. The results are shown in Table I.

From 10 to 14 hours after infection no dark inclusions could be observed in infected cells but at 15 hours a small number of cells showed the presence of dark inclusions. These inclusions were observed both in the nucleus and cytoplasm. As the infection proceeded, the number of cells showing inclusions increased and inclusions became

more numerous within each cell. However, the ratio of the number of cytoplasmic inclusions to the number of nuclear inclusions remained constant during the period of infection. It can be seen in Table I that at each time about 50% of the inclusions were found in the cytoplasm and 50% were in the nucleus. From these data it was concluded that the dark inclusions were formed at the same time both in the nucleus and cytoplasm of infected cells.

Fig 42

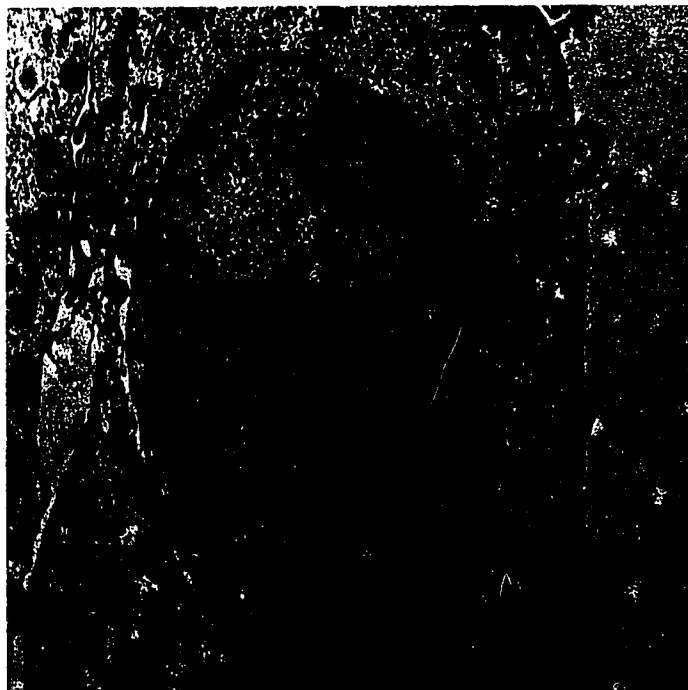


Fig 43





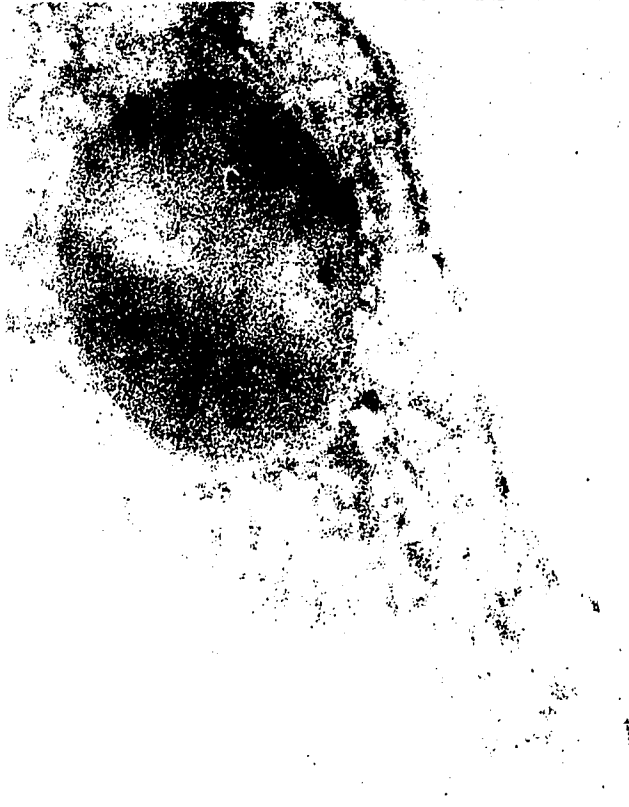


FIGURE 42

CYTOPLASMIC INCLUSION IN ICL VIRUS INFECTED CELL

MDCK cells were infected with ICL virus. Sixteen hours after infection cells were fixed in glutaraldehyde followed by osmium tetroxide and embedded in Epon 812. The section was stained with uranyl acetate and lead citrate. A dark inclusion is present inside the cytoplasm. X 12,000

FIGURE 43

STAINING OF CYTOPLASMIC INCLUSION WITH FERRITIN LABELED ANTIBODY

MDCK cells infected with ICL virus similarly to those in Fig. 42 were fixed in formaldehyde and embedded in GMA. A thin section was stained indirectly with ferritin labeled antibody using ICL virus hexon antiserum. Ferritin is attached to the inclusion. To enable the ferritin molecules to be visualized the section was not stained with lead citrate. X 65,000

TABLE I

DISTRIBUTION OF DARK INCLUSIONS IN MDCK CELLS AT DIFFERENT TIMES

AFTER INFECTION

<u>Time After Infection (hours)</u>	<u>Number of Cells Examined</u>	<u>% of Cells Showing Inclusions</u>	<u>% of Inclusions in Cytoplasm</u>	<u>% of Inclusions in Nuclei</u>
10	150	0	-	-
11	126	0	-	-
12	200	0	-	-
13	160	0	-	-
14	200	4	57	43
15	214	22	50	50
16	220	34	56	44
17	210	44	50	50
18	205	46	52	48
19	174	42	49	51

MDCK cells were infected with ICL virus. From 10 to 19 hours after infection, samples were taken at one hour intervals and prepared for electron microscopy. The above numbers of infected cells were examined from each sample. The number of cells showing the dark inclusions and the number of inclusions in the nuclei and cytoplasm were counted. The percentage of each was calculated.

### Purification of the Early and Ring Form Inclusions

The chemical composition of the early and ring form inclusions in ICL virus infected cells has been determined on the basis of cytochemistry and autoradiography studies (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971). For further analysis of the chemical nature of these inclusions and their role in virus multiplication, it was necessary to isolate these bodies from the infected cells.

#### A. Isolation

The essential step for purification of the early and ring form inclusions was to obtain an infected culture in which a majority of infected cells would show the presence of these inclusions. MDCK cells were infected with ICL virus. At various intervals samples were taken and stained with hematoxylin eosin and examined with the light microscope. It was found that a high multiplicity of infection (100 P.F.U. per cell) resulted in about 90-95% of the infected cells containing the early and ring form inclusions at 10 hours after infection. In the subsequent experiments the cells were infected with the above multiplicity and harvested at 10 hours. The next step was to disrupt the infected cells and to separate the inclusions from the cell components. Preliminary experiments revealed that sonication of the whole cell would cause contamination of inclusion preparation with cytoplasmic organelles such as mitochondria, lysosomes, etc. It was decided to remove the cytoplasmic portion of infected cells by a method which would preserve the intact nuclei containing inclusions. The details of the procedure employed were described in "Material and Methods". Fig. 44 is a photograph of the pure nuclear preparation which shows intact

nuclei containing early and ring form inclusions.

Once the nuclear fraction was prepared, it was sonicated briefly to break the nuclear membrane and release the inclusions. The final preparation of pure inclusions was obtained by differential centrifugation in sucrose followed by CsCl gradient centrifugation as described earlier.

To examine the degree of purity, a sample of final preparation was stained with methylene blue and examined in the light microscope. It was found that the preparation contained early and ring form inclusions with very low numbers of nucleoli (fewer than 0.5%; Fig. 45). Further examination of the purified early and ring form inclusions was done by fixing and embedding a sample for electron microscopy. Fig. 46 is an electron micrograph of the final preparation which shows typical early and ring form inclusions.

Fig 44

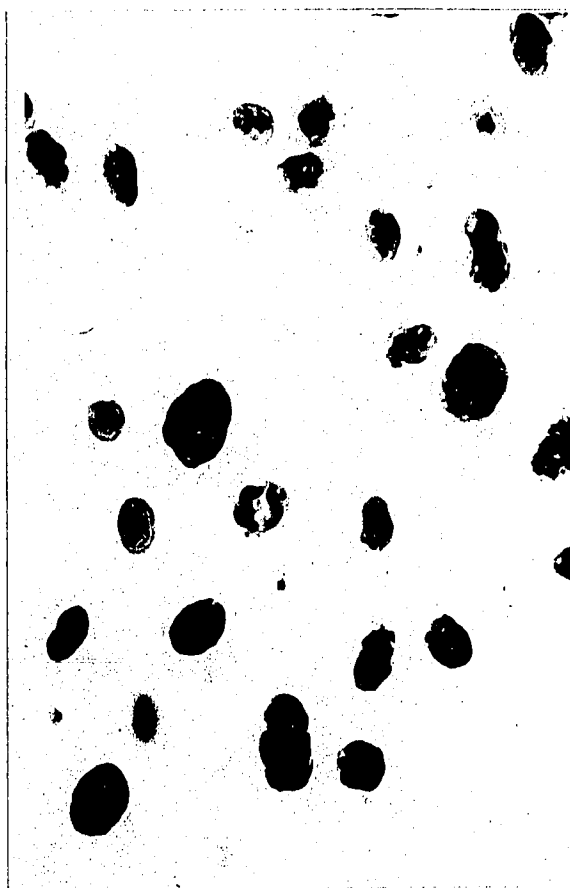


Fig 44

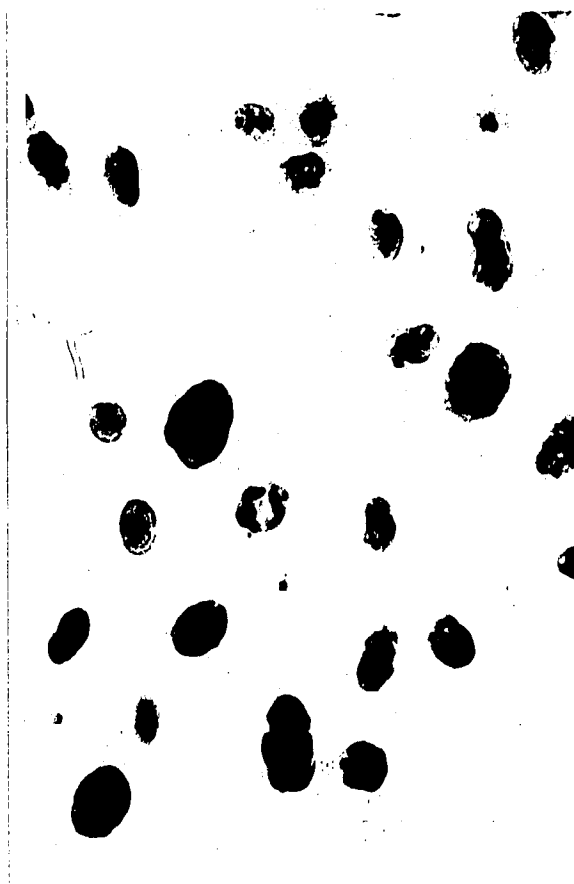


FIGURE 44

PURIFIED NUCLEI OF INFECTED MDCK CELLS

MDCK cells were infected with ICL virus with a multiplicity of infection of 100 P.F.U./cell. Ten hours after infection the cells were harvested and the nuclei were isolated according to "Material and Methods". A sample of the nuclear fraction was stained with methylene blue and examined in the light microscope. The purified infected nuclei contain early and ring form inclusions. X 2,000



Fig 45

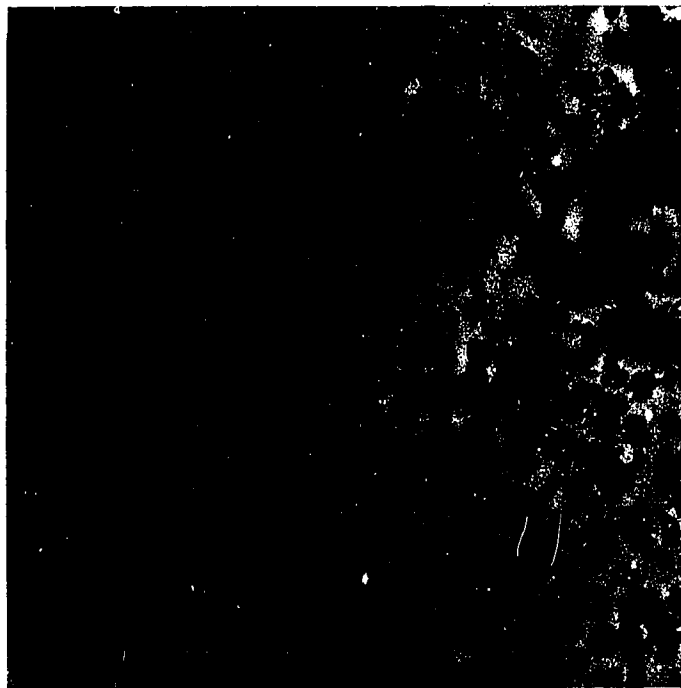


Fig 46



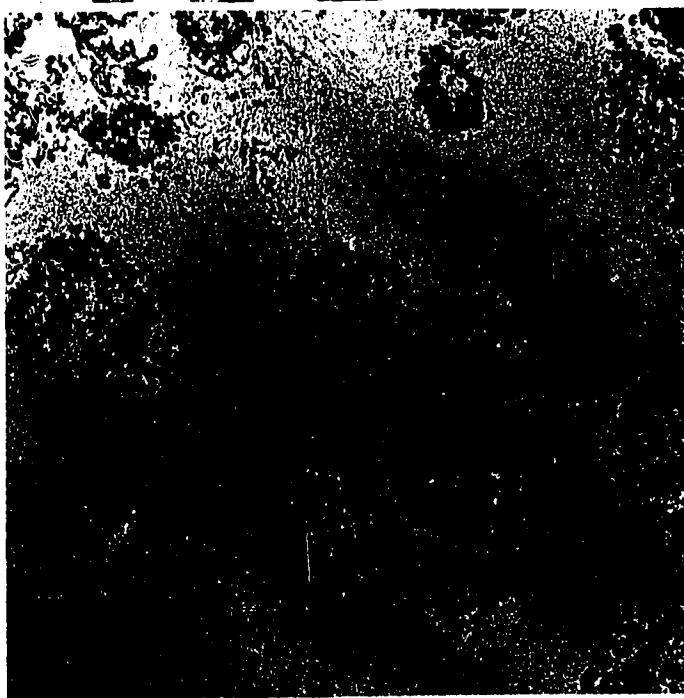
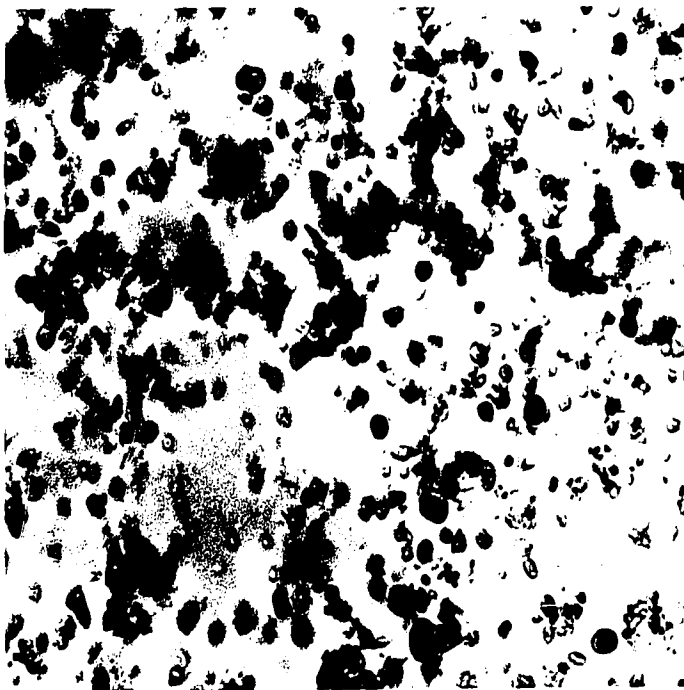


FIGURE 45

LIGHT MICROSCOPE PHOTOGRAPH OF PURIFIED INCLUSIONS

Inclusions were purified from the infected cells as described in "Material and Methods." A sample was stained with methylene blue and examined in the phase microscope. The preparation contains pure early and ring form inclusions. X 2,300

FIGURE 46

ELECTRON MICROGRAPH OF PURIFIED INCLUSIONS

A sample of purified inclusions was fixed in osmium tetroxide and embedded in Epon 812. The sections were stained with uranyl acetate and lead citrate. The photograph showing the ring form inclusions was taken randomly. X 13,000

B. Criterion of Purity of the Inclusion Preparation

Although the preparation seemed to be morphologically pure, it was decided to establish a criterion for purity by which the degree of purity could be measured quantitatively. Since the inclusions were prepared from the pure nuclei, the most probable source of contamination of the final preparation was thought to be cell chromatin. Experiments were designed to determine the amount of cell chromatin associated with the "pure" inclusions.

MDCK cells were grown in Roux bottles. Before a complete monolayer was formed, the cells were exposed to a medium containing 0.2  $\mu$ c per ml tritiated thymidine. After 48 hours incubation at 37°C, the radioactive medium was removed and the cells were washed 3 times with prewarmed medium. The labeled cells so obtained were divided into 2 groups. The first group of cells was incubated in fresh medium at 37°C. The second group was infected with ICL virus and then incubated similarly. Ten hours later, uninfected cells (first group) were harvested and their DNA was extracted. Infected cells were harvested at a similar time, the inclusions were harvested, and their DNA was extracted. Samples of both DNA from uninfected cells and DNA from the inclusions were counted in a liquid scintillation counter and their specific activity was calculated. The results were as follows:

Specific activity of DNA extracted from the

labeled cells ..... 2.5  $\mu$ c/10 mg DNA

Specific activity of DNA extracted from the

inclusions ..... 0.009  $\mu$ c/10 mg DNA

Thus, contamination with cell chromatin =  $\frac{0.009}{2.5} \times 100\% = 0.36\%$

As is shown, the amount of cell chromatin associated with pure inclusions was found to be 0.36%. In other words, the preparation of inclusions was 99.6% pure with respect to cell DNA.

C. Recovery of the Pure Early and Ring Form Inclusions from the Infected Cells

To calculate the recovery of pure inclusions from the infected cells, it was decided to label the infected cells with tritiated thymidine and determine the amount of radioactivity recovered in the inclusions.

Since only a certain portion of radioactive thymidine would be incorporated into the inclusions, it was necessary first to determine the percentage of  $^3\text{H}$ -thymidine incorporation into the inclusions of the total amount taken up by the infected cells.

MDCK cells were infected with ICL virus. Nine hours after infection the cells were pulse labeled with tritiated thymidine ( $50\text{ }\mu\text{C/ml}$ ) for 1 hour then used for electron microscope autoradiography as described in "Material and Methods". Quantitative autoradiography was applied to thin sections of infected cells. The number of grains over the different cell areas were counted. The results are shown in Table II. Assuming the incorporation of radioactive thymidine into the infected cells was 100%, about 75% of this amount was incorporated into the inclusions. This percentage of incorporation was used in the following experiment to calculate the recovery of the inclusions.

Approximately  $2 \times 10^8$  cells were infected with ICL virus; 9 hours later they were exposed to a medium containing tritiated thymidine at a concentration of  $0.5\text{ }\mu\text{C/ml}$  and incubated at  $37^\circ\text{C}$  for 1 hour. When the pulsing time was terminated, infected cells were harvested and washed 3 times in cold medium. The cells were resuspended in 10 ml of RSB. To determine the total radioactivity uptake of these cells,  $200\text{ }\mu\text{l}$  of the

cell suspension were added to 200  $\mu$ l of 1% SDS to lyse the cells. A 200  $\mu$ l sample of this lysate was counted in a liquid scintillation counter. From the remaining cells, inclusions were purified and suspended in 2 ml of RSB. 200  $\mu$ l of this preparation were treated as above with SDS and of this, 200  $\mu$ l were counted and the total radioactivity of the recovered, purified inclusions was calculated. The recovery of pure inclusions from the infected cells was calculated thus:

c.p.m. in total infected cells ..... 240,000

c.p.m. incorporated into the

inclusions (75% of the 240,000) ..... 180,000

c.p.m. in total purified inclusions ..... 40,000

$$\text{Recovery} = \frac{40,000}{180,000} = 22.5\%$$

TABLE II

INCORPORATION OF  $^3\text{H}$ -THYMIDINE IN INFECTED CELLS

(Distribution of Silver Grains over  
Different Regions of 98 Infected Cells Examined)

<u>Region of Cell Examined</u>	<u>Number of Grains</u>	<u>Per Cent Incorporation</u>
Whole cells	1,517	100%
Cytoplasms	140	9%
Nuclei	1,377	91%
Inclusions	1,138	75%

MDCK cells were infected with ICL virus. At nine hours after infection, they were labeled with  $^3\text{H}$ -thymidine for one hour and then used for electron microscope autoradiography. A number of cells were examined, the distribution of silver grains over different regions of infected cells was counted and the percentage of  $^3\text{H}$ -thymidine incorporation into the inclusions was calculated. (Background was less than one grain per cell area.)



Chemical Composition of the Inclusions

Composition of the inclusions was analysed by determining their DNA, RNA, and protein content. DNA was determined by the diphenylamine test, RNA by the orcinol test, and protein by Lowry's method. The amount of these macromolecules in 1 ml of suspension of inclusions (purified from about 30 Roux bottles of infected cells) is shown below.

<u>MACROMOLECULE</u>	<u>AMOUNT (<math>\mu</math>g)</u>	<u>PERCENT</u>
DNA	440	51
RNA	25	3
Protein	400	46

In agreement with the previous cytochemical study (Shahrabadi, 1969), it was found that these inclusions were composed of nucleoprotein with the DNA as a major constituent.

#### Isopycnic Centrifugation of DNA from the Inclusions

The DNA of human adenoviruses ranges in G + C content from 48-49% for highly oncogenic groups to 55-61% for nononcogenic groups (Green, 1970). ICL virus DNA has a G + C content of 56% and dog kidney cells have a G + C content of 42% (Gaunt, 1966). This high G + C content of the viral DNA gives it a higher density than the host DNA (Sueoka et al., 1959). We were interested in (i) separating the viral and host DNA on the basis of their densities, and (ii) analysing the DNA of inclusions and determining the relationship of its density to that of the viral and host DNA.

MDCK cells were grown in Roux bottles. Before a complete monolayer was formed, the cells were exposed to a medium containing 0.2  $\mu$ c per ml tritiated thymidine and incubated at 37°C. Forty-eight hours later the cells were infected with ICL virus and incubated in a medium containing 0.2  $\mu$ c/ml tritiated thymidine at 37°C. Sixteen hours after infection the cells were harvested and their DNA was extracted. This DNA was considered to be a mixture of viral and host DNA. To obtain labeled DNA from the inclusions, cells were infected with ICL virus as described in "Material and Methods". Eight hours after infection they were labeled with 0.5  $\mu$ c/ml tritiated thymidine for 2 hours. When the pulsing was terminated, infected cells were harvested, inclusions were purified as described, and their DNA was extracted by pronase-SDS treatment followed by phenol extraction according to "Material and Methods".

DNA from ICL virus was purified and used as a marker to identify the position of viral DNA after centrifugation. Samples of DNAs were

dissolved in CsCl solution and centrifuged as described in "Material and Methods". The results of the equilibrium sedimentation patterns of DNA from infected cells and inclusions are shown in Fig. 47. The position of viral DNA marker in CsCl after centrifugation was determined by measuring its optical density at 260 mμ. DNA from the infected cells was sedimented into two separate bands. The first band (from the bottom), which had the same density as the viral DNA marker, was considered to be viral DNA. The second band, with a lower density, was host cell DNA. DNA from the inclusions was banded in CsCl as a single component and cosedimented with viral DNA.

This experiment was repeated using an analytical ultracentrifuge. A mixture of MDCK cell DNA and viral DNA in CsCl was centrifuged at 44,000 r.p.m. to equilibrium. The DNA was banded in 2 separate bands (Fig. 48, top)

The density of each band was calculated according to the method of Chervenka described in A Manual of Methods For the Analytical Ultracentrifuge, Beckman, Spinco. The formulas used for buoyant density calculation were as follows:

$$\rho_s = \rho_e + \left( \frac{d\rho}{dr} \right) (r_s - r_e)$$

$$r_e = \sqrt{\frac{r_b^2 + r_m^2}{2}}$$

$$\left( \frac{d\rho}{dr} \right) = \frac{\omega^2 r_e}{\beta}, \quad \omega = \frac{2 \pi \text{ r.p.m.}}{60}$$

where  $\rho_e$  = initial density of CsCl = 1.724

$r_s$  = radial distance of sample meniscus = 6.644 cm

$r_m$  = radial distance of the center of the band = 6.063 cm

$r_b$  = radial distance of the bottom of the tube = 7.31 cm

$\beta$  = a factor which relates the composition of gradient  
to rotor speed and the physical properties of CsCl.

The value for  $\beta$  was obtained from the table of the  
book and equals  $1.190 \times 10^9$ .

$\rho_s$  = buoyant density of the sample

Using the above equations it was found that the first band from the left had a density of 1.716 which corresponds to the density of viral DNA. The density of the second band (MDCK cell DNA) was found to be 1.703. When the DNA from the inclusions was centrifuged under the same conditions, it banded as a single band with a density equal to that of viral DNA (Fig. 48, bottom). It is also shown that there was no detectable host DNA present in the DNA from inclusions. These experiments showed that on the basis of buoyant density, the DNA content of the early and ring form inclusions was viral.

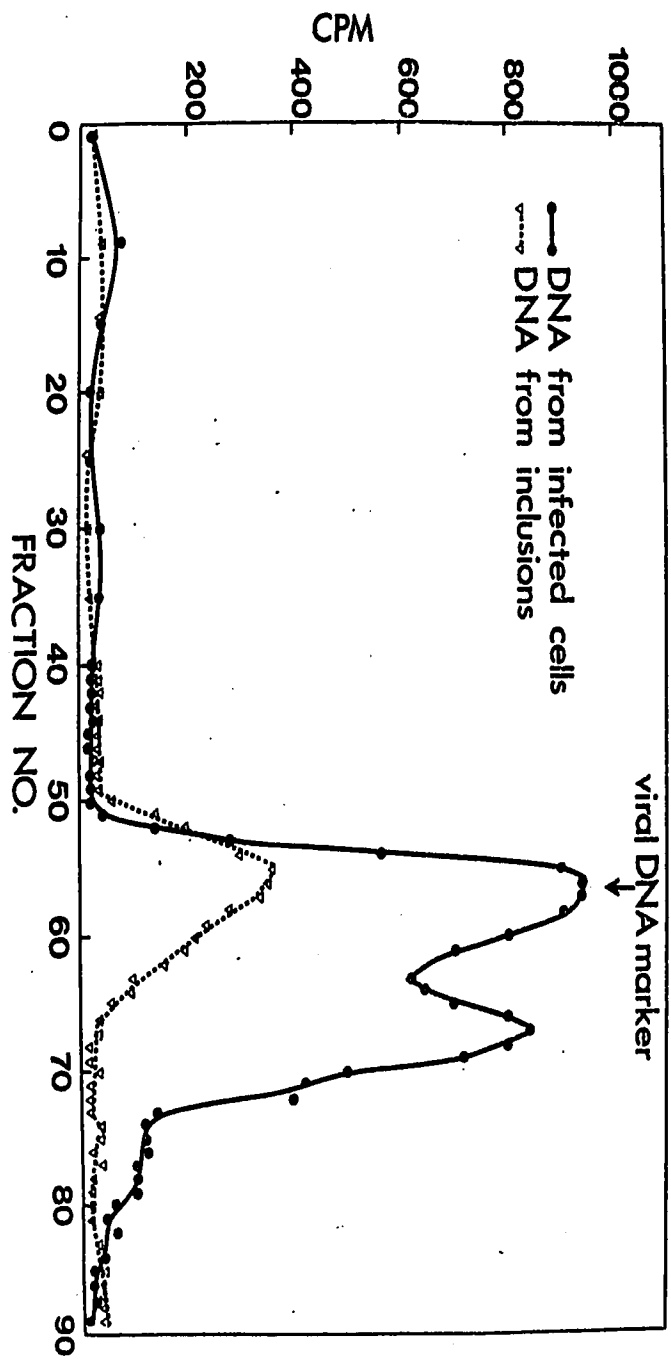


Fig 47

FIGURE 47

EQUILIBRIUM SEDIMENTATION PATTERN  
OF DNA FROM INCLUSIONS AND INFECTED CELLS

$^3\text{H}$  labeled DNA's from ICL virus infected cells and from purified inclusions were centrifuged in separate tubes in  $\text{CsCl}$  (density 1.7 g/ml) to equilibrium (38,000 r.p.m. for 50 hours at  $25^\circ\text{C}$  in an  $\text{SW}_{50,1}$  rotor). Viral DNA was also centrifuged in a separate tube as a marker. Three drop fractions were collected from all tubes and the radioactivity in the acid precipitable material of each fraction was determined.

Fig 48

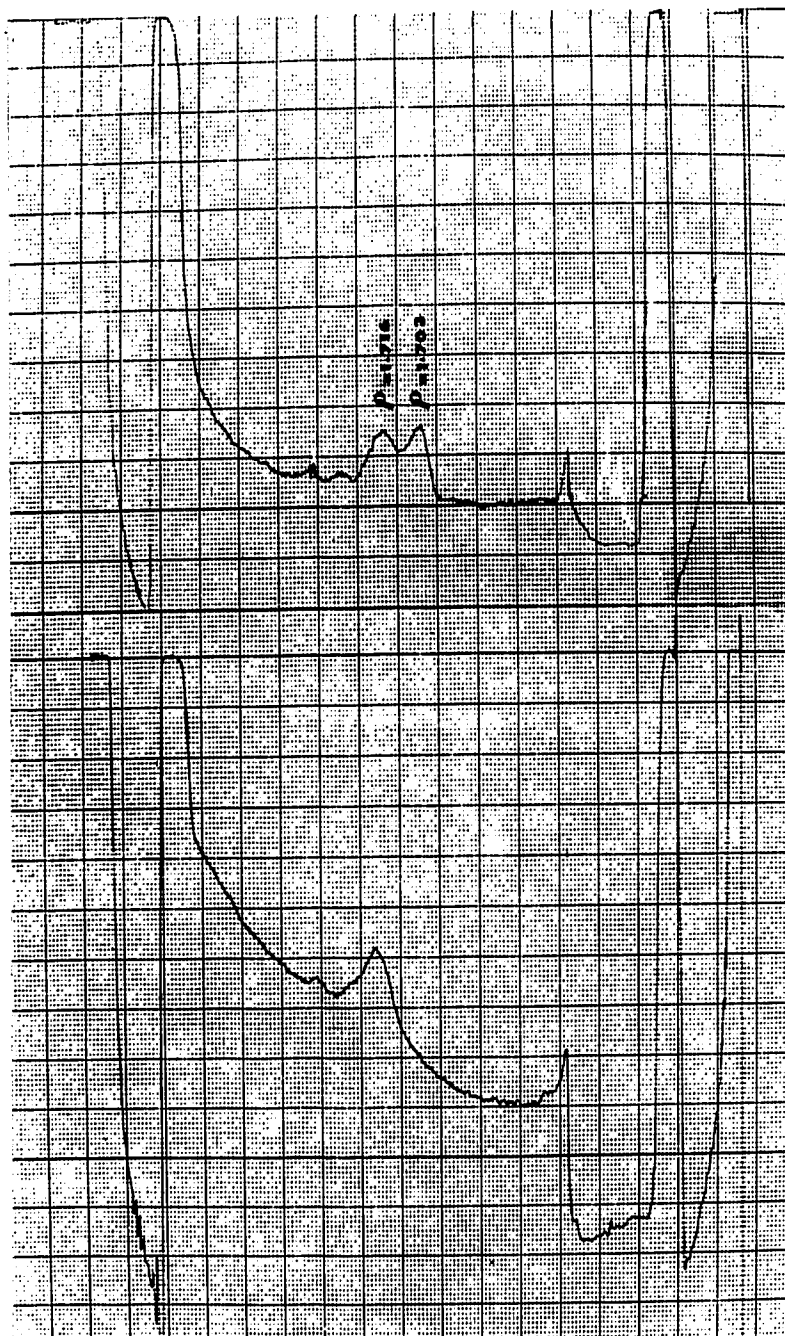


Fig 45

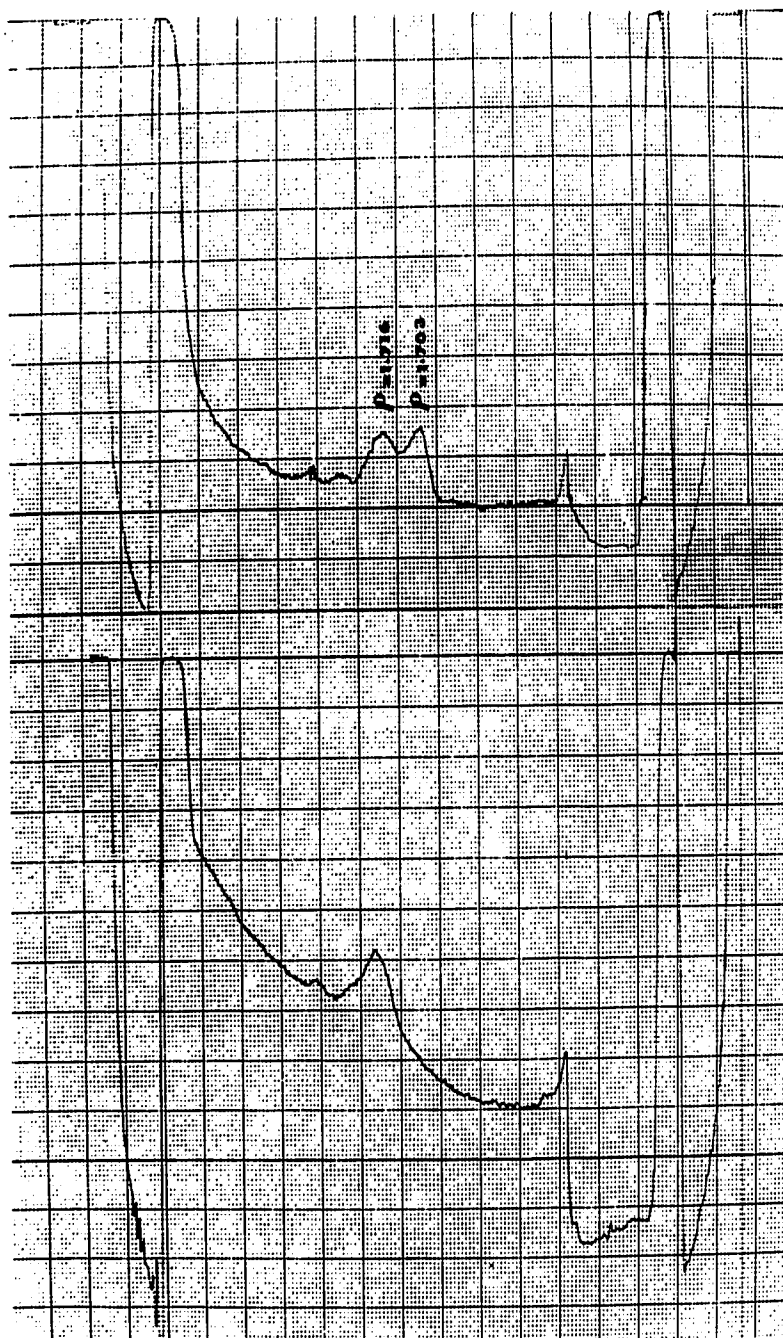




FIGURE 48

ISOPYCNIC CENTRIFUGATION OF DNA

Samples of DNA in CsCl were centrifuged in a Beckman Model E ultracentrifuge using an AN-D rotor. Centrifugation was performed at 44,000 r.p.m. for 24 hours at 25°C. Photographs were taken during the last 10 minutes of the centrifugation period and traced in a Beckman analytrol.

TOP: ICL virus DNA ( $\rho=1.716$ ) and MDCK cell DNA ( $\rho=1.703$ )

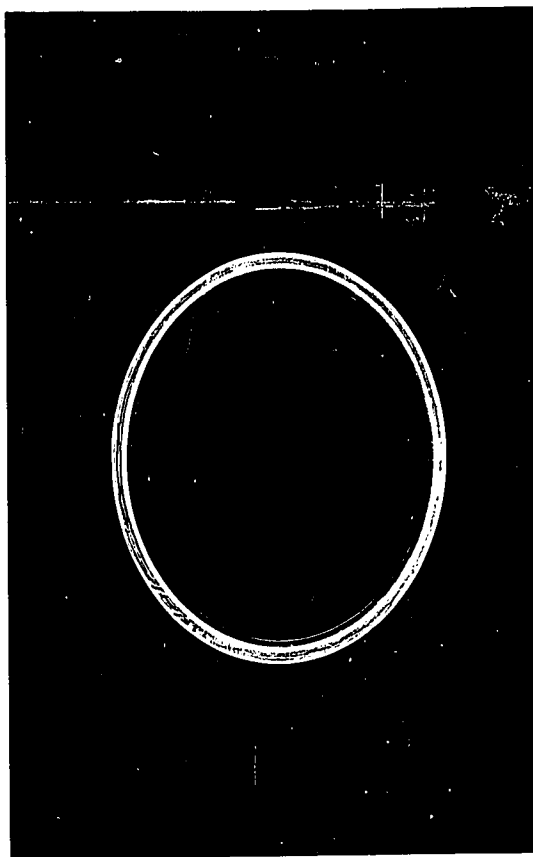
BOTTOM: DNA from inclusions

Presence of Viral Capsid Antigens in Purified Inclusions

In the previous experiments, on the basis of immunoferritin and immunofluorescent staining, it was found that the early and ring form inclusions contained ICL virus fiber antigen. It was of interest to determine the antigenic content of the purified inclusions with respect to the viral capsid proteins. Purified inclusions obtained from 10 hour infected cells were solubilized by treatment with 0.1% SDS at room temperature for 2 hours. They were examined by the immunogel diffusion test using ICL virus antiserum and antisera specific for viral capsid proteins as described in "Material and Methods". These antisera were the same as those used in the immunoferritin and immunofluorescent staining experiments. Furthermore, these antisera were tested against ICL virus soluble antigens in gel diffusion tests and shown to have approximately equal potency.

The immunodiffusion test with antiserum against the fiber antigen gave a single precipitin line (Fig. 49). Antiserum against the whole virus particles also gave a single precipitin line which cross reacted with the precipitin line of the fiber antiserum. However, there was no detectable reaction between the inclusion material and antisera against penton base and hexon antigens. The result suggested that the purified early and ring form inclusions contained only fiber antigen. This was in agreement with results of immunofluorescent and immunoferritin studies which showed the presence of only fiber antigen in the early and ring form inclusions.

Fig 49



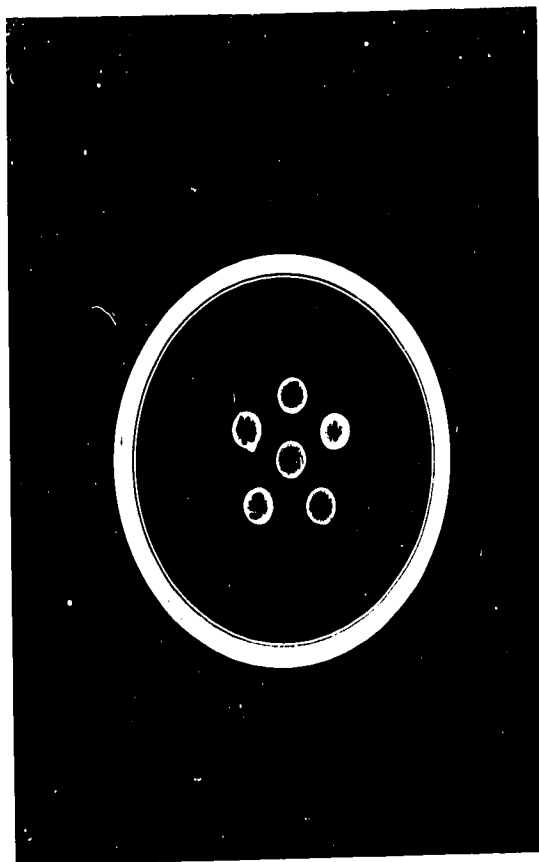


FIGURE 49

IMMUNOGEL DIFFUSION PATTERN OF PURIFIED INCLUSIONS

A sample of pure inclusions was solubilized with 0.1% SDS and tested against ICL virus capsid antisera. The photograph was taken four days after incubation at room temperature.

Center (I) ..... Inclusions

1 and 4 ..... ICL virus fiber antiserum

2 ..... ICL virus hexon antiserum

3 ..... Antiserum against whole ICL virus particles

5 ..... ICL virus penton base antiserum

#### The Site of Viral DNA Synthesis

In a previous study (Yamamoto and Shahrabadi, 1971) it was found that exposure of infected cells to tritiated thymidine for 1 hour would result in incorporation of this DNA precursor into the early and ring form inclusions. It was suspected that these inclusions were the site of viral DNA synthesis. If this were so, the rapid incorporation of thymidine into these bodies would be expected. Experiments were designed to label the infected cells in a short time and determine the site of thymidine incorporation.

MDCK cells were infected with ICL virus. Ten hours after infection the cells were exposed to  $^3\text{H}$ -thymidine for 4 minutes and then used for electron microscope autoradiography as described in "Material and Methods". Electron microscope examination of the autoradiograms showed that silver grains were mainly located over the early and ring form inclusions (Fig. 50). A total of 50 cells were examined and the number of grains over different cell areas counted. Of the total number of grains counted 88% of them were located over the early and ring form inclusions. This experiment showed that a major portion of thymidine taken up by the infected cells during the 4 minute period was incorporated into the early and ring form inclusions. The results suggested that these inclusions were the site of viral DNA synthesis.

Fig 50

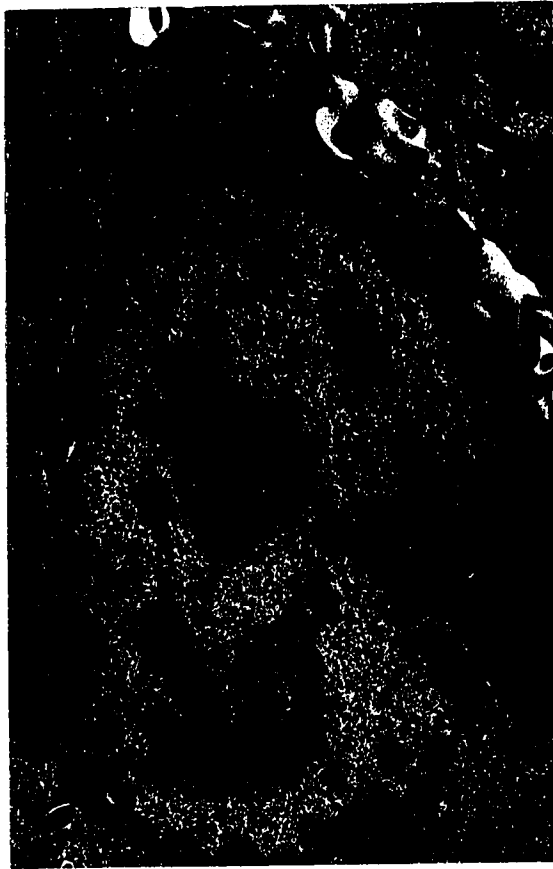


FIG. 1

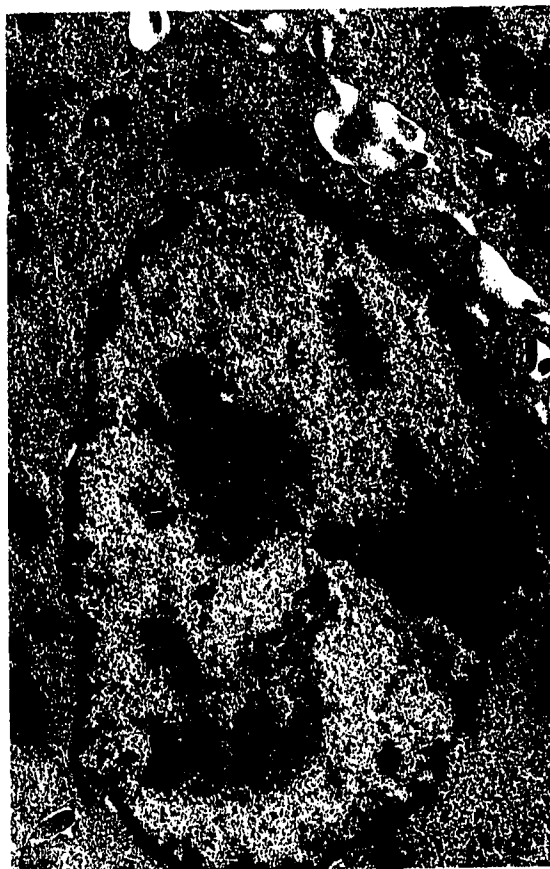




FIGURE 50

ELECTRON MICROSCOPE AUTORADIOGRAPH OF AN ICL VIRUS INFECTED CELL

MDCK cells were infected with ICL virus. Ten hours after infection the cells were labeled with  $^3\text{H}$ -thymidine for 4 minutes and used for electron microscope autoradiography. Silver grains are located over the inclusions. The nucleolus (right hand side) is pushed toward the nuclear membrane. X 18,000

Association of DNA Polymerase with the Early and Ring Form Inclusions

Since it was found that the early and ring form inclusions were the site of viral DNA synthesis, it was thought that these inclusions should contain enzymes involved in the synthesis of DNA. Therefore, the early and ring form inclusions were freshly purified from the infected cells and examined for the presence of DNA polymerase according to the "Material and Methods" description. Freshly prepared inclusions were used because it was found that the enzyme lost its activity after freezing and thawing. The incorporation of  $^3\text{H}$ -TTP into the acid insoluble material is shown in Fig. 51. The incorporation continued for 30 minutes then started to reach a plateau. To test the sensitivity of the acid insoluble product to digestion with DNase, at 40 minutes after incubation a sample was taken and treated with 5  $\mu\text{g}$  pancreatic DNase (Calbiochem, Los Angeles, California) at 37°C for 5 minutes. There was a loss of over 90% of the acid precipitable material. This experiment indicated association of a DNA polymerase with the early and ring form inclusions.

In the above experiment, the concentration of the cold nucleoside triphosphates used in the assay system was about 170 times that of  $^3\text{H}$ -TTP (see "Material and Methods"). In the next experiment extra amounts of cold TTP were added to the assay system. Addition of this extra cold TTP would lower the specific activity of the  $^3\text{H}$ -TTP and would decrease the effect of possible cross contamination of the commercial deoxynucleotide triphosphates (e.g. dATP can be contaminated slightly with dCTP; dGTP can be contaminated with dCTP or vice versa). The purpose was to add a certain amount of cold TTP into the assay

system and still retain the incorporation of  $^3\text{H}$ -TTP into the DNA at a high level. The result of such an experiment is shown in Fig. 52. Addition of 10  $\mu\text{M}$  cold TTP decreased the  $^3\text{H}$ -TTP incorporation to about 20% but the incorporation continued for 90 minutes. When 1  $\mu\text{M}$  TTP was added, about 70% of the initial  $^3\text{H}$ -TTP incorporation could be obtained.

In the subsequent experiment 1  $\mu\text{M}$  cold TTP was added to the assay system and the requirement for the 4 nucleoside triphosphates by the enzyme was tested. Four assay systems were prepared. One was the complete system and in each of the remaining 3, one of the deoxynucleoside triphosphates was omitted. It was found that in the absence of dGTP and dATP, almost no  $^3\text{H}$ -TTP was incorporated into the DNA (Fig. 53). In the absence of dCTP some incorporation was detected but compared with the complete system was very low (approximately 27%). This experiment indicated that the enzyme required all four nucleoside triphosphates for activity.

The relationship between the amount of inclusion input and the amount of  $^3\text{H}$ -TTP incorporation was also investigated. It was found that the enzyme activity was proportional to the amount of inclusion material added to the assay system. The incorporation of  $^3\text{H}$ -TTP into the DNA increased with increasing amounts of inclusion material up to about 250  $\mu\text{g}$  protein then started to reach a plateau (Fig. 54).

Since the inclusions contained a large amount of viral DNA, the exogenous template DNA was not used in the above experiments. To test the enzyme activity in the presence of exogenous DNA, 100  $\mu\text{g}$  of activated calf thymus DNA was added to the assay system. The result is shown in Fig. 55. Although in the presence of calf thymus DNA the

reaction continued for 45 minutes, the amount of  $^3\text{H}$ -TTP incorporation increased only about 60% (less than 2 fold).

Fig 51

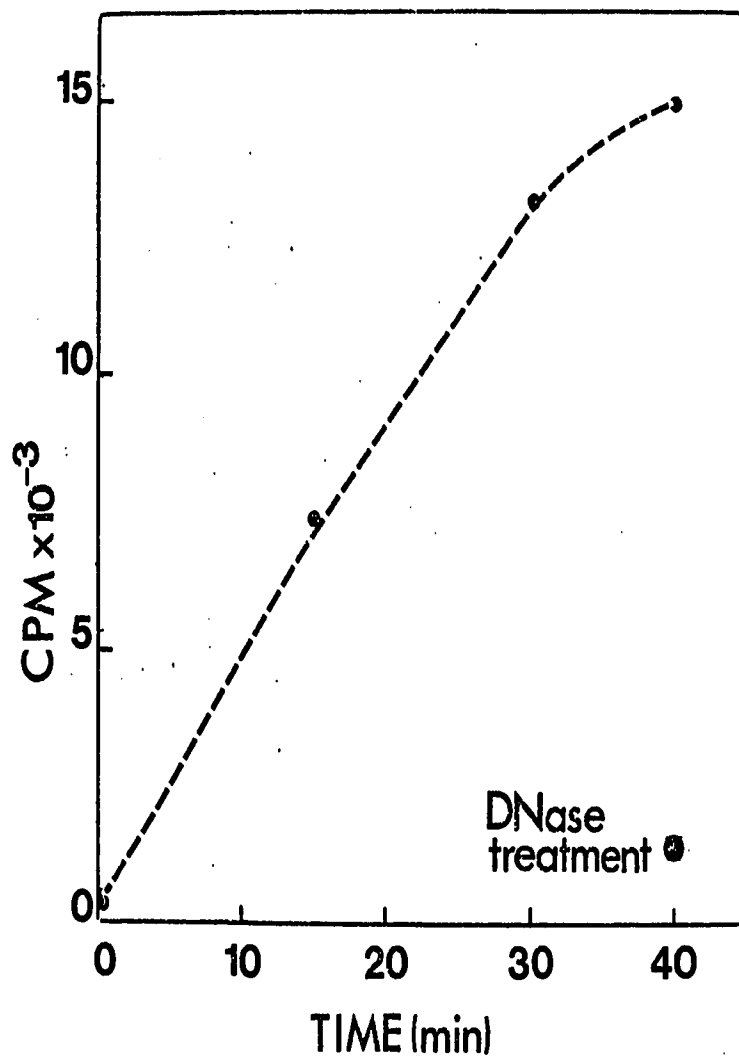


FIGURE 51

KINETICS OF DNA POLYMERASE OF INCLUSIONS

Four hundred  $\mu\text{g}$  of protein from purified inclusions was added to a reaction mixture containing dATP, dGTP, dCTP, and  $^3\text{H}$ -TTP in Tris buffer, pH 8.3. The mixture was incubated at  $37^\circ\text{C}$ . At the intervals shown, samples were removed and  $^3\text{H}$ -TTP incorporated into the acid precipitable material was determined. At 40 minutes a similar sample was removed and digested with DNase. The cpm in the acid insoluble material after DNase digestion is shown by a large circle.

Fig 52

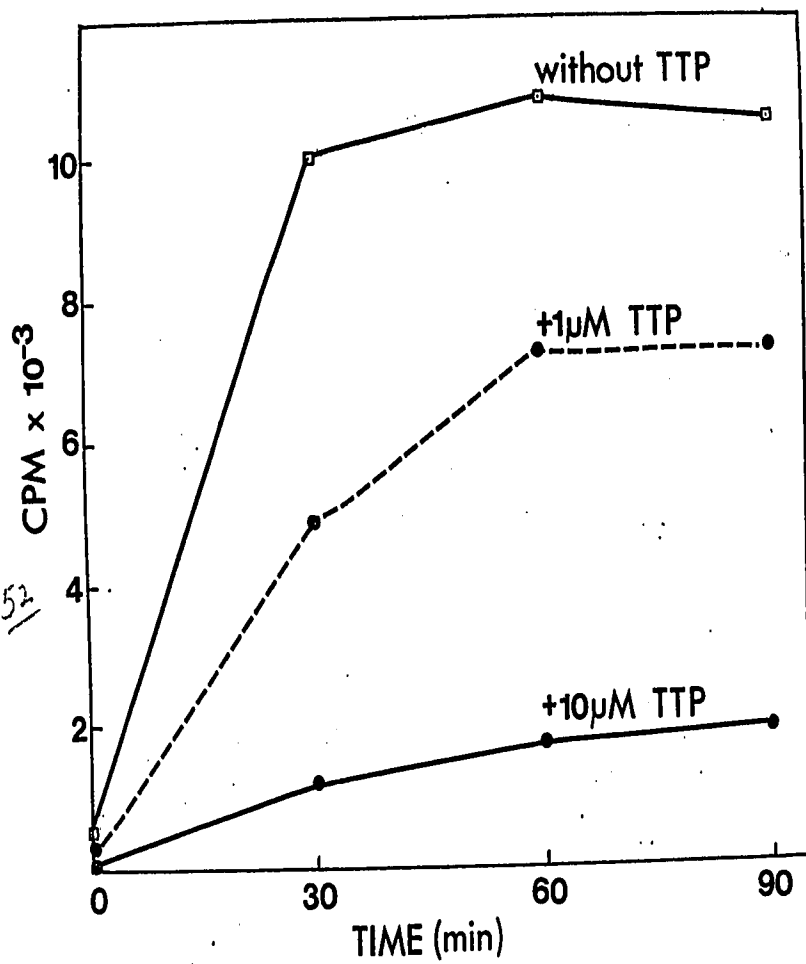


FIGURE 52

ENZYME ACTIVITY IN PRESENCE OF COLD TTP

The assay systems were similar to that in Fig. 51 except that different amounts of extra cold TTP were added to the reaction mixture.



Fig 53

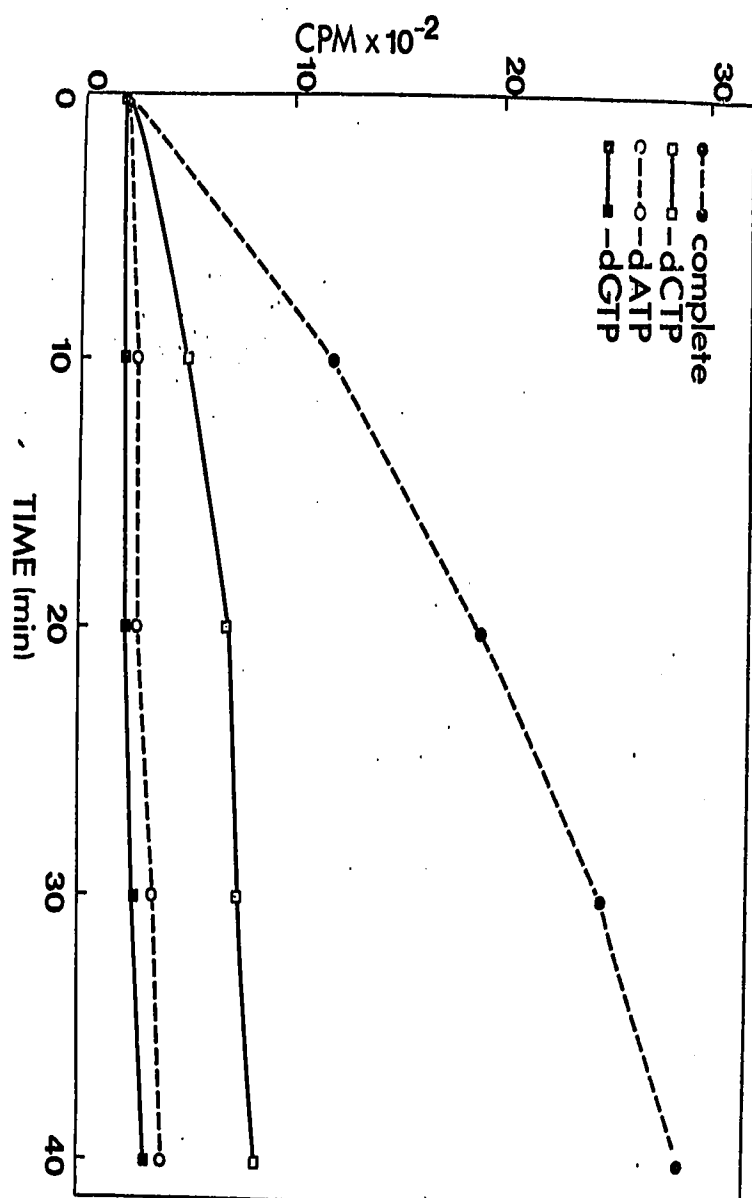


FIGURE 53

CHARACTERISTICS OF THE DNA POLYMERASE

Four assay systems each containing 130  $\mu$ g of protein from inclusions were prepared. One, the complete system, contained all the four deoxynucleoside triphosphates. In each of the remaining three, one of the cold deoxynucleoside triphosphates was omitted. C.p.m. represents  $^3\text{H}$ -TTP incorporated into the acid insoluble material.

Fig 54

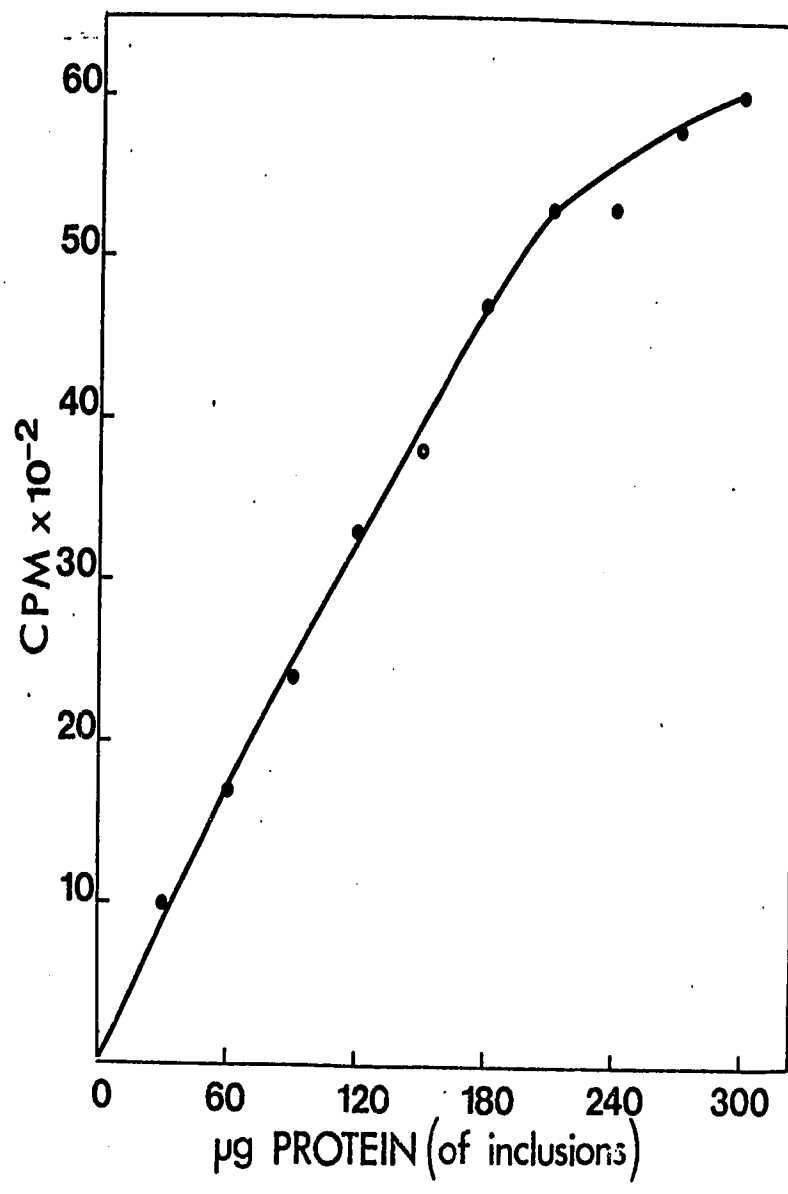


FIGURE 54

EFFECT OF INCLUSION CONCENTRATION ON  $^3\text{H}$ -TTP INCORPORATION

Different amounts of inclusions were added to the assay systems. The mixtures were incubated at  $37^\circ\text{C}$  for 30 minutes. Samples were taken at the end of the incubation period and  $^3\text{H}$ -TTP incorporation into the acid insoluble material was determined.

Fig 5b

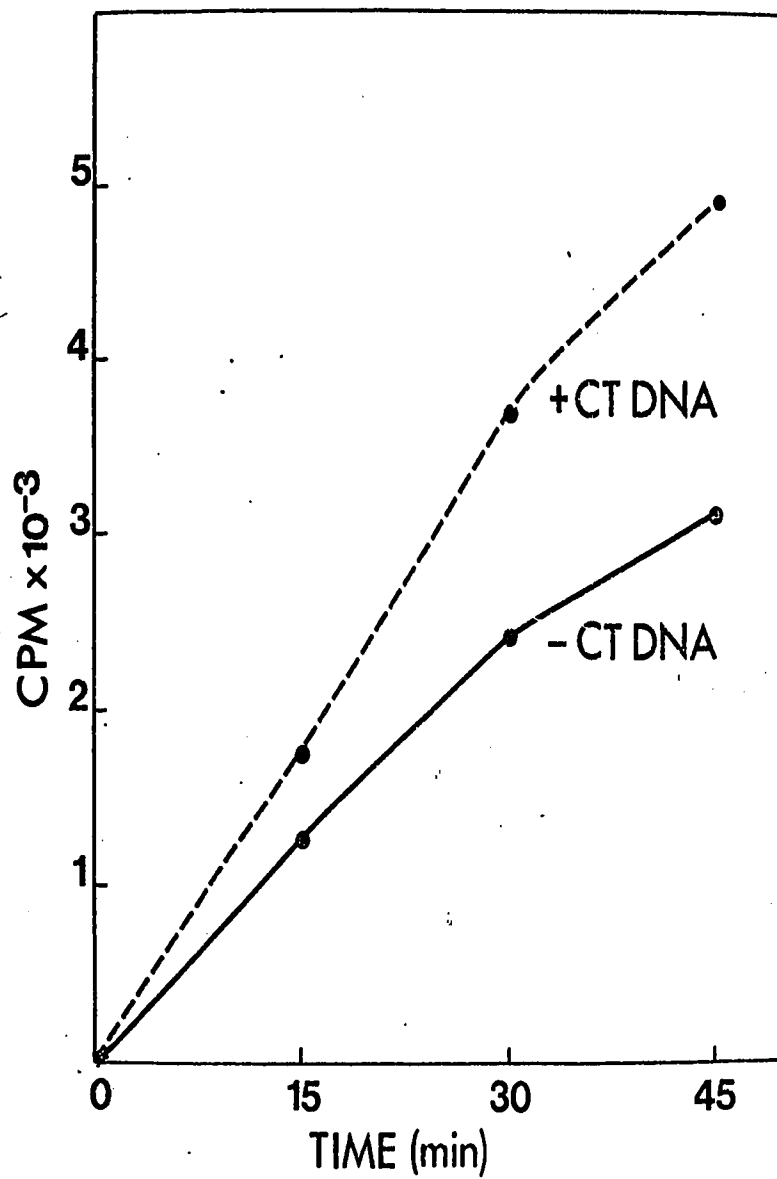


FIGURE 55

EFFECT OF CALF THYMUS DNA ON  $^3\text{H}$ -TTP INCORPORATION

Two assay mixtures, each containing 150  $\mu\text{g}$  of protein from inclusions, were prepared as described in "Material and Methods". To one of the two reaction mixtures, 100  $\mu\text{g}$  of activated calf thymus DNA was added.  $^3\text{H}$ -TTP incorporated into the acid precipitable material in both mixtures was determined.

#### Characterization of the Product of the DNA Polymerase

In the previous experiment it was found that the early and ring form inclusions contained viral DNA. In addition, the rapid incorporation of  $^3\text{H}$ -thymidine into these inclusions suggested that they were the site of viral DNA synthesis. Once the presence of DNA polymerase in these bodies was detected, it was decided to test the nature of the DNA product synthesized in vitro and to analyse its relationship with respect to viral DNA by equilibrium sedimentation in CsCl.

Four hundred  $\mu\text{g}$  protein of inclusions was added to the assay mixture and the concentration of  $^3\text{H}$ -TTP was increased to  $0.6 \mu\text{M}$ . The mixture was incubated at  $37^\circ\text{C}$  for 1 hour. The reaction was stopped by chilling the assay tube in an ice bath. Two hundred  $\mu\text{g}$  of host DNA was added to the mixture to facilitate the extraction of the DNA product from the assay mixture. DNA was extracted by pronase-SDS treatment followed by phenol extraction. Approximately 2 O.D. units of MDCK DNA was mixed with 15,000 c.p.m. of DNA product and the mixture was dissolved in CsCl. ICL virus DNA ( $150 \mu\text{g}$ ) was also used as a marker in a separate tube. The tubes were centrifuged to equilibrium as described in "Material and Methods". Three drop fractions were collected and the radioactivity and optical density ( $260 \text{ m}\mu$ ) of each fraction determined. Fig. 56 shows the equilibrium sedimentation patterns of the MDCK cell DNA and the product DNA. Cell DNA which was measured by its optical density alone banded in CsCl separately from the radioactive DNA (product DNA). It was found that the product DNA banded at the same position as the viral DNA marker and had the same density. These results indicated that the enzyme used viral DNA as a template and the product had a characteristic similar to that of viral DNA.

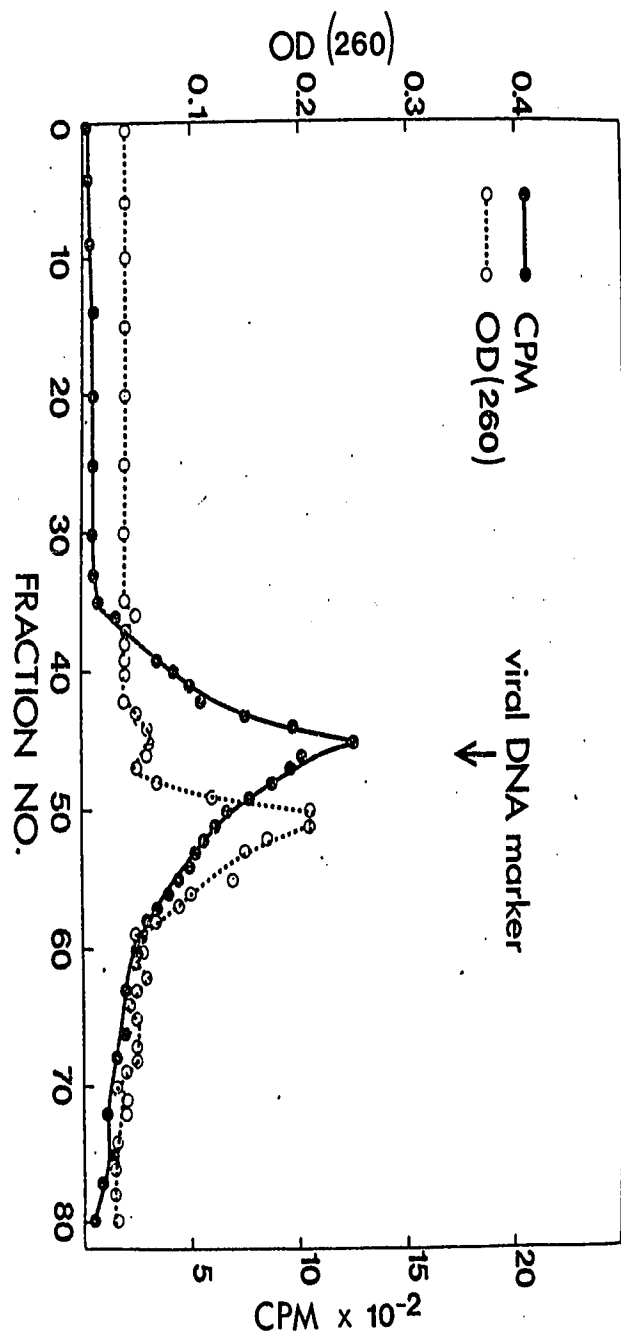


Fig 56



FIGURE 56

CHARACTERISTICS OF DNA PRODUCT

An assay mixture was prepared as described in the text. The mixture was incubated at 37°C for 1 hour. The reaction was stopped by cooling the mixture in an ice bath. The DNA product was extracted. A mixture of the labeled DNA product and unlabeled MDCK cell DNA was centrifuged in CsCl to equilibrium. ICL virus DNA was also centrifuged in a separate tube as a marker. Centrifugation was performed at 38,000 r.p.m. in an SW-51 rotor at 25°C for 50 hours. Fractions were collected from the bottoms of the tubes. C.p.m. in the acid insoluble material and the OD at 260 mμ of each fraction were determined.

MDCK cell DNA:	○-----○	OD <sub>(260)</sub>
DNA product:	○-----○	cpm

Treatment of Inclusions with Protamine Sulfate and Ammonium Sulfate

Since the enzymes bound to nucleoproteins can be liberated by treatment with high salt concentration (Morton, 1955), it was decided to release the enzyme from the inclusions by ammonium sulfate treatment. Inclusions were treated with 3 M ammonium sulfate according to the description in "Material and Methods". The mixture was centrifuged at 5,000 r.p.m. for 10 minutes. The pellet obtained was redissolved in Tris buffer and this was centrifuged as above. The final supernatant and pellet were assayed for the polymerase activity with and without calf thymus DNA (Fig. 57). Almost no activity was detected in the supernatant in the presence or absence of calf thymus DNA. In contrast, the polymerase activity was found to be associated with the pellet. The incorporation of  $^3\text{H}$ -TTP into DNA in the absence of calf thymus DNA showed that the DNA template was present in the pellet. Similar results were obtained when the inclusions were treated with protamine sulfate. Therefore, it would appear that the enzyme was bound to the DNA in the inclusions and could not be liberated from the DNA under the experimental conditions used. Whether different conditions of salt treatment would dissociate the enzyme from the nucleoprotein complex was not determined.

Fig

57

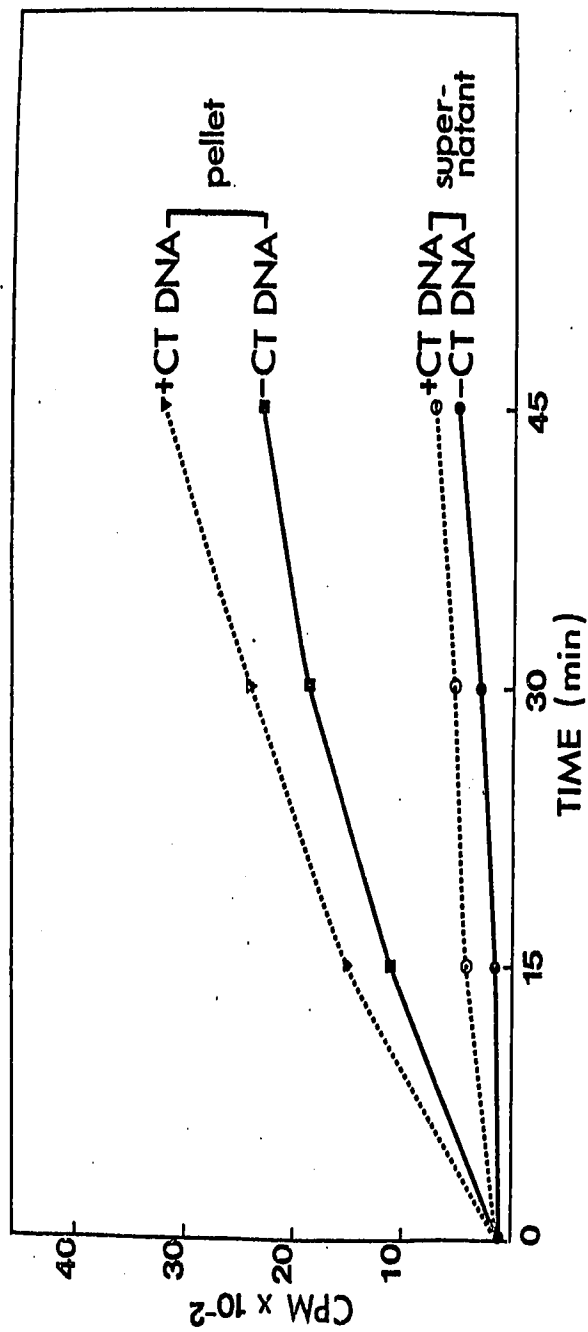


FIGURE 57

TREATMENT OF INCLUSIONS WITH AMMONIUM SULFATE

A suspension of inclusions containing 1 mg of protein was sonicated in the presence of 3 M ammonium sulfate then stirred in ice for 10 minutes. The mixture was centrifuged as described in "Material and Methods". The pellet was redissolved in Tris buffer and centrifuged again. The supernatant was saved and the pellet was resuspended in Tris buffer to a volume equal to that of the supernatant. Equal volume samples from the supernatant and the pellet were assayed for enzyme activity in the absence and presence (100  $\mu$ g) of calf thymus DNA.  $^3\text{H}$ -TTP incorporated into the acid insoluble material was determined.

DNA Polymerase in Infected and Uninfected MDCK Cells

It has been reported that the activity of DNA polymerase does not increase in KB cells infected with adenovirus type 2 (Green, 1962; Sheek, 1965). Since we found the association of a DNA polymerase with the early and ring form inclusions in ICL virus infected cells, it was of interest to determine the change in the level of DNA polymerase in MDCK cells after infection with ICL virus. To do so, the activity of the enzyme was measured in infected cells and compared with that in noninfected cells.

MDCK cells were grown in monolayers in 3 oz bottles. Before a complete monolayer was formed (cells were in the exponentially growing phase), they were divided into two groups. In one group, the cells were infected with ICL virus (moi of 100 P.F.U./cell). In the other group, the cells remained uninfected. Ten hours after infection both infected and noninfected cells were harvested and cells were separated from the supernatant by low speed centrifugation. The cell pellets were resuspended in 1 ml of Tris buffer containing KCl and  $MgCl_2$  (see the buffer system for DNA polymerase assay in "Material and Methods") and sonicated for 1 minute. Samples from both infected and uninfected cells were assayed for DNA polymerase in the presence of 100  $\mu$ g calf thymus DNA as described in "Material and Methods". This experiment was repeated exactly, except with MDCK cells in the stationary phase, to again compare the DNA polymerase activity in infected and noninfected cells.

Table III shows the results of such experiments. In exponentially growing cells there was no increase in DNA polymerase activity (measured

by  $^3\text{H}$ -TTP incorporation/mg protein/hour) after infection with ICL virus. In contrast, when the cells in stationary phase were infected with ICL virus, the enzyme activity doubled over that of noninfected cells.

Studies on the localization of DNA polymerase in mammalian cells have shown that DNA polymerase leaks out from the nuclei into the cytoplasmic fraction after breakage of cells in aqueous solution (Green, 1962; Sheek, 1966; Behki and Schneider, 1963). Since in MDCK cells infected with ICL virus the DNA polymerase was bound to the nuclear inclusions, it was decided to compare the level of the enzyme in the nuclei purified from infected and noninfected cells.

MDCK cells in the exponentially growing phase were infected with ICL virus (100 P.F.U./cell). A portion of cells was also kept uninfected. Ten hours after infection both infected and uninfected cells were harvested and the nuclei were isolated as described in "Material and Methods". The purified nuclei were sonicated for 30 seconds and samples from both infected and noninfected cells were assayed for DNA polymerase in the presence of calf thymus DNA. The results are shown in Table III. Although there was no difference in the enzyme activity of exponentially growing infected and noninfected whole cells, the level of the enzyme in the nuclei of infected cells was four times that in nuclei of noninfected cells.

In the above experiment, the activity of the enzyme was measured per mg protein per hour. When this experiment was repeated and the enzyme activity was calculated as cpm/nucleus/hour, it was found that the DNA polymerase content per infected nucleus was 5 times that of the noninfected nucleus.

TABLE III

DNA POLYMERASE IN INFECTED AND UNINFECTED CELLS

<u>State of Cells</u>	<u>Source of Enzyme</u>	<u>c.p.m./mg protein/hr</u>
Exponentially growing	Infected cells	7,684
	Uninfected cells	7,544
Stationary phase	Infected cells	8,148
	Uninfected cells	3,744
Exponentially growing	Infected nuclei	25,376
	Uninfected nuclei	6,528

MDCK cells in either the exponentially growing phase or in the stationary phase were divided into two groups. In one group the cells were infected with ICL virus for 10 hours. In the other group the cells were kept uninfected as controls. Cells were sonicated and assayed for DNA polymerase activity as described in the text. Nuclei of infected and uninfected cells in the stationary phase were isolated and similarly assayed for enzyme activity. The protein concentration of each sample was determined and the enzyme activity was calculated as c.p.m. <sup>3</sup>H-TTP incorporated into the acid insoluble material per mg protein of sample per hour.

RNA Polymerase in the Early and Ring Form Inclusions

Autoradiography studies on multiplication of ICL virus in MDCK cells (Yamamoto and Shahrabadi, 1971) showed the incorporation of uridine into the early and ring form inclusions which suggested the synthesis of RNA in these bodies. This led us to search for the presence of an RNA polymerase in the inclusions. An assay system was prepared as follows:

Purified inclusions .....	200 µg protein
GTP .....	0.3 mM
UTP .....	0.3 mM
ATP .....	0.3 mM
<sup>14</sup> C-CTP (Sp. Act. 60,000 c.p.m./n mole)....	0.1 mM
MgCl <sub>2</sub> .....	8 mM
MnCl <sub>2</sub> .....	1.5 mM
KCl .....	0.2 M
Mercaptoethanol .....	2 mM
Calf thymus DNA .....	200 µg

The mixture was made in 50 mM Tris buffer, pH 8, in a total volume of 1 ml and incubated at 37°C. For controls, similar assay systems were prepared. In one of the control systems inclusions were not added and in the other, the inclusions were first heated at 90°C for 15 minutes then added to the mixture. Samples were removed at 15 and 30 minutes and <sup>14</sup>C-CTP incorporated into the acid insoluble material was determined as described for the DNA polymerase assay. The results of such experiments were as follows:



<u>Assay System</u>	<u><math>^{14}\text{C}</math>-CTP Incorporation into the Acid Insoluble Material (picomoles/mg protein/hr)</u>
Complete	88
Without inclusions	3
With heated inclusions	4

These results suggested the presence of RNA polymerase in the early and ring form inclusions. Since further experiments were not carried out to characterize the enzyme, the nature of the product and the role of the enzyme in virus multiplication is unknown.

### Polyacrylamide Gel Electrophoresis of ICL Virus and Inclusions

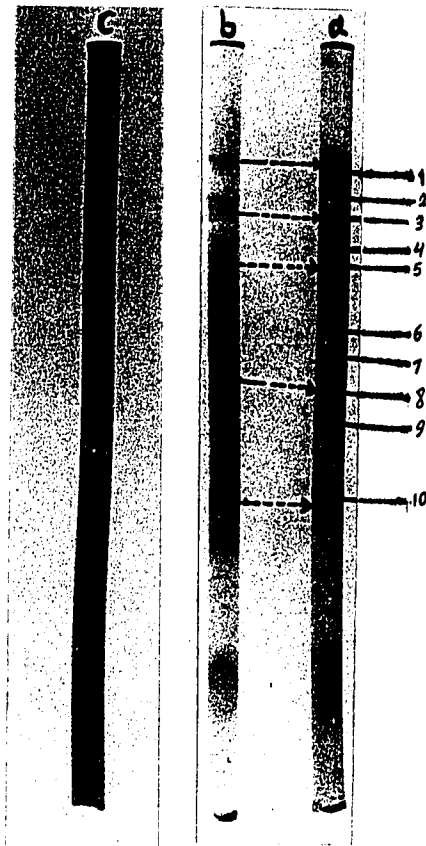
Purified early and ring form inclusions were analysed by polyacrylamide gel electrophoresis to characterize the enzyme DNA polymerase and to determine the total number of viral proteins present in these inclusions. ICL virus particles and the inclusions were treated with 1% SDS and electrophoresed in 10 cm gels composed of 5% acrylamide. The details of the procedures and the conditions of electrophoresis were described in "Material and Methods".

ICL virus showed 10 protein bands in acrylamide gel (Fig. 58a). Since the nature of these proteins was not determined, the results of these experiments were not conclusive. In relation to other electrophoresis studies performed in similar conditions on human adenoviruses (Laver *et al.*, 1968; Laver and Wrigley, 1969), it was assumed that band 1 (Fig. 58a) corresponded to hexon protein and bands 2 and 3 corresponded to penton base and fiber proteins, respectively. Band 10 which was a low molecular weight protein seemed to be one of the virus core proteins with high arginine content (Laver and Wrigley, 1969). Gel electrophoresis of the inclusions showed at least 7 bands (Fig. 58b). In comparison with the ICL virus gel (Fig. 58a) there was a trace of hexon protein present in the inclusions (band 1 in Fig. 58b). Band 2 in the inclusion gel also had similar mobility to band 3 in the ICL virus gel. There were other bands with similar mobilities to the proteins of ICL virus and these are shown by arrows. Since preliminary experiments revealed that DNA polymerase in inclusions was inactivated by SDS treatment, it was not possible to identify the enzyme after gel electrophoresis. The inclusion gel showed, however, the presence of a

protein (band 6, Fig. 58b) which corresponded to band 10 in the virus gel and presumably was virus core protein. The nature of bands 4, 5, and 7 could not be determined.

To support the assumption that band 1 of the virus gel was hexon protein, a small amount of ICL virus hexon material was prepared and tested as follows: ICL virus purified by CsCl centrifugation was dialysed against 2 liters of distilled water at 4°C for 40 hours (2 changes of distilled water). After dialysis, the virus suspension was centrifuged at 40,000 x g in an SW<sub>39</sub> rotor. The supernatant was saved and concentrated by polyethylene glycol. Electron microscopic examination of the supernatant revealed that it contained mainly hexon capsomers. When a sample of this preparation was tested by gel electrophoresis, a single band was obtained which had a similar mobility to band 1 in the ICL virus gel (Fig. 58c). This result suggested that band 1 in the ICL virus gel was hexon protein.

Fig 158



7

100 100

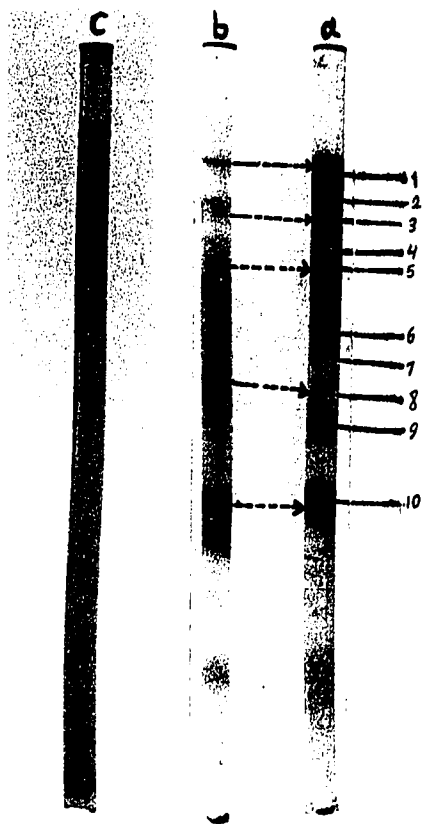


FIGURE 58

POLYACRYLAMIDE GEL ELECTROPHORESIS OF ICL VIRUS AND INCLUSIONS

ICL virus and purified inclusions were solubilized by treatment with 1% SDS. A sample of each preparation was electrophoresed in 5% acrylamide gel. The detail of the procedures and the conditions of electrophoresis were described in "Material and Methods". ICL virus hexons were prepared as described in the text and electrophoresed similarly.

a = ICL virus

b = Inclusions

c = ICL virus hexons

### DISCUSSION

Soon after the discovery of adenoviruses, Boyer et al. (1957) reported that adenoviruses induce morphological changes inside the nuclei of host cells. These morphological alterations were found to be the formation of intranuclear inclusion bodies which appeared at the early stage of infection. Since that time many adenovirus-cell systems have been examined both with the light microscope and the electron microscope and the presence of inclusions in infected cells has been demonstrated (Ginsberg and Dingle, 1965; Morgan et al., 1960; Martinez-Palomo et al., 1967). This may suggest that the formation of inclusions is an essential step in the developmental process of adenoviruses. To verify this suggestion it would be necessary to identify the nature of the inclusions with respect to their origin and chemical composition. Since no clear analysis of the inclusions has been reported, the role of these bodies in virus multiplication is still obscure.

ICL virus, like other adenoviruses, produces certain morphological alterations in the host cell. These sequential changes have been studied by the electron microscope (Yamamoto, 1969) and were reviewed in detail in the introduction. Enzyme cytochemistry and autoradiography studies on ICL virus infected cells (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971) showed that the early granular inclusions and ring form bodies which appeared at the early stage of infection were composed of nucleoprotein. The light staining inclusions and the dark spherical bodies which were found at the late stage were composed of protein. The results of the present study regarding the

multiplication of ICL virus in MDCK cells, the nature of inclusions and their role in virus multiplication will be discussed in relation to the previous studies (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971) of enzyme cytochemistry and high resolution autoradiography.

#### Growth of ICL Virus in MDCK Cells

The growth of ICL virus in MDCK cells was found to be similar to that of other adenoviruses (Ginsberg, 1958; Wilcox and Ginsberg, 1963). The virus had a long latent period (about 12 hours) and a release period of about 20 hours. Under the conditions employed, both the intracellular and extracellular virus hemagglutinins were detected at the time of appearance of infectious virus particles (Figs. 1, 2). Since penton monomers and free fibers of ICL virus do not directly hemagglutinate red blood cells under these conditions (Marusyk *et al.*, 1970), it seems that the appearance of hemagglutinin was due to the formation of mature virus particles.

#### Synthesis of Macromolecules in Infected Cells

The earliest detectable changes which occurred in ICL virus infected cells was an increase in the synthesis of macromolecules (Figs. 3, 4). An increase in the DNA content of infected cells was detected at 8 hours (4 hours before the formation of mature virus particles) and continued until 28 hours after infection. Similar results have been reported by Ginsberg and Dixon (1959) in HeLa cells infected with type 4 adenovirus and by Green (1962) in KB cells infected with type 2 adenovirus. These authors indicated that the increase in DNA content of adenovirus infected cells was due to the synthesis of viral DNA. We found that in ICL virus infected cells the DNA content of the inclusions



which appeared at 9-10 hours after infection was viral. This led us to the conclusion similar to that of the above investigators that the increase in DNA content of ICL virus infected cells was due to the synthesis of viral DNA.

The amounts of RNA and protein in ICL virus infected cells began to increase between 4 and 8 hours after infection. Since the samples were taken at 4 hour intervals, the exact time of increase in the number of macromolecules could not be determined. Everitt et al. (1971) found that synthesis of viral structural protein in KB cells infected with adenovirus type 2 began 9 hours after infection. Bello and Ginsberg (1967) found that host protein synthesis in adenovirus type 5 infected cells decreased at 16 hours and was completely inhibited by 20 hours after infection. They reported that most of the protein synthesized in adenovirus infected cells was viral coded. In ICL virus infected cells viral structural protein could be detected at 8 hours after infection (Fig. 32). It seems that the increase in the amount of protein in infected cells was mostly due to the synthesis of viral protein.

Increase in the RNA content of ICL virus infected cells was about 48% over that of noninfected cells. This agrees with the results obtained by Pina and Green (1969) who found a 30 to 80% increase in RNA content of KB cells infected with adenovirus type 2 and type 12. Whether this increase in RNA content is viral or host is not clear. It has been found that the synthesis of viral mRNA in adenovirus type 2 infected KB cells started at 8 hours after infection and continued until 18 hours (Rose et al., 1965). Recently Raska et al. (1971) reported that in BHK-21 cells infected with adenovirus type 12, RNA

synthesis increased 3 to 5 fold over that of noninfected cells. They found that part of this RNA was viral mRNA and the remaining was host ribosomal RNA. From these data it can be speculated that the increase in RNA content of ICL virus infected cells is probably due to the synthesis of both viral and host RNA.

#### Localization of Intracellular Antigens

In the initial experiments for localization of intracellular antigens, infected cells were exposed to ferritin conjugated antibody prior to fixation and embedding. Because of the lack of adequate penetration of the conjugate into the cells, the intracellular antigens did not react with the ferritin labeled antibody. It was decided to disrupt the cell membrane by quick freezing and thawing to allow access of the conjugate to the intracellular antigens. Although this method resulted in heavy staining of subcellular antigens with ferritin, the fine structure of cellular organelles was badly damaged. In addition, the large amount of nonspecific attachment of the ferritin to cellular components made it impossible to detect the specific antigen-antibody reaction. Therefore, an attempt was made to stain the antigens in thin sections with ferritin conjugate. The advantages of being able to stain with ferritin on thin sections are first, that the ferritin conjugate can attach directly to the antigens exposed at or near the surface of the sections without the destruction of membranes to allow access of the ferritin to the site of the intracellular antigens (Morgan et al., 1963), and second, that any number of antigen-antibody reactions can be carried out on a single block of cells.

Staining of thin sections of cells fixed in osmium tetroxide and

embedded in Epon 812 failed to give satisfactory results. No attachment of ferritin to the sections was observed. It was thought that the failure was due to the insolubility of the embedding medium which did not allow the access of ferritin conjugate to the antigens in thin sections. Other embedding media such as cross linked polyampholyte and glycol methacrylate were tried. Polyampholyte is known to be a water soluble medium and has been used by McLean and Singer (1964) for staining of antigens in thin sections with ferritin labeled antibody. Since this medium is highly hydrophilic, it was difficult to section. Instead, glycol methacrylate, which is also a water soluble medium, was used. However, staining of antigens fixed in osmium tetroxide was again unsuccessful. Since osmium tetroxide alters the structure of proteins to a large extent (Lenard and Singer, 1968), it was decided to use other fixatives. Glutaraldehyde, which is used in enzyme cytochemistry, is known to preserve the enzyme's activity to a low degree (see the review by Shnitka and Seligman, 1971). McLean and Singer (1970) used glutaraldehyde as a fixative and reported that the antibody combining site of cellular protein was preserved. In our system different concentrations of glutaraldehyde were used and it was found that this fixative largely inactivated the cellular antigens. However, formaldehyde fixation was found well suited to the preservation of the antigenic structure of the cell. When the cells were fixed in formaldehyde and embedded in glycol methacrylate specific staining of antigens in thin sections could be obtained. The next problem was to eliminate the non-specific attachment of ferritin to the supporting film on the grids. McLean and Singer (1970) coated the grids with bovine serum albumin (BSA) to overcome the problem of nonspecific staining. We found that the use

of BSA coated grids reduced the background of nonspecific staining, but did not eliminate it completely. Therefore, it was decided to stain thin sections with ferritin conjugate before putting them on the grids. This procedure (Fig. 9) was found to abolish the background staining. The only disadvantage of this method was the spreading of the sections when floated on water or phosphate buffer during the washing stages, resulting in some loss of preservation. This problem could be partially reduced by the use of low concentrations of BSA in the washing solutions. This method of staining thin sections of intracellular antigens with ferritin conjugated antibody was found to be quite specific. It was used successfully for antigens produced in eucaryotic and procaryotic cells (Figs. 10, 13) and probably is applicable to a variety of antigen-antibody systems. The major problem in this technique is the lack of good preservation of structural details of cells fixed in formaldehyde. In addition, some of the limited number of antigenic sites which are exposed to the surface of thin sections are probably inactivated during fixation and embedding. This means that if the amount of a specific antigen present in a cell is small, the retained antigenic determinants exposed to the surface of thin sections may be too few for successful localization.

#### Presence of Viral Antigen in Early and Ring Form Inclusions

The purpose of the development of the ferritin staining method was to determine the antigenic content of the ICL virus induced inclusions in infected cells. Although a number of immunofluorescent studies on localization and development of adenovirus antigens in

infected cells have been reported (Pereira et al., 1959; Boyer et al., 1959; Hayashi and Russell, 1968), the fluorescent staining antigens have not been clearly correlated with the inclusions observed with the electron microscope. Kalnins et al. (1966) and Levinthal et al. (1967) used ferritin conjugated antibody for localization of viral antigens in adenovirus type 12 infected cells. These investigators disrupted the cells by means of freezing and thawing to allow the access of ferritin conjugate to intracellular antigens. In addition to the damage to cellular organelles caused by freezing and thawing, the nonspecific attachment of ferritin to the cellular components and the problems of entrapment of excess ferritin in the cells makes the interpretation difficult. We used the method of indirect staining of intracellular antigens with ferritin conjugated antibody combined with fluorescent staining technique and determined the development and distribution of ICL virus capsid antigens in infected cells. Antisera against ICL virus capsid antigens used in these experiments had been prepared against purified components of ICL virus capsid proteins (see "Material and Methods"). In addition each of these antisera had been adsorbed to the heterologous antigens. The purity of the antisera was also checked by immunodiffusion and immunoelectrophoresis. Immuno-gel diffusion tests showed that only a single precipitin band was formed between each of these antisera and soluble antigens of ICL virus whereas antiserum against the whole virus particle produced at least three different bands. Similar results were obtained with immunoelectrophoresis tests. The purity of the antisera had been tested by Marusyk using specific ICL virus capsid antigens. Since we did not

have the purified capsid antigens, soluble antigens extracted from ICL virus infected cells were used. It has been shown that antisera against the whole adenovirus type 2 particle produced at least 3 precipitin bands against the virus soluble antigens, whereas specific antisera against individual capsid antigens produced only a single precipitin line (Laver and Wrigley, 1969). We also found a similar pattern of reaction in the ICL antigen-antibody system gel diffusion test. Knowing that these antisera had been pretested (personal communication with R. G. Marusyk, Department of Virology, Karolinska Institute, Stockholm) and considering the results of the immunogel diffusion test, we were satisfied with the purity and specificity of the antisera.

The early initial inclusions and ring form bodies which were stained with ferritin conjugated antibody contained fiber antigen. Using the fluorescent technique the fiber antigen was demonstrated as fine flecks and dots of fluorescent stain in the nuclei of 8 hour infected cells. These increased in size and appeared as rings by 10 hours after infection. The dots and rings of fluorescent stain correspond to the early and ring form inclusions which were stained with ferritin conjugated antibody using fiber antiserum (Figs. 17, 20, 33, 35). It was concluded that the early initial inclusions and ring form bodies observed in infected cells with the electron microscope contain fiber antigen. Although the amount of ferritin attached to these inclusions was low, the number of ferritin-conjugate molecules per unit surface of inclusions was significantly higher than in the surrounding area (background). The low attachment of ferritin conjugate to the inclusions was probably due to their low content of fiber antigen.

Chemical analysis of the purified inclusions showed that a major part of these bodies was composed of DNA and RNA. Recently Everitt et al. (1971) indicated that in KB cells infected with adenovirus type 2 there was an excess synthesis of fiber antigen over hexon and penton antigens during the early stage of infection. It would appear that the early and ring form inclusions not only contain viral DNA but are also depots for early synthesized fiber antigen.

The lack of ferritin attachment to the sections of early and ring form inclusions treated with hexon and penton base antisera is in agreement with the absence of hexon and penton base fluorescent antigens in 9 hour infected cells. Hayashi and Russell (1968) reported that in human adenovirus type 5 infected cells the hexon and fiber antigens appeared at the same time and the penton base antigen appeared one hour later. In ICL virus infected cells the fiber antigen was detected 2 hours before the appearance of the hexon and penton base antigens. The faintly diffuse fluorescent staining of penton base and hexon antigens at 10 hours after infection could be due to the presence of small numbers of virus particles since in sections of cells 11 hours after infection scattered virus particles could be observed which were stained with ferritin using hexon antisera (Fig. 22). It seems that at the early stage of infection the fiber antigen is synthesized and incorporated first into the early and ring form inclusions whereas the hexon and penton base antigens are assembled into the virus particles soon after synthesis and are not maintained in a pool during this stage of infection. The intensely fluorescent staining of hexon and penton base antigens at the periphery of the nuclear membrane which

appeared at 12 hours after infection was most likely due to the formation of virus particles since the ferritin staining of sections treated with hexon antiserum showed large numbers of virus particles in aggregate form close to the membrane and heavily labeled with ferritin conjugate.

Although the synthesis of adenovirus type 2 antigens has been shown to occur in the cytoplasm (Thomas and Green, 1966; Velicer and Ginsberg, 1968, 1970), there was very little detectable fluorescent staining antigen in the cytoplasm of ICL virus infected cells.

#### Presence of ICL Virus Capsid Antigens in Dark and Light Staining

##### Inclusions

At the late stage of infection (16 hours) the nuclei of infected cells were completely filled with fluorescent staining antigens and their relationship to the various nuclear inclusions could not be clearly distinguished. At the electron microscope level dark and light staining inclusions were observed in cells at the late stage of infection. On the basis of ferritin staining the light inclusions reacted with the hexon, penton base, and fiber antisera. It was concluded that these inclusions contained all the three viral capsid antigens. Virus particles in sections treated with penton base and fiber antisera did not react strongly with ferritin conjugate (Figs. 24, 26). This low attachment of ferritin to virus particles is probably due to the small number of penton base and fiber antigens exposed to the antibody molecules on the surface of thin sections. In contrast, the hexon antiserum reacted strongly with the virus particles as can be seen from the heavily stained virus particles (Fig. 29).

In a previous study (Yamamoto and Shahrabadi, 1971) it was shown



that the light staining inclusions resulted from the accumulation of some protein synthesized during the period of infection. Since they contained all three capsid antigens, it seems that some of the capsid antigens synthesized during infection are not assembled into virus particles but accumulate and appear as light staining inclusions. Initial experiments on the purification of light staining inclusions revealed that these inclusions disintegrated immediately after the disruption of infected cells. It is tempting to speculate that these inclusions represent the major source of soluble antigens which are released during the extraction of infected cells (Marusyk *et al.*, 1970) and their close spacial relationship to the virus particles (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971) suggests that they may have a role in virus assembly. In adenovirus type 5 infected cells, formation of paracrystalline inclusions composed of protein have been reported (Morgan *et al.*, 1957; Morgan *et al.*, 1960). Similar structures have been observed in a strain of adenovirus type 2 infected cells (Weber and Stich, 1968). On the basis of immunofluorescent staining these paracrystalline inclusions have been suggested to be of nonviral origin (Boyer *et al.*, 1959). These crystals were not found in ICL virus infected cells but may be analogous to the light staining inclusions.

The dark staining inclusions which have been found to be composed of protein (Yamamoto and Shahrabadi, 1971) did not contain penton base and fiber antigen but did contain hexon antigen as shown by ferritin staining (Fig. 30). In adenovirus type 12 infected cells the presence of dark granular inclusions susceptible to proteolytic enzyme digestion was reported by Martinez-Palomo *et al.* (1967) but the presence of

specific types of viral antigens in these inclusions was not determined although it was suggested that they contained viral structural antigens. In ICL virus infected cells the dark inclusions were only lightly labeled with ferritin conjugate. Since they appeared as compact structures with intensely packed granular proteins, it is most likely that in addition to the hexon antigen, the dark inclusions contain some other proteins whose nature is not known.

Bello and Ginsberg (1967) reported that in adenovirus type 5 infected cells the synthesis of host protein was decreased at 16 hours and completely inhibited by 20 hours after infection. Since the dark inclusions in ICL virus infected cells were synthesized from 14 to 20 hours after infection, it is possible that at this time the host protein synthesis was stopped; therefore, the protein synthesized during this period would be viral coded. If this possibility is true, the dark inclusions should contain only viral coded proteins. These proteins may be viral core proteins, viral coded enzymes, or some nonfunctional proteins. Further studies are necessary to elucidate this problem.

Adenoviruses are known to produce inclusions inside the nucleus of infected cells (see reviews by Ginsberg and Dingle, 1965; Rhodes and Van Rooyen, 1968; Fenner, 1969). In the present study it was found that the dark inclusions were present both in the nucleus and cytoplasm (Figs. 28, 42). Although some dark staining bodies present in the cytoplasm of cells infected with infectious canine hepatitis virus have been referred to as lysosomes (Givan and Jezequel, 1969) we found that the cytoplasmic inclusions in ICL virus infected cells resembled the

nuclear dark staining inclusions and were not found in noninfected cells. These cytoplasmic inclusions were synthesized at the same time as the nuclear inclusions (observations based on autoradiography experiments) and contained some hexon antigen. Since the origin of these inclusions was not clear, it was thought that they were formed first in the cytoplasm then transferred into the nucleus. If this were so, as the infection progressed the ratio of the number of nuclear inclusions to the number of cytoplasmic inclusions would increase. Analysis of the data obtained from the counting of inclusions (Table I) revealed that from 14 to 19 hours after infection the dark inclusions were present in both the nucleus and cytoplasm in almost equal number. The only conclusion which could be made from these data was that at a certain stage of infection some of the protein synthesized aggregated by an unknown mechanism and formed the dark inclusions. This aggregation could occur both in the cytoplasm and nucleus.

At the level of the electron microscope the inclusions found in the cytoplasm at the late stage of infection were labeled lightly with ferritin conjugate using hexon antiserum (Fig. 43). At this stage of infection the cytoplasm of some of the infected cells was stained faintly with fluorescein conjugated antibody. Since at this time the release of virus particles into the cytoplasm had begun, it was not possible to determine whether this faintly fluorescent stain in the cytoplasm was due to the presence of dark inclusions or to the scattered virus particles.

### Characteristics of the Early and Ring Form Inclusions

#### A. Purification

To analyse the nature of the early and ring form inclusions and to determine their role in virus multiplication it was necessary to isolate them from the infected cells. With the procedures described in "Material and Methods", these inclusions could be obtained in a pure form. The purity of the inclusions was checked both morphologically and quantitatively by isotopic labelling. It was found that the final preparation contained about 0.36% host chromatin and less than 0.5% nucleoli. In addition, density gradient centrifugation of DNA extracted from the inclusions showed that the DNA was banded as a single uniform band without any detectable host DNA. The yield of such a pure preparation was low (22.5% of the total amount of inclusions present in infected cells). The reasons for this low recovery were probably (i), most of the inclusions were broken down during sonication, and (ii), since these inclusions were heterogenous in size only the ones with a certain size could be purified by differential centrifugation in sucrose.

#### B. Chemical Composition

Cytochemical studies by Yamamoto (1969b) showed that the early granular inclusions and the ring form bodies in ICL virus infected cells were basophilic and Feulgen positive suggesting the presence of DNA in these bodies. Further studies using electron microscope autoradiography combined with enzyme cytochemistry (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971) revealed that the early and ring form inclusions were composed of DNA, RNA and protein. However, the amount of these macromolecules could not be determined quantitatively. In the

present study chemical analysis of purified inclusions showed that they contained about 51% DNA, 46% protein and 3% RNA. The nature of the DNA content of the inclusions was determined by density gradient centrifugation in CsCl (Figs. 47, 48). When a mixture of DNA extracted from MDCK cells and ICL virus was centrifuged in CsCl to equilibrium, it sedimented in two separate bands. MDCK DNA banded in a region with a density of 1.703. This density was similar to the density of KB cell DNA with a value of 1.702 (Green, 1962) and to the density of other human tissue culture cell DNA with a value of 1.703 (Sueoka, 1961). Viral DNA was found to have a density of 1.716 which is close to the density of adenovirus type 2 DNA with a value of 1.718 (Green, 1962). Since the DNA extracted from the early and ring form inclusions had a density of 1.716 which was equal to that of ICL virus DNA, it was concluded that these inclusions contained only viral DNA. This is in agreement with the results of previous studies (Shahrabadi, 1969; Yamamoto and Shahrabadi, 1971) which showed that the DNA content of the early and ring form inclusions was viral since it was incorporated into the virus particles.

The presence of RNA in the early and ring form inclusions also agrees with previous results of autoradiography experiments (Yamamoto and Shahrabadi, 1971) which showed incorporation of uridine into these inclusions. However, the nature of this RNA was not determined. Raska et al. (1971) reported that in BHK-21 cells infected with adenovirus type 12 the rate of host RNA synthesis increased several fold. A previous study on ICL virus infected cells (Yamamoto and Shahrabadi, 1971) showed that the nucleoli were actively involved in RNA synthesis during the

period of infection. Whether the RNA present in the inclusions is synthesized directly in these bodies or originates from the nucleoli is not quite clear. Since the presence of RNA polymerase in the inclusions was detected, and since these inclusions were shown to be a major source of viral DNA, it seems more likely that the RNA associated with these bodies was viral mRNA which was copied directly from the virus specific DNA. Hybridization experiments on the RNA of inclusions is required to elucidate the problem.

#### Site of Viral DNA Synthesis

In autoradiography experiments it was shown that the early and ring form inclusions contained newly synthesized viral DNA. Since the time of isotopic labeling was long (1 hour), the site of viral DNA synthesis could not be determined. Comings and Kakefada (1968) reported that the synthesis of cell DNA in synchronized human amnion cells was associated with the nuclear membrane. In ICL virus infected cells the question was whether the viral DNA is synthesized in association with the nuclear membrane or is synthesized inside the nucleus and then accumulated in the early and ring form inclusions. Autoradiography experiments on infected cells labeled with tritiated thymidine for a short time (4 minutes) revealed that radioactivity was mainly associated with the early and ring form inclusions (Fig. 50). This rapid incorporation of thymidine into the inclusions suggested that these bodies were the site of viral DNA synthesis. This suggestion encouraged us to search for the presence of DNA polymerase in the purified early and ring form inclusions. It was found that in the presence of the inclusions  $^3\text{H}$ -TTP was incorporated into the acid insoluble material. The product

was sensitive to digestion with DNase indicating that it had the properties of DNA (Fig. 51). The enzyme was inactivated by freezing and thawing and required all the four nucleoside triphosphates for activity. Since the enzyme activity did not increase substantially in the presence of activated calf thymus DNA, this suggested that the viral DNA present in inclusions was probably a natural template for the enzyme. It is also unlikely that the viral DNA has an advantage in competing with cell DNA for synthesis by the DNA polymerase. Green (1962) reported that the enzyme extracted from KB cells infected with adenovirus type 2 was not active in the presence of unheated viral DNA but heated DNA from adenovirus, KB cells and calf thymus were equally effective as templates for the DNA polymerase.

The origin of the enzyme associated with the inclusions is not clear but the following possibilities can be assumed:

I. The virus may induce the synthesis of a new enzyme like those observed in T-even infected E. coli (Kornberg et al., 1959) for the specific task of synthesizing viral DNA.

II. The existing host enzyme may be involved in the synthesis of viral DNA.

III. The virus may induce excess synthesis of the host enzyme to meet the needs for expanded DNA synthesis.

Ledinko (1968) reported that the DNA polymerase in human embryonic kidney cells infected with adenovirus type 12 had properties similar to that in noninfected cells. Since in ICL virus infected cells the in vitro product of the enzyme was found to be similar to viral DNA with respect to its density, it was concluded that the enzyme used viral DNA for template.

Reports on the DNA synthesizing system in adenovirus infected cells are controversial. In 1962 Green reported that there was no increase in the activity of DNA polymerase in exponentially growing KB cells infected with adenovirus type 2 whereas in vaccinia virus infected cells the level of the enzyme was increased 3 fold over that of noninfected cells. This experiment was repeated in 1964 by Green and Pina with similar results; that is, no increase in the DNA polymerase level in infected cells. Ledinko (1968) found a 1.5 to 3 fold increase in DNA polymerase in human embryonic kidney cells infected with adenovirus types 2 and 12 whereas Pina and Green (1969) observed no increase of the enzyme in exponentially growing KB cells infected with adenovirus types 31 and 12.

These controversial data have been interpreted thus: "... the degree of stimulation of enzyme activity in adenovirus infected cells appears to depend upon the cell type and its physiological state." (Green, 1970). This interpretation seems to be true in MDCK cells infected with ICL virus since the enzyme activity was equal in exponentially growing noninfected and infected cells but in cells in the stationary state the enzyme level in infected cells was two times that in noninfected cells.

In 1961 Smellie and Easton suggested that in mammalian cells the DNA polymerase was located in the cytoplasm and not the cell nucleus. Behki and Schneider (1963) and Smith *et al* (1963) found that the presence of DNA polymerase in the cytoplasm was due to the leakage of this enzyme from the nucleus into the cytoplasmic fraction during cell fractionation. Green (1962) reported that in noninfected KB cells DNA



polymerase was present mainly in the cytoplasmic fraction whereas in the cells infected with adenovirus type 2 the enzyme was associated with the nuclei. Similar results were reported by Sheek (1966) in KB cells infected with adenovirus type 2. They suggested the possibilities that either infection with adenovirus would cause the transfer of the enzyme from the cytoplasm to the nucleus or infection would alter the permeability of the nuclear membrane thus preventing the leakage of the enzyme into the cytoplasm.

We compared the level of DNA polymerase in purified nuclei of infected and noninfected cells and found a four fold increase in the enzyme activity in infected nuclei over that in noninfected nuclei whereas the enzyme activity in whole cells of both the preparations (infected and noninfected cells) was equal. This increase in DNA polymerase in infected nuclei was due to the association of the enzyme with the nuclear inclusions since it was found that the enzyme was tightly bound to the early and ring form inclusions. Whether the pre-existing host enzyme binds to the viral DNA or a new enzyme is induced upon infection is not clear.

#### Role of Inclusions in Virus Multiplication

From the available data it was concluded that the ring form inclusions in ICL virus infected cells are the site of viral DNA synthesis. The synthesized DNA accumulates in the inclusions and later on is incorporated into the virus particles (this was shown in the previous autoradiography studies by Shahrabadi (1969) and Yamamoto and Shahrabadi (1971). It seems that the starting point for virus assembly is the ring form inclusion. In addition to being the sites of synthesis

and accumulation of viral DNA, these inclusions are the sites of synthesis of an RNA which is presumable viral mRNA. They also contained several different proteins (Fig. 58). Some of these proteins which have the same mobility as the viral core proteins in acrylamide gel may have an important role in virus assembly. It is known that cells infected with adenovirus in the absence of arginine apparently produce both structural and nonstructural antigens and virus DNA but these are not assembled into infectious virus (Rouse and Schlesinger, 1967; Green, 1970). In vitro studies on the assembly of adenovirus type 5 by Winters and Russell (1971) showed that the appearance of infectious particles was dependent on the presence of an arginine rich factor in the reaction mixture. This arginine rich protein has been shown to be one of the constituents of the virus core (Russell et al., 1968). Recent cytochemical studies by Russell et al. (1971) showed that the ring form inclusions in adenovirus type 5 infected HEK cells were the sites of accumulation of arginine rich basic proteins.

From all the above data, it can be assumed that the viral DNA is made in the early and ring form inclusions where it accumulates and codes for viral mRNA. Some of the proteins synthesized by viral mRNA such as core proteins and fiber protein also accumulate in the inclusions and form a nucleoprotein complex. Once these starting materials for the formation of virus particles are made, some of the viral core proteins bind to viral DNA and change its configuration to that of a compact dense viral core. Capsid proteins which are synthesized in the cytoplasm are transferred into the nucleus and assembled into the preformed viral core. The formation of viral cores in the inclusions

is limited by some factors and cannot meet the demand of the expanded synthesis of viral capsid protein. Therefore, the excess of viral capsid antigens accumulates and appears as light staining inclusions.

Accepting the above assumptions there are still several questions which can be raised:

I. Why and how do the viral DNA and some viral protein accumulate and form the early and ring form inclusions?

II. Why do some of the proteins synthesized at the late stage of infection accumulate as dark inclusions?

III. Do these dark inclusions play any role in the formation or release of virus particles?

IV. What are the factors which limit the incorporation of the whole synthesized viral DNA and proteins into the infectious virus particle?

It is hoped that future studies will answer these questions.

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