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A STUDY OF THE CONTROL MECHANISMS IN POLIOVIRUS
INDUCED CYTOPATHOLOGY AS RELATED TO LYPOSOMAL
ENZYME RELEASE.

The Ohio State University, Ph.D., 1971
Microbiology

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A STUDY OF THE CONTROL MECHANISMS IN POLIOVIRUS
INDUCED CYTOPATHOLOGY AS RELATED TO
LYSOSOMAL ENZYME RELEASE

DISSEPTION

Presented in Partial Fulfillment
of the Requirements for the
Degree Doctor of Philosophy
in the Graduate School of
The Ohio State University

By

Louis Ernest Guskey, B. Sc., M. Sc.

* * * * *

The Ohio State University
1971

Advisor
Department of Microbiology
DEDICATION

To my wife, Carol, who deserves this degree more than I, for making it "two for the road".
ACKNOWLEDGMENTS

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INTRODUCTION

Host cell lysosomes have been found to play an integral part in certain cytolytic virus infections. Evidence indicates that hydrolytic lysosomal enzymes are released during certain viral infections, destroying the cell from within and establishing conditions aiding the eventual release of the virus. This latter phenomenon has profound and broad implications, since if lysosomal enzyme release can be controlled, perhaps virus disease can be controlled (A. Lwoff, 1969), perhaps even cell death. This statement assumes that lysosomal enzyme release is caused by virus infection. Although a great deal of evidence suggests that the virus is responsible for lysosomal enzyme release, it is difficult to prove. The mechanism responsible for lysosomal enzyme is probably one of the following: a viral derepression of its host cell genome; the lysosome interaction with the coat protein of the infecting virion; lysosome interaction with the RNA of the infecting virion; or lysosome interaction with a viral-specific substance produced prior to or during the synthesis of progeny components. These various mechanisms are
examined in this dissertation in the hopes of gaining a better understanding of the underlying mechanism governing virus-induced lysosomal enzyme release and cell destruction.
LITERATURE REVIEW

Because poliovirus is among the smallest known biological entities, it lends itself to the study of biological function in its simplest form. Since its genetic material, RNA, is limited, being about 1/500,000th the size of Escherichia coli DNA, its functions within a cell are also limited relative to bacteria and other uncomplicated biological forms. Cell death is either a direct or an indirect manifestation of the molecular events directed by poliovirus during its intracellular replication.

During the past ten years, these events have been nearly completely reconstructed as judged by research on the genetic capacity of poliovirus.

As in all areas of science, the advancement of virology is dependent on development of new concepts based on the predictability of old ones. In this sense, the work of Penman, Becker, and Darnell (1964) was instrumental in establishing the experimental approach that led to many significant discoveries. These investigators isolated membrane-bound structures called virus synthesizing bodies (VSB) from the cytoplasm of poliovirus-infected cells using
deoxycholate (DOC) to facilitate release from the membranes. Evidence was furnished that the VSB were the seats of virus assembly and synthetic activity.

Although a certain amount of controversy exists concerning some of the events leading up to poliovirus RNA synthesis, it is generally agreed that the parental virus RNA serves as its own messenger for protein synthesis. It was postulated that one early virus-mediated protein must, by necessity, be poliovirus RNA polymerase, and such an enzyme was recovered in poliovirus-infected HeLa cells (Baltimore, Eggers, Franklin, and Tamm, 1963). Tershak (1966) subsequently made a comparison of RNA polymerases isolated from cells infected with 5-fluorouracil (5-FU)-treated virus preparations and untreated virus preparations. Five-fluorouracil was chosen because it is erroneously incorporated into RNA molecules as an analog of uracil, thus leading to the synthesis of abnormal proteins (Altera and Moulton, 1966). The enzyme isolated from cells infected with 5-FU-treated virus was defective, indicating that the infecting viral RNA was necessary for synthesis of the enzyme. Virus RNA replication in HeLa cells is initiated during the first hour of infection and increases exponentially until after the third hour (Baltimore, Girard, and
Darnell, 1966). These events are marked by the formation of three types of poliovirus RNA: a double-stranded replicative form (RF) with a sedimentation coefficient of approximately 18 S in CsCl gradients (Bishop, Summers, and Levintow, 1965), a multi-stranded replicative intermediate (RI) which sediments heterogeneously between 15 S and 40 S, is precipitated by 2 M LiCl and is partially ribonuclease (RNase) resistant (Baltimore and Girard, 1966), and a single-stranded 35 S RNA which is identical to the RNA found in the mature virion and can be distinguished by the fact that it contains 29 moles percent of adenine (Summers and Levintow, 1965). These three classes of poliovirus RNA are intrinsically infectious (Koch and Bishop, 1968; Bishop and Koch, 1969). Viral RNA polymerase and a combination of double-stranded and single-stranded RNA have been found associated in a "poliovirus replication complex" which sediments after sodium deoxycholate (DOC)-treatment at an average sedimentation coefficient of 250 S (Girard, Baltimore, and Darnell, 1967). This is distinct from the viral synthesizing body (VSB) previously described by Penman et. al., (1964) which had a much higher sedimentation value. Because examination of the temporal appearance of the various poliovirus RNA molecules involved the use of radioisotope pulse-
chase studies, and because results of pulse-chase studies were clouded by large intracellular nucleotide pools (Baltimore and Girard, 1966), it was not until cell free (in vitro) systems were developed that the detailed mechanism of poliovirus RNA synthesis was revealed. Although reports were somewhat ambiguous, Baltimore (1968) showed evidence that the replicative intermediate consists of one complementary strand, one viral strand and an average of 6.5 growing RNA chains. Baltimore favored a semi-conservative mode of replication in which the parental plus strand is displaced from its association with the complementary strand by the continual synthesis of progeny plus strands, and the double-stranded replicative form is a by-product of the turnover of replicative intermediate. Bishop et al., (1969) reported the existence of two types of replicative intermediates, the major portion of which had a semi-conservative structure. Recently, Ehrenfeld, Maizel, and Summers (1970) successfully isolated an RNA-RNA polymerase complex from poliovirus-infected cells. This complex had a sedimentation coefficient of 70 S and was capable of synthesizing in vitro, RNase resistant material sedimenting at 20 S and RNase sensitive material sedimenting at 35 S. Although two different polymerase complexes, one capable of catalyzing the synthesis of the replicative form, the other capable of catalyzing synthesis of RNase-sensitive RNA, were found in extracts from
cells infected with foot-and-mouth disease virus (Arlinghaus and Polatnick, 1969), only one polymerase has thus far been reported in poliovirus-infected cells.

In addition to RNA synthesis as discussed above, the other aspect of poliovirus replication which attracted the attention of many investigators was the production of poliovirus-specific proteins. Using acrylamide gel electrophoresis, Summers, Maizel and Darnell (1965) revealed the presence of 12 to 14 virus-specific peptide chains in poliovirus-infected cells. By comparing these components to a polyacrylamide gel electrophoretic profile of capsid proteins obtained from whole pure virions, they found that four of these proteins were capsid proteins (VP-1, VP-2, VP-3, and VP-4) and the remaining ten were non-capsid polypeptides probably involved in the synthesis and assembly of the intact virion (Summers and Maizel, 1968). Some of these proteins arise by cleavage of unstable primary products of translation (Maizel and Summers, 1968). Jacobson and Baltimore (1968) suggested that all viral proteins may arise by cleavage of a single polypeptide that represents the genetic information of the entire viral RNA molecule. When Jacobson, Asso, and Baltimore (1970) used amino acid analogs, normal cleavage of the viral precursor proteins was prevented and an accumulation of a previ-
ously undetected protein with an equivalent molecular weight that exceeded 200,000 dalton units occurred. This furnished preliminary evidence that poliovirus RNA is translated as a polycistronic message. The synthesis of this protein follows the release of RNA from the replication complex and formation of poliovirus polyribosomes (Huang and Baltimore, 1970). Cesium chloride density gradient analysis of cytoplasmic extracts revealed polyribosomal precursors with densities of 1.4, 1.44, and 1.47 g/cm³. The 1.4 component was viral RNA probably complexed with protein, the 1.44 component was thought to be a single ribosome subunit bound to the 1.4 component, and the 1.47 complex represented a single ribosome attached to a molecule of viral ribonucleoprotein. Huang and Baltimore showed that radioactive uridine was incorporated first into the 1.4 component and then the 1.44 and 1.47 components. Since the message is polycistronic, it was postulated that only one site for ribosome attachment existed. Like the synthesis of poliovirus RNA, the synthesis of poliovirus proteins occurs in the cytoplasm of the cell, in association with large membraneous structures (Penman, Becker, and Darnell, 1964).

Both *in vivo* (Jacobson and Baltimore, 1968) and *in vitro* (Phillips, 1969) studies of poliovirus maturation and assembly indicated that a peptide cleavage, accompanied
by the appearance of capsid protein, VP-2, occurred at the time when RNA associated with the procapsid (73 S "top component") to form the virion. Furthermore, a prior transformation of 14 S particles into 73 S particles was detected by Phillips (1969). It was found that although protein synthesis was unnecessary for the formation of 73 S particles (empty capsids), disruption of lipid and protein structures by 0.5 percent DOC destroyed the component(s) in the cell extract responsible for virus assembly.

The ultimate goal of poliovirus research, whether by choice or by chance seems to be directed toward the duplication of cellular events in a cell-free system. The preceding in vitro and in vivo investigations concerning viral RNA synthesis, viral protein synthesis, and virus maturation and assembly have incriminated membranes as the common sites of poliovirus replication. Thus, recent evidence presented by Caliguiri and Tamm (1969) indicated that poliovirus RNA synthesis occurs in the smooth membrane fraction and poliovirus protein synthesis in the rough membrane fraction from infected cells. Subsequently, the sequence of membrane involvement was revealed through pulse-chase experiments (Caliguiri and Tamm, 1970a). After a 2.5 min pulse of $^3$H-uridine and $^{14}$C-labeled amino acids fraction 2 (smooth membranes), isolated after isopycnic
centrifugation in sucrose gradients, contained the bulk of viral RNA and viral RNA polymerase compared to the other 6 fractions. Fractions 5 and 6 contained most of the newly synthesized viral protein. These results were not compatible with Penman's description of a single structure, called a virus synthesizing body, that is capable of supporting all phases of virus replication. Further characterization of these membrane fractions revealed that the smooth membrane fraction sedimented at 130 S after DOC treatment and contained replicative intermediate RNA (RI), replicative form (RF), and ribosomal subunits. Fraction 3 sedimented at 230 S after DOC treatment and contained 74 S ribosomes, single-stranded RNA, and some RI and RF. Finally, Fraction 5, the rough microsomal fraction, sedimented at 320 S and contained viral polysomes, and single-stranded viral RNA (Caliguiri and Tamm, 1970b). Surprisingly, these membranes were also newly formed as indicated by increased $^3$H-choline incorporation into phospholipids between 3 and 5 hr after infection (Mosser, Caliguiri, and Tamm, 1971).

All of these findings are related and can be integrated with information concerning picornavirus-induced cytopathic effect (CPE). Dales and Amako (1967a) studied the effects of mengovirus plaque mutants on in vivo changes
in viral virulence and in vitro studies on changes in viral-induced cell toxicity. They concluded that a protein whose synthesis was viral induced, initiated cell degeneration. An extended study demonstrated that choline uptake was correlated with mengovirus-induced CPE (Amako and Dales, 1967b). In another independent study, Penman and Summers (1965) indicated that choline incorporation began approximately 2.5 to 3 hr post infection (PI) and required poliovirus RNA synthesis. Membrane involvement during poliovirus infection was substantiated by electron microscopic studies. Extensive areas of membrane bound bodies and cytoplasmic vesicles developed at 3 to 5 hr PI (Dales, et. al., 1965; Mattern and Daniel, 1965). Clusters of ribosomes were observed early in infection. These observations are compatible with the early light microscope and histochemical descriptions of CPE. The late manifestations of poliovirus-induced CPE were first characterized by Robbins, Enders, and Weller (1950) as a loss of typical staining properties and nuclear pyknosis and fragmentation. A cytoplasmic mass around a shrunken nucleus was described by Harding et. al., (1956) using time lapse photography. It seems reasonable to assume that the nuclear pyknosis, the cytoplasmic mass around the nucleus, the membrane infiltration, and the choline uptake are all manifestations of the same event as examined by more and more sensitive means.
Several mechanisms have been proposed as the cause of virus cytopathology - a shut-down in cellular macromolecular synthesis (Holland, 1963); production of a virus-coded protein (Bablanian et. al., 1965); or the release of hydrolytic enzymes from intracellular membrane-bound bodies called lysosomes (Allison and Sandelin, 1963). The first alternative was proposed by Holland (1963) who found that under the influence of guanidine HCl, which specifically inhibits poliovirus RNA synthesis (Eggers, 1962), all cellular RNA synthesis was depressed in addition to protein synthesis (Holland and Peterson, 1964). The early shutdown in host cell synthesis, however, has been the subject of several investigations. Penman and Summers (1965) found that the phenomenon is related to the number of initial infecting RNA molecules and is dependent on protein synthesis. Ghendon et. al. (1970) concluded that inhibition of cellular macromolecular synthesis by poliovirus was a property of the function of parental viral RNA molecules since no shutdown occurred when cells were challenged with UV-inactivated virus. The mechanism of host cell protein synthesis inhibition in poliovirus-infected cells was studied by Willems and Penman (1966). They attributed this inhibition to a product of the viral genome which was stable for 1 hr and was capable of disrupting host cell polyribosomes apparently by inactivating
m-RNA of the host cells. In another investigation (Mc Cormick and Penman, 1968), it was found that this RNA disrupting capability of poliovirus is highly specific for host cell RNA, since guanidine-treated cells that were infected by poliovirus and super infected by mengovirus, were capable of producing mengovirus. Although this shutdown in host cell synthesis implied inevitable cell death, when host cell RNA and protein inhibition was simulated with actinomycin D or puromycin, less drastic morphological changes occurred within the same period of time (Franklin and Baltimore, 1962). Thus, it seems unlikely that the shutdown of cellular synthesis contributes to the rapid cell destruction brought on by infection of cells by poliovirus.

Additional arguments concerning the mechanism of viral-induced CPE have been suggested by Bablanian et. al. (1965a), who reasoned that virus-induced metabolic depressions are not the primary factor causing cellular morphological changes since poliovirus-infected cells under the influence of guanidine do not show signs of CPE up to 16 hours beyond the appearance of CPE in untreated virus-infected cells. The key point of his argument was that even though guanidine is capable of inhibiting poliovirus, the agent does not prevent these virus-induced metabolic depressions. As an alternative, Bablanian and co-
workers (1965b) introduced evidence that poliovirus-induced CPE is caused by a virus-coded protein. These investigators approached the problem using combinations of the inhibitors guanidine HCl and puromycin, the latter a non-specific inhibitor of protein synthesis. When guanidine was added to cultures 3.5 hr post infection (PI), virus-induced morphological changes occurred even though virus reproduction was largely prevented. However, when puromycin was added at 3.5 hr PI, the major part of virus-induced CPE was prevented. It was concluded that virus capsid protein material, which is produced late in the virus replication cycle, was responsible for the cellular morphological changes. Some controversy has developed over the temporal appearance of this protein. Gauntt and Lockart (1968) used interferon to inhibit mengovirus replication in cells but reported that although reductions in synthesis of viral components occurred, interferon-treated cells underwent CPE at the same time as cells in infected untreated cultures. This they attributed to an early protein coded by the virus, but not a component of the mature virion. Blackman and Bubel (1969) established that events early in the infectious cycle of poliovirus-infected HEp-2 cells were responsible for cell damage and depended on viral protein synthesis. On the other hand, Amako and Dales (1967a) proposed that the cytotoxic principle responsible for initiating cell degeneration in
mengovirus-infected L cells is capsid protein (a late protein).

Although much of the evidence to date is circumstan­tial, several investigators have implicated lysosomal enzyme release as the factor responsible for viral-induced CPE (Allison and Sandelin, 1963). Actually, lysosomal involvement may occur at several levels of viral infection (Allison, 1967; Dales, 1969). Dales, Gomatos and Hsu (1965), for example, showed that reovirus was uncoated to nascent RNA within intracellular bodies later shown to be lysosomes (Silverstein and Dales, 1968). Owing to its double-stranded nature, however, it is believed that reovirus RNA has a selective advantage over other viruses in being able to withstand the action of host cell lytic enzymes. It was recently shown that poliovirus was uncoated by isolated membranes and, therefore, that lysosomes probably are not responsible for the uncoating of this virus (Chan and Black, 1970).

Allison and Sandelin (1963) furnished the first evidence that synthesis of viruses was accompanied by activation of lysosomal enzymes and that this activation preceded visible cell damage. This study involved the use of vaccinia and MHV-3 viruses.
Later, the biochemical studies of Wolff and Bubel (1964), Flanagan (1966) and Guskey, Smith, and Wolff (1970) on poliovirus-induced cell damage also showed that lysosomal enzymes were released in response to poliovirus and other virus infections resulting in rapid cytopathic effect but not during non-cytolytic virus infections. This release followed the onset of viral-induced CPE and occurred prior to maximum release of the virus (Guskey, Smith, and Wolff, 1970). Furthermore, lysosomal enzyme release and CPE were delayed or prevented when 2-(alpha-hydroxy-benzyl)-benzimidazole (HBB) or guanidine HCl were added up to 3 hr PI, but not beyond that time. The finding by Thacore and Wolff (1968) that a 3 hr PI extract from poliovirus-infected cells caused release of β-glucuronidase from isolated lysosomes, furnished added evidence for the existence of a lysosome-labelizing agent. Another approach was taken by Hotham-Iglewski and Ludwig (1966) and Macieira-Coelho et al. (1965) when they attempted to stabilize cellular lysosomes by the addition of cortisone. This led to reduced activation of lysosomal enzymes in response to mengovirus-infected L cells (Hotham-Iglewski and Ludwig, 1966) and poliovirus-infected WI-38 cells (Macieira-Coelho, et al., 1965). In addition, the latter study revealed that poliovirus-induced CPE was also inhibited in cortisone-treated cells.
Correlated histochemical-biochemical studies by La Placa et al., (1969) and Dusing and Wolff (1969) have confirmed these earlier contentions and re-emphasized that not all viruses are capable of releasing enzymes from lysosomes in infected cells.

While it now seems clear that protein synthesis and lysosomal enzyme release are involved in rapidly developing viral-induced cytopathologies, further evidence for these mechanisms is needed. The genetic basis for poliovirus-induced CPE and accompanying lysosomal enzyme release is poorly understood and two of the proposed mechanisms of poliovirus-induced CPE both share some credibility. It seems logical, therefore, to presume the presence of a viral-induced substance which is capable of altering the permeability of the lysosome membrane and forcing the cell into committing biochemical suicide.
MATERIALS AND METHODS

Cell cultures. HEp-2 cells (human epithelial carcinoma of the larynx) originally obtained from H. Stegmiller (Ohio State Department of Health Laboratory, Columbus, Ohio) were grown in either static (monolayer) or suspended cultures at 36 C. Cells were kept in the suspended state with a model G-25 gyratory incubator-shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) rotating at 125 rpm. Cell numbers were determined by counting in an eosinophile chamber. When cultures reached a population of 1.0 to 1.2 X 10^6 cells/ml after a 48 hour incubation period, the HEp-2 cells were pelleted in an International centrifuge, model CS, equipped with a swinging bucket rotor at 110 x g for 10 min. in 200 ml conical glass centrifuge bottles. The cell pellet was resuspended to a concn. of 0.5 X 10^6 cells/ml in 500 ml Erlenmeyer flasks containing 95 ml of Eagles Minimum Essential Medium (MEM) for Suspension Culture (Appendix A, Table 10) and 5 ml of 4 percent (50 cps) methocel (Dow Chemical Company, Midland, Michigan) in water. All cell culturing media and medium components were purchased
from Grand Island Biological Company, Grand Island, New
York, and filter sterilized using a .22 μ membrane filter
system. Monolayer cultures of HEp-2 cells were propagated
in glass culture bottles until a state of confluency was
reached. At this time, cells were removed from the glass
with the aid of a rubber policeman, resuspended in 5 ml
of MEM (Joklik-modified) for Suspension Culture (Appendix A,
Table 11) and distributed equally into two glass culture
bottles each containing 12.5 ml of MEM (Joklik-modified).
All the above medium was supplemented with 10 percent
inactivated sterile calf serum obtained from Flow Labora-
tories (Rockville, Maryland).

LM cells were provided by Dr. Merchant (W. A. Jones
Cell Science Center) and carried in the suspended state.
When cells reached a density of 1.5 to 2.0 x 10^6 ml, they
were pelleted by centrifugation and resuspended at a
density of 0.5 x 10^6 ml in 100 ml MEM (Joklik-modified) for
Suspension Culture containing 2 ml of 4 percent methocel
(W/V) and supplemented with 10 percent inactivated sterile
calf serum.

When experiments demanded large numbers of cells,
it was necessary to increase the suspension volumes up to
800 ml per 3-liter flask.
Viruses. Stocks of poliovirus, type 1, Mahoney strain, and mengovirus were provided by Dr. H. C. Bubel (Department of Microbiology, University of Cincinnati). Viruses were propagated in HEp-2 cell and LM cell monolayers respectively. To obtain high virus concentrations, approximately $1 \times 10^9$ cells were infected at a multiplicity of one plaque forming unit (PFU) per cell. After a 1 hr absorption period in a reduced volume at 36 C, infected cells were resuspended up to their original concentration ($1 \times 10^6$ ml). After an additional 2.5 hr incubation, cells were concentrated by centrifugation and finally resuspended in calf serum-free MEM for suspension cultures (MEM-S) at a 10 to 1 volume reduction. The infected cells were harvested at 14 hr post infection (PI), frozen and thawed three times, and clarified by centrifugation at $16,300 \times g$ for 10 min in the GSA rotor of the Sorvall RC 2B centrifuge. Virus suspensions prepared in this manner were stored at minus 20 C.

Virus titrations. Infectivity of virus samples was determined by the plaque formation technique (Delbecco, 1952). HEp-2 or LM cell confluent monolayers prepared in 60 x 15 mm Falcon plastic culture dishes and cultivated in an atmosphere of 5 percent CO$_2$ and 95 percent air, received 0.3 ml of a ten-fold serial poliovirus or mengovirus dilution. After a 1 hr adsorption period which was frequently interrupted by redistribution of the fluid over the
monolayers, each plate received 5 ml of an overlay medium consisting of equal volumes of 1.8 percent Ionagar #2 (Consolidated Laboratories, Chicago Heights, Illinois) and two-fold concentrated MEM supplemented with 0.05 percent yeast extract, 0.25 percent lactalbumin, 0.5 percent peptone (YELP), and 20 percent heat inactivated calf serum (Appendix B, Table 12). In the case of mengovirus titrations, the overlay also received 0.4 mg/ml protamine sulphate. The infected cell monolayers were incubated at 36 C in a humidified atmosphere containing 5 percent CO₂ and 95 percent air. After 36 hr, 2 ml of a 0.05 percent neutral red-saline solution were added to each plate and plaques were counted within 12 hr.

**Infectious center assays.** Infectious center assays were used on various occasions to determine the efficiency of infection under different conditions. Cells were infected and at one time or another after infection, were appropriately diluted and counted. A 0.5 ml aliquot was plated on each of no less than 5 preformed HEp-2 monolayers. To facilitate attachment, the plates were centrifuged at 700 rpm in an IEC centrifuge equipped with a swinging bucket motor. After a 10 min centrifugation plates received 5 ml of an MEM-YELP-Ionagar overlay. After approximately 36 hr incubation at 36 C in a CO₂ chamber, plates were overlayed with neutral red, developed, and counted. The
percent efficiency was determined by comparing the number of infectious centers to the number of cells plated.

**Cytopathic effect evaluation.** To evaluate the amount of cell damage induced by the virus, an equal volume of 0.05 percent erythrocin-B (Matheson Coleman and Bell, Cincinnati, Ohio) in phosphate buffered saline (PBS) was added to a volume of cells and the percent of uptake (dead cells) was determined with the aid of phase microscopy. To record the development of poliovirus-induced CPE, cell monolayers were established in Sykes-Moore chambers, infected, and the CPE recorded on Kodachrome II (35 mm film using a Zeiss photomicroscope with phase contrast objectives).

**Virus concentration and purification.** Subsequent to the ten-fold concentration by centrifuging infected cells as described above, the poliovirus suspension was treated with an equal volume of Freon 113 (Dupont de Nemours & Company, Inc., Wilmington, Delaware) to remove non-viral proteins and cellular debris (Porter, 1956), homogenized for 3 min in a micro-Waring blender, and the homogenate centrifugated at 4,080 X g in the Sorvall GSA rotor for 10 min (Guskey and Wolff, 1970). The supernatant fraction was collected and the pelleted material was
re-extracted with approximately 10 ml of 0.01 M tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.2). All supernatant fractions from the above extractions were pooled. Freon 113-treated virus suspensions were further concentrated by one of two methods – Diaflo ultra-filtration (Amicon Corp., Lexington, Mass.) or ultra-centrifugation onto a dense cushion of cesium chloride plated in the bottom of the centrifuge tube. This additional tenfold volume reduction was accomplished with an Amicon 200 ml UF cell diafiltration apparatus containing an XM-50 Diaflo membrane ultra-filter. The Freon 113-extracted virus preparation was forced against the membrane with 15 psi of nitrogen gas, and only material having a molecular weight below 50,000 passed through the membrane. The rest of the material including the virus and large contaminating molecules was concentrated to approximately 5 ml within 12 hr. This method provided a simultaneous dialysis of the concentrate. Centrifugation of a 32 ml suspension of poliovirus onto a 5 ml, 46 percent (W/W) CsCl cushion at 131,000 x g for 2 hr in the Beckman model L3-40 centrifuge, was used as a means of concentration. The band at the CsCl supernatant interface was collected and its density determined by weighing 100 µl aliquots.

Further concentration and purification of the Diaflo and cushioned preparations was accomplished through CsCl density gradient centrifugation. After the density of the
harvested virus band from the cushion was determined, the following formula was used to determine the weight in grams of additional CsCl needed to provide a final density of 1.34 gm/cc in the 2 inch x 1/2 inch cellulose nitrate tubes. 

\[ D_{\text{v}} (5\ X) = W_{1}; \ 2.38 - W_{1} = W_{2} \]

where \( D_{\text{v}} \) is density of virus on the cushion, \( 5 \) = volume in ml of sample to be centrifuged, \( X \) is the percent (W/W) of CsCl in the virus sample, \( W_{1} \) is weight in grams of 5 ml of the cushioned virus sample, 2.38 gm is the weight of CsCl in 5 ml sample with a density of 1.34 gm/cc, and \( W_{2} \) is the additional CsCl in grams needed to bring density of cushioned virus preparation to 1.34 gm/cc. Using the above formula, the required amount of additional CsCl was added to 4.6 ml of the cushioned preparation. This brought the total volume to 5 ml. Samples were then overlayed with heavy mineral oil to prevent sample loss by evaporation and centrifuged for 22 hr at 35,000 rpm in a Beckman SW 39 rotor. The same procedure was used for preparations from Diaflo ultrafiltration except that it was not necessary to calculate the base level of CsCl in the preparations. After the centrifugation run, photographs were taken of the gradients. The gradients were harvested by collecting fractions from a hole punctured into the bottom of the tubes. Fraction volumes were controlled through the use of a Buchler polystaltic pump, pumping mineral oil at a
constant rate to displace the gradient. Twenty-five to thirty fractions were collected from each gradient. Fractions containing the virus were dialyzed against two changes of 0.01 M Tris buffer (pH 7.2) over a period of 24 hr.

**Lowry protein determinations.** Protein determinations were done by the Lowry method (Lowry et al., 1951) using a bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) standard. Samples and standards were always run in duplicate. A standard curve is reconstructed in Figure 9 (Appendix B).

**Preparation of radioisotope-labeled virus.** At 2.5 hr PI, poliovirus-infected cells were harvested by centrifugation and resuspended at a 10:1 concentration in reconstituted MEM for suspension culture (Appendix A, Table 10) containing 1.25 μCi uridine -5-³H (specific activity = 21.7 Ci/mM Tracerlabs, Waltham, Massachusetts). At 14 hr PI, the infected cells were harvested by centrifugation, frozen and thawed three times and clarified by an additional centrifugation at 16,300 X g. Non-incorporated isotopes were removed by dialysis after collection of the virus on a 1.4 g/cm³ cushion.
Gradient analysis. Four criteria were used in the analysis of each fraction. A 100 µl sample was removed and weighed in a lambda pipette in order to determine the density of each fraction. Each fraction was then diluted 1:40 with 0.01 M Tris buffer and measured for ultraviolet absorption at 260 and 280 nm in a Gilford model 2400 spectrophotometer read against a distilled water blank. A 10 µl sample was removed and added to 10 ml of Brays solution prepared by adding 6 percent naphthalene, 0.4 percent PPO, 0.02 percent POPOP, 10 percent v/v methanol, 2 percent v/v ethylene glycol, and enough p-dioxane (1-4 dioxane) to bring volume up to 100 ml. These samples were analyzed for \(^3\)H-uridine incorporation with a Packard Tri-carb model 3375 liquid scintillation spectrometer. Fractions were also analyzed for the number of infectious viruses by the plaque formation technique.

Homogenization. Pelleted cells were resuspended to 2.5 ml with cold 125 M sucrose (4C in a Dounce "T" homogenizer). The homogenization tube was modified to fit a Sorvall SS-34 angle head rotor. The homogenate was examined from time to time by phase microscopy to determine the degree of disruption. Homogenization was considered complete when whole cells appeared in only 1 of every 10 1 mm
square fields. The homogenate was sedimented at 20,000 \( \times \) g in a Sorvall RC-2B centrifuge for 20 min. The supranatant fraction, containing the soluble cellular components, was collected and used for the assay of lysosomal enzymes. The sediment (lysosomal fraction), containing intact lysosomes and other cellular particulate matter, was resuspended to 3 ml with 0.2 percent v/v aqueous Triton X-100 and frozen and thawed three times before use.

**Isolation of lysosomes.** Lysosomes were isolated by first homogenizing 1 \( \times \) \( 10^8 \) HEp-2 cells in 3 ml of 0.125 M sucrose using the Dounce T homogenizer. The homogenate was centrifuged at 1085 \( \times \) g for 10 min in an SS-34 Sorvall rotor. After removing the supranatant fraction, the pellet was washed once with 0.125 M sucrose, recentrifuged, and the washing added to the supranatant fraction from the first centrifugation. The pellet, which contained nuclei and undisrupted whole cells, was discarded. Lysosomes were removed from the combined supranatant fractions by centrifugation at 20,200 \( \times \) g for 20 min in an SS-34 Sorvall rotor. This pellet was suspended up to 2 ml in 35 percent (W/V) sucrose, and two 0.7 ml aliquots were each layered over a stepwise 40 to 65 percent (W/V) sucrose gradients (5 percent steps in 1/2 inch x 2 inch tubes). Gradients were centrifuged at 35,000 rpm for 2.5 hr in a
Beckman SW 39L rotor. Bands were harvested either by removal with a Pasteur pipette or by forcing 77 percent (W/V) sucrose through the bottom of the 1/2 inch x 2 inch cellulose nitrate tube at the rate of 0.5 ml/40 sec. With the latter method, nine fractions were collected from the top of the gradient tube. After addition of an equal volume of 0.4 percent Triton X-100, each fraction or band was analyzed for the presence of β-glucuronidase and β-glucosaminidase.

Lysosomal enzyme release in infected cells. Lysosomal enzyme release was monitored in cells after virus infection. In a typical experiment, 9 X 10⁹ HEP-2 cells were infected by poliovirus at a multiplicity that averaged 25 PFU per cell. After a 1 hr absorption period, infected cells were washed two times with cell culture medium and equally distributed into nine, 500 ml Erlenmeyer flasks and each brought to a total volume of 100 ml with MEM-S containing 10 percent calf serum. After resuspension, aliquots were removed from each flask and counted in either an eosinophile counter or in an electronic cell counter (Cell-O-Scope, Model IIINS/TH Particle Data Inc., Elmhurst, Illinois). An uninfected control was prepared and treated in a similar manner. At indicated times, a 5 ml sample was removed for subsequent analysis of cell-associated and released virus. Simultaneously, a sample
was removed and stained with 0.5 percent erythrocyanin-B and examined by phase microscopy for cytopathic effect (CPE) evaluation and erythrocyanin-B uptake. The remaining cells in the flask were collected by centrifugation at 16,330 × g for 20 min. and homogenized as above. The soluble portion (supernatant fraction) and particulate portion (lysosomal fraction) were examined for lysosomal enzyme content and the percent of lysosomal enzyme released from the lysosomes into the cytoplasmic matrix was determined from the following formula (Wolff and Bubel, 1964):

$$\frac{X}{X + Y} \times 100 = \% \text{ enzyme released from lysosomes into cells}$$

where X is the optical density of supernatant fraction and Y is the optical density of lysosomal fraction.

**Enzyme assays.** Beta-glucuronidase was assayed by modification of the method described by Allison and Sandelin (1963). The reaction tubes contained 0.5 ml of 0.1 M sodium acetate buffer (pH 5.0), 0.1 ml lysosomal or supernatant fraction, and 0.1 ml of 0.01 M phenolphthalein glucuronidate (Sigma Chemical Company, St. Louis, Missouri). After 20 min. at 37 C, interrupted by frequent agitation with a Vortex mixer, the reaction was stopped by the addition of 2.0 ml of 0.4 M glycine buffer adjusted to pH 10.7 with 2 N sodium hydroxide. Suitable zero-time
controls were prepared by adding the glycine buffer before the addition of phenolphthalein glucuronidate in the reaction mixture. Sample and control tubes were centrifuged at 1,980 X g for 10 min. at 40°C in the Sorvall centrifuge and optical density of the supernatant fluid was measured at 545 nm in a Gilford model 2400 spectrophotometer against zero-time controls.

Beta-galactosidase was determined according to the method of Smith (1966) which was a modification of the Sellinger procedure (1959). The reaction tubes contained 0.5 ml of 0.1 M sodium acetate buffer (pH 5.0), 0.3 ml of the enzyme sample, and 0.2 ml substrate 188.5 mg p-nitrophenyl - β-D-galactopyranoside, (Sigma Chemical Company, St. Louis, Missouri) dissolved in 50 ml of 0.1 M sodium acetate buffer (pH 5.0). Reactions were stopped after one hr incubation at 37°C with 1.5 ml of 5 percent trichloroacetic acid. Suitable controls were prepared by precipitating enzyme and buffer mixtures after the incubation period with trichloroacetic acid, then adding substrate. All tubes were refrigerated to facilitate precipitation and centrifuged at 1,980 X g for 10 min. The color was developed by adding 1 ml of 0.4 M glycine buffer adjusted to pH 10.7 with sodium hydroxide, to 1.5 ml of the supernate. Optical density of the sample was read at 410 nm
in a Gilford model 2400 spectrophotometer against the appropriate control.

Because the β-glucosaminidase assay has such a high degree of sensitivity, all samples were first diluted 1:10 with 0.1 sodium acetate buffer (pH 5.0). A 0.2 ml sample from each dilution was delivered into each of two 13 x 100 mm sample tubes. After equilibration at 37 C, one tube received 0.8 ml of 7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosamine (substrate) from Pierce Chemical Company, Rockford, Illinois, dissolved in 0.1 M sodium acetate buffer (pH 5.0). After a 30 min. incubation at 37 C the reaction was terminated by the addition of 1.5 ml of 5 percent trichloroacetic acid to all tubes. All control tubes received 0.8 ml of the substrate and then were incubated with the reaction tubes for 1/2 hr to facilitate precipitation. Color was developed by adding 1.7 ml 0.4 M glycine-buffer (pH 10.7) to all tubes. After centrifugation at 1,980 X g for 10 min. in an SM-24 Sorvall rotor, the supernatant fluid was read at 420 nm in the spectrophotometer against the appropriate control.

**Antiserum production.** Poliovirus antiserum was prepared according to the method of Mandel (1965) by intravenous injection of poliovirus, prepared as described below, into white rabbits every seven days over a three-week
period. Other rabbits were also injected with an uninfected HEp-2 cell preparation to provide control sera. The virus used for injection was prepared by the centrifugation of a clarified virus suspension onto 2 ml of 0.6 percent gelatin cushion. After 2 hr. at 40,000 rpm in a type 40 Beckman centrifuge rotor, the poliovirus layered on the cushion was harvested and diluted 1:9 with serum-free MEM spinner medium. Seven days after the last injection with these preparations, the rabbits were sacrificed by exsanguination. After collection, the blood was allowed to clot for 4 days at 4 C in conical 140 ml centrifuge bottles pretreated with Siliclad (Clay Adams, Parsippany, New Jersey). The antiserum was sterilized through a Swinney type Gelman filter unit (Gelman Corp., Ann Arbor, Michigan) fitted with a type S-1 Seitz filter.

**Inhibitor studies.** Actinomycin-D (AMD) was purchased from Calbiochem (Los Angeles, California). A stock solution was prepared by dissolving 7.2 mg in 6 ml of serum-free MEM spinner medium. The AMD solution was sterilized by filtration through a Swinney type Gelman membrane filter (0.45 μm pore size), and appropriate dilutions were prepared. Suspended cells were exposed to these various concentrations and examined at various times after exposure for cytotoxic effects.
The toxicity of guanidine HCl was also examined in this manner. Concentrations of 200 µg/ml or less were not toxic to HEp-2 cells over a 24 hr. period. On the basis of these results, 200 µg/ml guanidine was used in subsequent experiments to inhibit poliovirus.

Cell Cloning experiments. The plating efficiency of cells treated with various concentrations of actinomycin-D was determined by cell cloning experiments. After a 3.75 hr. exposure to the agent cells were washed twice in Joklik medium by centrifugation and resuspended up to the original volumes. Cells were counted and approximately diluted so that 0.5 ml contained approximately 200 cells. All clumps were counted as one cell. Each of five 60 mm Falcon dishes received 0.5 ml of the cell suspension and was diluted up to 5 ml with Joklik medium. After 8 days of incubation, the medium was removed and replaced with 2 ml of 0.05 percent crystal violet in a formalin-PBS solution. Colonies were stained for 10 min., washed once in 2 ml distilled water, and drained dry. Clones were counted with the aid of a dissecting microscope.

Determination of radioactivity. Incorporation of $^3$H-uridine into host cell or poliovirus RNA was accomplished in one of two ways - (1) constant exposure to label or (2) pulse labeling experiments. For general labeling
experiments, $^3\text{H}$-uridine (Tracerlabs, Waltham, Massachusetts, specific activity = 21.7 Ci/mM) was added to cell suspensions at a concentration of 0.75 to 2 $\mu$Ci/ml, and a cell concentration of 0.3 to $1 \times 10^7$ /ml. When labeling poliovirus-infected cells for subsequent concentration, the culture was continuously labeled over a 14 hr period. In general, pulse labeling experiments were used only when it was necessary to demonstrate a shutdown in host cell synthesis, since great accumulations of labeled material would otherwise mask results. Pulse labeling was accomplished by adding $1.2 \times 10^7$ HEp-2 cells in a 2 ml volume of serum free MEM spinner medium lacking phenol red, to a 25 ml Erlenmeyer flask containing 10 $\mu$Ci of $^3\text{H}$-uridine in 2 ml MEM-S medium. The final concentrations of cells and label were $3.0 \times 10^6$ and 2.5 $\mu$Ci/ml respectively. After a 35 min. labeling period, duplicate 1 ml samples were removed and added to 12 X 100 mm tubes containing 10 $\mu$g of cold (unlabeled) uridine in 1 ml of water. The contents of the tube were rapidly frozen in an alcohol-dry ice bath and stored for further processing. For analysis of radioactivity, incorporated samples were thawed and each received 1 ml of 15 percent trichloroacetic acid. The precipitate was centrifuged at 1,980 X g for 10 min. and after removing the acid soluble fraction, washed two times with 2 ml 5 percent trichloroacetic acid.
After the last centrifugation, the recoverable precipitate was resuspended in either 0.5 ml 10 N NH₃OH and digested for 2 hr at 36 C, or 0.5 ml Soluene (Packard Inst. Co., Downers Grove, Illinois) and digested for 1 hr at 50 C. In the case of digestion by Soluene, the ratio of wet weight of whole cell or acid precipitable material to amount of Soluene, was critical. It was found that the acid precipitable material from 6 X 10⁷ cells or less was soluble in 0.5 ml Soluene at 50 C for 1/2 hr. All samples were transferred to glass counting vials containing either 10 ml Brays solution or 10 ml of a toluene base cocktail (0.7 percent PPO, 0.025 percent M₆PO POP) and counted in a Packard model 3375 Tricarb liquid scintillation spectrometer. Counting efficiency was determined by spiking several previously counted samples with a known amount of ³H-toluene standard. The automatic external standard (AES) ratio was then determined for each spiked sample, and counting efficiency was calculated by the following formula:

\[
\% \text{ Efficiency} = \frac{A_m}{A_o X_t} \times 100
\]

where \( A_m \) is the measured activity of the sample in counts per minute (CPM), \( A_o \) is the initial activity of the standard, and \( X_t \) is the fraction of standard activity at time \( t \). The maximum efficiency of counting in Brays solution was 27 percent (Appendix B, Figure 10) while the toluene cock-
tail provided a maximum efficiency of 39.9 percent (Appendix B, Figure 11).

Samples from labeling experiments were treated in the same way, however, because of the long exposure period, it was possible to reduce the label concentration to 1.25 $\mu$Ci $^3$H-uridine/ml.

**Acridine orange stain.** The acridine orange stain was used to demonstrate lysosomes in poliovirus-infected cells (Allison and Young, 1964). A 100 $\mu l$ sample of infected cells was added to an equal volume of 0.0005 percent acridine orange (Matheson Coleman & Bell, Cincinnati, Ohio) dissolved in MEM-S medium without phenol red. After 20 min., a drop of the acridine orange stained cells was added to a glass slide, overlayed with a glass coverslip, and examined by fluorescent microscopy using a dark field condenser. Excitation was accomplished with a Reichert UV-illuminator using Reichert filter #5970. The power supply was set at 3.4 A. C. amps. Photographs were taken with the aid of a Nikon AMF photomicroscopy system using manually controlled exposure time of 1/2, 1, 2 or 4 minutes. Kodak Ektachrome high speed film was used and developed at the recommended ASA.
Inactivation of poliovirus. To determine the kinetics of poliovirus inactivation by heat, a dialyzed poliovirus concentrate obtained from the ultrafilter or CsCl cushion described above, was diluted in 0.01 M tris buffer (pH 7.2) to yield $1.06 \times 10^{10}$ PFU/ml. The suspension was exposed to a temperature of 50°C for 2.5 hr and virus samples were removed at various times during the exposure period and immediately diluted 1:100 or 1:10 in serum-free MEM-S. Infectivity was determined by the plaque method. The rate of ultraviolet inactivation (Dulbecco and Vogt, 1955) was similarly determined.

A CsCl gradient concentrated preparation of poliovirus was dialyzed against 0.01 M tris buffer. Two milliliters from this suspension containing $1.06 \times 10^{10}$ PFU/ml were added to a 60 mm glass petri dish bottom, containing a teflon stirring bar. A UVS-12 ultraviolet light source (Ultra-Violet Products, Inc.) with a wavelength of 254 nm was elevated 32.8 cm from the virus suspension. The suspension was irradiated over a 20 min period during constant non-violent agitation. Samples were removed, sometimes in duplicate, from the irradiated suspension at various times during the exposure period and titrated by the plaque method as described previously.

Penetration of inactivated poliovirus. The uptake of inactivated poliovirus by HEp-2 cells was demonstrated
by first dividing a HEp-2 cell suspension into two equal parts, each part containing $1.2 \times 10^7$ cells. One aliquot was infected with heat inactivated $^{3}$H labeled poliovirus and the other aliquot by UV inactivated $^{3}$H-labeled poliovirus. After a one hour adsorption period, the infected cells were washed two times with MEM-S medium to remove unattached virus, and one-third of the infected cells were removed to determine the amount of tritium activity associated with the cell. To remove virus that was attached to the cell but did not penetrate, one-third of the remaining infected cells in each series was treated with 20 ml 1 percent chymotrypsin (Mann Research Labs., New York, New York) for 1/2 hr. at 37 C according to the method of Zajac and Crowell (1965). The remaining one-third was added to 100 ml 0.05 M glycine buffer (Holland, 1962) (pH 2.5) and incubated at ambient temperature. After 2 min the solution was neutralized by the addition of approximately 0.5 ml of 2.5 N NaOH. The glycine and chymotrypsin-treated cells were collected by centrifugation. The amount of tritium in the supernatant fraction (artificially released virus) was determined by adding an aliquot to 10 ml of Brays solution. Tritium activity incorporated by the cells was determined by dissolving the sedimented cells in 1/2 ml Soluene and counting in a toluene base cocktail.
RESULTS

Poliovirus isolation and concentration. Poliovirus was concentrated and partially purified according to the flow diagram shown in Figure 1. Each method used was evaluated in terms of convenience, efficiency of virus recovery, and degree of purification. Concentration was accomplished by a three step process: (1) a ten-fold reduction in cell culture fluid volume of poliovirus-infected HEp-2 cells before virus release; (2) further volume reduction by either Diaflo ultrafiltration or ultracentrifugation onto a CsCl cushion; (3) final concentration by isopycnic centrifugation in CsCl gradients. Plate I is a photograph of tubes obtained after an isopycnic centrifugation of poliovirus. The virus in the tube on the right was derived from a preparation previously centrifuged onto a CsCl cushion, and the tube on the left contains virus from a Diaflo ultrafiltration preparation. Two bands were resolved, a faint band near the top of the gradient tube (top component) and a heavier band at the center of the tube. Twenty-five to thirty fractions were collected from the bottom of the tube by displacement with heavy mineral oil pumped onto the top of the gradient. Four parameters
**FIGURE 1. POLIOVIRUS CONCENTRATION PROCEDURE**

**INFECTED CELLS**
- **CELL CONC. TO 1.2 X 10^7/ml by 600 X g at 3.5 HR PI**
- **FREEZE-THAW (3X) 10,000 X g 20 HR PI 10 MIN**
- **SUPERNATANT 1.97 X 10^9 PFU/ml**
- **FREON 4,080 X g**
- **AQUEOUS PHASE 7.58 X 10^8 PFU/ml**
- **DIAFLO ULTRAFILTRATION XM-50 FILTER**
  - **CONCENTRATE 6.67 X 10^9 PFU/ml**
  - **ULTRAFILTRATE 1.45 X 10^6 PFU/ml**
- **ULTRA CENTRIFUGATION 131,000 X g 90 min.**
  - **CsCl CUSHION SUPERNATANT 3.03 X 10^10 PFU/ml**
  - **SUPERNATANT 5.6 X 10^6 PFU/ml**
PLATE I

EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION OF DIAFLO ULTRAFILTRATION AND ULTRACENTRIFUGATION CONCENTRATE

Bands from cesium chloride equilibrium density gradient tubes formed from a poliovirus preparation after Diaflo ultrafiltration (left) or ultracentrifugation onto a cesium chloride cushion (right).
were usually used to determine the position of virus in the gradient tubes: (1) density, (2) UV absorption at 280 and 260 nm, (3) plaque forming units (PFU), and (4) radioisotope monitoring of $^3$H-labeled virus when applicable. Peak activity in each gradient (Plate IIa and b), as measured by the above parameters, was found in fractions 14 and 15 and corresponded with the position of the center band in Plate I. The densities of the material from the cushioned virus peak (Plate IIa) and the Diaflo virus peak (Plate IIb), were 1.34 g/cm$^3$ and 1.345 g/cm$^3$ respectively. The density of poliovirus in CsCl was previously reported as 1.34 g/cm$^3$ (Jamison and Mayer, 1966; Baltimore and Huang, 1968).

As an index of purification, the amount of protein at various steps in the concentration procedure was measured by the Lowry method as described in the previous section (Table 1). The PFU relative to protein concentration was two-fold higher after Diaflo concentration than after the cushion method. Concentration by Diaflo ultrafiltration, however, results in a high degree of protein contamination as indicated by UV absorption at 280 nm (Plate IIb). A summary of the advantages and disadvantages of each procedure is presented in Table 2. Diaflo ultrafiltration was best suited for the purpose of
FIG. a. ULTRACENTRIFUGATION CONCENTRATE

FIG. b. ULTRAFILTRATION CONCENTRATE

PLATE II

DISTRIBUTION OF POLIOVIRUS IN CESIUM CHLORIDE DENSITY GRADIENTS AS MEASURED BY PLAQUE FORMING UNITS, ULTRAVIOLET ABSORPTION DISTRIBUTION OF $^{3}$H-URIDINE AND DENSITY
TABLE 1

PURIFICATION AND CONCENTRATION OF POLIOVIRUS

<table>
<thead>
<tr>
<th>Concentration or purification step</th>
<th>Final volumetric concentration factor</th>
<th>PFU/mg protein</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell conc.</td>
<td>10.00:1</td>
<td>2.13 X 10^8</td>
<td>1.000</td>
</tr>
<tr>
<td>Genetron treat.</td>
<td>10.00:1</td>
<td>1.08 X 10^8</td>
<td>0.507</td>
</tr>
<tr>
<td>Diaflo conc.</td>
<td>62.50:1</td>
<td>1.62 X 10^10</td>
<td>76.000</td>
</tr>
<tr>
<td>Cushion conc.</td>
<td>31.25:1</td>
<td>1.40 X 10^10</td>
<td>65.700</td>
</tr>
<tr>
<td>Diaflo + d.g.c. b Prep.</td>
<td>1855.00:1</td>
<td>1.38 X 10^11</td>
<td>650.000</td>
</tr>
<tr>
<td>Cushion + d.g.c. Prep.</td>
<td>937.50:1</td>
<td>7.40 X 10^10</td>
<td>347.000</td>
</tr>
</tbody>
</table>

a The purification factor is based on increase in PFU per mg. protein over crude culture lysate.

b d. g. c. (density gradient centrifugation).
## TABLE 2

ADVANTAGES AND DISADVANTAGES OF TWO METHODS USED FOR CONCENTRATION OF POLIOVIRUS

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Diaflo Technique</th>
<th>Cushion Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time involved</td>
<td>12 hrs</td>
<td>26 hrs</td>
</tr>
<tr>
<td>Efficiency of recovery</td>
<td>320%</td>
<td>138%</td>
</tr>
<tr>
<td>Contamination of concentrate by macro molecules</td>
<td>large</td>
<td>small</td>
</tr>
<tr>
<td>Volume reduction</td>
<td>200 ml to 10 ml&lt;sup&gt;c&lt;/sup&gt;</td>
<td>115 ml to 35 ml</td>
</tr>
<tr>
<td>Interfering materials</td>
<td>serum, methocel</td>
<td>none</td>
</tr>
<tr>
<td>Osmotic inertness</td>
<td>isotonic</td>
<td>hypertonic due to CsCl</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data composite of three experiments.

<sup>b</sup> Includes time for dialysis.

<sup>c</sup> This reduction varied with the volume of unconcentrated preparations.
this study since it afforded the most efficient recovery while isotonicity was maintained.

Temporal appearance of lysosomal enzyme release.

Microscopy.

Collection of much data has established correlation between virus-induced CPE, lysosomal enzyme release, and viral synthesis. A phase-contrast microscope study of the progression of poliovirus-induced CPE in monolayers of HEp-2 cells infected at a multiplicity of 1,000 PFU per cell, is presented in Plate III. Uninfected cells contained a dark nucleolus within a spherical nucleus (Plate IIIe). Dark granules were evenly dispersed throughout the cytoplasm, and the cytoplasmic processes had well-defined borders. At 2.75 hr post infection (PI) the cell processes were beginning to retract judged by the darkened areas at the periphery of the cell (Plate IIIa). In addition, cytoplasmic granules had reoriented around the nucleus and several cells had prominent vacuolization. After an additional 1 hr 40 min incubation period, severe retraction occurred and nuclei appeared oval. Cytoplasmic granularity was much more obvious, and some cells in the same field underwent a more progressed infection as reflected by the detached rounded cells (Plate IIIb). In the next frame (Plate IIIc), the same field is shown after an additional 1.25 hr of infection (i.e., 5 hr 38 min PI).
PLATE III

POLIOVIRUS-INDUCED CYTOPATHIC EFFECT IN HEp-2 CELL MONOLAYERS

FIGURE a. 2 hr 45 min post infection (X 640)
Figure b. 4 hr 25 min post infection (X 640)
Figure c. 5 hr 40 min post infection (X 640)
Figure d. 9 hr post infection (X 640)
Figure e. uninfected control (X 640)
Cytoplasmic retraction was almost complete. Surrounding cells showed pyknosis of the nuclei. Finally, at 9 hr PI, infection was complete and characterized by displaced nuclei, complete rounding, and large cytoplasmic blebs (Plate IIIId). The cytoplasmic granularity which developed in response to poliovirus infection was examined more closely by using poliovirus-infected HEp-2 cell preparations stained with acridine orange (AO). Acridine orange used at very diluted concentrations will not kill cells and is considered a vital stain. Cells were infected at a multiplicity of less than 1 PFU and neutralizing amounts of specific anti-poliovirus rabbit serum were added after virus adsorption to assure a one-step replication cycle. At various times after infection, samples were removed and stained for 20 min with a 0.005 percent concentration of AO. Representative phase (right) and fluorescent (left) micrographs of the progression of the infection are shown in Plate IV. The lysosomes appear as bright orange bodies in the cytoplasm of the cells while the nuclei fluoresce dark green (Allison, 1969). As infection progressed, the stained bodies were larger and appeared to aggregate, particularly around the nucleus (Plate IVc). Lysosomes in the uninfected cells for the most part remained unclumped and diffuse throughout the cytoplasm (Plate IV e & f).
PLATE IV

DISTRIBUTION OF LYSOSONES IN POLIOVIRUS INFECTED
ACRIDINE ORANGE STAINED HEp-2 CELLS

FIGURE a. Fluorescent microscopy of acridine
orange stained cells at 3.5 hr PI.  
(X 355)

FIGURE b. Phase contrast microscopy of same
field as above.  (X 355)

FIGURE c. Fluorescent microscopy of acridine
orange stained cells at 11 hr PI.  
(X 355)

FIGURE d. Phase contrast microscopy of same
field as above.  (X 355)

FIGURE e. Fluorescent microscopy of acridine
orange stained uninfected cells.  
(X 355)

FIGURE f. Phase contrast microscopy of same
field as above.  (X 355)
PLATE IV

DISTRIBUTION OF LYSOSONES IN
POLIOVIRUS INFECTED ACRIDINE
ORANGE STAINED HEp-2 CELLS
**Multiplicity of infection.** Microscopy alone does not offer proof that the relationship between lysosomal enzyme release and CPE is one of cause and not of effect. One approach to solving this problem is to demonstrate that lysosomal enzyme release precedes virus release, but that virus infection is necessary for the release of lysosomal enzymes. It is first essential to assure that all cells are infected simultaneously so the events can be detected with precision. To determine the poliovirus multiplicity necessary for a maximum synchronous infection, cells were infected at multiplicities of 10, 25, 50, and 100 PFU per cell (Plate Vb), then the amount of lysosomal enzyme release determined by assaying for β-glucuronidase (Plate Va). At 25 PFU per cell, the efficiency of infection approaches 100 percent. The only observable difference in virus synthesis was that as the multiplicity increased (Plate Vb), it was increasingly difficult to decrease unattached virus to the same residual levels with the same number of washes. The slight differences in the rate of lysosomal enzyme release at different multiplicities was within experimental limitations as indicated by the values obtained from duplicate determinations of enzyme release in uninfected cells. To assure synchronous infection in all subsequent experiments, multiplicities of 25 PFU per cell or greater were used.
FIG. a. ENZYME RELEASE  
FIG. b. POLIOVIRUS REPLICATION

PLATE V

EFFECT OF VARIOUS MULTIPLICITIES OF POLIOVIRUS ON LYSOSOMAL ENZYME RELEASE IN HEp-2 CELLS
To add credence to the results obtained using \( \beta - \text{glucuronidase} \) and \( \beta - \text{galactosidase} \) as marker enzymes, a third lysosomal enzyme, \( \beta - \text{glucosaminidase} \) (Figure 2) was used in subsequent experiments. It was first necessary to show that \( \beta - \text{glucosaminidase} \) was found only in association with particulate lysosomes. Figure 2 shows that during isolation of lysosomes, the activity of \( \beta - \text{glucosaminidase} \) coincides with the activity of \( \beta - \text{glucuronidase} \), the marker enzyme used routinely. The isolation of lysosomes from a cell homogenate was accomplished on step-wise preformed sucrose density gradients (40 to 65% W/V). The top two bands contained 87.2% of the total \( \beta - \text{glucosaminidase} \) activity and about 64% of the total \( \beta - \text{glucuronidase} \) activity.

**Synchrony of infection.** In order to detect the earliest release of lysosomal enzymes in poliovirus-infected cells, three modifications of previous experimental protocol were used: (1) samples were taken at shorter time intervals; (2) cells were infected at a high multiplicity (215 PFU/cell) and at a temperature of 4°C, which arrests steps of infection beyond attachment (Mandel, 1967); (3) anti-poliovirus rabbit serum was added after the 30 min absorption period at 37°C to eliminate masking of virus production by unabsorbed virus. The activity of this anti-
FIGURE 2. LYSOSOMAL ENZYME ACTIVITY IN FRACTIONS ISOLATED FROM SUCROSE GRADIENTS.
serum was previously measured by the plaque neutralization test (Figure 3). The antiserum was prepared as outlined in the previous section and contained enough activity per ml to neutralize $2.5 \times 10^6$ PFU. To determine if all cells were infected synchronously, infectious centers were determined by counting infected cells then plating between 60 and 100 cells on each 60 mm monolayer culture, overlaying, and counting resultant plaques. Results in Table 3 indicate that at 15 min after addition, 90 percent of the cells were infected, but the efficiency of infection declined to 71.3 percent at 45 min after addition. Both β-glucuronidase and β-glucosaminidase release were first detected after 3.5 hr of incubation (Plate VIb). Between 3.5 and 4 hr after removal of unabsorbed virus and anti-serum, intracellular virus accumulated to 7.7 percent of maximum production (Plate VIA) while only 0.009 percent of maximum release occurred. This evidence indicated that release occurs after a substantial accumulation of intracellular virus. Little differences in virus release patterns were detected whether absorption was followed by treatment with antiserum or not. In an attempt to detect and quantitate the development of poliovirus-induced CPE, erythrocin B was added to cells and they were observed for uptake of the stain. The uptake, indicating membrane damage, was first detected at 6 hr after absorption and
FIGURE 3. NEUTRALIZATION OF POLIOVIRUS BY RABBIT ANTISERUM AS MEASURED BY THE PLAQUE REDUCTION METHOD.
### TABLE 3

THE EFFICIENCY OF ABSORPTION OF POLIOVIRUS INFECTION IN HEp-2 CELLS

<table>
<thead>
<tr>
<th>Time after addition of antiserum</th>
<th>Cell count</th>
<th>Infectious center count</th>
<th>Maximum Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>$8.9 \times 10^6$</td>
<td>$8.07 \times 10^6 \pm 0.66$</td>
<td>90.6%</td>
</tr>
<tr>
<td>45 min</td>
<td>$7.1 \times 10^6$</td>
<td>$5.06 \times 10^6 \pm 0.367$</td>
<td>71.3%</td>
</tr>
</tbody>
</table>

\(^a\) Anti-polio rabbit serum was added after a 30 min absorption period at 37 C.

\(^b\) Based on percent infectious centers formed from number of cells plated.
PLATE VI

THE ONSET OF LYOSOMAL ENZYME RELEASE
AND CPE AFTER SYNCHRONIZED
INFECTION BY POLIOVIRUS

FIGURE a. POLIOVIRUS PRODUCTION
IN THE ABSENCE AND PRESENCE OF
ANTISERUM

FIGURE b. CPE AND ENZYME RELEASE
FIG. a. POLIOVIRUS PRODUCTION IN THE ABSENCE AND PRESENCE OF ANTISERUM

FIG. b. CPE AND ENZYME RELEASE

a CPE determined by percent cells stained by erythrocin B, or showing morphological changes.

PLATE VI
and wash. Thus, morphological changes, lysosomal enzyme release, (Plate VIb) and maximum intracellular production (Plate VIa) occurred before cells became permeable to "vital staining" by erythrocin-B.

**Inhibition of host cell RNA synthesis.**

**Actinomycin-D studies.**

An attempt was made to find whether lysosomal enzyme release is under the genetic control of the virus or the cell. To help demonstrate this actinomycin-D (AMD) was used to inhibit DNA-dependent RNA synthesis (transcription) in HEp-2 cells. This agent has no direct inhibitory effect on poliovirus synthesis, as will be demonstrated. AMD was toxic to HEp-2 cells at concentrations of 0.25 \( \mu g/ml \) or higher over a period of 24 hr (Table 4), and cell division was severely depressed at concentrations above 0.01 \( \mu g/ml \). The ability of HEp-2 cells to support poliovirus replication after 24 hr in the presence of various concentrations of AMD was examined. The data in Table 4 indicate that even at 0.25 \( \mu g/ml \) AMD has an apparent inhibitory effect on the replication of poliovirus to the extent of 40 percent. Since this finding is not consistent with the mode of action of AMD, two further experiments are presented to clarify these results:
<table>
<thead>
<tr>
<th>AMD conc µg/ml</th>
<th>Cell Count X 10^6 a</th>
<th>Uninfected b Cell viability</th>
<th>PFU/viable cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>23 hr</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.06</td>
<td>1.75</td>
<td>93.0%</td>
</tr>
<tr>
<td>0.01</td>
<td>1.13</td>
<td>1.47</td>
<td>97.3%</td>
</tr>
<tr>
<td>0.025</td>
<td>1.22</td>
<td>1.48</td>
<td>97.2%</td>
</tr>
<tr>
<td>0.05</td>
<td>1.04</td>
<td>1.07</td>
<td>97.0%</td>
</tr>
<tr>
<td>0.10</td>
<td>1.09</td>
<td>1.06</td>
<td>96.0%</td>
</tr>
<tr>
<td>0.20</td>
<td>1.03</td>
<td>1.14</td>
<td>94.0%</td>
</tr>
<tr>
<td>0.25</td>
<td>1.22</td>
<td>0.68</td>
<td>67.0%</td>
</tr>
<tr>
<td>1.00</td>
<td>1.26</td>
<td>1.27</td>
<td>46.0%</td>
</tr>
<tr>
<td>5.00</td>
<td>1.18</td>
<td>1.29</td>
<td>26.0%</td>
</tr>
</tbody>
</table>

a Composite of three experiments.

b Determined by population of cells excluding erythrocitin B.

c AMD concentrations removed from cells after 24 hours and monolayers immediately infected with 1.75 X 10^8 PFU poliovirus.
(1) an infectious center experiment which measures the ability of AMD treated host cells to support poliovirus replication (Table 5) and a cell cloning experiment which demonstrates the effects of AMD on the cells (Table 6). Results from these experiments show that only 67 percent of the poliovirus infected, AMD-treated cells were capable of forming infectious centers (Table 5) at a concentration of 3 μg/ml, and virtually all of the cells at this or higher concentrations were ultimately killed after a 3.75 hr exposure period (Table 6). The reduction in virus production was, therefore, caused by direct toxic effects on the cells rather than biochemical inhibition of viral synthesis. The specificity of AMD on host cell RNA synthesis as measured by $^3$H-uridine-incorporation is demonstrated in Figure 4. Within one hr after addition of 5 μg/ml of AMD, $^3$H-uridine incorporation was reduced to less than 1 percent that of the untreated cells. To reach this same level of inhibition with 1 μg/ml AMD, a 2 hr 15 min exposure time was required.

In order to obtain rapid cessation of cell RNA synthesis and still maintain a high degree of cell viability, it was clear that further experimental work was necessary. A previous preliminary experiment indicated that prior to 7 hr exposure time, concentrations as high as 5 μg/ml AMD had no visual effects on the cells and from the above
### TABLE 5

**INFECTION CENTER EXPERIMENT**

<table>
<thead>
<tr>
<th>ACTINOMYCIN D/CONCENTRATION μg/ml</th>
<th>0</th>
<th>0.25</th>
<th>1.0</th>
<th>3.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO. INF CENTERS (^a)</td>
<td>360</td>
<td>300</td>
<td>367</td>
<td>241</td>
<td>260</td>
</tr>
<tr>
<td>NO. CELLS PLATED</td>
<td>360</td>
<td>360</td>
<td>360</td>
<td>360</td>
<td>360</td>
</tr>
<tr>
<td>PERCENT CELLS FORMING INFECTIOUS CENTERS (^b)</td>
<td>100%</td>
<td>83.2%</td>
<td>102%</td>
<td>67%</td>
<td>72.2%</td>
</tr>
</tbody>
</table>

\(^a\) \(3 \times 10^3\) per ml in undiluted sample.

\(^b\) All values adjusted to 100 percent efficiency in untreated control.
## TABLE 6

**PLATING EFFICIENCY EXPERIMENT**

<table>
<thead>
<tr>
<th>ACTINOMYCIN D CONCENTRATION μg/ml.</th>
<th>0</th>
<th>0.25</th>
<th>1.0</th>
<th>3.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RATIO NO. CLONES / NO. CELLS PLATED</td>
<td>120 / 120</td>
<td>70 / 120</td>
<td>4.2 / 120</td>
<td>.33 / 120</td>
<td>.35 / 120</td>
</tr>
<tr>
<td>PERCENT CELLS FORMING CLONES</td>
<td>100%</td>
<td>58.3%</td>
<td>3.5%</td>
<td>0.3%</td>
<td>0.3%</td>
</tr>
</tbody>
</table>

*a All values adjusted to 100% efficiency in untreated control.*
FIGURE 4. INCORPORATION OF $^{3}$H-URIDINE INTO HEP-2 CELLS TREATED WITH VARIOUS CONCENTRATIONS OF AMD.
results (Figure 4) only 1 hr exposure time was necessary to inhibit RNA synthesis by over 99 percent. The results presented in Figure 5 demonstrated that when AMD was removed from the cells after a 2 hr exposure period (arrow), inhibition of RNA synthesis continued at the 5 μg level. At the 2.5 μg/ml and 1 μg/ml levels, a slight reversal then gradual resumption of inhibition occurred. Six hr after removal of AMD, only 2 percent of the treated cells demonstrated damage (erythrocin-B uptake) as a result of AMD toxicity, however, when AMD was not removed from the cells, only 50 percent were viable. Thus, these levels of AMD used with appropriate time of exposure produced less cell damage and resulted in the reduction of not over 33 percent of the virus yield in untreated cultures (Table 5). The next experiments were designed to determine if cellular RNA synthesis is necessary for virus-induced CPE and lysosomal enzyme release. The first experiment involved the use of AMD under conditions which inhibit host cell RNA synthesis without any consequence to poliovirus development. Cells were pre-treated with 3 μg/ml AMD for 45 min, then infected with poliovirus (Plate VIIa) at a multiplicity of 54.7 PFU/cell. Patterns of release for β-glucuronidase and β-galactosidase from lysosomes are virtually identical (Plate VIIb). An increase in
FIGURE 5. INHIBITION OF $^{3}$H-URIDINE INCORPORATION INTO HEP-2 CELLS AFTER ADDITION AND REMOVAL OF AMD.

a Arrow indicates time of AMD removal.
FIG. a. POLIOVIRUS REPLICATION  
FIG. b. ENZYME RELEASE

FIG. c. POLIOVIRUS INDUCED CPE

PLATE VII

THE EFFECT OF AMD ON POLIOVIRUS REPLICATION CPE AND LYPOSOMAL ENZYME RELEASE IN HEP-2 CELLS
erythrocin-B uptake was first detected in AMD-treated cells at 6 hr PI (Plate VIIc). A corresponding increase did not occur in untreated infected cells until approximately 3 hr later. Morphological changes, however, were evident to about the same extent at 6 hr PI. Visual evaluation of CPE was not made before 6 hr PI, but the temporal profile (Plate VIIc) indicated that it began earlier. Thus, it seems clear that AMD may alter membrane permeability, but it does not change lysosomal enzyme release patterns.

Cell RNA inhibition by poliovirus.

Another method was developed to inhibit cellular RNA synthesis in an effort to substantiate results achieved with AMD. In this experiment of the series, poliovirus was used as an agent to inhibit cellular RNA synthesis in the presence of guanidine HCl (200 µg/ml). Under these conditions, the production of infectious virus was suppressed as well as development of CPE and lysosomal enzyme release. The experimental plan then called for the poliovirus-infected guanidine-inhibited cells to be challenged with mengovirus in an effort to elicit lysosomal enzyme release, since mengovirus infection is not inhibited by guanidine HCl. Before this experiment could be initiated, it was first necessary to establish the following criteria: (a) that complete poliovirus synthesis
does not occur in the presence of guanidine; (b) that mengovirus can replicate in HEp-2 cells with the release of lysosomal enzymes; (c) that guanidine has no effect on mengovirus replication; (d) that poliovirus can inhibit host cell RNA synthesis; (e) and that guanidine does not interfere with this function.

Suppression of host cell RNA synthesis by poliovirus under the influence of guanidine. -- Guanidine HCl has no effect on mengovirus replication while it does depress the formation of whole infectious poliovirus (Table 7) to 99.97 percent of untreated infected cells.

The incorporation of \(^3\)H-uridine into the RNA of HEp-2 cells was measured after poliovirus infection by pulse labeling in the presence of guanidine HCl. These results are presented in Table 8. Although incorporation is not completely inhibited, it is obvious that guanidine-treated poliovirus-infected cells can depress host cell RNA synthesis to 50 percent that of uninfected cells.

Since AMD specifically inhibited host cell RNA synthesis (Figure 4), it was possible to demonstrate the synthesis of viral specific RNA alone by measuring the accumulation of \(^3\)H-uridine incorporation (Figure 6) in the presence of the AMD. Viral RNA synthesis begins after
TABLE 7
EFFECT OF GUANIDINE HCl\textsuperscript{a} ON POLIOVIRUS
AND MENGOVIRUS REPLICATION

<table>
<thead>
<tr>
<th>Sample</th>
<th>PFU/ml</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mengovirus infected untreated LM cells</td>
<td>$1.93 \times 10^8$</td>
<td>0%</td>
</tr>
<tr>
<td>Treated mengovirus infected LM cells</td>
<td>$1.95 \times 10^8$</td>
<td>0%</td>
</tr>
<tr>
<td>Untreated poliovirus infected HEp-2 cells</td>
<td>$4.8 \times 10^7$</td>
<td>0%</td>
</tr>
<tr>
<td>Treated poliovirus infected HEp-2 cells</td>
<td>$1.33 \times 10^5$</td>
<td>99.97%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Guanidine concentration was 200 \( \mu \text{g/ml} \).
<table>
<thead>
<tr>
<th>Sample Description</th>
<th>% incorporation of $^3$H-uridine at time post infection $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 hr</td>
</tr>
<tr>
<td>Guanidine only</td>
<td>100%</td>
</tr>
<tr>
<td>Guanidine + poliovirus</td>
<td>76.2%</td>
</tr>
</tbody>
</table>

$^a$ Measured as portion of CPM uptake in treated infected cells as compared to control uninfected cells.
FIGURE 6. POLIOVIRUS PRODUCTION IN HEp-2 CELLS MEASURED BY $^3$H-URIDINE INCORPORATION AND PLAQUE FORMING UNITS.
1.5 hr PI and reaches a maximum peak at 7 hr PI, approxi-
mately 2 hr prior to maximum intracellular virus
production. On the other hand, when guanidine was added
at the time of infection, a peak in RNA synthesis occurs
at 3.5 hr PI and all further incorporation was inhibited.
Virus synthesis was not detected in the presence of
guanidine. The initial rise in RNA synthesis at 1.5 hr
PI was unexpected, but probably due to the lack of total
RNA synthesis cessation by AMD.

**Mengovirus infection of HEp-2 cells.**—After it was
established that guanidine inhibits poliovirus RNA synthesis
and that poliovirus is capable of inhibiting cellular RNA
synthesis (Tables 7 and 8), it was next necessary to test
whether mengovirus replicates in HEp-2 cells, and if there
is a release of lysosomal enzymes with the development of
CPE. As a control, LM-cells were infected with mengovirus
and samples were examined for virus production, CPE, and
lysosomal enzyme release (Plate VIII). In addition, HEp-2
cells were infected with either mengovirus or poliovirus
(Plate IV). The pertinent observations are as follows:
Once initiated, mengovirus replication occurred at a faster
rate in LM-cells (Plate VIIIb) than in HEp-2 cells (Plate
IXa). Virus release was virtually complete by 8 hr PI in
LM-cells, but in HEp-2 cells, virus release did not reach
completion within 12 hours after infection. Although
PLATE VIII

CYTOPATHIC EFFECT AND LYSOSOMAL ENZYME RELEASE IN MENGOVIRUS INFECTED LM CELLS

FIGURE a. CYTOPATHIC EFFECT INDUCED BY MENGOVIRUS IN LM CELLS.

FIGURE b. LYSOSOMAL ENZYME RELEASE AND MENGOVIRUS REPLICATION IN LM CELLS.
FIG. a. MENGOCVIRUS INDUCED CPE
Ratio of cells showing signs of CPE to number of cells counted.

FIG. b. ENZYME RELEASE AND VIRTUS PRODUCTION

PLATE VIII
FIG. a. VIRUS REPLICATION FIG. b. CYTOPATHIC EFFECT

CPE evaluated by ratio of stained cells to total cells.

FIG. c. β-GALACTOSIDASE FIG. d. β-GLUCURONIDASE RELEASE

PLATE IX

THE EFFECTS OF POLIOVIRUS OR MENGOVIRUS REPLICATION ON LYSOSOMAL ENZYME RELEASE AND CPE IN HEp-2 CELLS
lysosomal enzyme release began in both cell systems at approximately the same time, the release in LM cells reached 60 percent, but in HEp-2 cells β-glucuronidase and β-galactosidase reached only 26 percent and 44 percent respectively by 12 hr (Plate IX c and d). Poliovirus development preceded mengovirus development by 1.5 hr as indicated by peak virus release titer (Plate IXa). Likewise, lysosomal enzyme release occurred three hours prior to enzyme release induced by mengovirus. The above findings also correlated with the observations that CPE in mengovirus-infected HEp-2 cells developed 2.5 hr later than in poliovirus-infected cells (Plate IXb) and 1 hr later than in mengovirus-infected LM cells (Plate VIIib). These experiments, therefore, not only demonstrated that mengovirus replicates and produces CPE and lysosomal enzyme release in HEp-2 cells, but also strengthened the cause-effect relationship since the delay in CPE is accompanied by a delay in enzyme release.

The above findings can be used to explain differences in plaque sizes produced by these two viruses in HEp-2 cells. Plaques produced by poliovirus in HEp-2 cells average 2.1 mm in diameter, but plaques produced by mengovirus attained an average size of only about 0.35 mm (Plate X, a and b), even when permitted to develop 24 hrs longer. However, the mengovirus plaques that developed in LM cells
PLATE X

PLAQUE PRODUCTION BY MENGOVIRUS AND POLIOVIRUS IN HEp-2 CELLS

FIGURE a. POLIOVIRUS PLAQUES IN HEp-2 CELLS (left) AND IN LM CELLS (right).

FIGURE b. POLIOVIRUS AND MENGOVIRUS PLAQUES IN HEp-2 CELLS (left) AND AN UNINFECTED HEp-2 CELL MONOLAYER (right).

FIGURE c. MENGOVIRUS PLAQUES IN LM CELLS (left) AND UNINFECTED LM CELL MONOLAYER (right).
PLATE X

PLAQUE PRODUCTION BY MENGOVIRUS AND POLIOVIRUS IN HEp-2 CELLS
reached a diameter of 2.5 mm in 36 hours, and it was shown that lysosomal enzyme release in mengovirus-infected LM cells was higher. Thus, cell damage as measured by plaque size, is again correlated with extent of lysosomal enzyme release.

To recapitulate the findings of the above experiments, it was established that mengovirus is not sensitive to guanidine HCl, but can replicate in HEp-2 cells with accompanying lysosomal enzyme release. It can, therefore, be used as an agent for inducing lysosomal enzyme release in the presence of poliovirus, inhibited by guanidine HCl. It was also demonstrated that poliovirus RNA synthesis is sensitive to guanidine HCl at 200 µg/ml, that guanidine-inhibited polio-virus can inhibit incorporation of $^3$H-uridine into HEp-2 cell RNA, and that mengovirus plaques are much smaller in HEp-2 cells than are poliovirus plaques.

**Mengovirus superinfection of poliovirus-infected HEp-2 cells.**—In the following experiment, poliovirus-infected HEp-2 cells were treated with an inhibitory level of guanidine at 1.5 hr PI, then super-infected with mengovirus at 3 hr PI. These manipulations permitted the establishment of the poliovirus suppression of host cell macromolecular synthesis while simultaneously preventing the replication of the virus. Under these conditions, the replication of mengovirus was measured by two parameters:
(1) plaque formation (Plate XI a) and (2) $^3$H-uridine incorporation into newly synthesized RNA (Plate XII). It was possible to discriminate between poliovirus and mengovirus plaques on the basis of size in HEp-2 cells (Plate X). In addition, when duplicate samples were plated on both cell lines, numbers of mengovirus plaques on LM cells were similar to numbers on HEp-2 cells and confirmed the poliovirus plaque identification. At a multiplicity of 200 PFU/cell, poliovirus was not produced under the influence of 200 μg/ml guanidine (Plate XIa). That guanidine permitted some of the poliovirus functions to be manifested, was apparent from the suppression of host cell RNA synthesis (Plate XIB). At a time when less than 1 PFU of mengovirus per cell was detected (latent period), $^3$H-uridine incorporation in mengovirus superinfected cells was only 27 percent that of poliovirus-infected cells and 19 percent that of uninfected cells (Plate XII). Mengovirus RNA production began within 1 hr after superinfection during the decline of poliovirus RNA synthesis. Mengovirus RNA replication occurred over a 3.5 hr period, preceded whole virus production by approximately 1 hr, and reached a peak 4 hr after mengovirus infection. The most pertinent information gained from this experiment was that even though host cell RNA synthesis was inhibited, mengovirus induced the release of lysosomal
FIG. a. TOTAL VIRUS REPPLICATION

FIG. b. CYTOPATHIC EFFECT

Refer to Plate VIII, Figure a.

FIG. c. β-GLUCURONIDASE RELEASE

FIG. d. β-GLUCOSAMINIDASE RELEASE

PLATE XI

THE EFFECTS OF MENGOVIRUS SUPERINFECTION ON HEp-2 CELLS PREVIOUSLY INFECTED BY POLIOVIRUS
PLATE XII

RNA SYNTHESIS IN $^3$H-URIDINE PULSE-LABELED POLIOVIRUS INFECTED HEp-2 CELLS SUPERINFECTED BY MENGOVIRUS

FIGURE a. (top) TOTAL VIRUS PRODUCTION

FIGURE b. (bottom) RNA SYNTHESIS
enzymes (Plate XI c and d) and CPE development (Plate XI b) indicating that this phenomenon is directed by the virus and not the host cell.

Events directed by inactivated poliovirus. The above experiments suggested that some component of the virion or a product under the direction of the viral genome must be responsible for initiating the release of lysosomal enzymes. To determine what role, if any, the protein coat or the RNA of the virus plays in the initiation of lysosomal enzyme release, the following experiment was performed.

Poliovirus labeled with $^3$H-uridine was isolated by density gradient centrifugation as described above. Cesium chloride and unincorporated $^3$H-uridine were removed by dialysis at 4 C for 24 hr against 0.01 M Tris buffer (pH 7.2). Half of this preparation was exposed to heat at 50 C for 3 hr 30 min. Samples were removed throughout this time period to determine survival (Figure 7). After a 10 min exposure period, the number of survivors was less than 0.02 percent. The other half was treated with a UV light source (Mineralite model # UVS-12, 32.8 cm from the sample in an open petri dish). After 20 min at this height, the viability of the virus suspension was reduced from $1 \times 10^{10}$ to $1.3 \times 10^5$ PFU/ml (Figure 8). It was next necessary to show that heat inactivated and UV inactivated
FIGURE 7. HEAT INACTIVATION OF POLIOVIRUS AT 50° C
FIGURE 8. UV INACTIVATION OF POLIOVIRUS
virus could enter the cell. Table 9 shows the results of an experiment in which cells were exposed to heat and UV inactivated $^3$H-labeled virus. After a 1 hr absorption period, cells were treated with either 0.05M glycine (pH 2.5) or 1 percent chymotrypsin. These treatments were done in an effort to remove labeled virus which had not penetrated. The above methods are known to elute absorbed poliovirus (Zajac and Crowell, 1965; Holland, 1962). Even after elution with chymotrypsin and glycine, 18 and 29.1 percent of the labeled, UV inactivated virus entered the cell (Table 9). Forty-three percent and 16.9 percent of the heat inactivated virus entered the cell after chymotrypsin or glycine elution.

Another purified virus preparation was divided into 3 equal parts. One part was treated with UV light for 20 min, another with heat at 50 C for 10 min, and the last third remained untreated. These preparations were added to cells in concentration of 50 PFU/cell before inactivation and virus production, lysosomal enzyme release, and CPE were monitored. Since a small fraction of the virus suspension was not inactivated (Plate XIIa), however, a small amount of virus production occurred. Beta-glucuronidase and beta-glucosaminidase (Plate XIII b and c) release by the untreated virus preparation was detected after 3.5 hr PI. These enzymes were not released in response to
TABLE 9

PENETRATION OF HEAT AND UV INACTIVATED POLIOVIRUS INTO HEp-2 CELLS

<table>
<thead>
<tr>
<th>Activity associated with cell:</th>
<th>UV inact. virus</th>
<th>Heat inact. virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>chymot. treatment</td>
<td>glyc. treatment</td>
</tr>
<tr>
<td>CPM(^a) prior to treatment</td>
<td>1491</td>
<td>1491</td>
</tr>
<tr>
<td>CPM after treatment</td>
<td>273</td>
<td>434</td>
</tr>
<tr>
<td>% virus penetrated</td>
<td>18.3%</td>
<td>29.1%</td>
</tr>
</tbody>
</table>

\(^a\) CPM (counts per minute).
PLATE XIII

LYSOSOMAL ENZYME RELEASE IN RESPONSE TO INFECTION
BY UV AND HEAT-INACTIVATED POLIOMVIRUS

FIGURE a. REPLICATION OF UV AND HEAT
INACTIVATED POLIOVIRUS IN HEp-2 CELLS.

Uninactivated virus
Heat-inactivated virus
UV-inactivated virus
Cell associated virus
Released virus

FIGURE b. GLUCURONIDASE RELEASE IN
HEp-2 CELLS INFECTED WITH UV AND HEAT
INACTIVATED POLIOVIRUS.

Uninactivated virus
Heat-inactivated virus
UV-inactivated virus
Uninfected control

FIGURE c. GLUCOSAMINIDASE RELEASE IN
HEp-2 CELLS INFECTED WITH UV AND HEAT-
INACTIVATED POLIOVIRUS.

Uninactivated virus
Heat-inactivated virus
UV-inactivated virus
Uninfected control

FIGURE d. CYTOPATHIC EFFECT IN HEp-2
CELLS INFECTED BY UV AND HEAT-INACTI-
VATED POLIOVIRUS.

Uninactivated virus
Heat-inactivated virus
UV-inactivated virus
CPE by morphological changes
CPE by erythrocin B uptake
FIG. a. POLIOVIRUS REPLICATION FIG. b. $\beta$-GLUCURONIDASE RELEASE

FIG. c. $\beta$-GLUCOSAMINIDASE FIG. d. CYTOPATHIC EFFECT RELEASE

Refer to Plate VIII, Figure a.

PLATE XIII
infection by heat and UV inactivated virus, a finding which correlates with the inability to detect CPE induced by the inactivated virions (Plate XIII d).
DISCUSSION

Two reasonable hypotheses have been proposed to explain the mechanism of the poliovirus-induced cytopathic effect (CPE), and both are based on the supposition that the initiating factor is either an intimate part of the virion or is synthesized under the control of the viral genome.

Holland (1964) proposed that the virion carries the cytotoxic factor when he found that massive doses of poliovirus brings about CPE in the presence of RNA and protein synthetic inhibitors. Results presented here are in disagreement with Holland's work. Poliovirus inactivated by heat or ultraviolet (UV) irradiation is capable of infecting HEp-2 cells, as indicated by the cellular incorporation of $^3$H-uridine-labeled virus (Table 9). Whereas CPE, lysosomal enzyme release, and viral replication occurred in control cultures infected by a comparable amount of uninactivated poliovirus, none of these events occurred to a significant extent in cells infected by inactivated viruses over the same period of time (Plate XIII). Thus, the results indicate that intact
inactivated virions are not capable of inducing CPE when used at multiplicities of up to 50 PFU/cell.

The other hypothesis maintains that a product of viral replication, either viral RNA, or early or late viral proteins, is responsible for poliovirus-induced CPE. One early function of the viral genome is the rapid cessation of the host cell protein and RNA synthesis. It was originally proposed by Baltimore et al. (1963), that CPE is the result of early inhibitions of host cell macromolecular synthesis. Results presented from two experiments, one using actinomycin-D (AMD) (Plate VII), the other guanidine HCl (Plate XI) lead to doubt about Baltimore's proposal. Since AMD inhibits DNA-dependent RNA synthesis, its use simulated the early inhibitory effect of poliovirus on host cell RNA synthesis. Under these conditions, AMD was not capable of inducing the same morphological changes as poliovirus within the same period of time. In addition, under the influence of guanidine HCl, poliovirus, although incapable of replication, can inhibit host cell macromolecular synthesis. Cells show little signs of CPE after 6 hr in this state, but in a comparable time period, 83 percent of the untreated poliovirus infected cells showed signs of CPE. The inhibition of host cell macromolecular synthesis then is not solely responsible for poliovirus-induced CPE.
In studies involving the use of guanidine HCl and puromycin, Bablanian (1965b) found that poliovirus CPE could be prevented when guanidine was added before 3.5 hr PI. Puromycin, however, an inhibitor of protein synthesis, was able to prevent poliovirus CPE even when added at 3.5 hr PI. It was postulated from these results that virus coat protein caused the virus-induced morphological changes. Recent evidence, using a turn-off - turn-on approach with guanidine HCl and Streptovitacin, confirmed that CPE is associated with the onset of newly synthesized viral proteins (Bablanian and Shepler, 1971).

These previous studies have offered no experimental evidence that CPE is not the result of a host cell genetic expression function. The experiments presented here offer two lines of evidence that poliovirus-induced CPE is the result of information supplied by the viral genome and not the host cell genome. The first indication came through experiments using AMD which selectively shuts down host cell DNA-dependent-RNA synthesis. This approach, however, is valid only if AMD has no effect on viral RNA synthesis. Contrary to the report of Grado, Fischer, and Contreras (1965), results presented here indicate that AMD has no direct effect on poliovirus replication in an infected cell,
but that 30 percent of the cells appear to be refractile to poliovirus infection at concentrations of 3 \( \mu g/ml \) or over. Indeed, an exposure period of only 3.75 hr to 3 or 5 \( \mu g/ml \) AMD almost completely prevented subsequent cell growth, as shown by cloning experiments in Table 6. The previous investigations have ignored the direct effects of AMD on host cells and suggest that the agent exerts its effects on the replication of poliovirus even though the level of inhibition is only about 29 to 33 percent of infected untreated controls. Although findings presented here are not compatible with the above results, it must be conceded that direct comparisons of these results with those of others may not be possible since different strains of type 1 poliovirus were used. Since AMD had no direct effect on poliovirus in HEp-2 cells, the use of the agent indicated that poliovirus does not initiate a function of the host cell genome which can induce CPE and lysosomal enzyme release. In confirmation of these results, it was also shown in the present study that cellular RNA synthesis is inhibited by poliovirus under the influence of guanidine when poliovirus replication is non-permissive. When HEp-2 cells which had been treated with guanidine and then infected with poliovirus (non-permissive system) were challenged with a second virus, mengovirus, viral-induced CPE, lysosomal enzyme release, and mengovirus replication proceeded as in
the uninhibited system. Thus, again it was demonstrated that host cell RNA synthesis is not required for the expression of viral-induced CPE.

To answer the question concerning which phase of poliovirus replication initiates lysosomal enzyme release and accompanying CPE, inoculum encapsidated viral RNA was rendered non-functional by UV-inactivation, and the inactivated virions were added to a culture of HEp-2 cells. In this state, poliovirus was not capable of inducing lysosomal enzyme release or cytopathic affect at a rate comparable to cells infected with an equivalent number of infectious virus particles, (Plate XII b, c, and d). The same results were obtained using virus with capsid material made defective by heat inactivation. These experiments led to the conclusion that lysosomal enzyme release and accompanying CPE are not induced by the surface capsid material of the virus inoculum and that the inducing agent must be an expression of the functional viral genome. In previous investigations, it was shown that poliovirus-induced CPE and lysosomal enzyme release were inhibited when guanidine HCl or 2-(α-hydroxybenzyl) benzimidazole (HBB) independently were added at 2 hr or 3 hr PI respectively (Guskey, Smith, and Wolff, 1970). When added later in the infection, the agents were no longer effective. These
results indicate that the viral mediated function responsible for lysosomal enzyme release and CPE is produced prior to 3 hr PI, a time when the viral RNA is being synthesized and also directing the production of new virus coded proteins.

Poliovirus CPE may be conveniently divided into a two-step process; early CPE and late CPE. Morphologically early CPE is marked by cell swelling, increased nucleolar density and enlargement, marginal nuclear granularity, and nuclear distortion. Late CPE is characterized by damage to the peripheral part of the cell as indicated by cytoplasmic protrusions (blebbing), which result in a loss of cytoplasm and a decrease in cell volume. Under maximal conditions of infection (i.e., high multiplicities of infection and synchronized adsorption) experiments presented in this dissertation show that enzyme release can first be detected at 3.5 hr PI, a time which is subsequent to the early manifestations of CPE but prior to late manifestations. This redistribution of lysosomal enzymes from the particulate portion to the cytoplasmic matrix of the cell is also correlated with an increase in cell associated virus, and as indicated by other investigations, with the increased incorporation of $^3$H-choline (Penman and Summers, 1965; Mosser, Caliguiri, and Tamm, 1971) and the formation of newly developed cisternae in the centrosphere region of the
cell (Dales et al., 1965; Mattern and Daniel, 1965). It seems more than coincidental that lysosomes also accumulate in this region as revealed by infected HEp-2 cells vitally stained with acridine orange (Plate IV) and stained by specific cytochemical techniques which reveal the presence of lysosomal enzymes (Dusing and Wolff, 1969).

The late manifestations of CPE are correlated with virus release, lysosomal enzyme release, and increased uptake of erythrocin-B (Plate X b; Plate XIII d), a substance which proved to be unreliable for detecting cell death. This became evident when the uptake of erythrocin B was compared to cells comparably treated with AMD when evaluated for their ability to clone. The measurement of CPE by erythrocin B uptake in the presence of 5 µg/ml AMD (Table 4) indicated that 26 percent of the cells were viable (excluded the stain) but, when similarly treated cells were cloned for 8 days on 60 mm Falcon flasks (Table 6), only 0.3 percent of the cells plated, developed into colonies. Blackman and Bubel (1969) have also found that protein leakage and poliovirus release from infected HEp-2 cells were concomitant events occurring subsequent to lysosomal enzyme release.
The conclusions to be drawn from mengovirus plaque formation in HEp-2 cells are somewhat in accord with the above findings since plaque size and rate of formation can be correlated with virus synthesis and release rates. It was found that whereas poliovirus produced relatively large plaques in HEp-2 cells in a given time, mengovirus did not, even though the latter was capable of producing large plaques in LM cells. This phenomenon seems related to the rate of mengovirus release which was slower in HEp-2 cells (Plate IX a) than in LM cells (Plate VIII b). By 12 hr PI, only 18 percent of accumulated intracellular mengovirus was released from HEp-2 cells into the extracellular fluid. By contrast, in LM cells over 100 percent of the amount of intracellular mengovirus detected accumulated in the extracellular fluid by 8 hr PI. Lysosomal enzyme release was also delayed in HEp-2 cells, being detected after 4 hr PI in mengovirus infected LM cells but only after 6 hr PI in mengovirus-infected HEp-2 cells. By comparison, poliovirus release from HEp-2 cells was 100 percent efficient by 12 hr PI, and lysosomal enzyme release and CPE were detected after 3.5 hr PI. The release of mengovirus from HEp-2 cells, then appeared to be very inefficient. These findings implied that the release of lysosomal enzymes into the intracellular matrix facilities viral release from the host cells.
Recently, it was shown that the uptake of $^3$H-choline in poliovirus-infected cells can be chased from rough membranes where the viral proteins are made into smooth membranes (Mosser, Caliguiri and Tamm, 1971). Enzymatic activity is probably necessary to mobilize this membrane morphogenesis. Since lysosomes contain various hydrolytic enzymes such as phospholipase-C which is capable of liberating free phosphoryl choline from lecithin (Barrett, 1969) and in the light of recent findings concerning poliovirus replication and the data presented here, it seems reasonable to propose the following model of poliovirus-induced CPE: At approximately 3.5 hr PI lysosomal enzymes are released into the extralysosomal portion of the cell through the act of a labilizing agent induced directly by the virus or produced in response to viral infection. This initiating event may be due to a protein which directs the synthesis of the smooth membranes necessary for viral replication or may be due to the packing of the membranes against the lysosomes. The released lysosomal enzymes are then capable of mobilizing the rough membranes into smooth membranes or of acting on previously-formed rough membranes resulting in release of the completed virions. Concurrently or secondarily to these events, the released enzymes also act on the plasma membrane resulting in expulsion of the virus from the cell.
It is possible to develop a case for the existence of a poliovirus-induced protein capable of directly or indirectly labilizing lysosomes. Poliovirus RNA has an equivalent molecular weight of $2 \times 10^6$ dalton units and can direct the synthesis of about 7 proteins, 4 to 6 of which are structural proteins (Cooper, Summers, and Maizel, 1970). One of the non-structural proteins is the RNA-dependent RNA polymerase which functions in producing viral progeny single-stranded RNA. Another enzyme may be needed to produce the complementary strand of the replicative intermediate (Baltimore, 1969). A third protein is needed for the shut-down of host cell RNA synthesis (Willems and Penman, 1966). It is doubtful that a structural protein is responsible for lysosomal enzyme release since intact, inactivated poliovirus does not induce CPE or lysosomal enzyme release. Therefore, a cleavage product of one of the above proteins or a non-structural protein serving a dual function is probably responsible.

The basic structure of the proposed model needs no modification to be applicable to other picornavirus systems. Indeed, members of the picornavirus group show such consistent similarities that results derived from following studies are directly applicable toward substantiation of the poliovirus model. Thus, Amako and Dales (1967a) have noted similar cytopathic events in mengovirus-
infected L cells and have proposed that the agent which initiates the cytopathic degeneration is a protein produced about 4 hr PI - a time during which the synthesis of coat protein is rapid. In their studies, however, cytopathic effect was measured solely by the amount of erythrocinc uptake, a criterion which, as shown above, does not offer a reliable means for determining those cells that will ultimately die. The induction of CPE by echovirus 12 and coxsackie virus B4 was studied by Bablanian, Eggers, and Tamm (1966). Using the inhibitor 2- (α hydroxybenzyl) benzimidazole (HBB) to inhibit virus synthesis, they concluded that these cytopathologies are due to the expression of certain late viral functions (proteins) which accumulate in the presence of HBB. A similar finding was also noted in the poliovirus system. Electron microscopic studies by Bienz et. al. (1970) of coxsackie virus A1 in striated muscle of new born mice revealed that lysosomes play a part in the synthesis of new membranes which are involved in virus release, thus substantiating another aspect of the poliovirus model.

On the other hand, certain other types of viral pathologies do not exactly conform to the poliovirus model. Cytotoxic factors (proteins) directly responsible for CPE have been demonstrated in other groups of viruses. When large doses of adenovirus were added to susceptible cells, early cell clumping and detachment resulted (Pereira and
Kelly, 1957). The toxic factor was later identified as the penton antigen (Pereira, 1961). Early rounding of cells produced by large doses of vaccinia virus was reported by Mc Clain (1965). This property was susceptible to puromycin and, therefore, is dependent on protein synthesis (Bablanian, 1968). One of the most dramatic cytotoxic factors is produced in African green monkey kidney cells after infection by SV 40 (Mayor et. al., 1966). This CPE can be reversibly inhibited by fluorophenylalanine, an amino acid analog. It is evident from this discussion that although the proposed model based on studies of poliovirus infected HEp-2 cells has much bearing on cytopathologies induced by other picornaviruses, an analogous situation does not exist in the case of other virus groups, where patterns of cytopathic effect and their governing mechanisms seem to be somewhat different.
SUMMARY

The earliest detection of lysosomal enzyme release in poliovirus-infected HEp-2 cells was 3.5 hours after infection and concurrent with the onset of early CPE. Lysosomal enzyme release is not the result of host cell depression in response to viral infection, because cells in which DNA-dependent-RNA synthesis is inhibited are still capable of demonstrating redistribution of lysosomal enzymes during poliovirus infection. The inactive components of the virion such as denatured capsid and incapsidated RNA are not capable of initiating lysosomal enzyme release patterns. The initiating event seems to be a product of the functioning viral genome and is produced at approximately 3 hours after infection, a time prior to the onset of viral-induced CPE and virus release.
APPENDIX A

MEDIA COMPONENTS
### TABLE 10

COMPONENTS OF MINIMUM ESSENTIAL MEDIUM (EAGLE) FOR SUSPENSION CULTURE

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Gm/liter</th>
<th>Ingredient</th>
<th>Gm/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>6.8</td>
<td>L-methionine</td>
<td>0.051</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4</td>
<td>L-phenylalanine</td>
<td>0.033</td>
</tr>
<tr>
<td>MgSO(_4)·7H(_2)O</td>
<td>0.2</td>
<td>L-threonine</td>
<td>0.048</td>
</tr>
<tr>
<td>Na(_2)PO(_4)·2H(_2)O(^a)</td>
<td>1.25</td>
<td>L-tryptophan</td>
<td>0.010</td>
</tr>
<tr>
<td>NaHCO(_3)(^b)</td>
<td>2.2</td>
<td>L-tyrosine</td>
<td>0.36</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>L-valine</td>
<td>0.047</td>
</tr>
<tr>
<td>Phenol Red (^c)</td>
<td>0.01</td>
<td>Choline Cl</td>
<td>0.001</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>0.1</td>
<td>Folic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>Dihydro-Streptomycin</td>
<td>0.1</td>
<td>i-inositol</td>
<td>0.002</td>
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<tr>
<td>L-arginine.HCl</td>
<td>0.126</td>
<td>Nicotinamide</td>
<td>0.001</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.24</td>
<td>Ca-D-pantothenate</td>
<td>0.001</td>
</tr>
<tr>
<td>L-histidine HCl.H(_2)O</td>
<td>0.042</td>
<td>Pyridoxal HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.053</td>
<td>Riboflavin</td>
<td>0.0001</td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.054</td>
<td>Thiamine HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>0.073</td>
<td>Calf Serum (^d)</td>
<td>100 ml</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.292</td>
<td>qs ad H(_2)O 1.0</td>
<td>liter</td>
</tr>
</tbody>
</table>

\(^a\) The phenol red-free MEM contained 1.4 g Na\(_2\)PO\(_4\)·2H\(_2\)O

\(^b\) pH was adjusted to Ph 7.2 with .1 N NaOH before filtration.

\(^c\) Phenol red was not added to some batches of medium.

\(^d\) Calf serum inactivated at 56 C for 30 minutes.
### TABLE 11
COMPONENTS OF MINIMUM ESSENTIAL MEDIUM (JOKLIK-MODIFIED) FOR SUSPENSION CULTURE

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Gm/liter</th>
<th>Ingredient</th>
<th>Gm/liter</th>
</tr>
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<td>KCl</td>
<td>0.4</td>
<td>L-phenylalanine</td>
<td>0.032</td>
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<tr>
<td>MgCl₂·6H₂O</td>
<td>0.2</td>
<td>L-threonine</td>
<td>0.048</td>
</tr>
<tr>
<td>Na₂HPO₄·H₂O</td>
<td>1.33</td>
<td>L-tryptophan</td>
<td>0.010</td>
</tr>
<tr>
<td>NaHCO₃ a</td>
<td>2.0</td>
<td>L-tyrosine</td>
<td>0.036</td>
</tr>
<tr>
<td>Dextrose (anhyd.)</td>
<td>2.0</td>
<td>L-valine</td>
<td>0.046</td>
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<tr>
<td>Phenol Red</td>
<td>0.01</td>
<td>Choline Cl</td>
<td>0.001</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>0.05</td>
<td>Folic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>Dihydro-Streptomycin</td>
<td>0.05</td>
<td>i-inositol</td>
<td>0.002</td>
</tr>
<tr>
<td>L-arginine.HCl</td>
<td>0.105</td>
<td>Nicotinamide</td>
<td>0.001</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.024</td>
<td>Ca-D-pantothenate</td>
<td>0.001</td>
</tr>
<tr>
<td>L-histidine HCl·H₂O</td>
<td>0.31</td>
<td>Pyridoxal HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.52</td>
<td>Riboflavin</td>
<td>0.0001</td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.052</td>
<td>Thiamine HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-lysine</td>
<td>0.058</td>
<td>Calf Serum b</td>
<td>100 ml</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.294</td>
<td>qs ad H₂O 1.0</td>
<td>liter</td>
</tr>
</tbody>
</table>

a  PH was adjusted to PH 7.2 with 0.1 NaOH before filtration.

b  Calf serum inactivated at 56 °C for 30 minutes.
TABLE 12
COMPONENTS OF YELP MEDIUM

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Gm/liter</th>
<th>Ingredient</th>
<th>Gm/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>0.19</td>
<td>L-phenylalanine</td>
<td>0.032</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0</td>
<td>L-threonine</td>
<td>0.048</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4</td>
<td>L-tryptophan</td>
<td>0.001</td>
</tr>
<tr>
<td>MgSO$_4$·H$_2$O</td>
<td>0.2</td>
<td>L-tyrosine</td>
<td>0.036</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$·12H$_2$O</td>
<td>0.11</td>
<td>L-valine</td>
<td>0.049</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.06</td>
<td>Biotin</td>
<td>0.001</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1.0</td>
<td>Choline Cl</td>
<td>0.0013</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>Folic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.02</td>
<td>i-inositol</td>
<td>0.004</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>0.2</td>
<td>Nicotinamide</td>
<td>0.001</td>
</tr>
<tr>
<td>Dihydro-Streptomycin</td>
<td>0.2</td>
<td>Ca-D-pantothenate</td>
<td>0.0011</td>
</tr>
<tr>
<td>L-arginine.HCl</td>
<td>0.105</td>
<td>Pyridoxal HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.024</td>
<td>Riboflavin</td>
<td>0.0001</td>
</tr>
<tr>
<td>L-histidine HCl·H$_2$O</td>
<td>0.031</td>
<td>Thiamine HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.052</td>
<td>Yeast Extract</td>
<td>0.5</td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.052</td>
<td>Lactalbumin</td>
<td>2.5</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>0.08</td>
<td>Bactopeptone</td>
<td>5.0</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.3</td>
<td>Calf serum $^b$</td>
<td>100 ml</td>
</tr>
<tr>
<td>L-methionine</td>
<td>0.015</td>
<td>qs ad H$_2$O 1.0 liter</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ PH was adjusted to PH 7.2 with 0.1 N NaOH prior to filtration.

$^b$ Calf serum inactivated at 56 C for 30 minutes.
FIGURE 9. STANDARD CURVE FOR LOWRY PROTEIN DETERMINATIONS.
FIGURE 10. COUNTING EFFICIENCY OF TRITIUM-SPIKED SAMPLES IN BRAYS SOLUTION.

a AES (Automatic External Standard)
FIGURE 11. COUNTING EFFICIENCY OF TRITIUM-SPIKED SAMPLES IN TOLUENE.

a AES (Automatic External Standard)
LITERATURE CITED


Smith, P. C. 1966. M. S. Thesis. Biochemical studies of viral-induced cytopathologies. The Ohio State University, Columbus, Ohio.


