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THE CYTOTOXICITY OF ULTRAVIOLET LIGHT
IRRADIATED REOVIRUS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF
THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY
DECEMBER 1971

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DEDICATION

The author wishes to dedicate this work to the loving memory of his late parents. May they attain Nirvāṇā.
ACKNOWLEDGMENTS

I wish to thank Dr. Albert A. Benedict and the Department of Microbiology for the financial support, and all the members of the Doctoral Dissertation Committee for their patience and advice. I am thankful to Dr. Kenneth P. Camyre for his assistance in the RNA base composition studies, and Dr. T. Yamamoto for useful suggestions in the preparation of this manuscript. I wish to express my gratitude to the United States Education Foundation in Ceylon for the Fulbright travel award given.

I am indebted to my wife Kamala for her encouragement, understanding and sacrifice during my doctoral studies. Lastly, my son Senaka and daughter Udeni deserve special thanks for their understanding and sacrifice in their own little ways.
ABSTRACT

When reovirus type 2 was exposed to ultraviolet light (UV) for 2-5 minutes, it acquired a cytotoxic property to HeLa cells. Maximal toxicity was attained after 10 minutes irradiation and decreased to undetectable levels by 60 minutes. The incident photo energy dose at maximum cytotoxicity level was $6 \times 10^7$ ergs/cm$^2$. The production of UV-induced cytotoxicity was temperature dependent with a maximum at 37°C.

The toxic factor was found to be associated with the virus proteins since it was demonstrated that the "empty" particles of reovirus exposed to ultraviolet light were also toxic to HeLa cells. In addition, studies with virus protein components produced by selective chemical degradation of the mature reovirus with urea showed that the cytotoxicity was associated with the outer capsid proteins.

Virions in which the RNA polymerase enzyme was activated by brief heat treatment (70°C, 30 sec) were not toxic to HeLa cells. Extracted RNA exposed to UV-irradiation was also found not to be cytotoxic.

Examination of the cytotoxic virus particle indicated no detectable alterations in the following properties: adsorption rates to HeLa cell monolayers, hemagglutination of human "0" erythrocytes, interferon inducing ability, RNA transcriptase activity, virus architecture, buoyant density in Cesium chloride, capsid protein components, viral double-stranded RNA and adenine-rich RNA. In contrast, upon continued irradiation for 60 minutes the loss of cytotoxic property was accompanied by the following: loss of viral hemagglutination, reduction in interferon inducing ability, derangement of the virus architecture,
increase in buoyant density, change in electrophoretic mobility of the capsid protein components and alteration in the viral double-stranded RNA.

The effect of pre-infecting HeLa cells with viable reovirus before treatment with an equivalent multiplicity of UV-irradiated virus was determined. Viral antigen synthesis and the production of infectious virus was not interfered with to any significant level. Furthermore, the induction of cytotoxicity was not interfered with by pre-infecting the cells with homologous or heterologous viable reovirus.

Pre-treatment of mouse L cell cultures with interferon at concentrations which inhibited reovirus yields by 80-90% and vesicular stomatitis virus yields in excess of 99% failed to prevent the reovirus induced cytotoxicity.

The toxic property of UV-irradiated reovirus was not dependent on the cell system in which the virus was passed, but was a specific property of the virus itself. All three serotypes of reovirus acquired this toxic property on exposure to ultraviolet light.

On examination of the sensitivity of different cell types to the UV-irradiated reovirus induced toxicity, it was found that the established cell lines in general were more sensitive than the primary cell cultures.
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CHAPTER 1

General Introduction

Cytotoxicity is one of several possible events that could occur as a result of virus-cell interaction and it is used to describe the cytopathic changes occurring in the virus infected host cell usually in the absence of the synthesis of infectious virus.

The phenomenon of virus induced cytotoxicity has been observed in several virus cell systems (Pereira, 1961; Smith, 1969) and can be caused by (a) infectious virus, e.g. vaccinia (Bernkopff, et al., 1959; Brown, et al., 1959), rabbit pox (Appleyard, et al., 1962), adenovirus (Levy, et al., 1957; Pereira and Kelly, 1957), mumps (Henle, et al., 1954; Russell and Morgan, 1959), measles (Toyoshima, et al., 1960; Cascarido and Karzon, 1965), vesicular stomatitis virus (Cooper and Bellet, 1959; Huang and Wagner, 1965; Huang, et al., 1966), Newcastle Disease virus (Cantell, et al., 1962; Wilcox, 1959); (b) inactivated virus (by ultraviolet light, heat or chemical means), e.g. vaccinia (Hanafusa, 1960), adenovirus (Levy, et al., 1957), Newcastle Disease virus (Cantell, et al., 1962; Kohn, 1965), measles (Cascarido and Karzon, 1965), vesicular stomatitis virus (Huang and Wagner, 1965), reovirus (Oie and Loh, 1967); (c) defective viruses, e.g. "T" particles of vesicular stomatitis virus (Huang, et al., 1966); or (d) viral components, e.g. adenovirus (Everette and Ginsberg, 1958), poliovirus (Ackerman, et al., 1958), fowl plague virus (Scholtissek, et al., 1967); influenza virus (Henle, 1955).

Several criteria have been used to demonstrate that cytotoxicity (CT) was independent of the cytopathic effects (CPE) caused by virus
replication. Early onset, greater resistance to ultraviolet light (UV), inability for serial transfer and the requirement of a large multiplicity were the important characteristics of the former (Pereira, 1961). Virus induced CT in the above systems was either accompanied by partial production of virus products or none at all. In most cases the mechanism of CT was not understood. Even in the virus systems where attempts to elucidate the mechanism of toxicity were made, a satisfactory explanation is still wanting. Experimental evidence presented to explain a possible mechanism for these systems will be considered at this time.

In the myxovirus induced CT, evidence has been presented to show that neuraminidase enzyme was responsible for the phenomenon (Scholtissek, et al., 1967). It was postulated that the enzyme perhaps acts indirectly by attacking the lysosomal membranes and causing cell injury. In most other virus systems experimental evidence indicated that the phenomenon of CT was an intracellular event mediated by a structural component of the incoming virus or synthesized newly under its direction. A "late" protein synthesized under a viral function was reported to be responsible for mengovirus induced CT (Amako and Dales, 1967). It was hypothesized that this protein triggered cell injury by initiating a leakage of hydrolases from the cellular lysosomes. It was reported by Hanafusa (1960) that vaccinia virus induced CT was independent of virus replication and was mediated by a UV-resistant protein-like substance. He further indicated that CT due to heated vaccinia virus was different and was perhaps caused by some unknown function of the viral RNA. Later studies on vaccinia virus induced CT under conditions preventing virus replication showed that the toxic effects produced required the synthesis
of new virus related proteins (Bablanian, 1968).

Evidence for the disruption of macromolecular synthesis of the host cell by some structural component of the infecting virus has been shown in VSV and adenovirus infected cells (Huang, et al., 1966; Levine and Ginsberg, 1967). In the VSV both "B" and "T" particles were associated with cytotoxicity, and UV-irradiated virus particles were also capable of inhibiting cellular RNA synthesis (Huang and Wagner, 1965; Huang, et al., 1966). It was concluded that virus replication was not essential for the inhibition of RNA synthesis, and suggested further that the toxicity could be due to a virion component which deranges the cellular metabolism. In this case perhaps the shut off of the cellular RNA could have been the cause of cell injury. In the adenovirus system the fiber antigen was found to disrupt macromolecular synthesis of DNA, RNA and protein in virus infected or antigen treated normal cells and cause cytotoxicity (Levine and Ginsberg, 1967, 1968). It was concluded that the mechanism of CT in this case was probably the inhibition of the macromolecular functions of the cell.

Previous reports on UV-R2 induced CT showed that the toxicity was acquired by the virion after exposure to UV, and that the toxic factor was associated with the virus particle (Loh and Oie, 1969). It was further reported that the biosynthesis of cellular macromolecules (RNA, DNA and protein) was severely altered in HeLa cells exposed to UV-R2. It is, therefore, possible that UV-R2 induced CT could be mediated by a structural component(s) of the incoming virus which initiates a series of events leading to a shut off of RNA and protein synthesis in the host cell. Cytotoxicity induced by reovirus in the presence of a protein
antagonist cyclohexamide provides added evidence to show that a structural protein(s) could be responsible for cytotoxicity under such conditions (Loh and Crowley, 1967). It was shown subsequently that severe alterations in RNA synthesis occurred under these conditions (Loh, et al., 1970).

**Cytotoxicity Induced by Reoviruses.** The first report on some form of toxicity associated with reoviruses was by Burlingham and McKee (1965) who described that the Brown and Butner strains of reovirus isolated from infectious hepatitis patients produced a hemolysin when propagated in chick embryo chorioallantoic cavity or yolk sac. The hemolysin was dialyzable through dialysis tubing which retained molecules greater than 20,000 daltons. It was heat stable at 120°C for 20 minutes. The optimal conditions for hemolysis were: 30°C, pH 10 and 1-1.5% suspension of human erythrocytes. Hemolysis was enhanced by sodium thiocyanate but was inhibited by several heavy metal ions. The presence of erythrocyte receptors was not required for lysis and the site of attack was believed to be adjacent to the reovirus receptors on the erythrocyte surface. The authors did not however mention whether such a factor was toxic to cells in tissue culture.

Die and Loh (1967) from this laboratory first reported the phenomenon of CT in HeLa cells infected with a high multiplicity of UV-inactivated reovirus type 2. At multiplicities of 80 to 100 per cell cell cultures underwent rapid degeneration in the absence of virus multiplication. CT was observed as early as 3-4 hrs after infection at which time the cells became rounded and detached from the glass. Such morphological changes were indistinguishable from viral cytopathic
effects (CPE). However, no infectious virus inclusion bodies or viral antigens were produced. The CT was preceded by marked alterations in the synthesis of cellular RNA, DNA and protein. Cell survival curves suggested that the cell killing phenomenon was dependent on the multiplicity of infection and exhibited a multiple hit type of kinetics (Loh and Oie, 1969). The toxic factor could be prevented by the addition of antiserum to reovirus type 2 and the toxic effect could not be serially transferred. Reovirus heated at 56°C for 30 mins was not cytotoxic. The early data suggested that the toxic factor was associated with the UV-inactivated virus particle (Loh and Oie, 1969).

Cytotoxicity was observed in reovirus infected cell cultures treated with a protein antagonist cycloheximide (Loh and Crowley, 1967). In this study cell death occurred in 3-4 hrs. post infection (p.i.) under conditions where the synthesis of proteins, infectious virus or viral products were inhibited. Recent studies indicated that the phenomenon was affected by the kind of cell species employed, and that the effect could be reversed by removal of the antibiotic within one hour after exposure (Loh, et al., 1970). In addition, the biochemical evidence presented suggested that the accelerated CPE observed was the consequence of the combined inhibition of the synthesis of both cellular protein and RNA (Loh, et al., 1970).

Cytotoxicity in Other Virus-Cell Systems. Cytotoxicity has been observed in several virus cell systems as pointed out earlier in this chapter. The conditions under which each virus induced toxicity vary with each system. Therefore, a brief review of some of these systems is relevant and useful at this point.
Poliovirus infected HeLa cells were shown to undergo accelerated cytopathology when virus replication was inhibited by fluorophenylalanine (Ackerman, et al., 1954). This accelerated CPE was reported to be due to a "toxin" which was separable from the infectious virion, and was present in the medium from cell cultures infected with polio virus type 2 (Ackerman, et al., 1958). Accelerated cytopathology in polio virus infected cells using very high multiplicities (10,000 PFU/cell) and in the presence of guanidine which inhibited virus replication was reported (Holland, 1964).

Mengovirus, which is another member of the picorna virus group, has been studied recently for its virulence in animals and toxicity to cells in vitro (Amako and Dales, 1967). Experiments employing the protein inhibitor Streptovitacin A to interfere with virus multiplication and cell lysis have indicated that the cytotoxic factor was a "late" protein, probably a viral coat protein which had a capacity for induction of cell injury, controlling plaque size and virulence in animals.

Cytotoxicity accompanied by failure to produce infectious virus has been observed with several myxoviruses and paramyxoviruses. HeLa cells infected with large doses of influenza virus were seen to undergo cytopathic changes without the production of any infectious virus (Henle, et al., 1955).

Another member of the influenza group, viz: fowl plague virus, whose infectivity was destroyed chemically by ethylene iminoquinone (Bayer A 139) was reported to cause cell destruction (Scholtisske, et al., 1967). However, in contrast to the live virus, ten times as much inactivated virus was needed for cell destruction. The capacity of Bayer
A 139 treated virus to synthesize neuraminidase was reduced to 10% of the control cultures infected with live virus. It was concluded that the neuraminidase was responsible for toxicity.

Newcastle Disease virus (NDV) when added in large amounts to mouse L cell monolayers resulted in a non-transmissible cytopathic effect with the production of non-infectious hemagglutinins (Wilcox, 1959). This reaction was considered to be a toxic one because the cytopathic effects occurred in the absence of production of significant amounts of infectious virus. The ability of NDV to kill cells without multiplication in HeLa cells was also reported (Marcus, 1962). The introduction of NDV into mice intramuscularly resulted in their spleens exhibiting degeneration of follicles 6-12 hours post-infection. This property was also induced by virus inactivated by Bayer A 139 (Rott and Müller, 1965).

Infection of a number of cell lines (e.g. KB, Hep-2, FL and BHK-21) with high multiplicities of NDV caused fusion of cells resulting in the formation of polykaryocytes (Kohn, 1965). Virus rendered non-infectious by UV-irradiation also retained the ability to fuse these cells.

The infection of HeLa cells or monkey kidney cells with several strains of mumps virus resulted in the production of lesions resembling "giant-cells" when high multiplicities of infection were used (Henle, et al., 1954). These lesions were shown to be due to a cytotoxic property of the virus which was also related to its hemolytic property. Cytotoxicity was independent of virus multiplication and exhibited the following properties: (a) a greater resistance to UV inactivation as compared to infectivity, (b) inability for serial transfer of the toxic factor, and (c) a requirement of a large multiplicity of virus in order
to elicit the CT phenomenon. Mumps virus passed several times in chick embryos when inoculated onto HeLa or monkey kidney cells produced a predominantly cytotoxic response (Henle, 1955). Cytotoxic activity of mumps virus on four human epithelial cell lines was reported by Russel and Morgan (1959). Mouse L cells were not sensitive to the toxicity. Since the hemolytic property of mumps virus \textit{in vitro} was due to an enzyme (Moberly, et al., 1957), they concluded that the same enzyme might be responsible for cytolysis (cytotoxicity) as well. It was suggested that the difference in susceptibility of different cell types may be due to differences in availability of enzyme substrate.

Both viable and UV-inactivated measles virus produced giant cells in human FL cell cultures infected with high multiplicities of virus. Heat-inactivated virus did not produce the same effect under similar conditions; yet a cytotoxic effect was observed (Toyoshima, et al., 1960). A similar effect was seen in a line of human amnion cells (AV3) infected with measles virus, where cell limits became indistinct in thirty minutes and cell fusion was complete in five hours (Cascardo, et al., 1965). Measles virus rendered non-infectious by UV-irradiation also retained this property and was termed the fusion factor (FF). In contrast measles infected human diploid fibroblasts did not undergo fusion, but "stringy degeneration." Marked differences in cellular susceptibility to the production of this fusion factor was another interesting observation. These authors pointed out that cell injury occurred only to susceptible cells whose membranes contain appropriate receptor substance or substrate. Their studies further provided evidence to show that cell fusion or "stringy degeneration" was independent of the ability of the virus to produce infectious virus.
Suspensions of vesicular stomatitis virus prepared by infecting cell cultures with undiluted stock virus was found to be cytotoxic despite their low infectivity of such preparations (Cooper and Bellet, 1959). Mouse L cells treated with UV-inactivated NDV when challenged with VSV at multiplicities greater than one plaque forming unit (PFU) per cell were not protected from the effects of CT (Cantell, et al., 1962). These cells synthesized viral antigen and were destroyed without yielding infectious virus. With large doses of UV-inactivated VSV, cellular destruction occurred in both normal and pre-infected cells alike in the absence of detectable viral antigen synthesis. Toxic activity could not be separated from the virus particle (Cantell, et al., 1962). Both the B (infectious) particle and T (defective) particle of VSV were found to be cytotoxic (Huang, et al., 1966). Since UV-inactivated particles also possessed the capacity to affect RNA synthesis in the host cell, it was concluded that a preformed toxic component of the virus was responsible for the inhibition of RNA (Huang and Wagner, 1965; Huang, et al., 1966).

An early clumping and detachment of HeLa cell monolayer cultures infected with adenovirus 5 was reported to be due to a toxic effect (Pereira and Kelly, 1957). The toxic activity of the virus was distinguished from its infectious property by its greater resistance to UV-light, and the requirement of a large virus dose to elicit toxicity. The toxic factor was non-dialyzable, trypsin sensitive and was separable from the virus particle by centrifugation. Toxin damaged cells did not undergo nuclear change, and the toxin was rather heat stable (56°C, 60 minutes). Although the nucleases (DNase, RNase) had no effect, trypsin
(0.1 mg/ml) completely inactivated the toxin (Everette and Ginsberg, 1968). Adenovirus toxin was subsequently identified as the fiber antigen which also was responsible for virus attachment (Pereira, 1960). Adenovirus induced cell injury was attributed to two basic factors operating in virus-infected cells: (a) the synthesis and accumulation of virus specific material from inclusion bodies, and (b) direct injury caused by the toxin (Ginsberg, 1961). Recent evidence showed that purified fiber antigen could inhibit multiplication of adenovirus in infected cells, and also interrupt the biosynthesis of macromolecules in virus infected or non-infected cells (Levine and Ginsberg, 1967). The production of DNA, RNA and protein was suppressed within two hours of addition of the fiber antigen to the cell cultures. Based on recent evidence, it was suggested that cell injury could result through the complexing of the fiber antigen with the host cell DNA (Levine and Ginsberg, 1968).

The cytotoxic effect of both infectious and UV-inactivated pox viruses have been described in several systems. Vieuchange, et al. (1957) described an accelerated CPE occurring in vaccinia virus infected newborn rabbit kidney epithelial cells. Since the cytopathic changes were followed by a very small increase in virus titer, the authors considered it as a cytotoxic effect. Nishimi and Bernkopf (1958) confirmed the toxic nature of vaccinia virus in mouse and human leukocyte cultures. In such cell species they found that both live and UV-inactivated virus produced cytopathic changes in the absence of virus multiplication. The toxic principle was inseparable from the virus particle by centrifugation.
Amnion cells infected with UV-inactivated vaccinia virus caused extreme lengthening and thinning of the cytoplasm, and the cell sheet was transformed into a net-like structure (Bernkopf, et al., 1959). This indicated that toxicity could manifest itself in different forms. The toxic activity of vaccinia virus on foetal mouse lung cell cultures under conditions where virus multiplication was inhibited has been reported (Brown, et al., 1959). Toxicity caused by heat or UV-inactivated vaccinia virus in mouse L cells has also been described by Hanafusa (1960). All of these reports indicated that the cytopathic changes occurring in the host cell in the absence of virus replication was a toxic effect. The nature and mechanism of its action remain to be elucidated.

During his studies on the mechanism of vaccinia virus-induced cytopathology in LLC-MK-2 cells Bablanian (1968) was able to prevent virus induced CPE with very high concentrations of puromycin (330 μg/ml) as well as actinomycin D (5 μg/ml). The concentrations of puromycin used were 10 times greater than that required to prevent virus multiplication. He concluded that the early virus induced CPE required the synthesis of new protein(s). However, the evidence did not clarify whether the viral protein(s) induced were coded for by the virus or the host cell.

Toxic effect of yet another member of the pox virus group, namely rabbit-pox virus on embryonic rabbit kidney cells has been described by Appleyard, et al. (1962). Cell toxicity was observed in 1.5 to 2 hours after infection and the cells were completely rounded in about 8 hours. From 8 to 24 hours the cells began to form syncytia which later detached from the glass surface. Cytopathic changes caused by UV-irradiated virus did not however progress to fusion. Cytotoxicity was also observed in
cell cultures treated with azide at concentrations which inhibited virus multiplication. An interesting finding in this study was that UV-inactivated virus produced soluble antigens, and it was suggested that the toxic effect was a result of disturbances produced in the cell by the early stages of virus growth and could not occur in the absence of virus multiplication (Appleyard, et al., 1962).

Cytotoxicity Induced by UV-Irradiated Reovirus—A Unique Phenomenon.

Cytotoxicity exhibited by UV-irradiated reovirus is a unique phenomenon in that the virion acquires the toxic property only after exposure to UV-light (Oie and Loh, 1967). In contrast the non-irradiated virus does not exhibit cytotoxicity even when very high multiplicities of infection are employed. It is evident from the foregoing review of the other virus systems that cytotoxicity was associated with the virion even before UV-irradiation. In the reovirus system while losing its infectivity within 2 minutes of exposure to UV-light, the virus acquired the new property of cytotoxicity (Oie and Loh, 1967). Preliminary studies indicated that with continued irradiation over 10 minutes, the CT property decreased until it was completely lost in 60 minutes (Subasinghe and Loh, 1971). Earlier reports indicated that the CT was an integral part of the virus, and the toxicity could not be serially transferred (Loh and Oie, 1969). Pre-incubation of the UV-R2 particle with immune serum to reo-2 prior to infecting HeLa cells prevented the CT, further indicating that the toxic factor was on the virus particle (Loh and Oie, 1969). What remains to be elucidated is whether the toxic factor is the virus coat protein(s) or perhaps the UV-inactivated RNA. Acquisition of CT property is presumably accompanied
by certain alterations both in the virus capsid and the nucleic acid genome. Although nucleic acids are known to be more sensitive to UV, proteins can be affected mainly at higher levels of incident energy (Smith and Hanawalt, 1969). There could be a breakage in disulphide bonds and peptide bonds perhaps opening of the protein molecules exposing certain reactive groups. Perhaps these altered protein molecules may be responsible for inducing cytotoxicity.

**Objectives.** The main purpose of this study was to understand how the normally non-toxic reovirus acquired a CT property on exposure to UV-light, and to determine what component(s) of the virus was cytotoxic. Previous studies on the effects of UV-irradiation on the reovirus were confined to the loss of infectivity and the acquisition of cytotoxicity (Oie, et al., 1966; Oie and Loh, 1967; Loh and Oie, 1969). On the assumption that UV-irradiation would affect certain properties of the virion other than infectivity, the physical, chemical and biological properties of the UV-R2 were examined during the first phase of this study. The biological properties of the virus examined were: cytotoxicity, hemagglutination, adsorption to HeLa cells, interferon inducibility and transcriptase activity. The physical and chemical properties examined were: particle size of the UV-R2, sedimentation in sucrose gradients, buoyant density in CsCl, virus architecture, viral structural proteins and the RNA. Alterations in the above properties with varying doses of UV-irradiation were examined.

Experimental studies during the second phase of the investigation were directed to understand further on the nature of the toxic agent and the virus-cell interactions to elucidate further on the possible
mechanism involved in the cytotoxic phenomenon. Since the CT was previously reported only with reovirus serotype 2 (Loh and Oie, 1969) experiments were done to examine whether the CT phenomenon occurred with other serotypes of reovirus exposed to UV. Further investigations were carried out to determine whether the CT was dependent on the cell system in which the virus was passed or was a specific property of the virus independent of the cell in which the virus was produced.

In order to determine the toxic component of the virion the following were tested for cytotoxicity: UV-irradiated extracted RNA, reovirus subjected to brief heat treatment (70°C for 30 sec) where the virion polymerase was activated, empty particles of reovirus and structural proteins prepared by controlled degradation of the virus with urea.

The interactions of the toxic virus with the cells were examined during the next phase. Experiments were conducted to examine the role of temperature in the CT induction. In addition, the ability of live reovirus to interfere with CT-induction by UV-R2 were examined. In such experiments HeLa cells pre-infected with live homologous virus were challenged with UV-R2 and examined for the following: the effect on viral antigen synthesis, virus replication as determined by infectious virus production, and the effect on the cytotoxic response.

In order to examine whether viral or cell mediated proteins were involved in the CT phenomenon, cell cultures pre-treated with interferon were challenged with UV-R2, and observed for a possible inhibition of the CT response.

Lastly, the relative sensitivity of different species of cells in tissue culture and of mice to CT induction with UV-R2 were examined.
CHAPTER 2
Reoviruses—A Review

Introduction. A number of authoritative and comprehensive reviews on reoviruses have appeared recently (Stanley, 1967; Shatkin, 1968; Rosen, 1968; Shatkin, 1969; Spendlove, 1970; Joklik, 1970). Many of the reviewers have placed great emphasis in their own areas of research and therefore a comprehensive review on the subject will not be attempted at the present time. However, a brief discussion of the important properties of reoviruses and the recent pertinent findings on the subject will be considered.

Reoviruses are a group of animal viruses containing a double stranded ribonucleic acid genome protected by a two layered protein coat of icosahedral symmetry. They have a particle diameter of 60-75 nm and are resistant to ether. This group of viruses is ubiquitous in its geographic distribution, having been isolated from man and a variety of animal species (Rosen, 1968). However, their etiologic role in human disease is yet uncertain.

Classification. Reoviruses when initially isolated were erroneously classified as ECHO virus type 10 (Committee on ECHO Viruses 1955), because they satisfied the criteria accepted for the ECHO virus group. It was subsequently learned that the members of the "so-called" ECHO-10 group were larger and that they also produced cytoplasmic inclusions which were not seen with the other members of the ECHO virus group (Malherbe and Harwin, 1957; Drouhet, 1958; Shaver, et al., 1958). In 1959 Sabin therefore removed the members of the ECHO group and established a new group called the "reoviruses." The letters r e o
served to emphasize the respiratory and enteric sources of isolation of
the virus and their uncertain relationship to disease (o stands for
orphan).

Reovirus group comprises three serological types 1, 2 and 3 which
share a common complement fixing antigen, but are distinguished by
neutralization tests. All three serotypes agglutinate human erythrocytes.
Hemagglutination inhibition technique could therefore be used for their
serologic identification (Rosen, 1960). A group of antigenically unrelated
yet morphologically identical avian reoviruses have been isolated recently
(Kawamura, 1965).

Morphology. Reoviruses are icosahedral in structure possessing a
diameter of 60-80 nm (Jordan and Mayer, 1962; Vasquez and Tournier, 1962;
Gomatosis, et al., 1962; Loh, et al., 1965). The virion consists of a core
40 nm in diameter surrounded by an electron opaque inner layer 30-50 A°
wide and an outer capsid layer composing of 92 elongated hollow capsomeres
(Mayor, et al., 1965; Vasquez and Tournier, 1962; Loh, et al., 1965).
Recently Vasquez and Tournier (1964) have reinterpreted their electron
micrographs with respect to reo-3, and suggested that the capsid is not
composed of columnar capsomeres, but 180 solid truncated pyramids. The
pyramids share common structural subunits and are arranged in groups of
five or six about 92 holes on the surface of the virus particle. The
double layered capsid is considered to contribute to the thermo stability
of reoviruses (Gomatosis, et al., 1962; Rhim, et al., 1961).

Physico-Chemical Properties. Purified preparations of reovirus
type 3 have a buoyant density of 1.38 gm/cm$^3$, sedimentation coefficient
of 630 S and a diffusion coefficient of $8 \times 10^{-8}$ cm$^2$/sec. corresponding
to a minimum estimated molecular weight of $70 \times 10^6$ daltons (Gomatosis and
Tamm, 1963). The virion contains 15% RNA by weight. Since the virus particle is resistant to ether, it is considered free of lipids. The virus particle consists of a helical double stranded RNA genome (Langridge and Gomatos, 1963) and a single stranded adenine rich ribopolymer (Bellamy, et al., 1967; Bellamy and Joklik, 1967; Shatkin and Sipe, 1968).

**Viral RNA.** The double stranded RNA genome of reovirus has a molecular weight of approximately $15 \times 10^6$ daltons (Shatkin, et al., 1968). Although electron microscopic evidence showed that the double stranded RNA extracted from purified reovirus was a single molecule of 7-8 microns long (Dunnebacke and Kleinschmidt, 1967; Granboulan and Niveleau, 1967) when the RNA was extracted from purified virus or infected cells by a variety of procedures, a mixture of genome segments falling into 3 sizes having molecular weights of 2.5, 1.4 and 0.8 $\times 10^6$ daltons was obtained (Bellamy, et al., 1967; Gomatos and Stoeckinius, 1964; Kleinschmidt, et al., 1964; Shatkin, 1965; Watanabe and Graham, 1967; Iglewski and Franklin, 1967; Shatkin, et al., 1968). Each of these RNA fragments had a unique base sequence, thus indicating that they were not products of random breakage, but a result of cleavage at predetermined weak points in the RNA molecule (Watanabe and Graham, 1967; Bellamy and Joklik, 1967; Watanabe, et al., 1967). Reovirus messenger RNA was transcribed in three size classes corresponding to the three sizes of the genome RNA molecule, and they hybridized specifically with the corresponding genome segment only, further confirming the uniqueness of each segment (Bellamy and Joklik, 1967; Watanabe, et al., 1967).

Double-stranded RNA extracted from all three serotypes of reovirus
has been resolved into 10 discrete segments by polyacrylamide gel analysis. They fall into 3 large, 3 intermediate and 4 small size fragments adding up to a total molecular weight of 15 x 10^6 daltons (Shatkin, et al., 1968). It is not known yet how these fragments are joined together in the intact molecule.

The presence of a non-infectious defective virion in reovirus type 3 preparations propagated in mouse L cells was reported recently (Nonoyama, et al., 1970). These virions were lacking in the largest of the ten segments of the double stranded RNA. These defective virions were found to arise even from cloned preparations of infectious virus after seven serial passages in L cells (Nonoyama and Graham, 1970).

**Adenine-Rich RNA.** In addition to the RNA genome reoviruses contain an adenine-rich single stranded RNA (Bellamy and Joklik, 1967; Shatkin and Sipe, 1968). This RNA comprises of about 25-30% of the total viral RNA. The estimated molecular weight is about 5000 daltons, corresponding to a chain length of 15 nucleotides. More than 90% of the bases in the polymer was adenylic acid, a small amount of uridylic acid and cytidylic acid were also present (Shatkin and Sipe, 1968). Bellamy and Joklik (1967) reported that the ribo-polymer is probably of the order of 60 nucleotides in length, with a remarkably uniform electrophoretic behaviour. A subsequent report (Bellamy and Hole, 1970) however shows that the molecule is highly heterogenous with respect to size and base composition, overall size ranging from 2 to 20 nucleotides.

The adenine-rich (A-rich) RNA was found associated with the subviral particle (SVP) though to a lesser extent (Loh and Shatkin, 1968). Hybridization of A-rich RNA to polyuridylic acid has been demonstrated,
but hybridization or linking with dsRNA has not been shown (Bellamy and Joklik, 1967). It has been reported that A-rich RNA can be removed from reoviruses without decreasing their specific infectivity (Krug and Gomatos, 1969). However, the function of A-rich RNA still remains unknown.

Structural Proteins of the Virion. The genome of reovirus having a molecular weight of approximately 15 million daltons contains sufficient information to code for a large number of proteins. Structural proteins of all three serotypes of reoviruses have been studied using polyacrylamide gel electrophoresis, after solubilization of purified viruses with Sodium dodecylsulfate (SDS), 2-mercapto-ethanol (ME) and Urea (Loh and Shatkin, 1968; Smith, et al., 1969). These studies indicate that the virion is comprised of at least 7 polypeptides: 3 major components (I, II and IIIb) and 4 minor components (M1, M2, M3 and IIIa). Structural proteins of the virion are distributed among the various components in the following manner: Component I, 20%; II, 31%; IIIb, 27%; the four minor components comprise the remaining 22% of the virus proteins.

Molecular weights of the various components were determined by their relative electrophoretic mobilities in comparison with proteins of known molecular weight. They are in excess of 100,000, 83,000 and 40,000 daltons for components I, II and IIIb, respectively, while component IIIa is 44,000.

Subviral particles obtained by selective degradation of the virion by brief treatment with 4M urea at 4°C comprise of only two proteins: Components I and IIIa. Approximately 31% of the total proteins of the virion are contained in the subviral particle, indicating that the
remaining 69% is the outer capsid (Loh and Shatkin, 1969).

Although Smith, et al. (1969) have given a different nomenclature to the protein components of reoviruses analyzed on acrylamide gels, their results were essentially in agreement with those of Loh and Shatkin (1968). According to Smith, et al. (1969) there are two large polypeptides \( \lambda_1 \) and \( \lambda_2 \) (M.W. 150,000 and 140,000) two medium size polypeptides \( \mu_1 \) and \( \mu_2 \) (M.W. 80,000 and 72,000) and three small polypeptides \( \sigma_1, \sigma_2 \) and \( \sigma_3 \) (M.W. 42,000, 38,000 and 34,000).

**Hemagglutination.** Hemagglutinating property of reoviruses was used to obtain much of the early information on the virus capsid. All three serotypes of reoviruses agglutinate erythrocytes of the human ABO groups (Brubaker, et al., 1964), but not all serotypes do agglutinate erythrocytes from monkeys, rabbits, guinea pigs, mice, rats and chickens (Lerner, et al., 1963; Beno and Edwards, 1966).

The type of cell system used for the production of virus was an important factor in the development of high hemagglutination titers (Rhim, et al., 1965).

Reovirus types 1 and 3 elute from group 0 human red cells upon prolonged incubation (Lerner, et al., 1963). After elution of reovirus type 3, the cell receptors for all three serotypes remain intact, indicating that the release differs from the influenza virus hemagglutination which involves neuraminidase enzyme (Gottschalk, 1959). Agglutination of bovine erythrocytes by reoviruses was restricted to type 3 (Eggers, et al., 1962). This interaction differs from agglutination of human erythrocytes. Receptor-destroying enzyme (RDE) or neuraminidase treatment renders bovine erythrocytes inagglutinable by reovirus type 3, but does not have this effect on human erythrocytes.
Agglutination of bovine red cells was also inhibited by ovomucin, yet it was not effective against the agglutination of human 0 group red cells with reovirus type 2. It was further reported that treatment of reovirus type 3 with trypsin (0.5 mg/ml) or parachloromercurobenzoate (PCMB) reduced hemagglutination titers with bovine red cells, suggesting the involvement of a protein component and sulfhydryl group in reovirus-3 and ox red cell interaction (Gomatos and Tamm, 1962).

When reovirus was pretreated with periodate, HA titers of all three serotypes were destroyed (Tillostson and Lerner, 1966). Incubation with ROE did not however alter the HA titers of types 1 and 2 (Lerner, et al., 1963) although PCMB (Buckland, 1960) and iodoacetamide (Allison, et al., 1962) reduced the titers of both types. PCMB effect was reversed by glutathion (Lerner, et al., 1963).

It was concluded from the foregoing evidence that the mechanism of attachment of reovirus types 1 and 2 to human 0 group erythrocytes involves the presence of a sulfhydryl containing glycoprotein on the virus surface, and the union was non-enzymic (Lerner, et al., 1963). Subsequent studies showed that hemagglutination of human 0 group erythrocytes with reoviruses was inhibited by pretreatment of the virus with salivary mucoproteins (Lerner, et al., 1966) or with N-acetyl-D-glucosamine (Gelb and Lerner, 1965).

The sugar presumably occupied the hemagglutinating sites on the surface of the virus, blocking the interaction with the glycoproteins on the red cell surface.

**Interferon Induction.** A wide variety of DNA and RNA viruses are known to be inducers of interferon, both in tissue culture and in the
whole animal (Ho, 1967). Yet the interferon inducible property of reoviruses in tissue culture was not demonstrated until recently, when Oie and Loh (1968) reported the production of an interferon in a stable line of human amnion cells, using reovirus type 2 as inducer. They also reported on the sensitivity of reoviruses to this interferon.

Production of serum interferon in rabbits to purified reovirus-3 double stranded RNA was reported by Tytell, et al. (1967). He showed evidence that naked RNA was more efficient as an inducer than the complete virion in the whole animal.

Reoviruses have been shown to induce interferon in poikilothermic animal cells (fathead minnor—-a line of fish cells) even though the virus did not replicate in this cell line (Oie and Loh, 1971).

Virus Infectivity. Reoviruses replicate in a wide variety of primary cells and continuous cell lines of human and animal origin. Due to their broad host range this could be expected.

Plaque assays using mouse L cells and monkey kidney cell cultures have been described (Franklin, 1961; Rhim and Melnik, 1961). Although virus assay by the plaque method is slower, it is more sensitive than hemagglutination. One hemagglutinating unit was equivalent to $6.2 \times 10^6$ PFU (Gomatos and Tamm, 1962). Certain strains of reoviruses are poor plaque formers. Infectivity assay of such strains are determined by immunofluorescent plaque technique (Spendlove and Lenette, 1962). This method detects viral protein formation in infected cells, and may not necessarily be equivalent to the production of infective virus. Strains of reoviruses that can be plaqued usually produce small plaques of about 1-5 mm in diameter 4-6 days after infection. The size of plaques can be
enhanced by incorporating pancreatin in the agar overlay medium (Wallis, et al., 1966). Pancreatin treated virus has also yielded higher virus infectivity titers in monkey kidney cells (Wallis, et al., 1966). Similar enhancement of infectivity has been observed by heating reo-1 infected cell lysates at 55°C in 2M MgCl₂ with no change in HA titer (Wallis, et al., 1964, 1965).

Enhancement of infectivity by chymotrypsin was first seen in the preparations of purified reovirus used in electron microscopic studies, when the enzyme was used in the purification procedure (Kleinschmidt, et al., 1964). Subsequently, infectivity titers of most strains of all three serotypes of reovirus were shown to have increased several fold by incubation with low concentrations (20 μg/ml) of chymotrypsin, papain or trypsin. "Potentially infectious virus" found in abundance in infected cell lysates were converted to infectious virus by the enzyme treatment (Spendlove, et al., 1965; Spendlove, et al., 1966). The enzyme enhancement of infectivity was found to be due to the removal of or alteration of the outer layer of the viral double capsid (Spendlove and McClain, 1967; Spendlove and McClain, 1968). Proteolytic enzymes remove the outer layer of capsomeres leaving the virus core enclosed only by the inner layer or the "subcapsid layer" (Spendlove and McClain, 1967). Infection with such preparations of virion cores result in the shortening of the eclipse phase.

Enhancement of infectivity titers in reovirus type 2 was obtained by sonication of virus suspensions (Oie, 1968). Two minutes of sonication time was sufficient to give increased titers. The enhancement effect was considered to be due to breaking up of virus aggregates or by
releasing cell bound virus (Oie, 1968).

Reoviruses are relatively stable to heat and a wide range of pH changes (Usmankhodzhayer and Zakskelskaya, 1964; Kelter, et al., 1962). It was reported from this laboratory that the infectivity of reovirus-2 was stable at 25°C, less stable at 37°C and rapidly inactivated at 60°C (Oie, 1968). Its half life at 37°C and 60°C was 5 days and 60 seconds, respectively (Oie, 1968). His findings further confirmed the pH stability of the virus, infectivity being virtually unchanged over a pH range of 2 to 9, suffering only 1 to 1.7 log reduction in titer over a period of seven days.

Studies on photoinactivation of reovirus type 2 by UV-light showed that the virion infectivity was completely destroyed within 2 minutes (Oie, et al., 1966). A reduction in viral infectivity of reovirus type 1 to 1% within 6 minutes of UV-irradiation was reported by McClain and Spendlove (1966). They also reported on a multiplicity reactivation occurring in FL cells infected with reovirus type 1 which had $10^2$ to $10^3$ survivors after exposure to UV light. The rates of photo inactivation of reoviruses and DNA viruses were similar, but greater than the rate for certain single stranded RNA viruses (Hiatt, 1961). In contrast Rauth (1965) found that reovirus-3 was less sensitive to UV-light than viruses containing single stranded nucleic acid.

Loss of virus infectivity as well as hemagglutinating activity was observed in reoviruses treated with potassium periodate and PCMB (Lerner, et al., 1963), whereas with beta propiolactone only the infectivity was lost (Server, et al., 1964).

Proflavin neutral red and toludine blue binds to reovirus type 1.
Infectivity of reovirus was destroyed after exposure of dye-virus complex to light presumably as a result of the dye binding to the viral nucleic acid (Wallis and Melnick, 1964). Permeability of reoviruses to photosensitizing dyes was less than other viruses tested by Hiatt (1961).

**Virus Replication.** Reoviruses can replicate in a variety of cell cultures. In contrast to many single stranded RNA viruses reoviruses have a long infectious cycle and an eclipse period varying from 6-9 hours, depending on the reovirus serotype and the cell system used. Mouse L cells infected with reovirus type 3 was observed to have an eclipse period of 6 hours (Gomatos, et al., 1962). Similar results were obtained with reo-3 infected RA cells (Oie, et al., 1966), and reo-1 infected monkey kidney cells (Rhim, et al., 1962) or HeLa cells (Spendlove, et al., 1963). But reo-2 infected RA cells had a longer eclipse period of 9 hours (Oie, et al., 1966).

Mouse L cells infected with reo-3 adsorbed 50% of the virus in 1 hour, and 90% in 3 hours (Kudo and Graham, 1965; Gomatos, et al., 1962). Adsorption of reo-2 to RA cells was maximum at about 75-90 minutes after exposure of the cells to the virus (Oie, et al., 1966). Subsequent adsorption and elution studies with reo-2 in HeLa cells indicated that 75-90% of the virus was adsorbed in 2 hours at 37°C, when the cell cultures were infected at a multiplicity of 10 I.U./cell (Loh, et al., 1967). However, when the adsorption was carried out at 4°C, and the virus cell complex was subsequently incubated at 37°C, more than 25% of the virus was eluted within 5 minutes, but no further elution occurred. Since the amount of virus eluted was negligible when lower multiplicities of infection (0.1 to 1 I.U./cell) were employed, the elution phenomenon
appeared to be multiplicity dependent. The eluted virions were found to be biologically and physically unaltered (Loh, et al., 1967).

Maximum virus yields were obtained in 36 and 26 hours with reo-2 and reo-3 respectively, in virus infected RA cell cultures (Oie, et al., 1966). The long latent period required for reovirus, replication may be due to slow adsorption and penetration of the virus, or due to slow degradation of the infecting virion and asynchronous release of the virus nucleic acid (Dales, et al., 1965).

Penetration of reoviruses appear to occur by "viropexis" as seen through electron microscopic studies (Dales, et al., 1965). Although virus attachment takes place at 4°C, incubation at 37°C is a step required to initiate rapid entry of virus into the cell (Silverstein and Dales, 1968). Electron microscopic studies on reo-3 infected L cells reveal that 20% of the cell-associated virus was enclosed as single particles in phagocytic vacuoles in 15 minutes, while approximately another 15% was present in dense lysosomes. The fraction of virus found in lysosomes increased to 80% in 1 hour.

Virus particles found in lysosomes frequently appeared as nucleoids lacking a capsid (Silverstein and Dales, 1968). These observations suggest that the adsorbed virus particles get enclosed within phagocytic vacuoles as they enter the cell and are transported to the lysosomes where uncoating occurs. It has been suggested that some pre-existing cellular enzymes are responsible for the release of the reovirus genome from the virus particle (Dales, et al., 1965). That no new or virus mediated protein synthesis is required for uncoating was indicated by the following evidence: (a) streptovitacin A an inhibitor of protein
synthesis did not interfere with the release of SVP cores (Dales, 1965), (b) the presence of puromycin during the first hour of infection did not delay the formation of progeny virus, and (c) the levels of lysosomal hydrolases in infected and uninfected cells were similar (Silverstein and Dales, 1968).

The fate of the parental viral RNA and the protein capsid has been studied using uridine-3H labeled purified reovirus, and electron microscopic autoradiographic technique (Silverstein and Dales, 1968). Parental viral RNA was conserved in a macromolecular form for at least 5 hours. In reovirus-2 infected HeLa cells the parental RNA was conserved for at least 12 hours post infection, and was degraded later (Loh, et al., 1968). However, the viral capsid proteins were gradually hydrolyzed to acid soluble material (Silverstein and Dales, 1968).

A condition similar to viral uncoating occurred in vitro when reoviruses were incubated with chymotrypsin (20 μg/ml), papain, or trypsin (Spendlove, et al., 1966). Electron microscope examination of these preparations showed that the conversion of the virion to particles with higher levels of specific infectivity was accompanied by reduction in size to a diameter of approximately 50 nm. The enzyme digested the outer layer of capsomeres. The SVP thus produced was found to contain RNA polymerase activity, and was able to synthesize reovirus specific single stranded RNA in vitro from the 4 triphosphate nucleotides in the presence of Mg++ and an energy source (Shatkin and Sipe, 1968). The RNA polymerase activity was found in enzyme-treated preparations of all three serotypes of reovirus. It was postulated that the virion cores produced by lysosomes in the infected cells presumably possess the RNA
polymerase activity, and was able to synthesize the viral messenger RNA. Light and electron microscopic studies (Gomatos, et al., 1962), Drouhet, 1958; Drouhet, 1960; Macriae, 1962) fluorescent antibody staining (Oie, et al., 1966; Rhim, et al., 1962; Spendlove, et al., 1964) and autoradiography (Loh and Soergel, 1965; Dales, et al., 1965) of reovirus infected cells indicated that the synthesis of viral RNA and protein as well as virus maturation occurred in the cytoplasm of the infected cell. Involvement of the nucleus was not seen in these studies.

A unique feature in reovirus replication is the association of the foci of virus development with the mitotic apparatus. Spindle fibers in infected dividing cells were coated with viral antigen which fluoresced when stained with fluorescein conjugated anti-reovirus serum (Spendlove, et al., 1963). However, other sites can be involved in the virus replication. Electron microscopic studies revealed irregular and dispersed foci of virus replication in the infected cells. These foci were discrete and compact and the replicative sites were scattered in the cytoplasm (Dales, 1963). Virus replication did not depend on spindle formation because treatment of infected cells with colchicine (a mitotic inhibitor) did not reduce yields of infectious virus (Spendlove, et al., 1964; Dales, 1963). However, it prevented the reticular appearance of the virus antigens, being replaced by large clumps of viral antigen (Spendlove, et al., 1963; Spendlove, et al., 1964).

Another interesting feature noted recently is the role of the amino acid lysine in the replication of reoviruses. Lysine was found to be essential for the production of infectious reoviruses (Loh and Oie, 1969). Omission of this amino acid from the experimental medium resulted in the
enhanced production of "empty" virion particles essentially free of RNA and sedimenting at a buoyant density of 1.29 g/cm$^3$ in CsCl density gradients. This effect was reversible if lysine was added to the medium early after the infection (Loh and Oie, 1969).

**Effect of Virus Replication on Host Cell Metabolism.** Synthesis of DNA in reovirus-3 infected L cells was inhibited to the extent of about 80% in about 12 hours after infection (Gomatos and Tamm, 1963; Ensminger and Tamm, 1969). Protein synthesis was partly inhibited after reo-3 infection of L cells in suspension culture (Kudo and Graham, 1965; Ensminger and Tamm, 1969), but was unaffected in monolayer cultures (Gomatos and Tamm, 1963; Ensminger and Tamm, 1969). However, inhibition of both cellular DNA and protein occurred in reo-2 infected RA cells in monolayer cultures (Loh and Soergel, 1967). In contrast cellular RNA synthesis of reovirus infected cells was not altered (Loh and Soergel, 1965; Kudo and Graham, 1965). Since approximately 25% of the protein synthesized in reovirus infected cells are virus specific, it follows that infection would result in at least a partial inhibition of cell protein synthesis.

**Viral RNA Synthesis.** Since the virus directed RNA synthesis comprises only a small fraction of the total RNA being produced in reovirus infected cells, Gomatos and Tamm (1963) could not detect its synthesis readily. However, Loh and Soergel (1965) were able to show an increase in incorporation of $^3$H-uridine into acid-insoluble RNA fraction of the cytoplasm in RA cells infected with reovirus type 2. This effect commenced at about 6-7 hours after infection reached a maximum in approximately 15 hours and declined soon after. They obtained similar
results with reovirus-2 infected HeLa cells maintained under non-growing conditions.

Replication of reo-3 in L cells and reo-2 in HeLa cells was inhibited by actinomycin D at concentrations of 1-2 µg/ml (Gomatos, et al., 1962; Loh and Soergel, 1965). However reovirus replication occurred optimally when low concentrations of actinomycin D (0.1 - 0.5 µg/ml) were employed, although cell RNA synthesis was reduced by about 90% (Loh and Soergel, 1966; Kudo and Graham, 1965; Shatkin, 1965). With reo-2 enhanced production of infectious virus was reported (Loh and Soergel, 1966). Under these conditions of selective inhibition, reovirus-3 infected L cells produced two species of virus specific RNA beginning from 6 to 8 hours after infection (Kudo and Graham, 1965; Shatkin and Rada, 1967). One species of RNA was sensitive to RNAse, while the other was resistant. The resistant fraction was double stranded RNA and was approximately 40% of the RNA formed up to 16 hours after infection.

Double-Stranded RNA. Synthesis of both species of RNA was inhibited by 5 µg/ml of actinomycin D added early in the infection, but when the antibiotic was added 6-8 hours after infection (when the production of virus specific RNA has begun), no inhibition occurred (Kudo and Graham, 1968; Shatkin and Rada, 1967). Reovirus induced RNA extracted from reo-3 infected mouse L cells during the infectious cycle consisted of both single- and double-stranded RNA at all times, both being formed simultaneously (Kudo and Graham, 1965; Shatkin and Rada, 1967; Prevec, et al., 1968). Bellamy and Joklik (1964) however, detected single stranded RNA before the appearance of double stranded RNA early in the infectious cycle. The ratio of single stranded (ssRNA) to double stranded RNA (dsRNA) formed during one hour interval was about 3:1 and
increased to 5:1, as determined by using a 30 minute pulse (Bellamy and Joklik, 1967). The purified dsRNA extracted from infected cells was similar in size and base composition to virus RNA. This dsRNA is primarily incorporated into the mature virion (Shatkin and Rada, 1967).

The two types of virus RNA synthesized in reovirus infected cells have been separated by a variety of methods namely: (a) differential precipitation of the single ssRNA in 2 M Li Cl (Bellamy, et al., 1967), (b) varying the conditions of phenol extraction eliminating SDS, whereby only dsRNA can be extracted (Kudo and Graham, 1965), (c) chromatography on MAK columns (Kudo and Graham, 1965; Shatkin and Rada, 1967) and (d) electrophoresis on polyacrylamide gels (Bellamy and Joklik, 1967; Watanabe, et al., 1967; Prevec, et al., 1968).

Double stranded RNA fragments of three different sizes have been isolated from reovirus infected cells, and separated on methylated albumin-kieselguhr (MAK) columns and sucrose gradients. These pieces sedimented at 14-15 S, 12-13 S and 10.5 S corresponding to a molecular weight of approximately 2.5, 1.4 and 0.8 x 10^6 daltons (Watanabe and Graham, 1967). These S values and the molecular weights correspond to those obtained for dsRNA segments extracted from purified virus particles (Shatkin, 1968; Watanabe and Graham, 1967; Bellamy, et al., 1967; Prevec, et al., 1968). Furthermore, dsRNA extracted from reovirus infected cells can be separated by polyacrylamide gel electrophoresis into 10 segments falling into 3 different sizes (Prevec, et al., 1968).

The dsRNA fragments were denatured by exposure to dimethyl sulfoxide (DMSO) (Bellamy, et al., 1967; Watanabe and Graham, 1967) or by heating (Iglewski and Franklin, 1967). The resulting single strands were
half the molecular weights of the duplex (Bellamy, et al., 1967; Watanabe and Graham, 1967). The denatured RNA could be renatured up to 60-90% by annealing in the presence of 0.3 M NaCl. Annealing occurred only with the strands of the corresponding size (Watanabe and Graham, 1967; Shatkin and Rada, 1967).

Double stranded RNA from infected cells was acid precipitable like that from purified virus particles, and remain the same after exposure to RNase under conditions which would degrade single stranded RNA completely (2 μg/ml, 37°C, 30 min, 0.15 M NaCl) (Kudo and Graham, 1965; Shatkin and Rada, 1967).

**Single-Stranded RNA.** Single stranded RNA isolated from reovirus infected cells also consisted of a mixture of fragments of three different sizes and was RNase sensitive (Watanabe and Graham, 1967; Bellamy and Joklik, 1967; Gomatos, 1967). Their sedimentations in sucrose gradients were 24 S, 18 S and 14 S, which correspond to the large, medium and small pieces of the dsRNA, respectively, evidence from electrophoretic studies on scrylamide gels also confirmed the above data (Watanabe, et al., 1967). Available evidence indicate that each segment of the dsRNA acts as a template for the corresponding single stranded RNA fragments. Supporting evidence was provided by hybridization experiments where each class of single stranded messenger RNA (mRNA) hybridized exclusively with a denatured dsRNA fragment of equivalent length (Bellamy and Joklik, 1967; Watanabe, et al., 1967; Gomatos, 1967; Prevec, et al., 1968). Single stranded RNA did not self anneal with L cell DNA or heterologous RNA from rice dwarf virus (Bellamy and Joklik, 1967; Watanabe, et al., 1967; Gomatos, 1967; Shatkin and
Rada, 1967). It has therefore been concluded that the base sequence of each of the 3 species of single stranded RNA was homologous to the corresponding dsRNA fragment.

Although the sum of guanine and cytosine (G+C) content of each ssRNA species was similar to that of dsRNA, the guanine content was considerably higher than that of cytosine, in ssRNA (Shatkin and Rada, 1967). It has therefore been surmised that there is preferential copying of one strand or alternate regions of the two strands of the dsRNA during the transcription process (Shatkin and Rada, 1967).

Transcription of the Reovirus Genome. Information encoded in the reovirus genome cannot be directly translated by ribosomes of the infected cell. The dsRNA must be transcribed into ss messenger RNA. This is achieved by an RNA dependent RNA polymerase found associated with reovirus virion cores (Borsa and Graham, 1968; Shatkin and Sipe, 1968; Skehel and Joklik, 1969; Benarjee and Shatkin, 1970; Gomatos, 1970). The polymerase is activated by chymotrypsin treatment (Shatkin and Sipe, 1968; Skehel and Joklik, 1969) or by brief heat treatment at 70°C (Borsa and Graham, 1968) in the in vitro situation. Polynucleotides are rapidly synthesized from nucleotide triphosphates in the presence of the activated enzyme, and an energy generating system such as phosphoenol pyruvate (Shatkin and Sipe, 1968; Skehel and Joklik, 1969). The event specifically associated with the activation of the enzyme is probably a change in configuration of the inner core shell which can be triggered either by removal of a minimum number of outer shell capsomeres or by brief heating at 70°C (Joklik, 1970). Genome RNA added to cores was not transcribed, indicating that the enzyme sites were on the inside.
(Skehel and Joklik, 1969). Density of reovirus cores increased from 1.44
to 1.47 g/ml within 2 minutes of the addition of the 4 nucleotide
triphosphates to the cores, but the density was restored to 1.44 by
sonication or RNase treatment (Skehel and Joklik, 1969). This was
interpreted to indicate that nascent RNA is extruded through the core
shell while being transcribed, remaining associated with the cores
(Skehel and Joklik, 1969).

The RNA polymerase transcribes one strand of each of the 10 genome
segments by a conservative mechanism in vitro (Benarjee and Shatkin,
1970; Skehel and Joklik, 1969). Evidence for the above has been supplied
by sucrose gradient sedimentation studies and hybridization experiments
with the dsRNA.

Although the polymerase in vitro copies all the double stranded
segments of the genome in reovirus infected cells (Watanabe, et al.,
1968), in the early part of the infectious cycle, only a limited number
of genome segments were transcribed (Skehel and Joklik, 1969). A
subsequent paper (Zweerink and Joklik, 1970) reported that the rate of
transcription during the early period was exceedingly low, and that
when transcription commenced all ten genome segments were transcribed
simultaneously, relative rates of transcription being constant from 2-8
hours after infection.

The relative efficiency of the polymerase from virion particles
exposed to chymotrypsin treatment, to that of the polymerase from
infected cells was found to be similar and probably identical in their
properties (Gomatos, 1970). In addition to the enzyme RNA polymerase
four base-specific nucleoside 5'-triphosphatases have also been found
to be associated with the virus particle (Kapuler, et al., 1970). These phosphatases are responsible for hydrolyzing polymerase substrates to diphosphates. Their activities are functionally separate from that of the polymerase and are probably associated with a single protein. They may be involved in processing the mRNA, regulating the transcriptase or reorganizing the ten genome segments into the virion (Kapuler, et al., 1970).

A double stranded RNA polymerase from reovirus infected L cells was also isolated very recently (Watanabe and Sakuma, 1971). The dsRNA polymerase was template-bound and was separated from the ssRNA polymerase by controlled chymotrypsin digestion, followed by centrifugation in CsCl gradients. Single stranded RNA polymerase was recovered in a band of subviral particles while the dsRNA polymerase-template was distributed over the gradient (Watanabe and Sakuma, 1971). They concluded that dsRNA polymerase-template complex is an entity distinct from the ssRNA polymerase associated with the subviral particle.

**Viral Protein Synthesis.** In early studies immunologic techniques were used to observe the newly formed virus related proteins in reovirus infected cells (Spendlove, et al., 1963; Oie, et al., 1966). Virus antigens were first detected in the cytoplasm in 4–8 hours after infection, in HeLa cells infected with reovirus type 1 (Spendlove, et al., 1963) and RA cells infected with reovirus type 2 (Oie, et al., 1966) by the fluorescent antibody technique. Viral antigen which was detected as fine discrete particles originating in the perinuclear region as early as 3–6 hours post infection showed multiple foci at about 12 hours. These multiple foci enlarged to form a "collar" around the
nucleus in 24 hours (Oie, et al., 1966).

More exacting biochemical studies on the temporal synthesis of the reovirus structural proteins have been undertaken recently (Loh and Oie, 1969; Zweerink and Joklik, 1970). Of the 4 structural proteins which constitute the reovirus particle (Loh and Shatkin, 1968) most of the major proteins of reo-2 were formed early in the infection (Loh and Oie, 1969). These structural proteins were synthesized as early as 1-2 hours after infection and proceeded at a rapid rate until 8-10 hours reaching a maximum in 12 hours. An exception was major polypeptide IIIb, an outer capsid component, which was not made until 4 hours after infection. Synthesis of minor components were not seen early but small amounts were detected in 2-4 hours and 4-6 hours after infection. Components I and IIIa reached a peak at 8 hours and began to decline, whereas components II and IIIb continued to be synthesized at a rapid rate even at 12 hours but declined by 18 hours post infection (Loh and Oie, 1969). They further reported the presence of at least 6 new protein peaks other than structural viral proteins or cell proteins in cytoplasmic extracts of reo-2 infected HeLa cells from their polyacrylamide analysis. It was believed that these new peaks could be non-structural viral proteins (Loh and Oie, 1969). Zweerink and Joklik (1970) found no evidence for the synthesis of non-structural viral peptides. They stated that the synthesis of reovirus capsid polypeptide species were formed synchronously in the infected cells, and that there was no evidence that some begin to be formed significantly earlier than any others. However, these authors pointed out that at very early times after infection when the rates of synthesis of polypeptides were very low, it was not possible
to establish the above fact rigorously (Zweerink and Joklik, 1970). 

Subsequently, Joklik (1970) reported that there was clear evidence for the synthesis of a non-structural virus-coded polypeptide which belongs to the μ family whose function is not known.

The various structural polypeptides are not synthesized in equal amounts and these differences are due to the fact that both transcription and translation of the reovirus mRNA are regulated in the virus infected cell (Zweerink and Joklik, 1970) in contrast to the in vitro transcription of the reovirus genome RNA (Skehel and Joklik, 1969).

**Summary.** Reoviruses are ubiquitous in nature. The virion has an average particle diameter about 70 nm and is composed of an inner core plus a double capsid layer. The capsid is comprised of 92 capsomers arranged in an icosahedral pattern. Reoviruses are relatively stable to heat and a wide range of pH changes. They are resistant to ether.

Reovirus group comprises of 3 serotypes, namely 1, 2 and 3 which share a common complement fixing antigen, but are distinguished by neutralization tests. All three serotypes agglutinate human erythrocytes. Reoviruses replicate in a wide variety of mammalian cell species, producing inclusions in the cell cytoplasm. Foci of virus development are often associated with the mitotic apparatus.

Purified reovirus particle has a buoyant density of 1.38 g/cm³ and an estimated molecular weight (M.W.) of 70 x 10⁶ daltons. The virion has a helical double stranded RNA genome of M.W. 15 x 10⁶ daltons, and a single stranded adenine rich ribopolymer of approximate M.W. 5 x 10³ daltons, and of no known function. The dsRNA consists of 10 discrete segments which fall into three sizes: 3 large (L), 3 medium (M) and 4
small (S). The structural proteins of the virion capsid have been characterized into at least 3 major and 4 minor species of polypeptides.

Associated with the virion core is found an RNA dependent RNA polymerase which can be activated by a variety of methods in vitro. The polymerase transcribes one strand of each of the 10 segments of the dsRNA genome by a conservative mechanism to produce viral messenger RNA (mRNA) which codes for the various species of viral proteins.

Another group of enzymes associated with the virion is the four nucleoside 5' triphosphatases, which hydrolyze the substrates of RNA polymerase, and are associated with the subviral cores. A more recent finding is the detection of double stranded RNA polymerase in reovirus infected L cells, which would perhaps lead to a better understanding of the replicative mechanism of reovirus.

Of the several proteins found in the reovirus, most of the major proteins are synthesized early after infection reaching a maximum in 12 hours. All the structural proteins are made in 6-8 hours. Several new non-structural proteins also appear in reovirus infected cells. Their role and function remains to be defined.
Materials and Methods

Cell Cultures. Of the following cell cultures described, HeLa cells were used mainly for the production of reovirus pools and in cytotoxicity studies. The BSC-1 cells were occasionally used for the same purpose. RA cells were primarily used for reovirus titration and for the production of interferon. They were also used for the production of vesicular stomatitis virus pools. The CCL-1 strain of mouse L cells were mainly used to determine the effect of interferon on the cytotoxicity and for the assay of mouse interferon. The cell cultures were grown in monolayers in bottles using Eagle's basal medium (EBM) (Eagle, 1955) supplemented with 5% fetal calf serum (FCS).

HeLa: A stable line of human cells derived from carcinoma of the cervix.

RA: A stable line of human amnion cells, originally obtained from Dr. Robert Atkinson, University of Pittsburgh.

BSC-1 cells: A continuous line of African green monkey kidney cells originally obtained from Dr. Edwin H. Lennette of California State Department of Public Health, Berkeley, California.

CCL-1: A strain of mouse L cells kindly supplied by Dr. Samuel Baron of the National Institute of Health, Bethesda, Md.

The following cell cultures were used to test the sensitivity of the different cell systems to reovirus toxicity:

WI-38: A diploid line of human embryonic lung cells kindly supplied by Dr. Leon Rosen of the Pacific Research Section, National Institute of Health, Honolulu, Hawaii.
AGMK cells: Primary cells of African green monkey kidney obtained from Grand Island Biological Co., Grand Island, New York.

MEF: Primary mouse embryo fibroblast cultures prepared by trypsinizing mouse embryos (at 37°C) obtained by Caesarean section of pregnant mice.

BHK-21: An established line of baby hamster kidney cells, obtained from the laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

FHM: A continuous line of (fish cells) Fathead Minnow cells obtained from Microbiological Associates, Inc.

Viruses: Reovirus: Serotype 1 strain Lang (reo-1), serotype 2 strain D-5 Jones (reo-2), and serotype 3 strain Abney (reo-3) were passed several times in HeLa or BSC-1 cells before virus pools were prepared. Reo-1 was obtained from Dr. A. J. Shatkin of the National Institute of Health, Bethesda, Md., while reo-2 and reo-3 were obtained from Dr. Leon Rosen, Pacific Research Section, N.I.H., Honolulu.

Vesicular Stomatitis Virus (VSV): Indiana strain was obtained from Dr. Samuel Baron of the N.I.H., Bethesda, Md., and passed in RA cells before fresh pools were made.

Interferon: Mouse serum interferon used in this study was kindly supplied by Dr. S. Baron of the N.I.H. The interferon was titered in CCL-strain of mouse L cells by the plaque reduction technique (titer = 16,000 units/ml).

Radioactive isotopes: Carrier free $^{32}$P orthophosphate was obtained from International Chemical and Nuclear Corporation, Irvine, California.
3H-Leucine (40 Ci/m mole) and 3H-labeled reconstituted protein hydrolysate were obtained from Schwartz Bioresearch, Inc., Van Nuys, California.

Chromatography paper for RNA base analysis: Whatman DE81-DEAE cellulose paper was obtained from H. Reeve Angel & Co., Clifton, N. J.

Chemicals. The Poly I:C preparation (1 mg/ml) used for interferon induction was obtained from Microbiological Associates, Bethesda, Md.

Bovine pancreatic ribonuclease (A grade) was obtained from Calbiochem., Los Angeles, California.

Sodium Dodecyl Sulphate (SDS) was obtained from Fisher Scientific Co.

Urea (ultrapure) was from Mann Research Laboratories, N. Y.

2-mercaptoethylamine-HCl-B grade (2ME) was from Calbiochem., Los Angeles, California.

Virus-Pools. Reoviruses (serotypes 1, 2 or 3) were prepared in 4-5 day old monolayer cultures of HeLa or BSC-1 cells in 32 oz prescription bottles. The cell cultures were infected at a multiplicity of approximately 10 infectious units (I.U.) per cell using an inoculum of approximately 2 ml per bottle and maintained at 37°C in EBM containing 0.1% FCS. When infected cultures showed over 90% cytopathic effects (CPE) (in about 36 to 48 hours) the virus was harvested in the following manner: Infected cultures were frozen and thawed 3 times (-20°C and 25°C) and sonicated for 2 minutes at 20 kc in the cold using a Branson sonifier. The cell debris was removed by centrifugation at 10,000 rpm in an International centrifuge HR-1 using rotor No. 856 at 4°C for 20 minutes. The supernate containing semipurified virus was stored at -70°C in 2 to
10 ml aliquots after the virus titer was determined. Such preparations were used as stock virus and usually contained infectious titers of about $3 \times 10^7$ to $2 \times 10^8$ I.U. per ml (determined by the immuno-fluorescent plaque technique). When necessary, these pools were concentrated 5 to 10 fold by centrifuging the virus at 30,000 rpm in the SW30 rotor (78,600 $\times$ g) for 2 hours in the Spinco Model L and resuspending the pellet of virus in EBM containing 0.1% FCS.

For preparation of VSV pools, monolayers of RA cells were infected at a multiplicity of 1-5 plaque forming units (PFU) per cell and adsorbed at 37°C for 1 hour. Unadsorbed virus was removed by washing monolayers twice in BSS. Infected cultures were maintained in EBM containing 0.1% FCS for 24-36 hours until maximum CPE was attained. The virus was harvested by freezing and thawing the infected cultures twice and centrifuging down the large cell debris at 2000 rpm in an International centrifuge model CS for 10 minutes. Virus containing medium was stored frozen at -70°C. After determining the virus titer by plaque assay, the pool was redistributed into 1 ml quantities and kept at -70°C. Such pools had virus titers ranging from $6.6 \times 10^6$ PFU to $2 \times 10^7$ PFU/ml.

**Infection Procedure.** Unless otherwise specified, the infection procedure adopted in all experiments using reovirus was as follows: monolayer cultures were washed twice in BSS and the virus inoculum introduced. Virus was allowed to adsorb for 2 hours at 37°C with intermittent rocking to redistribute the virus inoculum evenly on the monolayer. At the end of the adsorption period monolayers were washed again to remove the unadsorbed virus, and fresh EBM containing 0.1% FCS introduced. The infected cultures were then incubated at 37°C.
Purification of Virus. The procedure described by Loh and Oie (1969) was followed for the purification of reovirus pools. Reoviruses grown in monolayer cultures of HeLa cells were harvested after 36-48 hours. The cells were frozen and thawed twice, and the virus was pelleted by centrifugation at 78,000 x g for 2 hours. The pellet was resuspended in calcium-free Dulbecco's phosphate buffered saline (pH 7.2). The virion preparation was extracted with fluorocarbon Genetron 113 (Allied Chemical Co.) by mixing with half its volume cold fluorocarbon and homogenizing in a Virtis "45" mixer at a rheostat setting of 40 for 2 minutes in an ice bath. The virus-containing aqueous supernatant was recovered by centrifuging the homogenates at 2000 rpm for 15 minutes at 4°C in an International centrifuge model PR-1. The virion was concentrated by overlaying the fluorocarbon-treated suspension onto a cushion of cesium chloride (CsCl) (1.39 g/cm³) and centrifuging at 64,000 x g in the SW25 rotor of the Spinco model L for 1 hr at 4°C. The band containing reovirus was collected by puncturing the tube from the bottom and re-banded in CsCl (1.37 g/cm³) at 33,000 rpm in the SW39 rotor for 24 hrs at 4°C. The band of virus was collected from the bottom. Some preparations had a band of "empty" virion particles banding at about 1.5 cm above the band of complete virus. This band was collected with a syringe and needle from the side of the tube immediately below the band. The virion collected was dialyzed against Ca²⁺ free Dulbecco's phosphate buffered saline (D-PBS) (pH 7.2) at 4°C. The density of the complete virion in CsCl solution was 1.38 g/cm³ and that of the "empty" particle was 1.29 g/cm³. Purity of the preparation was checked by examination of the particles under a Hitachi HU-11 electron microscope.
**Virus Titration Methods.** Immunofluorescent plaque technique: Titration of reoviruses serotypes 2, as well as 1 and 3, was performed by the immunofluorescent plaque method (Spendlove and Lennette, 1962) using monolayers of RA cells grown on 15 mm round coverslips placed inside 35 x 10 mm Falcon plastic petri plates containing EBM with 5% FCS. Cell cultures were incubated at 37°C in a Hot Point incubator in an atmosphere of 5% CO₂. Monolayers on coverslips were washed twice with BSS and each of duplicate coverslips was infected with 0.02 ml of the appropriate virus dilution. Virus adsorption was done at 37°C for 2 hrs in the CO₂ incubator. Infected cultures were washed 2x with BSS, fed with EBM containing 0.1% FCS and incubated at 37°C in the CO₂ incubator for 18-20 hours. The infected coverslip cultures were washed twice with Dulbecco's phosphate buffered saline (D-PBS) and fixed in cold acetone for 10 minutes. The direct fluorescent antibody (FA) technique of Coons and Kaplan (1950) was employed for the visualization of viral antigens. Non-infected cultures on coverslips served as controls. Two drops of fluorescein isothiocyanate labeled reovirus antibody was spread on the coverslip and incubated at 37°C for 45 minutes. Excess antibody was removed by washing 3 times in Dulbecco's PBS. Coverslips were mounted on a glass slide in a 50% glycerol-saline mixture and the fluorescent cells in each coverslip culture was examined and enumerated by the use of a Zeiss fluorescent compound microscope assembly. Only duplicate coverships containing from 10-200 fluorescing cells were considered for counting.

was used to titrate the vesicular stomatitis virus. RA cell monolayers were grown in either 1 oz prescription (Rx) bottles or 35 x 10 mm Falcon plastic petri dishes. Monolayers were washed in BSS and replicate cultures were infected with 0.1 ml of the appropriate virus dilution and incubated at 37°C for 1 hour. Unadsorbed virus was removed by washing once in BSS, and EBM containing 0.75% Bacto agar and 2% FCS was introduced. The cultures were incubated at 37°C in the CO₂ incubator for 48 hours at which time plaques appeared. The agar overlay was gently removed and the monolayers containing plaques were stained with 0.1% crystal violet for 10 minutes. The stain was washed off and the plaques were enumerated. Cultures having 10 to 100 plaques were used for the calculation of virus titers.

Assay for Cytotoxicity. Cytotoxic studies were performed on monolayer cultures of HeLa cells unless specified otherwise. HeLa cells were grown in 1 oz prescription bottles, or on 15 mm round coverslips. Cell numbers were usually estimated by a cell count of trypsinized replicate cultures before the beginning of the experiment. The one oz prescription bottle cultures used in most experiments generally had an average cell count of about 8 x 10⁵ cells/bottle, while the coverslip cultures had about 1.5 x 10⁵ cells/coverslip.

One oz prescription bottle cultures: When 0 oz prescription bottle cultures were used they were washed with BSS and infected with a multiplicity of about 50 infectious units (I.U. present before UV-inactivation) in a volume of 0.1 to 0.2 ml. In all experiments estimating the degree of cytotoxicity, except where the effect of UV dose was being determined, virus exposed to 10 minute UV-light was used
because such preparations were found to be the most efficient in cell-killing property. The virus was allowed to adsorb at 37°C for 2 hours, and the bottles were rocked intermittently to redistribute the inoculum over the monolayer. At the end of the adsorption period, the cultures were washed and fed with EBM containing 0.1% FCS and returned to the 37°C incubator. At different times post infection (p.i.) the cultures were examined under a Unitron inverted microscope for the appearance of cytotoxicity (CT). The degree of cytotoxicity was estimated by the percent of cells rounded and detached from the glass surface. This was measured in comparison to the uninfected cell cultures and cell cultures infected with non-irradiated reovirus at similar multiplicities. Cytotoxicity was scored in the following manner: 4+ = 100% CT, 3+ = 75% CT, 2+ = 50% CT and + = 25% CT.

Coverslip cultures: HeLa cells were grown in duplicate coverslips placed in 35 x 10 mm Falcon plastic petri dishes in a 37°C CO₂ incubator. The virus inoculum in 20-40 μl volume per coverslip was added and was allowed to adsorb under conditions previously described, except that the cultures were incubated in a CO₂ atmosphere. Employment of this technique facilitated the use of smaller volumes of purified and degraded virus samples.

Assay for viable cells by trypan blue: The method described previously by Payne, et al. (1958) was used. Normal and R2 or UV-R2 infected HeLa cell monolayers in 1 oz bottles were washed twice with BSS at pre-determined time periods, and trypsinized (0.025% trypsin, 37°C, 10 mins). The trypsinized cells were washed once and re-suspended in 2 ml BSS. A sample (0.5 ml) of the cell suspension was allowed to react
with an equal volume of 0.02% trypan blue in BSS for 10 mins. Only the cells that did not take up the stain were enumerated. Mean cell counts from replicate monolayers were determined. In calculating the percent viable cells in UV-R2 infected cultures, mean viable cell count of the normal cell cultures at the corresponding time was taken as 100%.

**Interferon Assay.** The plaque reduction method described by Baron (1969) with few modifications were used. Depending on the species of interferon being assayed (human or mouse), monolayer cultures of RA or mouse L cells (CCL-1) grown in 1 oz prescription bottles or 35 x 10 mm plastic petri dishes were exposed to varying dilutions of interferon for 18-24 hrs at 37°C. These cultures were then washed 3x in BSS and challenged in the same manner. After an adsorption period of 1 hr at 37°C, the unadsorbed virus was removed and washed once in BSS. Agar medium containing 0.75% Bacto agar was gently removed, the cultures were stained with 0.1% crystal violet and the plaques were counted. Cultures which showed 50% inhibition of plaques in comparison to the control cultures was considered to have 1 unit of interferon per ml at that dilution.

**Hemagglutination Procedure.** The method described by Rosen (1960) was adopted to assay the hemagglutination (HA) titers of reovirus. The test was carried out in 12 x 75 mm test tubes. Virus dilutions were made in phosphate buffered saline pH 7.2. To each of 0.4 ml amounts of serial two fold dilutions of the virus, 0.2 ml of a 0.75% suspension of human type "0" erythrocytes in PBS was added, mixed and allowed to sediment at room temperature for 1 hour and the agglutination titer was determined. The highest virus dilution showing maximum agglutination (4+) pattern
was considered the end point of the titration.

**Electron Microscopy.** Reovirus-type 2 purified by sedimentation in CsCl gradients was suspended in D-PBS (pH 7.2) and used for electron microscopical examination by the negative-staining technique described by Brenner and Horne (1959). A loopful of the concentrated purified virus suspension was added to a carbonized collodion coated grid, and after a brief period (30 seconds) a loopful of the negative stain containing 2% phosphotungstic acid (PTA) containing 0.4% sucrose at pH 6.0 was added. Excess fluid was removed after one minute by touching the sides of the grid with a filter paper, and the film of liquid on the grid was allowed to dry. The grids were then examined in a Hitachi HU-11 electron microscope.

**UV-Inactivation of the Virion.** Ultraviolet light irradiation of reovirus was performed with a General Electric 15 watt germicidal lamp according to the procedure described earlier (Loh and Oie, 1969) with a few modifications. Preparations of virus stock to be irradiated were exposed to UV light in 2 ml amounts in an open glass petri dish (40 mm diameter) placed at a distance of 4 cm from the lamp. The sample was rocked gently during irradiation by means of a Yankee rotator. In other experiments 1 ml preparations of reovirus purified by banding in CsCl gradients were inactivated in a 10 ml glass beaker under the same conditions. For cytotoxicity studies the virus samples were irradiated for 10 minutes, and for the biological and physico-chemical characterization of the CT-inducing virus, the samples were irradiated from 5 to 60 minutes. All such preparations did not contain any residual infectivity as determined by the immunofluorescent plaque method described in this
The amount of energy incident on the virus preparation under this set up was equivalent to: $10^5$ ergs/sec/cm$^2$.

**Preparation of Radioactive Reovirus.** Viral RNA, protein or dual-labeled reovirus was prepared according to the procedures described before (Loh and Shatkin, 1968; Loh and Oie, 1969).

$^{32}$P-labeling of viral RNA: HeLa cell monolayer cultures grown in 32 oz prescription bottles were infected at an input multiplicity of approximately 10 I.U. per cell; adsorbed at 37°C for 2 hrs. Fresh low phosphate EBM containing $^{32}$P-orthophosphate containing 12-17 μCi/ml and 0.1% FCS was added to the infected cultures and incubated at 37°C for approximately 36 hrs until maximum CPE was observed. The virus was harvested and purified as described elsewhere in this chapter.

Labeling of structural proteins of the virion with $^3$H-protein hydrolysate or $^3$H-leucine: HeLa cell monolayers grown in 32 oz prescription bottles were infected at an input multiplicity of 10 I.U./cell and adsorbed for 2 hrs as before. At the end of the adsorption period the cultures were fed with EBM containing only 10% of the normal concentration of amino acids, $^3$H-labeled protein hydrolysate at a concentration of 2 μCi/ml, and 0.1% FCS. The virus was harvested and purified as described above.

Dual labeling of protein and RNA of the virion: the procedure adopted here was similar to the above except that in addition to the $^3$H-labeled protein hydrolysate $^{14}$C-uridine at a concentration of 0.3 μCi/ml was added to the medium before incubation. The harvesting and purification of the virus was done in the same manner.
Solubilization of Virus. Radio-active labeled and unlabeled purified preparations of reovirus type 2 were degraded to viral proteins according to the methods described before (Loh and Shatkin, 1968). Briefly, virus samples containing 150-600 μg/ml protein suspended in phosphate buffered saline (pH 7.2) were degraded by treatment with 2% sodium dodecyl sulphate (SDS), 1% 2-mercapto-ethanol (2-ME) and 8 M urea, for 2 to 3 hours at room temperature and were then dialyzed for 2 to 3 hours against 0.1% SDS, 0.1% 2-ME and 8 M urea in 0.01 M phosphate buffer (pH 7.2). Sucrose was added to a final concentration of 10% and the samples applied to polyacrylamide gels for electrophoresis.

Controlled Degradation of Reovirus. Controlled degradation of purified preparations of reovirus type 2 to subviral particles (SVP) and outer capsid proteins (OCP) was performed according to the method described by Loh and Shatkin (1968). Purified preparations of reovirus suspended in Dulbecco's Ca++-free phosphate buffered saline (D-PBS, pH 7.2) were treated with 4 M urea for 3 minutes at 4°C and the reaction halted by the rapid addition of CsCl solution (590 mg/ml). Subviral particles were separated from the outer capsid proteins by centrifugation in CsCl isopycnic density gradients for 24 hours at 33,000 rpm in a Spinco SW-39 rotor. The tubes were punctured at the bottom and 8 drop fractions were collected. Radioactivity and/or optical density in each fraction was measured. Peak fractions at the bottom of the gradient were SVP, while the outer capsid proteins (OCP) settled in a peak at the top of the gradient. The peak fractions were dialyzed against 2 changes of D-PBS in the cold and concentrated when necessary before using for cytotoxicity studies.
Extraction of Viral RNA. $^{32}$P-labeled viral RNA was extracted from purified reovirus type 2-D, by one of two methods: Sodium dodecyl sulphate (SDS) and phenol method (Shatkin and Rada, 1967) or SDS and urea method (Bellamy, et al., 1967) modified by Loh (Personal Communication).

Sodium dodecyl sulphate--phenol method: The virus sample was suspended in 0.01 M acetate buffer (pH 5.1) containing 0.1 M NaCl and 0.001 M MgCl$_2$ (final volume 3 ml). 0.15 ml of 10% SDS and 40 mg polyvinyl sulphate (PVS) were added. An equal volume of water saturated hot phenol (55°C) was added next and was shaken for 5 minutes in a 55°C water bath. The mixture was centrifuged at 2000 rpm in an International Centrifuge Model CS for 5 minutes at 25°C. The aqueous layer on the top was recovered with a Pasteur pipette. The phenol layer was re-extracted with 1 ml of acetate buffer (pH 5.1) by shaking in the 55°C water bath for 2.5 minutes and centrifuging as in the initial step. The aqueous layer from the second extraction was pooled with that of the first extraction. To this was added potassium acetate (to a final concentration of 2%) and 10-20 μg/ml PVS and 3 volumes of cold absolute ethanol. RNA was allowed to precipitate overnight at -20°C. The precipitate was recovered by centrifugation at 10,000 rpm in an International Centrifuge Model HR-1 for 20 minutes at -20°C, was washed in cold absolute ethanol, and redissolved in the appropriate buffer.

SDS-urea method: To a suspension of reovirus in PBS (pH 7.2) SDS was added to a final concentration of 1%, and urea to 4 M concentration. The mixture was gently shaken to dissolve the urea. A sufficient volume of 12 x Tris-acetate EDTA buffer (pH 7.8) was added to the mixture to give a 1 x concentration, and incubated for 10 minutes at 37°C. The
recovery of RNA by this method was very efficient. The extracted RNA was added directly onto polyacrylamide gels for electrophoretic analysis.

**Polyacrylamide Gel Electrophoresis of Virus Proteins.** Gels were made as described previously (Summers, et al., 1965) and consisted of 10% acrylamide, 0.26% N, N'-bis-methylene acrylamide, 0.1% SDS and 0.1 M phosphate buffer (pH 7.2). The acrylamide gel electrophoresis of reovirus proteins has been described (Loh and Shatkin, 1968). Samples of solubilized viral proteins (100–300 µg) in 0.05 to 0.2 ml of buffer containing 10% sucrose were electrophoresed for 24 hours at 4 mamps per gel at 20°C. After electrophoresis the gels were fixed in 10% trichloracetic acid (TCA), stained with 0.025% Coomassie blue and destained in 7% acetic acid (Maizel, 1966) when unlabeled virus was used. Gels having radioactive proteins were sliced into 1.3 mm sections and digested in hydrogen peroxide at 60°C overnight. On occasion the solubilized gel slices were allowed to absorb onto filter paper placed in glass vials and allowed to dry.

Scintillation fluid (2,5-bis-[2-(5-tert-Butyl benzoxazolyl)] Thiophene in toluene) was then added and radioactivity counted in a Packard liquid scintillation spectrometer (Shatkin, et al., 1968).

**Polyacrylamide Gel Electrophoresis of Viral RNA.** The electrophoresis of viral RNA was performed according to the methods described (Loening, 1967; Shatkin, et al., 1968). ³²P-labeled viral RNA extracted either by using SDS and phenol (Shatkin and Rada, 1967) or SDS and urea (Loh, personal communication) was electrophoresed on 11 cm long 4% polyacrylamide gels containing 0.025% agarose. The electrophoretic buffer was 0.04 M Tris, 0.02 M sodium acetate and (2mM) EDTA (pH 7.8).
Electrophoresis was conducted at 4 mamps per gel for 24 hours at 18-20°C. After electrophoresis the gels were sliced and processed in the same manner as for the radioactive labeled proteins described in this chapter and counted in a Packard liquid scintillation spectrometer.

**Base Composition Analysis of Viral RNA.** $^{32}$P-labeled purified preparations of reovirus type 2 were digested in 0.3 M KOH overnight at 37°C and neutralized with 11.9 M perchloric acid, as described before (Katz and Comb, 1963). Base analysis of such preparations were performed on sheets of DEAE cellulose according to the method of Jacobson (1962).

Briefly: 60 μl of a mixture of unlabeled nucleotides (A, U, G and C containing 1 mg/ml of each) were spotted at a distance of 23 cm from one side of each of the replicate strips of DEAE-cellulose sheet (2.5 cm x 56 cm) which was previously treated with 1 M formic acid. Having allowed the nucleotide standards to dry, 60 μl of the digested RNA sample containing 50-100 x $10^3$ cpm were spotted at the same place and allowed to dry. The strips of chromatography paper were allowed to hang down from the trough of the chromatography cabinet. The papers were irrigated with 0.05 M formic acid in a descending manner until the cytidylic acid (C) moved about 20 cm (90 minutes). The irrigant was then removed and papers allowed to dry. Adenylic acid (A) also moved 10 cm to 15 cm by this time, while the Uridylic acid (U) and guanylic acid (G) remained unseparated at the origin. The papers were examined in the dark under a UV lamp to identify the spots and were cut between the UV-adsorbed spots of GU and A. Spots A and C were also cut and saved at this time. A 5 cm long strip of chromatography paper was attached onto the paper containing spot GU, placed in the chromatography cabinet
and irrigated with 4 M formic acid. Chromatography in the reverse direction was conducted for 50 minutes. At this time the acid was removed and the papers allowed to dry inside the cabinet, and examined under the UV lamp. Spots G and U were cut out. The papers containing A, U, G and C were then placed in scintillation vials containing liquifluor-toluene and the amount of radioactivity determined in a liquid scintillation spectrometer. The base composition was obtained by calculating from the average cpm of replicate samples.
CHAPTER 4

Properties of the UV-irradiated Reovirus

Sensitivity of viruses to ultraviolet light (UV) has been reviewed by several workers (Kleczkowski, 1957; Stent, 1958; Barron, et al., 1959). However, most of the early work was done with bacterial and plant viruses. Exposure of viruses to UV could produce a series of changes in the virion, with loss of infectivity being one of the first. Loss of viral infectivity principally yields a nucleic acid action spectrum. This has been shown with several bacterial, plant, and animal viruses such as T2, ØX 174, MS2, TMV, VSV, vaccinia, polyoma, EMC, reo-3 and influenza virus (Smith and Hanawalt, 1969; Rauth, 1965). Viruses with single stranded nucleic acid were ten times more sensitive to UV than those with double stranded nucleic acid. The above was true for both RNA and DNA viruses. Since reo-3 was found to have a lower ratio of inactivation cross section (at 2650 Å to 2250 Å) than single stranded viruses, it was concluded that reovirus proteins were relatively more sensitive to UV (Rauth, 1965).

Many viral properties were retained after the loss of infectivity, for example: cytotoxicity of adenovirus, VSV and reovirus (Cantell, et al., 1962; Huang and Wagner, 1965; Loh and Oie, 1969), cell fusion by measles virus (Cascardo and Karzon, 1965), interferon induction by myxoviruses (Kohn, 1965), hemagglutination by influenza virus (Tamm and Fluke, 1950), transformation of human fibroblasts by SV40 (Aaronson, 1970), induction of an uncoating protein by pox virus (Joklik, 1964), immunogenecity of poliovirus and influenza (LeBouvier, 1955; Tamm and Fluke, 1950). Although the above biological properties were less
sensitive to UV than infectivity, these functions could also be affected by higher doses of irradiation.

When influenza virus was exposed to UV the different properties of the virus were lost in the following sequence: infectivity, toxicity, immunizing capacity and hemagglutinating property (Henle and Henle, 1947). Although infectivity was one of the earliest properties of the virus to be lost on UV irradiation, certain alterations have been detected before the loss of infectivity with phages, namely a delay in multiplication of coliphage, decrease in stability and photoreactivation in rhizobium bacteriophage, and increased frequency of genetic recombination in Escherichia coli bacteriophage (Kleczkowski, 1957). Similar findings have not been reported with animal viruses.

Some of the biological effects of UV could now be explained in terms of specific chemical and physical changes occurring at the molecular level. Perhaps one of the important findings in this area of research was the discovery of thymine dimers in DNA by Beukers and Berends (1960). Several other photo-biological products have been discovered since and their biologic importance was determined in some cases. Recent reviews on photochemical changes occurring in the proteins and nucleic acids show that a significant number of photoproducts have been identified (Smith, 1969; Smith and Hanawalt, 1969; Setlow, 1966; McLaren and Shugar, 1964; Yearger, 1969). Photochemical products found in the nucleic acids were dimers and hydrates of pyrimidines, DNA or RNA-protein cross links, chain breaks and the formation of DNA-DNA cross links. Chain breaks and interchain cross links were not considered biologically important because they were formed only at high doses of UV. RNA-protein cross
links and pyrimidine dimers have been observed in UV-irradiated TMV (Setlow, 1966).

The sensitivity of proteins to UV depends mainly on the amino acid composition. Aromatic amino acids are photochemically important because they strongly absorb wavelengths above 2400 Å. The most important chromophores in proteins are: cystine, tryptophan, phenylalanine, tyrosine, peptide bonds and histidine. UV-irradiated proteins have been found to undergo certain changes in electrophoretic mobility, ultracentrifugal sedimentation, molecular weight and enzyme inactivation, etc. (Smith and Hanawalt, 1969). However, the fundamental chemical mechanisms involved in these changes are largely unknown. Photochemical studies on individual amino acids and peptides could perhaps be useful in understanding some of the biological effects of UV on proteins, and could be extended to viruses.

Viruses can be used as a simple model system for the study of the effects of UV on macromolecular structure and functions, since they may be considered as organized packages of biologically active molecules, which could be obtained in purified form, and are lacking in metabolic processes within their systems to complicate any reaction. Such advantages are not available even in a simple living system such as a bacterial cell.

It was pointed out in Chapter 1 that reoviruses acquired a new cytotoxic property on exposure to UV inspite of the loss of infectivity under such conditions (Oie and Loh, 1967). In addition, it was also shown above that many animal viruses, rendered non-infectious by UV, retained several biological properties. It was of interest in this
study to examine the effects of UV on the biological and physico-
chemical properties of reovirus, and the dose dependency of such effects. 
It was of further interest to correlate some of these effects with the 
acquisition of cytotoxicity.

Biological Properties of UV-R2

The Cytotoxic Property of the UV-irradiated Reoviruses and the 
Kinetics of Cell Killing. Previous reports have shown that reovirus 
type 2 acquired a cytotoxic property after exposure to ultraviolet 
light (UV) for 2-5 minutes (Oie and Loh, 1967). No attempts were made 
to determine the optimum dose of UV-irradiation necessary for the 
acquisition of maximum cytotoxic (CT) property by the virion particle in 
such studies. The following experiment was done to determine that 
optimum dose of UV, and also to study the effects of continued 
irradiation on the virus particle.

Procedure: Reovirus type 2 was irradiated for varying periods of 
time from 0 to 60 minutes (0', 5', 10', 30' and 60') as described in 
Materials and Methods (Chapter 3). Replicate monolayer cultures of HeLa 
cells grown in 1 oz prescription bottles were exposed to these UV-R2 
preparations at an infection multiplicity of 50 I.U./cell (i.e. infectivity 
before inactivation). Control cultures were inoculated with EBM 
containing 0.1% FCS and treated in the same manner. After adsorption of 
the virus for 2 hours at 37°C, the cultures were washed in BSS and 
returned to the 37°C incubator with EBM containing 0.1% FCS. At pre-
determined times post-infection (i.e. 0, 2, 4, 8 and 12 hrs) replicate 
experimental and control cultures were washed in BSS to remove dead
cells and the surviving number of viable cells was determined after trypsinization of the monolayer as described before (Payne, et al., 1958).

Results: The results indicated that the CT property of UV-R2 increased with increasing dose of UV-irradiation until a maximum toxic response was obtained by R2 exposed to 10 minutes UV under the experimental conditions employed (Fig. 1). The amount of photoenergy incident on the virus sample during the period of 10 minutes was calculated to be approximately $6 \times 10^7$ ergs/cm$^2$. At a multiplicity of 50 I.U./cell of the 10 minute UV-irradiated virus sample (UV10-R2), a 52% cell death was observed at 12 hrs p.i. Upon continued irradiation the CT induction capacity of the virus decreased in efficiency until the cytotoxic property was completely lost after 60 minutes exposure to UV. The percent cell death at 12 hrs p.i. was 24% with the 30 min UV-irradiated virus (UV30-R2) and fell to undetectable levels with the 60 minute sample (UV60-R2) (Fig. 1). In contrast to UV-R2, non-irradiated virus exhibited no CT. The kinetics of cell-killing by virus exposed to various UV doses showed that in all UV-R2 samples, very rapid cell killing occurred between 2-4 hrs p.i. and became slow thereafter. Beyond 8 hrs the rate of cell-killing was extremely low (Fig. 2). These data confirm the previous report on the kinetics of cell killing where R2 exposed to a single dose of UV (2-5 mins) was used (Loh and Oie, 1969).

It was concluded that the CT acquired by reovirus was dependent on the dose of exposure to UV, and that maximum toxicity was acquired when the virus was irradiated for 10 minutes at an incident photoenergy dose of $10^5$ ergs/sec/cm$^2$. 
Figure 1. The effect of UV dose on reovirus (type 2) induced cytotoxicity in HeLa cells. Percent cell death at 12 hrs p.i. are plotted against the dose of UV. Reovirus preparations were exposed to UV-irradiation for 0, 5, 10, 30 and 60 mins. under the conditions described in the Materials and Methods. Replicate monolayer cultures of HeLa cells in 1 oz bottles were exposed to R2 or each of the UV-R2 samples at a multiplicity of 50 I.U./cell. At 12 hrs p.i. infected and non-infected cultures were washed trypsinized and viable cell counts were made. The per cent cell death was estimated from the mean difference in surviving cells of the non-infected controls and each of the infected cultures.
Figure 2. Kinetics of cell death caused by reovirus type 2 exposed to various doses of UV (0 mins •, 5 mins ●, 10 mins ○, 30 mins □ and 60 mins △). Percent surviving cells in cell cultures exposed to R2 or each of the UV-R2 samples are expressed as a function of time. Replicate monolayer cultures of HeLa cells in one oz bottles were exposed to R2 or each of the UV-R2 samples at a multiplicity of 50 I.U./cell. At various intervals after infection each set of infected and non-infected cultures was washed, trypsinized and a viable cell counts made. The percent of cells surviving was calculated from the mean cell counts using the cell counts from non-infected controls as 100%.
Adsorption Property of UV-irradiated Reovirus. Virus adsorption is the primary step in the process of virus-cell interaction. The mechanism of adsorption of reovirus to tissue culture cells is largely unknown. Drawing an inference from hemagglutination studies, it was suggested that surface glycoproteins could perhaps play an important role (Shatkin, 1968). The experiments described below were done to examine the adsorption property of the cytotoxicity (CT)-inducing UV-irradiated reovirus (UV-R2), and also to study the effect of further irradiation on this property of the virus.

Procedure: Replicate cultures of HeLa cell monolayers grown in 35 x 10 mm Falcon plastic petri dishes were exposed to purified $^3$H-leucine-labeled reovirus type 2 (4788 cpm in 0.5 ml) that was UV-irradiated for 5 and 60 minutes as described in Materials and Methods (Chapter 3). Non-irradiated $^3$H-labeled R2 was used as control. Virus adsorption was conducted at 37°C. At specific times post-infection the unadsorbed virus inoculum was removed, each monolayer was washed once with 0.5 ml of BSS, and pooled with the removed inoculum. The radioactivity in the unadsorbed inoculum and wash was determined by spotting 0.1 ml samples on strips of filter paper (5 cm x 2.5 cm) and counting in a Packard liquid scintillation spectrometer. The amount of labeled virus adsorbed at different times after infection was calculated from the difference in the radioactivity in the supernates between time 0 and subsequent time periods.

Results: It was found that 51% of the inoculated non-irradiated virus was adsorbed in 2 hrs, whereas within the next 2 hrs adsorption occurred at a decreasing rate when only 6% of the labeled virus was adsorbed (Table I). The adsorption rates of UV-R2 samples showed no
TABLE I. ADSORPTION OF $^3$H-LEUCINE LABELED REOVIRUS TYPE 2 TO HEla CELL MONOLAYERS

<table>
<thead>
<tr>
<th>Samples</th>
<th>% Radioactivity Adsorbed at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>R2</td>
<td>34</td>
</tr>
<tr>
<td>UV5-R2</td>
<td>31</td>
</tr>
<tr>
<td>UV60-R2</td>
<td>23</td>
</tr>
</tbody>
</table>

R2 = reovirus type 2, unirradiated
UV5-R2 = virus irradiated for 5 mins.
UV60-R2 = virus irradiated for 60 mins.

The various $^3$H-leucine-labeled virus samples in 0.5 ml volumes were added to monolayers of HeLa cells in 35 x 10 mm plastic petri dishes and the cultures were incubated at 37°C. At intervals the virus inoculum was removed from each of the duplicate plates used per variable, the plates were washed in 0.5 ml BSS and the radioactivity in the removed inoculum and the washing determined for each plate. The mean value for each group are recorded. Input radioactivity per petri dish was 4788 cpm.
significant difference from the controls. At 2 hrs p.i. the amount of
radioactivity adsorbed from the 5 min irradiated virus (UV5-R2) and 60 min
irradiated virus (UV60-R2) were 48 and 43% respectively. The rate of
adsorption became slower after 2 hrs, in the UV-R2 as well.

It was concluded that exposure to various doses of UV-irradiation
did not alter the adsorption capacity of reovirus to any detectable
level. Although UV60-R2 has been previously shown to be no longer
cytotoxic, this loss of CT property cannot be associated with a loss in
adsorption capacity to the cells. This experiment has shown that the
adsorption rate of UV60-R2 (non-cytotoxic) was not different to that of
UV5-R2 (which is toxic) or non-irradiated R2. Therefore, it appears
that the initiation of the cytotoxic event is not just the simple
adsorption of virus to sensitive cells.

**Hemagglutinating Property of UV-irradiated Reovirus.** Hemagglutination
is a property of reovirus, due to the presence of a glycoprotein on the
virion (Lerner, et al., 1963) and is often used to determine the amount
of virus present in a given preparation. However, it is not as sensitive
a method as the titration of virus infectivity. A detailed discussion
on reovirus hemagglutination was presented in Chapter 2 of this
dissertation. The experiments discussed below were conducted to study
the effect of UV-irradiation on the hemagglutinating capacity of the
virus and its correlation to cytotoxicity.

**Procedure:** Purified preparations of R2 were exposed to varying
doses of UV-irradiation as described in Materials and Methods (Chapter
3). Two-fold dilutions of such UV-R2 preparations were allowed to react
with a standard suspension of human "0" group erythrocytes at room
temperature (25°C) and the hemagglutination (HA) titers were determined according to the procedure described in the Materials and Methods.

Cytotoxicity-inducing property of the UV-R2 preparations were studied by exposing monolayer cultures of HeLa cells to UV10-R2 at multiplicities of 60-80 I.U./cell and observing the CT at 4, 8 and 12 hrs post-infection (as described in Chapter 3).

Results: The results indicated clearly that reovirus lost its HA property with increase in dose of UV-irradiation (Table II). At a UV dose of 10 mins (6 x 10^7 ergs/cm^2) HA titer was reduced to 25% of its original value. However, the CT property of the UV-R2 at the same dose of UV was found to be the maximum observed (i.e. 80-90% cell death in 12 hrs). Virus that received a dose of 30 min UV under the same conditions (1.8 x 10^8 ergs/cm^2) had lost its hemagglutinating property completely, while the property of CT was present to the extent of about 25%. No HA activity or CT was observed with virus exposed to UV doses of 40 mins or more.

It was concluded that the loss in HA property can not be correlated to the acquisition or loss of CT property of the virus since the loss in HA property of reovirus preceded that of CT property, the former property appeared to be more sensitive to UV than the latter and they are apparently independent of one another. Since the glycoproteins involved in the hemagglutination of reovirus are present on the surface of the virion, perhaps these molecules are affected by UV-irradiation earlier and undergo certain alterations resulting in a decrease in HA activity. On the other hand the cytotoxic component might be a protein(s) deeply seated in the capsid of the virion and might also present a smaller
### TABLE II. EFFECT OF UV-IRRADIATION ON HEMAGGLUTINATION OF REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>Dose of Irradiation (mins)</th>
<th>HA Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cytotoxicity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64</td>
<td>±</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>3+</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>&gt;3+</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>2+</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reciprocal of highest virus dilution showing complete agglutination.

<sup>b</sup> Cytotoxicity at 12 hrs post infection. MOI = 60-80 I.U./cell (infectivity before exposure to UV)

Purified R2 was exposed to UV for varying periods of time from 0 to 60 mins. under the standard conditions described earlier. Several two-fold dilutions of the virus were allowed to react with 0.2 ml of a 0.75% suspension of human "O" erythrocytes at 25°C. The highest dilution of virus which showed maximum agglutination after 1 hr was taken as the end point containing 1 HA unit.

The CT inducing capacity of the various UV-R2 preparations were determined by exposing duplicate monolayer cultures of HeLa cells in 1 oz bottles to the various UV-R2 preparations at a multiplicity of 60-80 I.U./cell according to the standard procedure described before. CT observed at 12 hrs p.i. is shown in the table (+ = 25% CT; - = no CT observed).
target. Such a component(s) would therefore require a high dose of UV in order to render it non-cytotoxic.

**Induction of Interferon by UV-irradiated Reovirus.** The production of an antiviral substance with the properties of interferon, in a stable line of human amnion (RA) cells was previously described (Oie and Loh, 1968).

Further studies were undertaken to determine (a) whether UV-irradiated reovirus, retained the interferon (IF) inducing property, and (b) the effect of UV dose on the IF induction ability of the virion.

**Procedure:** The method of Oie and Loh (1968) was followed for IF induction. Replicate cultures of RA cell monolayers grown in 12 oz prescription bottles were exposed to non-irradiated reo-2 or reo-2 irradiated for 5, 10, 30 or 60 minutes (as described in Chapter 3) at multiplicities of 1-4 I.U./cell. After 2 hours virus adsorption at 37°C, the unadsorbed inoculum was washed twice with BSS and fed with EBM containing 0.1% FCS. The cultures were incubated at 37°C for 48 hours. At 12 hours post-infection monolayers were examined to see whether there was cytotoxicity. Since the multiplicity was low CT was less than 5%. After 48 hours incubation, the cultures were frozen and thawed 2x. The medium was centrifuged for 2 cycles at 78,000 x g for 2 hrs in the Spinco to remove any remaining virus particles from the supernate containing the virus inhibitor.

Oie and Loh (1968) had shown that the antiviral inhibitor produced by reo-2 in RA cells satisfied the criteria for interferon. Therefore, all the tests required to prove that the inhibitor was interferon were not repeated. Nevertheless, when tests for pH stability, non-sedimentability at 78,000 x g, non-dializability, non-toxic nature and
host specificity were made they confirmed that the inhibitor was interferon. The assay for interferon yield was done by treating replicate RA cell monolayers grown in 1 oz bottles or 10 x 35 mm plastic petri plates for 18-24 hrs, and challenging them with 100 PFU of VSV after washing the interferon treated cultures thrice with BSS. The details of the method are described in Chapter 3.

Results: Interferon yields obtained in this and other experiments using RA cells were low. However, the results indicated clearly that reo-2 rendered non-infectious by exposure to UV retained its ability to induce interferon in RA cells. The percent plaque reduction caused by a standard dilution of the inhibitor (1:4) further indicated that the interferon induction ability decreased with increasing doses of UV-irradiation from 5 to 60 mins (Table III). Virus irradiated for 60 mins showed only 50% of the activity present in non-irradiated virus. Maximum yields observed were produced by R2 exposed to a UV dose of 5 mins. Even such preparations of interferon had only 20 units of interferon per ml of the undiluted sample. The low IF inducing ability of R2 sample irradiated for 60 mins (UV60-R2) was perhaps due to inefficiency of adsorption or to UV damage of the dsRNA. Since the previous experiment on the adsorption capacity of UV-R2 did not show such a difference the likely cause might well be the latter. Further evidence for the above is shown by the polyacrylamide data presented in another section of this Chapter which shows chain breaks in the dsRNA extracted from UV60-R2 particles.

Effect of UV-irradiation on RNA Polymerase Activity. RNA polymerase (transcriptase) resides in the subviral particle of the reovirus virion
### TABLE III. EFFECT OF UV-IRRADIATION ON INTERFERON INDUCTION BY REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>UV Dose (min)</th>
<th>Mean Plaque Number</th>
<th>Percent Plaque Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>30</td>
<td>68</td>
<td>32</td>
</tr>
<tr>
<td>60</td>
<td>81</td>
<td>19</td>
</tr>
</tbody>
</table>

Interferon was induced by exposing monolayer cultures of RA cells to R2 or UV-R2 for 48 hrs at a multiplicity of 1-4 I.U./cell. The IF preparations were subjected to 2 cycles of centrifugation at 28,000 x g for 2 hrs and the supernate containing IF was saved. For assay of IF, monolayer cultures of RA cells in 35 x 10 mm plastic petri plates were treated with a standard dilution (1:4) of each IF sample for 18-24 hrs and challenged with 100 PFU of VSV. The cultures were overlayed with agar medium and the plaque numbers scored after 48 hrs. The mean plaque count and the corresponding % plaque reduction obtained with each IF preparation is shown. Mean plaque number of the virus control was 100.
(Borsa and Graham, 1968; Shatkin and Sipe, 1968). Its function is to transcribe the dsRNA viral genome to produce single stranded viral specific messenger RNA from the ten segments of the genome. Studies described here were directed to examine the fate of this enzyme in UV-irradiated reovirus.

Procedure: Purified reovirus type 2 preparations exposed to 10 and 60 minutes UV-irradiation under the conditions described in Chapter 3 were used with unirradiated virus as controls. The method of Banerjee and Shatkin (1970) was used for polymerase assay. The complete reaction mixture contained the following in a final volume of 0.25 ml: purified virus, ATP, GTP, CTP, UTP (each 0.2 μ moles); MgCl₂ (3 μ moles); Tris-HCl (25 μ moles, pH 8.0). ³H-labeled CTP was used. The mixture was incubated for 40 minutes at 37°C and the reaction was terminated by adding 1 ml ice cold 5% TCA (trichloracetic acid). The precipitate was collected by filtration on Millipore filters (0.45 μ pore size) and washed thoroughly with 5% TCA. The nitro-cellulose filters were dried and radioactivity counted in Liquifluor-toluene in a Packard liquid scintillation counter. The amount of label incorporated into the RNA produced was a measure of the enzyme activity.

Results: The results showed that the RNA polymerase activity was resistant to UV irradiation even up to a period of 60 mins. The UV60-R2 samples in fact exhibited a higher enzyme activity than UV10-R2. In contrast the chymotrypsin treated UV60-R2 particles showed a still higher enzyme activity (Table IV). Although either brief heat treatment (70°C, 30 secs) or chymotrypsin (CT) treatment (50 μg/ml), 1 hr, 37°C) of the virion was normally necessary to activate the polymerase of reovirus, in the present experiment the enzyme activity was unmasked by UV treatment
### TABLE IV. EFFECT OF UV-IRRADIATION ON VIRAL RNA TRANSCRIPTASE ACTIVITY OF REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>Conditions</th>
<th>CPM Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2 - 4°C</td>
<td>960</td>
</tr>
<tr>
<td>R2</td>
<td>910</td>
</tr>
<tr>
<td>Heat treated R2</td>
<td>16,750</td>
</tr>
<tr>
<td>UV10-R2</td>
<td>2,294</td>
</tr>
<tr>
<td>Heat treated UV10p-R2</td>
<td>956</td>
</tr>
<tr>
<td>CT-UV10-R2</td>
<td>15,886</td>
</tr>
<tr>
<td>UV60-R2</td>
<td>8,892</td>
</tr>
<tr>
<td>Heat treated UV60-R2</td>
<td>1,601</td>
</tr>
<tr>
<td>CT-UV60-R2</td>
<td>16,256</td>
</tr>
</tbody>
</table>

R2 = reovirus type 2. Heat treatment = 70°C for 30 secs.
CT = Chymotrypsin, 50 µg/ml, 1 hr, 37°C

The complete reaction mixture contained in a final volume of 0.25 ml = purified virus, ATP, GTP, UTP (each at 0.2 µ moles); MgCl₂ (3 µ moles); tris-HCl (25 µ moles, pH 8.0). ³H-labeled CTP was used. Incubation was for 40 min at 37°C and the reaction was terminated by adding 1 ml of ice cold 5% TCA. The precipitate was collected by filtration on Millipore filters (0.45 µ), washed thoroughly with 5% TCA and then counted.
alone. Polymerase activity increased with increasing UV dose. However, in contrast to non-irradiated reovirus, additional heat treatment of the UV-R2 resulted in the rapid inactivation of the enzyme activity. The half life of the reovirus RNA polymerase has been reported to be less than 1 min at 70°C (Kapuler, et al., 1970). As will be shown later, increasing UV-dose resulted in the greater disarrangement of viral capsid structures which could then account for the activation of the polymerase without prior heat or chymotrypsin treatments.

Since the polymerase was maximally active after irradiation of the virus for 60 minutes when the cytotoxicity has been reduced to a minimum, it can be concluded that the virion polymerase is not directly involved in the production of cytotoxicity. However, the possible role of a gene product induced by the RNA polymerase in the induction of cytotoxicity cannot be ruled out.

Physico-Chemical Properties of UV-R2

**Filtration Studies to Estimate Virion Particle Size.** Viruses are often filtered through membrane filters of known pore diameter in order to estimate their particle size. Since reoviruses have a particle diameter of 60-75 nm they would normally be retained in filters of 50 nm pore diameter. Therefore, a filtration experiment was set up to determine any change in the size of the virion that may occur as a result of UV-irradiation.

**Procedure:** Purified $^{32}$P-labeled R2 samples were irradiated for periods of 10, 30 and 60 minutes (as described in Chapter 3) and 0.5 ml aliquots containing 35,000 cpm were filtered through Millipore filters of
50 nm pore diameter fitted to a Sweeny syringe. One ml of PBS was filtered after each sample in order to wash any virus left in the syringe. The filter membranes were then dried and assayed for radioactivity. The amount of label retained on the filter pads were determined by counting the samples in a Packard scintillation counter and represented the virus retained on the filter.

Results: The results showed that the filter retained 99% of the radioactivity of the non-irradiated virus and the virus sample exposed to a UV dose of 10 mins (Table V). However, virus samples exposed to higher doses of UV, i.e. 30 and 60 mins, were not retained and only 19 and 5% of the label, respectively, were found on the filter membrane. The results suggested that reovirus exposed to 10 mins UV irradiation had not undergone any significant alteration in particle size. In contrast the virus samples exposed to higher doses of UV had undergone a drastic change so that 81-95% of the label could now pass through the filter. The 1% of the label that passed through the filter in the non-irradiated and the R2 sample exposed for 10 mins could be due to the break down of the particles during the process of purification, and subsequent manipulations.

A possible explanation for the phenomenon suggests that the chemical and electrostatic bonds holding together the structural subunits of the virion coat may have been disrupted by ultraviolet light, and that dissociation of these subunits could be further facilitated by the physical pressure exerted through filtration. Also it is possible that the viral RNA could have leaked out of the virion core through the UV-damaged capsid, and consequently was found in the filtrate. Support
**TABLE V. EFFECT OF UV-IRRADIATION ON THE PARTICLE SIZE OF REOVIRUS TYPE 2**

<table>
<thead>
<tr>
<th>UV Dose (mins)</th>
<th>CPM Retained on Filter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>34,794</td>
<td>99</td>
</tr>
<tr>
<td>10</td>
<td>34,878</td>
<td>99</td>
</tr>
<tr>
<td>30</td>
<td>6,620</td>
<td>19</td>
</tr>
<tr>
<td>60</td>
<td>1,834</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Millipore filter 50 nm pore diameter.

<sup>32</sup>P-labeled reovirus type 2 was exposed to UV for 0, 10, 30 and 60 mins under the standard conditions described in Materials and Methods. 35,000 cpm in 0.5 ml aliquots were filtered through a Millipore filter fitted to a Sweeny syringe. An additional 1 ml of PBS was passed through to wash the syringe. Radioactivity in each filter and the filtrate was assayed in a liquid scintillation spectrometer.
for the above hypothesis was provided when electron microscopic studies were made on the UV-irradiated samples. This will be discussed in a subsequent section of this Chapter.

Sedimentation of UV-R2 in Sucrose Gradients. The effect of UV-irradiation on the sedimentation behavior of reovirus in sucrose gradients was examined. In some experiments purified $^{32}\text{P}$-labeled reovirus type 2 was used, and in others $^{14}\text{C}$-Uridine and $^{3}\text{H}$-protein labeled reo-2 was used.

Procedure: Purified radioactive labeled virus preparations were exposed to 10 and 60 minutes UV-irradiation under conditions described in Chapter 3. Samples of non-irradiated and UV-irradiated virus were layered on pre-formed 4 ml sucrose gradients (15-45% w/w) and centrifuged for 1.5 hrs at 28,000 rpm in the SW39 rotor in the Spinco model L. The centrifuge tube was pierced from the bottom with a 25 gauge hypodermic syringe needle and 5 drop fractions were collected. The radioactivity in each fraction was assayed by spotting 20 μl samples on strips of filter paper, air dried, and counted in a liquid scintillation spectrometer. In the experiment using $^{32}\text{P}$-labeled R2 0.1 ml of virus sample containing $7 \times 10^5$ cpm were put on each gradient. In the double labeled experiment the amount of cpm put on each gradient was as follows: $^{3}\text{H} = 27144$ and $^{14}\text{C} = 226$ in 0.1 ml volume.

Results: In the experiments using the double labeled virus both the non-irradiated virus and the virus sample exposed to a UV dose of 10 mins (UV10-R2) sedimented in a single peak at fractions 4 and 5 from the bottom. This indicated that there was essentially no change in the sedimentation pattern of the virus as a result of UV-irradiation (Fig. 3).
Figure 3. Sucrose gradient sedimentation of purified $^3$H-protein and $^{14}$C-Uridine labeled reovirus type 2 before irradiation (A) and after UV-irradiation for 10 mins (B) and 60 mins (C). Closed circles: $^3$H-protein; open circles: $^{14}$C-uridine. UV-irradiated and non-irradiated virus samples were put on sucrose gradients (15 to 45%) and centrifuged for 1.5 hrs at 28,000 rpm in SW39 rotor of Spinco Model L. Five drop fractions were collected from the bottom of the tube, and the radioactivity of each fraction assayed in a liquid scintillation counter.
However, the sedimentation pattern was completely altered with the UV60-R2 sample. Virus proteins appeared to be distributed in a wide area of the gradient. Seventy-six percent of the $^{14}$C-uridine and 8% of the $^3$H-protein sedimented at the top of the gradient, while 20% of the $^{14}$C and 85% of the $^3$H were distributed in a broad area of the gradient spreading from fractions 2 through 9 from the bottom.

Sedimentation profiles (Fig. 4) of the experiments using $^{32}$P-labeled R2 showed similar results. Non-irradiated R2 sedimented at fractions 5 and 6 from the bottom of the tube while UV10-R2 also sedimented at the same place. However, as was found previously, the sedimentation pattern of UV60-R2 was completely altered. Most of the $^{32}$P activity was distributed near the top of the gradient in fractions 10 through 13.

The results showed that the sedimentation pattern of the CT-inducing UV10-R2 particle was similar to that of the non-irradiated reovirus. In contrast, prolonged irradiation (60 mins) resulted in an extreme change in sedimentation behavior. The virus particles sedimenting near the top of the gradient would normally be smaller than those that sediment to the middle of the gradient. This was confirmed by electron microscopy. A high percent of subviral particle-like structures and disintegrated capsid materials was observed in samples taken from the top of the gradient (Fig. 5). It is probable that the resulting degradation of the UV60-R2 particle may not be solely due to UV damage. The physical stresses exerted during centrifugation on the UV damaged virus particle may have caused severe alterations to the structure of the virus.

**Buoyant Density Studies on UV-irradiated Reovirus.** The preceding sucrose gradient sedimentation studies on UV-R2 show that the integrity
Figure 4. Sucrose gradient sedimentation of purified $^{32}$P-labeled reovirus type 2 non-irradiated (A) and UV-irradiated for 10 mins (B) and 60 mins (C). UV-irradiated and non-irradiated virus samples were put on sucrose gradients (15-45%) and centrifuged for 1.5 hrs at 28,000 rpm in SW39 rotor of Spinco Model L. Five drop fractions were collected from the bottom of the tube and the radioactivity of the fractions were counted in a liquid scintillation spectrometer.
Figure 5. Electron micrograph of UV-irradiated (60 min) reovirus type 2 after sedimentation in sucrose gradient. Virus sample was taken from the top fraction of the gradient and stained with 2% PTA. Original magnification x 74,000.
of the virus was altered when the virion particle was exposed to a higher dose of UV (60 min), although no detectable alterations were observed with a low dose of UV (10 min) under similar conditions. The effect of the high dose of UV on the virion was reflected in the altered sedimentation pattern. It was of interest to examine the effect of UV on other physical properties of the virion. In this experiment the effect of UV-irradiation on the buoyant density of the virus in a cesium chloride gradient was examined.

Procedure: Purified $^{32}$P-labeled reovirus type 2 were exposed to 0, 10 or 60 minute doses of UV light under the standard conditions described earlier. The samples were put on CsCl isopycnic gradients (600 mg/ml) and centrifuged at 33,000 rpm in the SW39 rotor of Spinco model L for 24 hrs. Four drop fractions were collected by puncturing the centrifuge tube at the bottom. The samples were assayed for radioactivity in a liquid scintillation counter. Refractive index measurements ($\eta_D$) of non-peak fractions were taken in a refractometer, and the buoyant density ($\rho$), was calculated by the application of the following formula by Ifft, et al. (1961): 

$$\rho^{25°} = 10.8601\eta_D^{25°} - 13.4974.$$  

Results: In the non-irradiated virus sample 82% of the recovered cpm banded at the center of the gradient in a single peak at fraction 9. The buoyant density of the particles at this peak was 1.38 g/cm$^3$ (Fig. 6A). The sedimentation profile of the UV10-R2 showed two populations of particles. One population of particles sedimented in a peak at fraction 8 from the bottom while the other peak was at fraction 2 (Fig. 6B). The buoyant density of the particles that sedimented in fraction 8 was 1.39 g/cm$^3$ and the density of the particles that sedimented near the bottom
Figure 6. Isopycnic sedimentation of $^{32}$P-labeled reovirus in CsCl after exposure to UV-irradiation for 0 min (A) 10 min (B) and 60 min (C). Purified $^{32}$P-labeled reovirus samples after exposure to UV were put on CsCl gradients (600 mg/ml) and centrifuged at 33,000 rpm for 24 hrs in SW39 rotor of Spinco Model L. Four drop fractions were collected from the bottom of the tube and radioactivity of the fractions were counted in a liquid scintillation spectrometer. Densities were calculated from refractive index measurements of the fractions.
of the gradient was 1.44 g/cm$^3$. Reo-2 exposed to 60 min UV exhibited a complete change in the sedimentation pattern, and banded to the bottom of the gradient as a single peak in fraction 3 (Fig. 6C). The density of this population of particles was 1.44 g/cm$^3$.

The buoyant density of non-irradiated R2 obtained in this experiment is in agreement with the results of other workers (Loh and Shatkin, 1968). The population of virus particles that sedimented at a density of 1.44 g/cm$^3$ in the UV10-R2 represented a new population of heavier particles, while those that sedimented at a density of 1.39 g/cm$^3$ corresponded to the normal virus. It appeared that a small fraction of the virus population was converted to these new particles after 10 mins UV-irradiation and centrifugation. On the other hand, with continued irradiation (60 mins) the entire population of virus particles was converted to this new population having a density of 1.44 g/cm$^3$. Since the density of subviral particles prepared by controlled degradation of purified reovirus with urea was found to be 1.46 g/cm$^3$ (Loh and Shatkin, 1968), it was inferred that this new population of virus particles formed from UV60-R2 in the CsCl gradient was approximately equivalent in size to subviral particles. Examination of negatively stained samples from the peak fractions in the UV60-R2 gradient supported this inference (Fig. 7). The particle diameter was about 47 to 59 nm, similar to that produced by degradation with urea.

It can be concluded for this experiment that the acquisition of CT is not accompanied by a change in buoyant density of the virion. At lower doses of UV (5-10 mins) with the acquisition of CT property there was little or no change in the buoyant density of UV-R2. In contrast,
Figure 7. Negatively stained preparations of purified reovirus type 2 after exposure to UV for 60 mins and sedimented in isopycnic CsCl gradient. After exposure of purified reovirus to UV, the sample was centrifuged at 33,000 rpm for 24 hrs in CsCl gradient and fractionated as described in the text. Sample of virus from the peak (bottom) of the gradient was stained with 2% PTA and examined in the electron microscope. Original magnification x 84,000.
at the higher dose of UV (60 mins), when the CT property of the virion particle was lost, the entire population was converted to structures possessing a higher density.

**Morphological Changes in UV-irradiated Reovirus.** Sucrose gradient sedimentation and buoyant density studies of UV-R2 did not show any detectable alterations in the above properties of the virus that was exposed to 10 mins UV. In contrast, exposure to a high dose of UV (60 mins) resulted in drastic changes in the sedimentation pattern and buoyant density of the virus. It was further observed that the new population of virus particles (with a density of 1.44 g/cm³) obtained by centrifugation of UV60-R2 in the CsCl density gradient was similar in size and density to the subviral particles prepared by urea degradation. The present experiment was performed to examine whether the UV-irradiated CT-inducing reovirus underwent any detectable morphological changes in its architecture.

**Procedure:** Preparations of purified reo-2 were exposed to UV light for varying periods of time from 0 to 60 mins under the standard conditions described earlier. These preparations were immediately stained with 2% phophotungstic acid (PTA) according to the technique described in Chapter 3, and examined in a Hitachi HU-11 electron microscope.

**Results:** The virus preparations which were irradiated for 5 to 10 minutes did not show any detectable change from the non-irradiated virus except that there were a few more virus particles with centers taking up PTA stain and consequently resembling "empty" particles lacking the core (Fig. 8). It was previously shown that cytotoxicity was obtained with virions irradiated for 10 minutes. Since R2 particles irradiated for
Figure 8. Negatively stained preparations of purified reovirus type 2 before UV-irradiation (A) and after exposure to UV-irradiation for 10 mins (B) 30 mins (C) and 60 mins (D). The virus samples were stained with 2% PTA before irradiation or immediately after exposure to UV and examined. Magnification of A:x 60,000; B:x 60,000; C:x 80,000; D:x 92,000.
5 or 10 minutes did not show any morphological change in the virion capsid architecture, it can be concluded that the acquisition of CT was not accompanied by morphological alterations in the virus detectable by this technique.

The higher doses of UV (30 and 60 min exposures) caused progressive damage to the virion architecture (Fig. 8, C and D). The capsomeres were displaced to varying degrees, and permitted the PTA stain to penetrate into the virion core causing the appearance of an "empty" virus particle. The degree of disorganization in the virus capsid was greater in the UV60-R2 than in the UV30-R2 sample. Although the capsomere arrangement in the UV60-R2 particle was highly disorganized, the capsomeres continued to be associated with the virus particle (Fig. 8D).

In contrast to the electron micrographs of the R2 exposed to a UV dose of 60 mins and examined immediately, the virus particles from the peak (fraction 3 from bottom) in the CsCl gradient showed that the outer capsid layer was stripped away, leaving only the subviral particle (SVP) (Fig. 7). Since the electron micrographs of the uncentrifuged UV60-R2 showed "complete" virus particles, it could be assumed that the conversion of these particles to smaller "SVP-like" structures was facilitated by centrifugal forces and other conditions of physical stress exerted on the UV damaged particle during isopycnic centrifugation.

**Effects of UV on the Structural Proteins of Reovirus.** Although greater emphasis was placed on the role of nucleic acids in the photo-biological effects of UV-irradiation in the past, the importance of proteins cannot be overlooked. Some of the important changes that could occur to proteins after UV-irradiation are: induction of
electrophoretic heterogeneity, formation of high and low molecular weight products, changes in antigenic properties, inactivation of enzymes, denaturation, and cross linking of proteins to nucleic acids (Smith and Hanawalt, 1969).

In addition to alterations in biological properties such as HA which is directly associated with the protein capsid of the UV-R2, centrifugation studies of the UV60-R2 have shown that distribution of $^3$H-labeled virus proteins occurred in a wide area of the sucrose gradient. Such a distribution could be due to the heterogeneity in the molecular weights of the proteins formed as a result of irradiation. It was therefore of interest to examine the effects of UV on the capsid proteins using the polyacrylamide gel electrophoresis technique.

Structural proteins of reoviruses were characterized into seven components by electrophoresis on polyacrylamide gels, namely: I, II and IIIb, and four minor components m1, m2, m3 and IIIa (Loh and Shatkin, 1968). The effect of UV irradiation on these polypeptide components were examined.

Procedure: Samples of purified $^3$H-protein-labeled reovirus type 2 were exposed to UV-irradiation for given periods of time (0, 10, 30 and 60 mins) according to the standard procedure adopted in other experiments. These virus samples were then solubilized by treatment with SDS, 2-ME and SM urea as described in Chapter 3. The samples of solubilized virus were put on 10% acrylamide gels and electrophoresed for 24 hrs at 4 mamps per gel. The gels were then sliced into 1.3 mm fractions in a gel slicer, solubilized in H$_2$O$_2$, and assayed for radioactivity in a liquid scintillation spectrometer.
Results: Comparison of the electrophoretic migration patterns of the proteins of non-irradiated virus with those of UV-R2 shows a number of changes occurring in UV-R2 (Fig. 9). A new slowly migrating protein component ("X") appeared in the UV-R2 samples. This new component was initially small in UV10-R2, but became larger in virus samples exposed to higher doses of UV (30 and 60 mins). With increasing irradiation there was a reduction in the amount of major component I (Fig. 9, C and D). After 60 mins irradiation, except for component "X", the other viral protein components have almost disappeared. The origin of the new protein component "X" remains to be explained. Such a protein component may be formed by the disaggregation and reaggregation of viral polypeptides, as a result of UV-irradiation. Its slower migration in contrast to the other polypeptides suggests a high molecular weight. However this change in electrophoretic mobility may also be due to the formation of RNA-protein cross links as a result of exposure to UV. Regardless, the acquisition of cytotoxicity does not appear to be related to any of the changes observed in the proteins. It is unlikely that the new protein "X" in the UV10-R2 is responsible for CT, since the new protein is present in even greater quantity in UV60-R2 (which had no CT property) than the UV10-R2 which possessed the maximum toxicity.

Viral RNA of UV-irradiated Reovirus: (a) Electrophoretic Studies. Double-stranded RNA of reovirus contains 10 segments which fall into 3 sizes: large (L), medium (M) and small (S). A single segment from each of these classes have a molecular weight of $2.5 \times 10^6$, $1.4 \times 10^6$, and $0.8 \times 10^6$ daltons, respectively. Large and medium segments each have 3 pieces of equal size, while the small segment has 4 pieces (Shatkin, et al., 1968).
Figure 9. Polyacrylamide gel electrophorograms of dissociated proteins from purified $^3$H-protein-labeled reovirus type 2 before UV-irradiation (A) and after UV-irradiation for 10 mins (B) 30 mins (C) and 60 mins (D). Samples of non-irradiated and UV-irradiated virus were solubilized as described in Materials and Methods and applied to 10% gels. Electrophoresis was for 24 hrs at 4 mamps per gel. After electrophoresis the gels were sliced, solubilized in $H_2O_2$ and the radioactivity of the gel fractions were assayed in a liquid scintillation spectrometer. Migration is from the left to right (anode).
The previous experiment showed certain alterations occurring to the virus proteins of the UV-R2. Since the infectivity of the virus was the first property that was lost as a result of UV-irradiation, certain chemical changes in the RNA of the UV-R2 could be expected. In the following experiment the RNA of reovirus was examined to determine whether it has undergone any detectable physio-chemical changes. It was of further interest to study the effects of irradiation dose on the RNA.

Procedure: Purified $^{32}$P-labeled reovirus type 2 was exposed to 0, 10 or 60 minutes of UV-irradiation under the standard procedure. Double-stranded RNAs were extracted from UV-irradiated and non-irradiated R2 by one of two methods using SDS and urea or SDS and phenol, as described in Chapter 3. The extracted RNA samples were put on 11 cm long 4% polyacrylamide gels containing 0.025% agarose and electrophoresed for 24 hrs at 4 mamps per gel as described in the Materials and Methods. The gels were then removed and sliced into 1.3 mm slices and solubilized in $H_2O_2$ at 60°C. Radioactivity of each of the gel slices was assayed in a liquid scintillation spectrometer.

Results: Examination of the electrophoretic migration patterns of the RNA of non-irradiated R2 and UV10-R2 showed no detectable difference (Fig. 10). The three main segments L, M, and S and their sub-segments were separated in the same manner as the controls. However, certain changes in the migration pattern were noticeable in the dsRNA extracted from UV60-R2. Although the main segments (L, M and S) were identifiable, a number of additional peaks (2 to 4) were seen in each of them. Perhaps the higher dose of UV used may have caused chain breaks within the L, M and S segments of the RNA resulting in slight changes in the migration
Figure 10. Polyacrylamide gel electrophorograms of double stranded RNA extracted from purified $^{32}$P-labeled reovirus type 2 before UV-irradiation (A) and after UV-irradiation for 10 mins (B) and 60 mins (C). The RNAs were extracted from the virus samples with 1% SDS and 4 M urea as described in Materials and Methods and applied to 4% polyacrylamide gels containing 0.025% agarose. Electrophoresis was for 24 hrs at 4 mamps per gel and the electrophoretic buffer was a Tris-acetate-EDTA buffer (pH 7.8) containing 0.2% SDS. After electrophoresis, the gels were sliced and solubilized in $\text{H}_2\text{O}_2$. The radioactivity of the gel fractions were assayed in a liquid scintillation spectrometer. Migration is from the left to right.
pattern.

It can be concluded that, if the acquisition of cytotoxicity was accompanied by certain chemical changes in the RNA genome, then the changes must be minor ones which can not be detected by the present technique. The UV-irradiation of RNA is known to result in the formation of photo-products, such as pyrimidine hydrates and dimers and RNA-protein cross links. The chemical nature of such cross links is not understood yet. Cross links between chains and chain breaks have been observed in UV-irradiated DNA. Since these lesions were observed only at high doses of UV their biological significance was considered to be minor. However, similar lesions could occur in irradiated RNA as well. In order to examine whether any of the above mentioned lesions do occur in the RNA of UV-irradiated reovirus, further biochemical studies will have to be undertaken.

(b) **Viral RNA of UV-irradiated Reovirus: Resistance to Ribonuclease.** The previous experiment indicated that although no alterations were detectable in the dsRNA of reovirus exposed to a UV dose of 10 mins, certain alterations which appear to be breaks in the RNA chain were observed with higher doses of UV (60 mins). The present experiment was done to determine the susceptibility of the viral dsRNA from UV-R2 to pancreatic ribonuclease. Reovirus dsRNA is normally resistant to degradation by ribonuclease under conditions which would digest single stranded nucleic acid (Gomatos and Tamm, 1963). The viral dsRNA becomes susceptible only after strand separation under conditions of, e.g., low salt solutions. Therefore, if the two strands of the viral RNA becomes separated as a result of UV-irradiation, they would be
susceptible to the enzyme.

Procedure: Viral RNAs were extracted from purified $^{32}$P-labeled non-irradiated R2 and R2 exposed to 10 and 60 mins, by the phenol-SDS method described in Chapter 3. Each RNA sample was treated with 5 μg/ml of bovine pancreatic ribonuclease at 37°C for 1 hr in 1 x SSC (Standard saline citrate) buffer pH 7. The RNA was precipitated in 5% cold TCA filtered and dried. The samples were assayed for radioactivity in a liquid scintillation counter.

Results: The results (Table VI) showed that the dsRNA extracted from UV-irradiated reovirus remained resistant to the RNase in 1 x SSC buffer like the non-irradiated controls. No difference existed between UV10-R2 and UV60-R2 samples. It can be concluded that the two complementary strands of the dsRNA genome were held together even after exposure to a high dose of UV.

(c) Viral RNA of UV-irradiated Reovirus: Base Analysis. The two preceding experiments showed the effect of UV-irradiation on the electrophoretic mobility of the RNA and the double stranded property of the RNA of UV-R2. Base composition studies of viral RNA extracted from UV-irradiated reo-2 were undertaken to examine whether irradiation had any effect on the base composition of the viral RNA and also to study the fate of adenine-rich RNA in UV-R2.

Procedure: Purified $^{32}$P-labeled R2 was exposed to varying doses of UV (0, 10, 30 and 60 mins) as described before. The RNA was extracted from these samples and base composition analysis was done by chromatographic separation on sheets of DEAE-cellulose as described in Materials and Methods (Chapter 3). In another study a filtration
TABLE VI. EFFECT OF RIBONUCLEASE ON VIRAL DOUBLE STRANDED RNA OF UV-IRRADIATED REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>CPM of RNA Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min(^a)</td>
</tr>
<tr>
<td>SSC buffer only</td>
<td>38,840</td>
</tr>
<tr>
<td>RNase(^b) in 1 x SSC buffer</td>
<td>38,849</td>
</tr>
<tr>
<td>RNase in distilled water</td>
<td>22,362 (43%)</td>
</tr>
</tbody>
</table>

\(^a\)Exposure time of reovirus to UV
\(^b\)5 μg/ml, 37°C, 1 hr

RNAs were extracted from purified \(^32\)P-labeled R2 and UV-R2 by the phenol-SDS method and each sample was treated with pancreatic ribonuclease (5 μg/ml) for 1 hr at 37°C in 1 x SSC buffer pH 7 or distilled water. The RNA was precipitated in 5% cold TCA filtered, dried and radioactivity assayed. Mean value of duplicate samples are given.
experiment such as the one described earlier in this Chapter to determine the changes in particle size of the UV-R2 was done, and base composition studies were made on the RNAs extracted from the filter pad, after filtration of non-irradiated R2 and UV-R2 samples through 50 nm Millipore filters.

Results: The results of several determinations did not show any significant change in the base composition of the viral RNA of UV-R2 from that of the non-irradiated R2. Adenylic acid was about 40 moles/100 moles, while purine:pyrimidine ratios (A+G/U+C) were greater than unity (1.3 to 1.4), indicating that the adenine rich-RNA was still associated with the UV-irradiated virus. Table VII gives the results.

In the filtration experiments (Table VIII) the values for adenylic acid and for the purine:pyrimidine ratios of the RNAs from R2 and UV10-R2 retained on the filter were similar. In contrast, lower adenine values and purine:pyrimidine ratios of less than one were obtained with the RNAs extracted from UV30-R2 and UV60-R2. This alteration in adenine content may be due to the loss of the A-rich RNA as a result of high UV-dose. It was previously shown that UV irradiation for 30 to 60 mins results in disruption of viral architecture. Under such circumstances the small adenine-rich polynucleotide molecules could have leaked out. That such leakage has occurred was substantiated when the base composition of the filtrates were analyzed. The results show correspondingly higher values of adenine, and purine:pyrimidine ratios for UV30-R2 and UV60-R2. The higher values in this case accounts for the presence of adenine that leaked out of the virus on the filter pad.
TABLE VII. EFFECT OF UV-IRRADIATION ON THE BASE COMPOSITION OF REOVIRUS TYPE 2 RNA

<table>
<thead>
<tr>
<th>UV Dose (min)</th>
<th>Adenine</th>
<th>Uridine</th>
<th>Guanine</th>
<th>Cytosine</th>
<th>A+G&lt;sup&gt;b&lt;/sup&gt;/U+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40.2</td>
<td>25.2</td>
<td>17.5</td>
<td>17.0</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>39.2</td>
<td>27.2</td>
<td>17.0</td>
<td>16.6</td>
<td>1.3</td>
</tr>
<tr>
<td>30</td>
<td>40.3</td>
<td>27.0</td>
<td>15.7</td>
<td>16.3</td>
<td>1.3</td>
</tr>
<tr>
<td>60</td>
<td>41.8</td>
<td>23.7</td>
<td>16.6</td>
<td>17.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Value of A, U, G and C are expressed as moles per 100 moles of nucleotide.

<sup>b</sup>Purine:pyrimidine ratio

RNAs were extracted from <sup>32</sup>P-labeled purified reovirus type 2 before and after UV-irradiation for 10, 30 and 60 mins, and base composition analysis was done on chromatographic separation on sheets of DEAE cellulose as described in Materials and Methods.
TABLE VIII. BASE COMPOSITION ANALYSIS OF RNA extracted from UV-IRRADIATED REOVIRUS TYPE 2, AFTER MILLIPORE FILTRATION

Base Analysis of RNA Retained on Filter

<table>
<thead>
<tr>
<th>UV Dose (min)</th>
<th>Adenine</th>
<th>Uridine</th>
<th>Guanine</th>
<th>Cytosine</th>
<th>A+G&lt;sup&gt;c&lt;/sup&gt; U+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>36.4</td>
<td>28.8</td>
<td>16.2</td>
<td>18.6</td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>35.7</td>
<td>28.9</td>
<td>16.9</td>
<td>18.6</td>
<td>1.1</td>
</tr>
<tr>
<td>30</td>
<td>26.6</td>
<td>28.9</td>
<td>22.3</td>
<td>22.2</td>
<td>0.9</td>
</tr>
<tr>
<td>60</td>
<td>24.3</td>
<td>30.3</td>
<td>21.9</td>
<td>23.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Base Analysis of RNA in the Filtrate

<table>
<thead>
<tr>
<th>UV Dose (min)</th>
<th>Adenine</th>
<th>Uridine</th>
<th>Guanine</th>
<th>Cytosine</th>
<th>A+G&lt;sup&gt;c&lt;/sup&gt; U+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>36.5</td>
<td>27.5</td>
<td>17.8</td>
<td>18.3</td>
<td>1.2</td>
</tr>
<tr>
<td>10</td>
<td>44.5</td>
<td>22.9</td>
<td>15.7</td>
<td>17.0</td>
<td>1.5</td>
</tr>
<tr>
<td>30</td>
<td>44.4</td>
<td>21.4</td>
<td>17.1</td>
<td>17.1</td>
<td>1.6</td>
</tr>
<tr>
<td>60</td>
<td>41.7</td>
<td>22.2</td>
<td>17.0</td>
<td>19.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>value for K, U, G and C are expressed as moles per 100 moles of nucleotide

<sup>b</sup>50 nm pore diameter Millipore filter

<sup>c</sup>purine:pyrimidine ratio
It can be concluded that UV-irradiation did not alter the base composition of the RNA of the virus. However, the damage caused to the virion capsid by high doses of UV did facilitate the leakage of the A-rich viral RNA.
CHAPTER 5
Nature of the Toxic Agent

Based on centrifugation studies and neutralization with specific immune serum it was previously reported that the cytotoxic property of the UV-irradiated reovirus was associated with the virus particle (Loh and Oie, 1969). Although no additional evidence was presented, they postulated that some structural component(s) of the virion might be responsible for the cytotoxicity. In other virus systems, such as vesicular stomatitis virus and adenovirus, the role of structural components in the regulation and shut off of macromolecular synthesis and cytotoxicity have been shown (Huang, et al., 1966; Yaoi, et al., 1970; Levine and Ginsberg, 1967, 1968; Moss, 1968). It was of interest to examine which component of the UV-irradiated reovirus was toxic. Results of studies conducted in this area will be presented in this chapter.

Toxicity Studies with UV-irradiated Double Stranded-RNA. In order to determine which component of the UV-irradiated virus was toxic, an experiment was done to determine whether RNA extracted from reovirus type 2 and exposed to UV-irradiation was toxic to HeLa cells.

Procedure: Viral RNA was extracted from purified reovirus type 2 by the phenol-SDS method described in Chapter 3. The extracted RNA preparation containing 420 μg/ml was exposed to 5 minutes UV under the standard conditions described in Materials and Methods. Replicate cultures of HeLa cells grown in 15 mm round coverslips containing approximately 1.5 x 10^5 cells per culture were exposed to UV-irradiated RNA in a volume of 20 μl/coverslip. The controls used in the experiment
were: non-infected cell controls, non-irradiated RNA at the same concentration as UV-RNA (8.4 μg/20 μl), and UV-R2 at a multiplicity of 50 I.U. per cell. After an adsorption period of 1 hr at 37°C, the cultures were washed in BSS and fed with EBM containing 0.1% FCS and the cultures were returned to the incubator. At various times post-infection (2, 4, 8 and 12 hrs) the cultures were examined for the appearance of cytotoxicity. Assay for CT was done as described in Chapter 3.

Results: The results showed that neither UV-irradiated nor non-irradiated RNA extracted from virion exhibited any toxicity to HeLa cells, when the positive controls infected with UV-R2 exhibited a CT response greater than 3+ in 12 hours post-infection (Table IX). It was concluded that UV-irradiated RNA was not toxic to HeLa cells at least at concentrations used in this experiment (i.e. 5 x 10^-5 μg/cell).

Toxicity Studies with Reo-2 Subjected to Brief Heat Treatment. A previous report has shown that reovirus type 2 heated for 30 minutes at 56°C was not cytotoxic (Loh and Oie, 1969). Since the polymerase of reovirus can be activated by brief heat (30 sec at 70°C; 60 sec at 60°C) treatment (Borsa and Graham, 1968; Banerjee and Shatkin, 1970), and since UV-irradiation also was found to be capable of activating the enzyme and that such UV-R2 preparations were still cytotoxic, it was therefore of interest to determine whether the virion polymerase (activated by heat) played a role in cytotoxicity.

Procedure: Purified samples of reovirus type 2 were heated to a temperature of 70°C for 30 seconds or 60°C for 1 minute in a stoppered tube. Replicate cultures of HeLa cells grown in one oz bottles were exposed to each of the two samples at a multiplicity of 50 I.U./cell.
### TABLE IX. EFFECT OF UV-IRRADIATED REO-2 RNA ON HELA CELLS

<table>
<thead>
<tr>
<th>Experimental Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Appearance of Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>UV-R2</td>
<td>-</td>
</tr>
<tr>
<td>R2-RNA</td>
<td>-</td>
</tr>
<tr>
<td>UV-R2-RNA</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>UV-R2 = 10<sup>-1</sup>-UV-inactivated reovirus type 2 (MOI = 50 I.U./cell)  
R2-RNA = RNA extracted from purified R2 (8.4 µg/20 µl/cover slip culture). UV-R2-RNA = R2-RNA exposed to 10 mins UV.
Positive controls were infected with UV-R2 at the same MOI, while the cell controls were kept uninfected. Virus adsorption and the assay for cytotoxicity was performed according to the standard method described before. The cultures were examined for toxicity at various time periods after infection.

Results: The cell cultures exposed to the heat-treated R2 (Δ-R2) did not show any CT even at 12 hrs p.i. In contrast, the UV-R2-treated cultures exhibited a 3+ CT at this time (Table X).

It was therefore concluded that under the experimental conditions employed, reo-2 exposed to brief heat treatment was not toxic to HeLa cells. Thus the virion polymerase is not directly involved in the induction of cytotoxicity.

Cytotoxic Studies with Reovirus "Empties." It was previously reported that except for a smaller content of RNA all of the structural components found in the complete reovirus particle were present in the "empty" particle (Loh and Oie, 1969). To investigate the possible role of some incoming structural protein(s) on the induction of reovirus toxicity, it was decided to examine the toxic property of the purified "empties" exposed to UV. In addition, the CT of non-irradiated "empties" in the presence of protein antagonist cycloheximide was examined.

Experimental procedure: Purified CsCl banded preparations of reo-2 "empties" containing 500 μg/ml protein was used. To eliminate any contamination of the empties with complete virions, the top band containing the empties was collected from the side of the cellulose nitrate centrifuge tube with a syringe. HeLa cell monolayers in 1 oz Rx bottles containing an average of 800,000 cells per bottle were exposed to
TABLE X. EFFECT OF HEAT TREATED REO-2 ON HELA CELLS

<table>
<thead>
<tr>
<th>Experimental Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Appearance of Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>R2</td>
<td>-</td>
</tr>
<tr>
<td>UV10'-R2</td>
<td>-</td>
</tr>
<tr>
<td>HT-R2(1)</td>
<td>-</td>
</tr>
<tr>
<td>HT-R2(2)</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>R2 = reovirus type 2; UV10'-R2 = R2 UV-inactivated for 10 mins.
MOI = 50 I.U./cell. HT-R2(1) = Heated R2 (60°C, 1 min)
HT-R2(2) = Heated R2 (70°C, 0.5 min)
non-irradiated or UV-irradiated (10 mins) empties at a dose of 20 μg/800,000 cells in a volume of 0.2 ml. To examine the toxicity of empties in the presence of protein inhibitor cycloheximide, another set of cultures were incubated with cycloheximide and actinomycin D at a concentration of 10 μg/ml and 0.05 μg/ml respectively. The experimental set up used was the standard procedure described in Materials and Methods. Cytotoxicity was observed at varying periods after infection.

The results (Table XI) indicated that UV-irradiated empties were toxic to HeLa cells, while non-irradiated empties were non-toxic. Toxicity appeared in about 3-4 hrs and over 50% of the cells were dead in 12 hrs. Both UV-irradiated and non-irradiated empties were toxic in the presence of actinomycin D and cycloheximide. However, UV10-E was toxic in the presence of either of the antibiotics or in combination. It was further observed that in the presence of both antibiotics the toxic response was enhanced. The residual amount of infectivity present in the empties (assayed by the immunofluorescent plaque method) was 7 x 10^4 I.U./ml. Hence the multiplicity of infectious particles in such preparations of UV10-E was at the most approximately 1 per 1000 cells. Since the "empties" contain little or no RNA the role of viral nucleic acid in eliciting the CT could therefore be ruled out. Furthermore, the previous experiment had shown that the UV-irradiated RNA was not toxic to HeLa cells. The evidence thus far presented strongly suggest the role of some structural viral protein(s) in the CT phenomenon. Whether this toxic protein(s) acts directly or through some indirect regulatory mechanism to produce the CT remains to be elucidated.

**Toxicity Studies with Capsid Proteins.** Since the previous experiment provided evidence indicating the importance of the structural
### TABLE XI. EFFECT OF REOVIRUS "EMPTIES" ON HELA CELLS

<table>
<thead>
<tr>
<th>Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Appearance of Cytotoxicity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Cell control</td>
<td>-</td>
</tr>
<tr>
<td>AD &quot;</td>
<td>-</td>
</tr>
<tr>
<td>Cy &quot;</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
</tr>
<tr>
<td>E + AD</td>
<td>-</td>
</tr>
<tr>
<td>E + Cy</td>
<td>-</td>
</tr>
<tr>
<td>E + AD + Cy</td>
<td>-</td>
</tr>
<tr>
<td>UV10'-E</td>
<td>-</td>
</tr>
<tr>
<td>UV10'-E + AD</td>
<td>-</td>
</tr>
<tr>
<td>UV10'-E + Cy</td>
<td>-</td>
</tr>
<tr>
<td>UV10-E + AD + Cy</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>AD = Actinomycin D at 0.05 μg/ml. Cy = cyclohexamide at 10 μg/ml. E = "empties" of purified reovirus type 2, at a protein concentration of 20 μg/800,000 cells. UV10E = empties exposed to 10<sup>4</sup> UV.

<sup>b</sup>Cytotoxicity (CT) was recorded as follows: + = 25% cell death, 2+ = 50% CT, 3+ = 75% CT and 4+ = 100% CT.
viral proteins in the induction of cytotoxicity, the next experiment was designed to examine whether the proteins of the outer capsid or the subviral particle itself were toxic.

Procedure: Subviral particles and outer capsid proteins were prepared from a mixture of purified $^{14}$C-leucine labeled and unlabeled reovirus type 2 by controlled degradation of the virion with 4M urea at 4°C as described in Chapter 3. The unlabeled purified sample of reo-2 contained about 900 $\mu$g of virus protein and the $^{14}$C-leucine labeled reo-2 contained 25,000 cpn in a total volume of 0.3 ml. This mixture was subjected to urea degradation as described in Chapter 3 and was put on a CsCl isopycnic density gradient (590 mg/ml). A control sample containing PBS in place of virus was treated in the same manner. Both experimental and control tubes were centrifuged at 33,000 rpm in the SW39 rotor in a Spinco Model L for 24 hrs. After centrifugation the tubes were punctured from the bottom and 8 drop fractions were collected. Fifty $\mu$l samples of each fraction were spotted on filter paper and the radioactivity determined in a liquid scintillation spectrometer. Two peaks of radioactivity were obtained. The refractive indices of selected "non-peak" fractions were taken in a refractometer and their densities calculated. Density measurements confirmed that the bottom peak contains the subviral particles. Figure 8 is a radioactivity profile of the subviral particles (SVP) and the outer capsid proteins (OCP) in a CsCl density gradient. The buoyant density of the SVP was 1.47 g/cm$^3$. In other experiments when radioactive-labeled virus was not used as a marker, subviral particles and the OCP were monitored by optical density measurements.
Figure 11. Isopycnic sedimentation of subviral particles and outer capsid proteins prepared from $^{14}$C-leucine-labeled reovirus type 2 in CsCl. $^{14}$C-leucine-labeled reovirus was mixed with purified unlabeled reovirus type 2 and exposed to 4 M urea for 3 mins at 4 C, diluted with a solution of CsCl (590 mg/ml) and centrifuged at 33,000 rpm for 24 hrs as described in the text. Fractions were collected from the bottom of the centrifuge tube, and the radioactivity was counted in a liquid scintillation spectrometer.
Assay for toxicity: HeLa cells grown in 15 mm round coverslips placed in plastic petri plates were used for toxicity assay as described in Chapter 3. UV-irradiated and non-irradiated samples of SVP and OCP in volumes of 20 μl were inoculated into each of 4 replicate cultures. Irradiated and non-irradiated reo-2 at a MOI of 50 I.U./cell were used as controls. In addition corresponding samples of SVP and OCP from the control gradient (c/SVP and c/OCP) were also tested for toxicity. The purpose being to examine whether the urea used for virus degradation or CsCl from the gradient was toxic to cells under the conditions of the experiment. Inoculated cell cultures were washed with BSS after adsorption and returned to the incubator after introduction of EBM with 0.1% FCS. At pre-determined times p.i. the cultures were examined for CT.

The results showed that the outer capsid proteins exposed to UV under the standard conditions used were toxic to HeLa cells (Table XII). Irradiated SVP also exhibited some CT but to a much lesser degree. Perhaps the CT of SVP could be due to some of the proteins of the outer coat remaining attached to the subviral particles. Capsid proteins were found to exhibit the CT regardless of whether the proteins were exposed to UV after mild degradation with urea or degraded after exposure of the virion to UV light.

Non-irradiated OCP or SVP were found not to be toxic. The non-toxicity of the controls containing no virus indicated that the urea or CsCl at concentrations used in the experiments were not toxic to HeLa cells. Previous reports have indicated that 75% of the virion proteins were contained in the outer capsid which is made up of at least five
TABLE XII. EFFECT OF UV-IRRADIATED CAPSID PROTEINS AND SUB-VIRAL PARTICLES ON HELA CELLS

<table>
<thead>
<tr>
<th>Conditionsa</th>
<th>Appearance of Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 2 4 8 12 24 hr hr hr hr hr hr</td>
</tr>
<tr>
<td>R2-SVP</td>
<td>- - - - - +</td>
</tr>
<tr>
<td>R2-UV-SVP</td>
<td>- - ± + &gt;+ 2+</td>
</tr>
<tr>
<td>R2-OCP</td>
<td>- - - - ± +</td>
</tr>
<tr>
<td>R2-UV-OCP</td>
<td>- + 2+ &gt;2+ 3+ &gt;3+</td>
</tr>
<tr>
<td>UVR2-SVP</td>
<td>- - - + + 2+</td>
</tr>
<tr>
<td>UVR2-OCP</td>
<td>- - 2+ &gt;2+ 3+ &gt;3+</td>
</tr>
<tr>
<td>Cell control</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>R2</td>
<td>- - - ± &gt;+ 4+</td>
</tr>
<tr>
<td>UV-R2</td>
<td>- ± &gt;+ &gt;2+ 3+ &gt;3+</td>
</tr>
</tbody>
</table>

aR2 = purified reovirus type 2; R2-SVP = subviral particles from R2; R2-UV-SVP = SVP exposed to 10' UV; OCP = outer capsid proteins from R2. R2-UV-OCP = OCP exposed to 10' UV. UVR2-SVP and UVR2-OCP = SVP and OCP made from UV-irradiated R2. R2 and UV-R2 MOI = 50 I.U. per cell.

coverslip cultures of HeLa cells were exposed to 20 µl of each of the reactants as described in the text, and CT was observed at various times according to standard procedure.
molecular species of polypeptides (Loh and Shatkin, 1968). It could be concluded from the results of this experiment that one or more of these proteins when exposed to UV-light somehow induces or acquires the cytotoxic property. In future studies it would be interesting to examine which of these proteins are toxic and/or what kind of photo-chemical changes occur which causes these protein molecules to be cytotoxic.

**Toxic Dose of Virus Protein.** Loh and Oie (1969) reported through kinetic studies that a minimum of 5 to 10 "infectious" particles (infectivity before inactivation) must hit a single cell to cause UV-R2 induced cell death. Purified virus preparations contain several fold more virion particles than what is revealed by infectivity titrations. This is due to the presence of non-infectious particles. As we have obtained substantive evidence to show that complete virions are not necessary for the induction of cytotoxicity, and that incoming virion protein(s) could elicit this phenomenon, an attempt was made to estimate the toxic dose of the protein(s).

Experiments were done with UV-inactivated purified complete reovirus (type 2) particles. HeLa cell monolayers containing about 150,000 cells each were grown on 15 mm round coverslips placed inside 35 x 10 mm Falcon plastic petri plates. These coverslip cultures were exposed to serial dilutions of the virus whose protein concentration was predetermined by the Lowry's method (1951). The virus sample was irradiated for 10 minutes according to the standard procedure described in Chapter 3, and dilutions made in EBM with 0.1% FCS before inoculating the cultures. Each coverslip was inoculated with 20 μl of virus dilution. After virus adsorption at 37°C in the usual manner described earlier, the cultures
were washed in BSS, fed with experimental medium and placed in the CO$_2$ incubator (37°C). At specified times after infection the cultures were observed for the presence of CT. The highest dilution of virus which exhibited a 2+ toxicity in 8 hrs was taken to be the minimum toxic dose (MTD).

Twenty μl of a $10^{-2}$ dilution of virus containing 1.9 μg/ml exhibited a 2+ toxicity 8 hrs after infection. Since each coverslip culture contained about $1.5 \times 10^5$ cells, the toxic dose of protein calculated was about $2.5 \times 10^{-6}$ μg of virus protein per cell. This estimation would only be approximate due to the intrinsic errors involved in the technique. However, the importance is that it gives an indication of the infinitesimally small amount of protein required to induce the toxic response. A more accurate quantitation could only be done by a particle count of the virion sample.
The two preceding chapters dealt with the changes in the reovirus after UV-irradiation, and the nature of the toxic agent. It was observed that whatever photochemical change that accompanied the conversion of the virion from a non-toxic particle to a toxic one must have been subtle, such that it was undetectable with the techniques employed in this study. The only major changes noted in the toxic virus were the loss of infectivity and the change in toxic property with increase in UV-irradiation. In this instance, the loss in CT property was accompanied by changes in several physico-chemical and biological properties of the virus.

It was established in Chapter 5 that the toxic agent associated with the UV-R2 was not the RNA but the capsid protein(s) of the virus.

It was of interest at this time to examine some of the cell interactions with the toxic agent in order to understand the probable mechanism involved in the toxic response, the nature of the toxic response in different cell species and to determine the toxicity of other serotypes of reovirus. Studies conducted along such parameters will be described in this chapter.

**Effect of Temperature on the Toxic Response.** Adsorption studies presented in Chapter 4 showed that UV-irradiated reovirus adsorbs onto HeLa cells at 37°C with the same efficiency as non-irradiated virus. Although reovirus type 2 attaches onto HeLa cells at 4°C (Loh, et al., 1968), virus penetration is a temperature dependent step which does not occur at 4°C. The purpose of this experiment was to determine whether
the UV-R2 induced toxicity is either primarily a surface phenomenon independent of incubation temperature, or a temperature sensitive reaction.

Procedure: Replicate cultures of HeLa cells grown in 1 oz prescription bottles were exposed to UV-R2 at a multiplicity of 50 I.U. per cell. The virus in three sets of cultures was adsorbed at 4°C, 22°C and at 37°C. Adsorption was carried out for 2 hrs with intermittent rocking of the cultures to redistribute the inoculum. The cultures were washed with BSS at the end of the adsorption period and incubated at the respective temperatures after feeding with EBM containing 0.1% FCS. The cultures were examined for cytotoxicity at various time periods. The cultures infected at 4°C were subsequently shifted to 37°C at 4 hrs and at 6 hrs after infection, and then examined for the appearance of CT.

Results of the experiment are shown in Table XIII. No toxicity was observed in cultures incubated at 4°C. However, in the cultures that were shifted up to 37°C, the CT response began to appear after a lag of 3-4 hrs from the time it was shifted. This suggests that the virus which was attached to the cell at 4°C required a temperature dependent step (37°C) for penetration in order to elicit the toxic response. Hence the delay in the appearance of CT after the shift up in temperature. The cultures that were kept at room temperature (22°C) took approximately 12 hrs or more to exhibit a one + CT reaction.

The above results strongly suggest that UV-R2-induced toxicity is a temperature dependent reaction involving a step beyond the simple attachment of virus to cell. This step requires the penetration of virus into the cell. Maximum CT response is observed only at 37°C with a lag period of 3-4 hrs prior to the appearance of toxicity.
TABLE XIII. EFFECT OF TEMPERATURE ON CYTOTOXICITY INDUCED BY UV-INACTIVATED REOVIRUS ON HELA CELLS

<table>
<thead>
<tr>
<th>Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Appearance of Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Temperature</td>
</tr>
<tr>
<td>Normal</td>
<td>4°C</td>
</tr>
<tr>
<td>UV-R2</td>
<td>4°C</td>
</tr>
<tr>
<td>Normal</td>
<td>4°C + 37°C</td>
</tr>
<tr>
<td>UV-R2</td>
<td>4°C + 37°C</td>
</tr>
<tr>
<td>Normal</td>
<td>4°C + 37°C</td>
</tr>
<tr>
<td>UV-R2</td>
<td>4°C + 37°C</td>
</tr>
<tr>
<td>Normal</td>
<td>22°C</td>
</tr>
<tr>
<td>UV-R2</td>
<td>22°C</td>
</tr>
<tr>
<td>Normal</td>
<td>37°C</td>
</tr>
<tr>
<td>UV-R2</td>
<td>37°C</td>
</tr>
</tbody>
</table>

<sup>a</sup>UV-R2: Reovirus type 2 exposed to 10 mins UV-inactivation. MOI = 50 I.U. per cell (i.e. infectivity before UV-inactivation)

<sup>b</sup>, <sup>c</sup>Time at which the culture was shifted from 4°C to 37°C
Effect of UV-R2 on HeLa Cells Pre-infected with Infectious R2. It was previously demonstrated in Chapter 4 that UV-R2 particles adsorb onto HeLa cells at the same rate as non-irradiated reovirus. Earlier studies have also shown that CT can be prevented by incubating the UV-R2 with antiserum to R2 prior to infection (Loh and Oie, 1969). The prevention of CT by antiserum was interpreted to mean that virus adsorption was interfered with. The previous experiment indicated that virus penetration was a required step for the induction of CT. All of these evidence strongly suggest the involvement of some intracellular event. Therefore, it was of interest to determine whether the synthesis of viral components such as proteins or of infectious virus in HeLa cells pre-infected with R2 would be inhibited or interfered with by super infection with UV-R2 at different times in the replicative cycle. These experiments will be described now.

Effect of UV-R2 on Viral Antigen Formation. HeLa cells were grown on rectangular (9 x 22 mm) coverslips contained in Leighton tubes. Five experimental cultures in replicate were infected with live reovirus type 2 at a multiplicity of 20 I.U./cell, two were infected with UV-R2 at a similar multiplicity and the controls were inoculated with EBM. After 2 hrs adsorption at 37°C the cultures were washed in BSS and one set of cultures were super-infected with UV-R2 at the same MOI (at 0 time p.i.). The other cultures were fed with EBM containing 0.1% FCS and returned to the 37°C incubator. At predetermined times post infection (2, 4 and 6 hrs) each set of cultures was super-infected with UV-R2, adsorbed as before and washed in BSS. The cultures were then fed with EBM containing 0.1% FCS and returned to the 37°C incubator. At 10 hrs after live virus
infection all the cultures were washed in BSS and fixed in cold acetone for 10 minutes. These fixed cultures were kept at 4°C until they were stained with fluorescein conjugated antibody to reovirus type 2 (FA). The coverslip cultures were stained with anti-reo-2 FA according to the standard procedure described in Materials and Methods. The number of fluorescing cells in five different fields of 100 cells each were counted from each of duplicate coverslips. The percent mean of the number of fluorescing cells out of a total of 1000 cells in each experimental set was determined.

Results: The FA technique showed that HeLa cells infected with UV-R2 did not produce new viral antigen and confirmed the earlier report (Loh and Oie, 1969). At 10 hours post-infection 71.4% of the cells infected with live reo-2 alone showed pin-head size sites of antigen synthesis in the cytoplasm around the nucleus. Although cells super-infected with UV-R2 also exhibited similar size antigen-forming sites scattered in the cytoplasm, there was a reduction of about 10-14% in the number of fluorescing cells observed (Table XIV). The significance of this is not clear at the present time. However, this reduction may represent cells lost through the action of CT since such an event is not interfered with. As will be seen later the pre-infection of cells with live reovirus did not interfere with the induction of CT.

No significant difference in the formation of antigen was detectable among the cultures that were challenged with UV-R2 at later periods.

Since viral antigen synthesis by live virus was not interfered with to any significant level in cells super-infected with UV-R2, it suggested that the UV-R2 did not compete with live R2 for any cellular components.
TABLE XIV. SYNTHESIS OF REO-2 ANTIGEN IN HEla CELLS SUPER-INFECTED WITH UV-INACTIVATED REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Time of Super-infection</th>
<th>Percent Fluorescing Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>-</td>
<td>71.4</td>
</tr>
<tr>
<td>UV-R2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>0</td>
<td>57.1</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>2</td>
<td>61.0</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>4</td>
<td>61.7</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>6</td>
<td>58.3</td>
</tr>
</tbody>
</table>

\(^a\)R2 = Reovirus type 2; UV-R2 = inactivated R2. MOI of R2 as well as UV-R2 was 20 I.U. per cell.

\(^b\)R2 infected cultures were super-infected with UV-R2 at the specified times after infection with live R2.

\(^c\)Infected cells were fixed at 10 hrs p.i., stained with FA and fluorescing cells counted as described in the text.
such as amino acids or ribosomes used for viral antigen formation. Conversely, the pre-infection of HeLa cells with live R2 does not interfere with the induction of CT. Furthermore, no viral antigen formation was observed in HeLa cells infected with UV-R2 alone. Therefore, it appears that the mechanism of cytotoxicity is independent of the synthesis of structural viral proteins.

**Effect of UV-R2 on the Production of Infectious Virus.** The previous experiment demonstrated that UV-R2 particles did not interfere with the production of viral antigen synthesis in R2 infected HeLa cells. This experiment was carried out to determine whether there was any effect on the production of infectious virus.

Procedure: HeLa cell monolayers were grown in 1 oz prescription bottles. Six cultures were infected in duplicate with reovirus type 2 at a multiplicity of 20 I.U./cell. One set was infected with UV-R2 at a MOI of 20 I.U./cell and the cell controls were inoculated with EBM containing 0.1% FCS. After virus adsorption for 2 hrs at 37°C, the cultures were washed with BSS. One of the pre-infected cultures was super-infected with UV-R2 at a MOI of 20 I.U./cell (i.e. infectious titer prior to UV-irradiation; irradiation time: 10 mins) and the others were fed with medium and returned to the 37°C incubator. Each set of pre-infected cultures were challenged with UV-R2 (MOI 20 I.U./cell) at predetermined times after infection with live reo-2 (2, 4, 6 and 8 hrs). The UV-R2 was permitted to adsorb for 2 hrs at 37°C, washed with BSS, and returned to the incubator with EBM containing 0.1% FCS.

At 24 hours after infection all cultures were frozen at -20°C. The infected cultures were frozen and thawed twice and centrifuged at 10,000
rpm for 20 mins in an IEC model HR-1 to separate the virus from cell debris. The supernatant medium containing virus was saved and frozen at -20°C until infectivity measurements were made. Infectivity titrations were done on pooled duplicate samples by the immunofluorescent plaque assay described in Chapter 3.

Results: Cell cultures infected with UV-R2 alone exhibited 3+ cytotoxicity in 24 hrs p.i. and those that were pre-infected with infectious virus showed nearly 4+ cell destruction. This could be due to the additive action of UV-R2 and R2 infection on the cells.

The HeLa cultures infected with R2 only had a virus titer of 2.4 x 10^7 I.U./ml, whereas those that were infected with UV-R2 alone showed less than 1 infectious unit in 20 μl of undiluted sample. Variation in virus titers in the pre-infected cultures was from 1.9 x 10^7 to 7.5 x 10^7 I.U./ml (Table XV). No inhibition of virus yield was observed as a result of super-infection with UV-R2, irrespective of the time in the replication cycle at which the infected cultures were challenged with UV-R2.

It was concluded that the synthesis of infectious virus was not interfered with by super-infection of the cultures with cytotoxic doses of UV-R2, either as early as 0 hrs p.i. or as late as 8 hrs p.i.

The Effect of Pre-infecting HeLa Cells with Infectious Reovirus on UV-R2 Induced Cytotoxicity. The two previous experiments showed that viral antigen formation and virus replication in reovirus infected HeLa cells was not interfered with by super-infection with UV-R2 at equivalent multiplicities. This experiment was done in order to determine whether cytotoxicity could be interfered with or arrested by pre-infected the cells with an equivalent multiplicity of infectious reovirus.
## TABLE XV. FORMATION OF INFECTIOUS REO-2 IN HELA CELLS
SUPER-INFECTED WITH UV-INACTIVATED REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Time of Super-infection (hrs)</th>
<th>Virus Titer at 24 hrs (I.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>-</td>
<td>(2.4 \times 10^7)</td>
</tr>
<tr>
<td>UV-R2</td>
<td>-</td>
<td>0(^d)</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>0</td>
<td>(2.1 \times 10^7)</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>2</td>
<td>(2.1 \times 10^7)</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>4</td>
<td>(7.5 \times 10^7)</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>6</td>
<td>(6.8 \times 10^7)</td>
</tr>
<tr>
<td>R2 + UV-R2</td>
<td>8</td>
<td>(1.9 \times 10^7)</td>
</tr>
</tbody>
</table>

\(^a\) R2 = reovirus type 2; UV-R2 = reovirus type 2 UV-inactivated for 10 mins under standard conditions described before.

\(^b\) HeLa cultures pre-infected with R2 was super-infected with UV-R2 at the same MOI (20 I.U./cell) at specified times after pre-infection.

\(^c\) Virus was harvested 24 hrs post-infection and titrated by the immunofluorescent technique described before.

\(^d\) Less than 1 I.U. per 20 μl of undiluted sample.
Procedure: HeLa cell monolayers grown in 1 oz prescription bottles were infected with reo-2 at a multiplicity of 30 I.U./cell. Five sets of experimental cultures were infected in this manner. The controls were inoculated with EBM. After virus adsorption for 2 hrs at 37°C, the cultures were washed in BSS. One set of infected cultures and one set of un-infected cultures were super-infected with UV-R2 immediately at the same multiplicity and allowed to adsorb for 2 hrs at 37°C. The rest of the cultures were incubated at 37°C with ERM containing 0.1% FCS. The UV-R2 infected cultures were washed with BSS at the end of the adsorption period and returned to the incubator with EBM containing 0.1% FCS. Out of the remaining 3 sets of R2 infected cultures each set was super-infected with UV-R2 (MOI=30) at 1/2 hr, 1 hr and 2 hrs after infection with live reo-2 and treated in the same manner as before. All cultures were kept at 37°C and examined for the appearance of CT at hourly intervals after infection.

Results: Cytotoxicity appeared at 4 hrs both in cell cultures infected with UV-R2 alone or those that were pre-infected with R2. No detectable difference in the progress of the CT was seen among the UV-R2 infected and the R2 + UV-R2 infected cultures (Table XVI). In other experiments cell cultures pre-infected with either reovirus serotypes 1 or 3 were immediately challenged with equivalent doses of UV-R2 after virus adsorption. Under these conditions again CT induction was not interfered with. It was concluded that pre-infection of HeLa cells with homologous or heterologous reovirus at equivalent multiplicities did not interfere with the cytotoxic response. Previous experiments on virus adsorption and temperature sensitivity of the UV-R2 induced CT showed
TABLE XVI. EFFECT OF PRE-INFECTION WITH LIVE REO-2 ON UV-R2 INDUCED CYTOTOXICITY IN HELA CELLS

<table>
<thead>
<tr>
<th>Pre-Infecting Sample</th>
<th>Superb Infecting Sample</th>
<th>Time Super-Infected (hr)</th>
<th>Appearance of Cytopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>UV-R2</td>
<td>0</td>
<td>- ± &lt;2+ 2+ &gt;2+ 3+ 3+ &gt;3+</td>
</tr>
<tr>
<td>R2</td>
<td>UV-R2</td>
<td>1/2</td>
<td>- + 2+ &gt;2+ 3+ 3+ &gt;3+</td>
</tr>
<tr>
<td>R2</td>
<td>UV-R2</td>
<td>1</td>
<td>- + 2+ &gt;2+ 3+ 3+ &gt;3+ 4+ 4+</td>
</tr>
<tr>
<td>R2</td>
<td>UV-R2</td>
<td>2</td>
<td>- - + &gt;2+ 3+ 3+ &gt;3+ 4+ 4+</td>
</tr>
</tbody>
</table>

^a^MOI of 30 I.U. per cell adsorbed at 37°C, 2 hrs.

^b^MOI of 30 I.U. per cell infected at given times and adsorbed at 37°C, 2 hrs.

^c^0 time after adsorption of UV-R2 in each case.
that attachment and penetration of the UV-R2 particle were necessary to elicit the CT response. This experiment indicated that the pre-infecting live virus did not interfere with the UV-R2 in adsorption or compete with it for any other common intracellular component so as to prevent the toxic response.

**Effect of Interferon on Cytotoxicity.** The preceding experiments showed that a temperature dependent intracellular event was required for the induction of CT. Also, it was shown that UV-R2 did not interfere with either viral antigen formation or the production of infectious virus in reo-2 infected cells. A further observation was that the CT was not prevented by pre-infecting the cells with infectious virus. Viral antigen formation was not detected in UV-R2 infected HeLa cells by the FA technique. However, since the virion transcriptase was found to be active in UV-R2 the possibility of the transcription of a viral messenger RNA coding for a new viral protein(s) on a functional segment of the genome must be considered. It was therefore of interest to examine whether such virus induced protein(s) coded for by the genome of the UV-R2 particle was responsible for the induction of CT.

Previous studies have shown that virus-directed protein synthesis could be inhibited by pre-treatment of infected cell cultures with the cellular protein interferon. The present concept on the mechanism of action of interferon is that the inhibition of the synthesis of virus is achieved by the prevention of the translation of the viral messenger RNA through the action of a translation inhibitory protein (TIP) produced by the host cell in response to interferon treatment (Marcus and Salb, 1966; Joklik and Merigan, 1966).
In the present study, it was hypothesized that if CT was induced by a newly synthesized viral protein, then interferon pre-treatment of the cell cultures would prevent the CT phenomenon from occurring. The following experiments were done to examine this possibility.

Procedure: Mouse serum interferon and the CCL-1 strain of mouse L cells were used in these experiments. Two sets of replicate cultures of mouse L cells grown in 1 oz prescription bottles were treated for 24 hrs at 37°C with mouse serum interferon at a concentration of 160 units per ml of EBM. The control cultures were treated with EBM containing no interferon (IF). The cultures were washed thrice with BSS and duplicate bottles of IF-treated and non-treated cultures were challenged with live reovirus type 2 (R2), UV-R2 or vesicular stomatitis (VSV) at the following multiplicities of infection: R2 = 0.0025 and 50 I.U./cell; UV-R2 = 50 I.U./cell; VSV = 0.0025 and 10 I.U./cell. Normal cell controls were inoculated with EBM. Reo-2 was adsorbed for 2 hrs and VSV for 1 hr, both at 37°. At the end of adsorption the cultures were washed and returned to the 37°C incubator with EBM containing 0.1% FCS. All the cultures were then examined for cytopathology at various times after infection. At 24 hrs p.i. the cultures were frozen at -20°C and the virus was harvested. The virus yield in each experimental set of cultures was determined by the standard titration procedures described in Materials and Methods. Reo-2 was titrated by the immunofluorescent plaque technique, while the VSV was titrated by the agar overlay plaque technique.

Results: The results (Table XVII) showed that UV-R2 induced CT was not inhibited in interferon treated L cell cultures. The IF treated
TABLE XVII. EFFECT OF INTERFERON ON UV-R2 INDUCED CYTOTOXICITY AND YIELD REDUCTION

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MOI per Cell</th>
<th>Cytopathology&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Infectious Virus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Percent Yield Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 12 24</td>
<td>8 12 24</td>
<td>pfu/ml</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>- - - -</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>0.0025</td>
<td>- - + - -</td>
<td>4.3x10^7</td>
<td>2.2x10^6</td>
</tr>
<tr>
<td>R2</td>
<td>50</td>
<td>- ± 3+ - -</td>
<td>6.0x10^7</td>
<td>1.3x10^7</td>
</tr>
<tr>
<td>UV-R2</td>
<td>50</td>
<td>2+ &gt;2+ 3+ 2+ &gt;2+ 3+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VSV</td>
<td>0.0025</td>
<td>- - &lt;+ - -</td>
<td>2.6x10^5</td>
<td>2.7x10^2</td>
</tr>
<tr>
<td>VSV</td>
<td>10</td>
<td>2+ 3+ &gt;3+ 2+ &gt;2+ 3+</td>
<td>2.1x10^6</td>
<td>1.1x10^4</td>
</tr>
</tbody>
</table>

<sup>a</sup>R2 = reovirus type 2; UV-R2 = R2 UV-inactivated for 10 mins under the standard conditions described earlier.

<sup>b</sup>Mouse L cell cultures (strain CCL-1) were treated with 160 units of interferon (IF) for 24 hrs. Interferon treated and control cultures were washed 3 x in BSS and challenged with R2, UV-R2 or VSV at the MOI specified.

<sup>c</sup>Virus yields were titered after 24 hrs.
cultures exhibited the same CT changes as in the non-treated control cultures. Nevertheless, a marked reduction of virus yield was observed in cell cultures infected with infectious reo-2 as well as VSV. Even the cultures infected with high multiplicities of virus showed marked reduction in virus yields. With low multiplicity of infection the per cent yield reduction of R2 was 95 whereas with a high MOI (50 I.U./cell) the per cent yield reduction was 80. In VSV infected cultures the per cent yield reductions were 99.9 and 99.5 with low and high multiplicities of infection, respectively. The results indicated a greater sensitivity of VSV to interferon than R2.

It was concluded that mouse interferon at a concentration which caused 80-95% yield reduction of R2 and over 99% in VSV infected mouse L cells, was incapable of blocking the CT response.

**Effect of Different Concentrations of Interferon on Cytotoxicity.** Since the previous experiment did not prevent the CT response in mouse L cells treated with interferon at a concentration of 160 units/ml, this experiment was done to determine whether higher doses of interferon would at least block the CT.

Mouse L cell monolayers grown in 1 oz prescription bottles were exposed to different concentrations of mouse serum interferon varying from 160 to 800 units/ml of EBM for 24 hrs at 37°C. The cultures were washed as before and challenged with UV-R2 at a multiplicity of 50 I.U./cell. The appearance of CT was observed at various times post-infection, in comparison to the controls.

**Results:** The results indicated that IF concentrations even as high as 800 units per ml of medium did not block the CT in mouse L cells
(Table XVIII). It was concluded that regardless of the IF concentration used the CT could not be prevented by IF pre-treatment.

**Effect of Poly I:C on UV-R2 Induced Cytotoxicity.** The previous experiments showed that mouse serum interferon did not prevent the CT response in mouse L cells despite the high concentrations of IF used. It was of interest to examine whether human IF induced in RA cells would show a similar effect on CT in human (RA) cells. In this experiment the synthetic polynucleotide poly I:C, a known inducer of IF was used (Field, et al., 1968).

Monolayer cultures of RA cells in 2 oz prescription bottles were treated with poly I:C at a concentration of 20 to 40 µg/ml for 18 hrs at 37°C and challenged with either 4,000 PFU of VSV per culture (MOI = 0.005/cell) or UV-R2 at a multiplicity of 50 I.U./cell. In another experiment RA cell cultures in 1 oz prescription bottles were treated with undiluted preparations of poly I:C induced interferon (in RA cells) for 24 hrs and challenged with VSV or UV-R2 as done previously.

Results: The results indicated that in both experiments the cultures treated with poly I:C and challenged in 24 hrs, or interferon treated cultures were protected against the CPE caused by VSV but not the cell destruction caused by UV-R2.

It was concluded that irrespective of the nature of the IF or the host cell used, CT cannot be inhibited by interferon. The foregoing experiments with IF strongly suggest that the CT phenomenon does not involve the synthesis of new virus coded protein(s). Furthermore, it strongly points to the involvement of a protein component(s) of the incoming virion.
TABLE XVIII. EFFECT OF INTERFERON CONCENTRATION ON UV-R2 INDUCED CYTOTOXICITY

<table>
<thead>
<tr>
<th>Sample</th>
<th>Interferon Units</th>
<th>Appearance of Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Normal</td>
<td>320</td>
<td>-</td>
</tr>
<tr>
<td>UV-R2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>UV-R2</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>UV-R2</td>
<td>320</td>
<td>-</td>
</tr>
<tr>
<td>UV-R2</td>
<td>800</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)CCL-1 strain of mouse L cell monolayers were exposed to varying concentrations of mouse serum interferon for 24 hrs, washed 3 x in BSS and challenged with UV-R2 at a MOI of 50 I.U. per cell.

\(^b\)UV-R2: Reovirus type 2 inactivated by UV for 10 mins under standard conditions described earlier.

\(^b\)Cytotoxicity was observed at the given times post-infection with UV-R2 according to the previously described procedure.
Toxic Property of Reo-2 Passed in BSC-1 Cells. In all of the previous studies on UV-R2 induced cytotoxicity, the reovirus used had been passed in HeLa cells (Loh and Oie, 1969). It was of interest to examine whether the toxic property was dependent on the cell species in which the inducing virus was passed. If it was a property of the cell species, then the virus passed in other cell species should lose this toxicity.

Reovirus type-2 was passed several times in an established line of African green monkey kidney cells (BSC-1). Purified preparations of this virus were irradiated for 10 minutes in the usual manner. HeLa cell monolayers grown in 1 oz prescription bottles were exposed to this UV-irradiated virus at a multiplicity of 30-40 I.U./cell. R2 passed in HeLa cells and uninfected cultures were used as controls. Infected cultures were examined at various times p.i. for the appearance of CT.

Results: The results showed a 3+ CT at 12 hrs p.i. in both the cell cultures infected with BSC-1 passed R2 as well as HeLa passed R2. The two preparations of virus were equally toxic to HeLa cells. It was concluded that the toxic property of R2 was not dependent on the cell system in which the virus was passed, but a specific property of the virus.

The Toxicity of UV-Irradiated Reovirus Type 1 and 3. Up to the present time the cytotoxicity induced by UV-irradiated reovirus was studied only with reovirus type 2. The previous experiment indicated that the toxicity was a specific property of the virus, and was independent of the cell system in which the virus was passed. Therefore it was of interest to examine whether the toxicity was a property common
to all serotypes of reoviruses or peculiar to serotype 2 only.

Reovirus serotypes 1 and 3 were passed several times in HeLa cells. Preparations of these two serotypes as well as reo-2 passed in HeLa cells were irradiated for 10 minutes according to the standard procedure described. HeLa cell monolayers grown in 1 oz bottles were exposed to the above virus preparations at multiplicities of 30-40 I.U. per cell and adsorbed for 2 hrs as usual at 37°C. The cultures were washed with BSS after adsorption and incubated at 37°C with EBM containing 0.1% FCS. The cultures were then examined at various times for the appearance of CT.

Results: All 3 serotypes exhibited more than 25% cell death in 4 hrs and about 3+ toxicity in 12 hrs. Toxicity was observed up to 24 hrs. The corresponding live virus of each serotype did not exhibit accelerated cytopathology. It was concluded that serotypes 1 and 3 acquired a cytotoxic property with UV-irradiation, as did reo-2. The toxic property is therefore common to all three serotypes of reoviruses.

Sensitivity of Different Cell Species to UV-R2 Induced Cytotoxicity.
Except in a few experiments where the effect of mouse interferon was being tested in all of the previous studies on cytotoxicity, HeLa cells were used as the test cell system. HeLa cells were selected for such studies because preliminary observations of Oie (1968) and the author's studies indicated that they were the most sensitive of the cells tested for UV-R2 induced CT. Variation in the sensitivity of different cell species to cytotoxicity has been reported in other virus-cell systems, e.g.: mumps (Russel and Morgan, 1969), and measles virus (Cascardo, et al., 1965) as well as for R2 induced accelerated cytopathology in the
presence of cycloheximide (Loh, et al., 1970). It was of interest to
examine the sensitivity of a number of cell cultures from human and other
animal species to the UV-R2 induced CT.

Procedure: Monolayer cultures of the following cells were grown in
1 oz prescription bottles: HeLa, RA, WI-38, BSC-1, MKC, Mouse-L, CCL-1,
MEF, BHK-21 and FHM. These cultures were exposed to UV-R2 at a
multiplicity of 50 I.U./cell and allowed to adsorb for 2 hrs. At the
end of adsorption the cultures were washed in BSS and re-incubated at
37°C with EBM containing 0.1% FCS. The cultures were then examined for
the appearance of CT at various times post-infection.

Results (Table XIX) indicated that of the human cell lines tested
HeLa cells were the most sensitive. It was observed that established
human amnion (RA) cells were also sensitive although the CT was of a
lesser degree than HeLa cells. In contrast diploid human embryonic lung
cells (WI-38) were more resistant, exhibiting less than 1+ CT at 12 hrs
as compared to a greater than 3+ in HeLa cells.

Of the primate cells tested the established monkey cell line BSC-1
was less sensitive than the human RA cells exhibiting less than 2+ CT
response at 12 hrs p.i. In contrast the primary monkey kidney cells
exhibited little or no CT.

An established line of murine cells (L) was found to be almost
equivalent to HeLa cells in its sensitivity to UV-R2 induced CT, whereas
another strain (CCL-1) of mouse L cells was relatively less sensitive.
In contrast, primary mouse embryo fibroblasts were totally resistant to
CT.
TABLE XIX. TOXIC RESPONSE IN DIFFERENT CELL SPECIES TO UV-INACTIVATED REOVIRUS TYPE 2

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Cell Type</th>
<th>Virus</th>
<th>Appearance of Cytopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Human</td>
<td>HeLa</td>
<td>UV-R2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>UV-R2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>WI38</td>
<td>UV-R2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Monkey</td>
<td>BSC-1</td>
<td>UV-R2</td>
<td>-</td>
</tr>
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*Each cell culture was treated as follows: (1) no treatment, (2) UV-inactivated reovirus type 2 at a MOI of 50 I.U./cell (infectivity prior to inactivation).*
The two established cell lines, baby hamster kidney (BHK-21) cells and Fathead minnow (FHM) cells, were completely resistant to UV-R2 induced CT.

Although the CT response in the different cell types examined varied widely, all of the cells tested with the exception of FHM cells support reovirus replication (Loh, et al., 1970). Since the FHM cells were capable of adsorbing R2 and also producing interferon (Oie and Loh, 1971) they were used in the present study to examine their sensitivity to UV-R2 induced CT. It was concluded that HeLa cells and mouse L cells were the most sensitive of all the cell types that were tested for UV-R2 induced cytotoxicity. In general, primary cell cultures were more resistant than the established cell lines to UV-R2 induced CT. However, BHK-21 cells, an established cell line, were totally resistant. Perhaps the reasons for this resistance could be related to the origin of the cell or its fibroblastic nature, since the mouse cells which were sensitive to CT were epithelioid cells. It appears that the origin of the cell as well as the genetic state of the cell have important roles in the UV-R2 induced CT.

**In-Vivo Studies: Effect of UV-R2 in Swiss White Mice.** All of the previous experiments on UV-R2 induced CT were done in tissue culture. It was of interest to examine whether UV-R2 would be toxic in the whole animal. Therefore, the following in vivo studies were done on live mice as test animals.

Studies with adult mice: 14 day old Swiss white mice having an average weight of 10 grams were used in this experiment. Fifteen mice were divided into 3 equal size groups. One group was given semi-purified live reovirus type 2 (0.5 ml/mouse) by the intravenous route, while the
second group was given a similar dose of UV-inactivated virus. The third group was given a placebo (EBM). Infectivity titer of the virus before inactivation was $1.3 \times 10^8$ I.U./ml. The mice were kept in separate cages and observed for signs of toxicity or any abnormal behavior for 5 days.

Results: All animals were apparently normal until 24 hrs, when the mice that received live reo-2 were lethargic, and had ruffled hair. On the other hand the group that received UV-R2 remained normal like the control group. After 48-72 hrs all of the sick mice recovered. The other experimental animals appeared to be in normal health up to 5 days when the experiment was ended. It was concluded that UV-inactivated reovirus given intravenously was not toxic to adult mice at the tested dose. In contrast, corresponding similar doses of live virus caused obvious signs of illness in the animals.

Studies with baby mice: 7 day old Swiss baby mice having an average body weight of 4 grams each were used in this experiment. Each of 6 baby mice was given 0.2 ml of a UV-R2 preparation containing $1.3 \times 10^8$ I.U./ml by the intraperitoneal (i.p.) route. The six mice in the control group were given 0.2 ml of EBM each i.p. The animals in the experimental group did not show any difference in behavior to those of the control group. The animals were kept under observation for 5 days and were disposed of. It was concluded that UV-R2 was not toxic to baby mice at the dosage levels tested. It is unlikely that they would have responded even to higher doses for we have shown in the previous experiments that primary mouse embryo cultures in vitro are resistant to UV-R2 induced cytotoxicity.
CHAPTER 7
Discussion

The cytopathology induced by UV-irradiated reovirus in tissue culture is in effect a "true" cytotoxicity, in terms of the criteria described by Pereira (1961), i.e. early onset, non-production of infectious virus and the multiplicity dependency. Reovirus exposed to UV for 2 minutes lost its infectivity completely, but acquired a CT property (Loh and Oie, 1969). The CT induced by UV-R2 in HeLa cell monolayers was multiplicity dependent, appeared as early as 2-3 hrs after infection, and lacked the production of infectious virus. In addition to new findings presented in the previous chapters of this dissertation, the present study has confirmed the above criteria reported previously (Loh and Oie, 1969).

Cytotoxicity associated with other animal viruses reported previously, for example, are vaccinia (Hanafusa, 1960), adenovirus (Levy, et al., 1957; Pereira and Kelly, 1957), Newcastle Disease virus (Cantell, et al., 1962) measles (Cascardo and Karzon, 1965) and vesicular stomatitis virus (Huang and Wagner, 1965; Wagner, et al., 1965). However, these viruses were cytotoxic even prior to UV-irradiation. Ultraviolet light irradiation only enabled the delineation of cell injury due to some component(s) of the incoming virion from the cytopathology which usually accompanied virus multiplication. The CT of R2 was unique since the toxic property was acquired only after exposure to UV, unlike in the case of the other viruses mentioned. The CT induced by reo-2 was also induced by UV-irradiated reovirus serotypes 1 and 3. Since the CT property of the virus did not appear to be dependent on the cell species
in which the virus was passed, it reaffirmed that the CT was a specific property of the virion and not a virion-associated cellular derived component.

An important new finding of this study was the dependency of the acquisition and loss of CT on the dose of UV. Non-irradiated virus which did not exhibit any toxic property acquired a cytotoxic property in 2-5 minutes when exposed to UV at a dose rate of $10^5$ ergs/sec/cm$^2$. This toxic property was maximum when irradiation was extended to 10 mins. Continued exposure of the virus to UV resulted in partial loss of CT in 30 mins, and a complete loss in 60 mins. The rate of cell killing by the samples of virus exposed to various doses of UV showed similar kinetics to those reported previously with R2 exposed to 2-5 mins UV (Loh and Oie, 1969).

The present studies confirm the previous reports that the toxic property was associated with the UV-irradiated virus (Loh and Oie, 1969). Since the extracted double-stranded RNA was found not to be toxic after exposure to UV, the quest for a toxic protein was the next obvious step. Previous reports showed that reovirus virion transcriptase can be activated by either brief heat treatment or by chymotrypsin (Borsa and Graham, 1968; Shatkin and Sipe, 1968). In addition, the present study showed that it could be activated by UV. If the transcriptase was directly responsible for CT, then virion transcriptase activated by heat also would have induced CT. However, R2 after exposure to brief heat treatment ($70^\circ$C, 30 secs) did not induce CT. Therefore it was concluded that the virion transcriptase was not directly responsible for CT. The experimental results presented here also showed that although R2 exposed
to UV for 60 mins had lost its CT property the virus still retained its transcriptase activity. This finding further confirmed the above conclusion.

One of the most interesting findings of this study was the CT exhibited by CsCl gradient purified reovirus "empties" after exposure to UV. As in the case of whole virus, non-irradiated "empties" were non-toxic in the absence of protein antagonist cycloheximide. "Empties" are virus particles essentially free of RNA (Loh and Oie, 1969; Smith, et al., 1969). Since it was pointed out earlier that UV-irradiated RNA was not toxic, the importance of some capsid protein(s) of the virion became further evident. Except for some quantitative differences the polypeptide components of the reovirus empties were identical to those of the whole virus (Loh and Oie, 1969; Smith, et al., 1969). Hence, whatever component that was toxic in the whole virus should have been present in the empties as well. Cytotoxicity induced by the fiber antigen of adenovirus (Levine and Ginsberg, 1967) and T-particles of VSV (Huang, et al., 1966) provided the best evidence for the role of structural proteins in the phenomenon of CT. However, a major difference between these systems and the reovirus is that the reovirus proteins must undergo irradiation by UV before becoming CT, whereas the proteins of VSV or adenovirus do not have to be irradiated.

The probable involvement of structural protein(s) in the induction of reovirus induced accelerated CPE in the presence of protein antagonist cycloheximide has been proposed (Loh and Crowley, 1967; Loh, et al., 1970). In these studies the phenomenon was seen only when virus replication was interfered with. The present studies again emphasized
the role of the structural proteins in the CT phenomenon. The "empties" which do not contain a functional genome apparently elicited a similar accelerated cytopathology under conditions which inhibit cellular protein synthesis. Furthermore, UV-irradiated "empties" by themselves were found to be cytotoxic.

The toxicity of UV-irradiated capsid proteins prepared by urea degradation of purified reo-2 provided evidence to show which part of the virion structure was toxic. The experiments showed that the toxic factor was associated with one or more of the proteins of the outer shell. The outer capsid layer is composed of at least 5 polypeptide components of which two are major proteins: I and IIIb, and three are minor ones: ml, m2 and m3. Either one of these singly or in combination might be cytotoxic. It would be interesting to separate the proteins on acrylamide gels or on sephadex columns and examine each one of these proteins for cytotoxicity. Practical difficulties which might arise in obtaining sufficient amounts of the minor proteins should be taken into consideration. Attempts with the major proteins in the first instance may prove fruitful.

Examination of the biological and physico-chemical properties of the toxic virion did not indicate any detectable alterations, to which the CT could be attributed. However, since these studies have established that structural protein(s) of the virus are responsible for inducing the CT, these proteins presumably undergo some subtle photochemical alterations on exposure to UV. A very minor change in the proteins such as disruption of some disulfide bonds or peptide bonds, etc., could perhaps convert a non-toxic polypeptide to a toxic one.
Since the CT property is acquired only on exposure of the virus or the proteins to UV, the conferring of toxicity must be due to some selective adsorption property of the molecular structure of reovirus proteins. Rauth (1965) in his studies on the relative sensitivity of several viruses to UV found that for wavelengths below 2650 Å the inactivation cross section for reo-3 increased rapidly in relation to other double stranded nucleic acid containing viruses like vaccinia and polyoma. He suggested that this peculiar behavior may reflect a role of the proteins in the sensitivity of reo-3 to UV. Powell (1959) who had similar findings with herpes simplex invoked an equivalent role for the virus proteins to explain his results. Since the amino acid composition of reovirus proteins has not been analyzed at the present time, it is difficult to speculate why reovirus proteins should have a greater sensitivity to UV. Whether these proteins are rich in aromatic amino acids or in other effective chromophores such as histidine have to be determined.

Although cytotoxicity was reported with several viruses as pointed out earlier in Chapter 1, a satisfactory explanation for the mechanism of CT is still lacking. In the mengovirus induced CPE it was reported that a late protein synthesized under the direction of the virus triggered the cell injury by initiating a leakage of hydrolases from the lysosomes (Amako and Dales, 1970). UV-irradiated vaccinia induced CT was reported to be due to some UV-resistant protein like substance, while the CT due to heated vaccinia was presumably due to an unknown function of the viral nucleic acid (Hanafusa, 1960). However, no mechanism of action was suggested. In subsequent studies, Bablanian
(1968) has suggested that vaccinia induced CT was due to virus induced proteins. On the other hand, studies with VSV and adenovirus (Huang and Wagner, 1965; Huang, et al., 1966; Pereira and Kelly, 1957) have shown that the cytotoxicity induced by these viruses was due to a protein component of the incoming virion. Since both T particles of VSV and fiber antigens of adenovirus inhibit macromolecular synthesis, it was suggested that cell injury could result from such a process (Levine and Ginsberg, 1967, 1968; Huang and Wagner, 1965; Huang, et al., 1966). Previous studies indicated that a marked suppression of RNA, DNA and protein synthesis occurred in UV-R2 infected HeLa cells as well (Loh and Oie, 1969). A similar mechanism of action could, therefore, be suggested for UV-R2 induced cytotoxicity.

It was established that an incoming protein of the virion capsid altered in some manner by UV was responsible for induction of UV-R2 cytotoxicity. Adsorption studies and temperature shift up experiments provided further evidence to show that adsorption and penetration of the UV-R2 were necessary for the CT phenomenon to occur. Lack of hemolysis of RBC by UV-R2 (Loh and Oie, 1969) and the fact that CT does not occur at 4°C are indicative of the CT being not a cell surface phenomenon. Although these evidences may not be conclusive, the temperature dependence of the CT phenomenon indicate that virus penetration does take place. Therefore, an intracellular site of action for the incoming proteins could be postulated. Ultrastructural studies currently in progress would provide conclusive evidence to show that the UV-R2 particles penetrated the cell membrane.
Several possible theories for the mechanism of action could be postulated. Cell injury may be caused by direct interaction of the toxic protein with the lysosomal membranes resulting in the release of cellular hydrolases into the cell milieu. If this were the case, perhaps alterations in the lysosomal membranes or evidence of their rupture might be seen through thin section. This possibility is being investigated at the present time. Another possibility would be that the incoming toxic protein shuts off the macromolecular synthesis of the host cell, perhaps by complexing with the cell genome. Evidence for macromolecular shut off was provided in previous studies (Loh and Oie, 1969), and recent $^{3}$H-uridine incorporation studies. Recent studies indicated that RNA synthesis was shut off in UV-R2 infected HeLa cells beginning at 2 hrs, whereas R2 infected cells or non-infected controls did not show such a change. Early reports also indicated that host cell RNA synthesis remained unaffected in reovirus infected cells (Kudo and Graham, 1965; Loh and Soergel, 1966). The 2-3 hr time lag necessary after infection with UV-R2 to elicit the toxic response coincided with the commencement of the shut off of cellular RNA synthesis. Whether the shut off of RNA alone is sufficient to cause cell injury has to be determined. The latter hypothesis appears to be the more attractive one. Nevertheless the cytotoxic proteins could act through any one of the postulated mechanisms or in combination to cause cell death.

Since RNA transcriptase activity was present in UV-R2 particles, the possibility of a viral protein coded for by a functional segment of the genome interfering with the host cell metabolism was considered as a probable cause of CT. However, HeLa cells infected with UV-R2 did not
produce any viral antigens detectable by the fluorescent antibody technique. Furthermore, pretreatment of host cells with interferon prior to viral infection did not prevent the occurrence of CT. If the production of CT necessitated the synthesis of new virus protein(s) the synthesis of such a protein could be inhibited by interferon. The present concept of the action of interferon is that the synthesis of viral protein is inhibited by an interferon induced translation inhibitory protein (TIP). The TIP prevents the translation of the viral messenger RNA (Marcus and Salb, 1966; Joilik and Merigan, 1966; Friedman, 1968). Thus, if CT was caused by a viral induced protein, then the pretreatment of cell cultures with interferon would prevent its appearance when challenged with UV-R2.

During this study it was found that pretreatment of mouse L cells with interferon at concentrations which inhibited reovirus yields by 90 to 95% or VSV yields over 99% failed to prevent the cytotoxic response. Therefore, the possibility of a newly synthesized viral protein as the cause of UV-R2 induced CT is remote. Furthermore, since CT has been reported to occur in the presence of actinomycin D and cycloheximide at concentrations which inhibited cell RNA and protein synthesis (Loh, et al., 1970), the possibility of a virus induced cell protein eliciting the toxic phenomenon is unlikely. Yamazaki and Wagner (1970) reported that pretreatment of rabbit kidney cells with interferon at concentrations which inhibited VSV yields by 95 to 99% failed to prevent the switch off of host cell protein synthesis. In addition, even large doses of interferon caused only a slight delay in the appearance of cytopathology. Inhibition of host cell RNA synthesis by UV-irradiated VSV in Krebs-2-carcinoma cells was reported earlier (Huang and Wagner, 1965). Their
results provided evidence to show that in the VSV the toxic component was an incoming structural protein which inhibited viral RNA and protein synthesis of the host cell. Our results also appear to be quite similar to what they obtained with VSV.

Different cell species showed a wide variation in sensitivity to UV-R2 induced CT. In general, the established cell lines were more susceptible than the primary cells tested. Similar results were obtained with R2 induced accelerated CPE in the presence of the protein antagonist cycloheximide (Loh, et al., 1970). A difference in sensitivity of various cell species to a measles virus "fusion factor" was also reported (Cascardo and Karzon, 1965). In this study, primary cells of human, monkey and lower animals were found to be insensitive, as compared to established cell lines, and they suggested that cell injury occurred only in susceptible cells which contained the appropriate receptor substance. Differences in cytolytic activity of mumps virus in human epithelial and mouse L cells were reported by Russel and Morgan (1959). They suggested that since the same enzyme which caused hemolysis was responsible for cytolysis, the differences in susceptibility of the different cells may be due to the availability of enzyme substrate. Differences in the sensitivity of the various cell species to UV-R2 induced CT may have been due to the differences in their ability to adsorb the virus or the genetic state of the cell. Failure to exhibit a toxic response in mice injected with UV-R2, through intra-peritoneal or intravenous route, may reflect a relationship to the insensitivity of primary cell cultures to UV-R2 induced toxicity.

Except for the loss of infectivity no other changes were detectable in the toxic virion. Apparently the changes occurring to the virion on
exposure to UV were very subtle. Nevertheless, the effects of UV dose on the cytotoxic property itself and the other biological as well as physico-chemical properties were interesting and informative. The difference in sensitivity of the various properties and functions would depend not only on their capacity to absorb UV-energy and target size, but also on their ability to undergo photochemical changes. Adsorption capacity of reo-2 to HeLa cells was not affected even by high doses of UV (60 mins). Perhaps the receptors on the virus were not affected to any appreciable extent in order to show a change. Gradual loss of HA property of the virus with increasing dose of UV was observed, although no correlation did seem to exist between the loss of HA property and the acquisition of CT. Loss of HA property before the loss of CT property indicated a greater sensitivity of the former to UV and may reflect a role of the location or photosensitivity of the surface glycoproteins of reovirus involved in hemagglutination.

Induction of interferon by reovirus type 2 in RA cells was reported previously (Oie and Loh, 1968). Double stranded RNA of reo-3 has also been used to induce interferon in rabbits (Tytell, et al., 1967). The present study indicated that the interferon induction property of reovirus was retained after exposure of the virus to low doses of UV which destroyed virus infectivity. This property was lost on continued irradiation for 60 mins, at which time the structure of the double stranded RNA was affected. This appears to indicate the importance of the integrity of the double stranded RNA for the induction of interferon.

Morphological studies of the reovirus after exposure to UV for 60 mins indicated that high doses of UV did seriously disorganize virion architecture. Buoyant density studies further indicated that the damage
caused by high dose of UV to the virion was serious. However, it was interesting to find that virion polymerase was resistant to inactivation even after such a high dose. There is at present no valid explanation. Perhaps the resistance is due to its location within the SVP, its size, or its amino acid composition.

The induction of CT by the new protein component observed in the acrylamide gel analysis of UV-R2 proteins is unlikely since the new protein appears in greater quantity after longer irradiation (60 mins).

Although no detectable changes were seen in the RNA with low doses of UV, the exposure of virus to 60 mins irradiation affected the double stranded RNA. The large, medium and small segments of the RNA were broken up into a greater number of smaller pieces. Instead of the usual total of 10 pieces, approximately 16 pieces were observed. Perhaps this may be due to chain breaks occurring from the high dose of UV. Although there appeared to be such breaks the viral RNA remained resistant to pancreatic ribonuclease. The interchain bonds were presumably unaffected. Although the base composition of its dsRNA was not altered, the A-rich RNA component appeared to have leaked out of the virion after exposure to the high dose of UV (60 mins).
SUMMARY

The present studies have established that when the reovirus was exposed to ultraviolet light it acquired a cytotoxic property within 2-5 minutes, after irradiation. Cytotoxicity was maximum after 10 minutes irradiation and was reduced to undetectable levels after 60 minutes. This toxic property was found in all three serotypes of reovirus, and was independent of the cell species in which the inducing virus was passed. Toxicity was temperature dependent, occurring maximally at 37°C. Toxicity did not occur at 4°C although the virus was adsorbed at that temperature.

Reovirus subjected to brief heat treatment (70°C, 30 mins) was not cytotoxic even though the virion transcriptase was activated under such conditions. The extracted viral RNA after exposure to UV also was found not to be cytotoxic.

Experiments with UV-irradiated "empties" and chemically degraded proteins of reovirus demonstrated that the toxic factor was associated with a structural protein(s) present on the virion.

The effect of varying doses of UV on the following properties of the virus were examined: virus adsorption, hemagglutination of human "O" erythrocytes, interferon inducibility, RNA transcriptase activity, virus morphology, buoyant density in CsCl, sedimentation in sucrose gradient, capsid proteins, double stranded RNA and A-rich RNA.

The acquisition of toxicity was not accompanied by changes in any of the above properties except for the loss of infectivity. However, on prolonged exposure to UV for 60 minutes the following changes were observed: loss in cytotoxicity, loss in HA property reduction in
interferon inducibility of the virus, derangement of capsid architecture, increase in buoyant density from 1.38 to 1.44, partial degradation of the virus in the sucrose gradient, and alterations in the electrophoretic patterns of the structural viral proteins and RNA also occurred.

Production of infectious virus or viral antigens was not interfered with in cell cultures superinfected with UV-R2. The cytotoxicity could not be prevented by preinfecting HeLa cells with live homologous or heterologous reovirus. Pretreatment of mouse L-cells with interferon which reduced reovirus yields by 80-90% failed to prevent the UV-R2 induced cytotoxicity.

Examination of the sensitivity of different cell species indicated a greater susceptibility of the established cell lines to the UV-R2 induced cytotoxicity than the primary cells examined. UV-irradiated virus was not toxic to live mice. On the basis of the evidence presented a probable mechanism for UV-R2 induced cytotoxicity is proposed.
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