THE UNIVERSITY OF MICHIGAN

INDUSTRY PROGRAM OF THE COLLEGE OF ENGINEERING

HOST-CELL METABOLISM DURING VIRAL SYNTHESIS

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1959

January, 1959

IP-350

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ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. W. W. Ackermann for the inspiration and advice given during the course of this investigation. Appreciation is extended to Dr. F. E. Payne and Dr. H. F. Maassab who, through their assistance, criticisms, and suggestions, have aided in the completion of this work, to Dr. Thomas Francis, Jr. for his generous support especially during the latter stages of this project and to Dr. Gordon C. Brown for his part in editing the manuscript.

The author takes pleasure in acknowledging the constant encouragement and faith of his wife, Susie.

He is indebted to the Industry Program of the College of Engineering, The University of Michigan, for support in the final stages in the preparation of this dissertation.

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THE PROBLEM

Since the genesis of virology, which began with the demonstration of the filterability of the infectious agents of tobacco mosaic diseases, attention has been directed to characterizing the viruses and investigating the epidemiologic aspects of their associated disease states. Early in the history of this field the need for intact host cells for virus reproduction was recognized and considerable information has accumulated regarding the fundamental relationships between viruses and cells, but generally these studies have largely centered around the virus rather than the cell. In recent years new impetus was given to studies of virus-host cell interrrelationships by the development of tissue culture techniques. This has allowed for more exacting observations on virus multiplication, especially with respect to the nature of the infected cell.

It is generally accepted on the basis of present knowledge that viral infection of a cell may be divided into four states: 1. adsorption; 2. penetration; 3. intracellular multiplication; 4, release of virus particles. The time required to complete a single cycle of virus growth depends on the properties of both the virus and the host cell, and on the environmental conditions.

Least is known concerning the third stage of the infectious process, that is, intracellular virus multiplication. One characteristic feature of this stage of replication by small viruses is the "eclipse period" during which it is impossible to recover infectious viral particles

by breaking open the infected cells. This suggests that actively multiplying vegetative virus is structurally different from mature virus particles. A virus, once within its host cell, seems to behave as a component of an integrated functional cell system, yet it retains its genetic and biochemical individuality.

Since viruses have been found to be essentially nucleoprotein in composition, it is reasoned, largely by analogy with bacteriophage, that the viral nucleic acid separates from the viral protein, and hence the viral nucleic acid, ribonucleic acid or deoxyribonucleic acid, forms the initiating element for viral multiplication. Once the viral nucleic acid is within the cell, there exists a new unit for consideration, not the virus nor the cell, but the infected cell.

This unit will function to produce in all cases, in addition to other variable viral materials, viral nucleic acid. Thus nucleic acid metabolism of the infected cell is an absolute requirement for viral production and will be the major subject of the present investigation.

To investigate this, cultures undergoing single cycles of infection have been used allowing interpretations in terms of a single infected cell. Further, so that the biochemical processes under study were meaningful, they were related in time to parameters such as the appearance, accumulation and release of infectious virus.

In planning this investigation consideration was given to the following questions. Are the biochemical reactions of the infected cell unique in synthesizing viral materials, or do they occur in the uninfected cell? In which morphologic component of the cell does this

"synthesis" occur? In the synthesis of virus are there intermediate levels of organization of precursor materials which in a step-wise fashion become more and more virus-like? The data in the present investigation indicate that after infection of cells by poliovirus, a considerable synthesis continues of materials which do not constitute virus and might not be destined to be virus.

HISTORICAL REVIEW

Introduction:

The process of viral multiplication within the host cell is the central problem of academic virology. There is already a large volume of information bearing on the changes that take place in infected cells particularly with respect to morphology. Only within the last decade has advancement begun in the study of the biochemistry of intracellular viral replication. Both aspects have progressed through improvement of virological and biochemical techniques.

The behavior of viruses within their host cells can be studied most ideally under conditions where a known number of susceptible cells can be exposed simultaneously to a known number of fully infectious virus particles, and the resulting events followed quantitatively. For a long time these conditions have been fulfilled most easily in the case of bacterial viruses, which consequently became a model system for the study of virus multiplication. However, with the advent of the tissue culture technique, many of the same experimental conditions could be established with viruses of animal cells. Virus-host cell interactions can now be studied at the cellular level. The tissue culture system is also advantageous in that it eliminates secondary responses, such as, inflammation, which may obscure the measurement of changes that the infected cell may be undergoing.

The life history of a virus can be divided formally into a number of phases. First, there is the attachment of the virus to the surface of its host cell, followed by penetration. There follows a

period during which the virus can no longer be detected by infectivity measurements, and during which the intracellular development and maturation of the virus occur. In a later phase viral release from the host cell occurs. Numerous investigations have been conducted on all these phases, but in this review only the literature pertinent to the phase of intracellular development and maturation will be discussed. The information will be derived chiefly from experimental data obtained from representative animal virus and bacteriophage systems. This review will attempt to relate the available biochemical information concerning the intracellular development of viruses to that obtained through morphological, cytochemical and immunologic means. Effort will be made to describe these particular observations recorded in the literature without prejudice as to their ultimate validity.

(a) Fate of the Infecting Particle: One of the most significant accomplishments of modern virus research has been the elucidation of the fate of the infecting virus particle. Non-recoverability or the "eclipse phase" of the infecting virus inoculum has been reported for the influenza virus system by Henle and Henle (1949), and Ackermann and Maassab (1954); the psittacosis virus system by Sigel, et al., (1951), and Girardi, et al., (1952); vaccinia virus system by Crawford and Sanders (1952); Western equine encephalitis virus system by Dulbecco and Vogt (1954); poliovirus system by Ackermann, et al., (1954), and Howes and Melnick, (1957); herpes simplex virus system by Kaplan (1957), and Gostling (1956); and phage systems by Putnam and Kozloff (1950), and Doerman (1952), and appears to be a general phenomenon. However, the underlying mechanisms for each of the above systems may be different.

At present little or no information, concerning the fate of the virus particle, can be derived from electron microscopical, cytological or immunological studies. However, these studies do confirm the disappearance of the virus particle, for virus or virus-like particles are not observed until late in the intracellular phase of the multiplication process (Wyckoff, 1948; Morgan, et al., 1956; Buckley, 1956; and Breitenfeld and Schäfer, 1957).

Through chemical and isotopic means, Hershey and Chase (1952) and French (1954), have shown that intra-bacterial growth of T2 is initiated, not by the penetration into the bacterium of the phage particle as a whole, but by injection of some "priming material". The primer consisted of all the DNA and only about 4 per cent of the protein of one phage particle (Hershey, 1955). Analyses of the protein material in the primer reveals two distinct polypeptide components (Hershey, 1957). However, both are not transferred together with the DNA from parental to offspring phage.

Among animal virus systems, Hoyle and Frisch-Niggemeyer (1955), employing P^{32}-labeled influenza virus, found that on entry into the host cell (chorioallantoic membrane), the virus particle disintegrates, and the isotope derived from virus phospholipid can be recovered from the infected membranes in the form of small molecular weight compounds, which are not precipitated by protein precipitants or sedimentable at high speeds. Although a small fraction of labeled nucleic acid is recoverable from the infected membrane with physiological saline, a larger part of it can only be recovered if the cell nuclear material is dissolved in molar sodium chloride. This latter fraction is present partly

as a nucleoprotein and partly as free nucleic acid. The authors suggested that on entry into the cell, the virus particle is broken down, the phospholipid destroyed, and the nucleoprotein disintegrated with the release of free nucleic acid, which enters into a close relationship with the cell nuclear material.

Indications that the virus after entrance into the cell has now assumed a new identity, have been shown by its greater resistance to irradiation as compared to the free virus. This has been seen in several viral systems and using different radiation sources (Latarjet, 1953; Dulbecco and Vogt, 1955; and Franklin, 1958). Benzer (1952) has suggested that changes in radiation sensitivity of the virus-host cell complex may represent a progression from one step to another in the development of new virus.

Several lines of indirect evidence employing various approaches indicate the possible existence of a "naked nucleic acid phase" at the beginning of the growth cycle of the virus. The intracellular multiplication of both influenza and tobacco mosaic viruses has been shown by Le Clerc (1956) and Hamers-Castermann and Jeener (1957) respectively, to be suppressed by ribonuclease if the latter is given up to two hours after infection. The free virus is resistant to the action of this enzyme.

With the use of anion-exchange chromatography, Cochran and Chidester (1958) have been able to differentiate an infectious component containing RNA from the mature nucleoprotein particle where both are present in homogenates of leaves infected with tobacco mosaic virus. The relative contribution to the total infectivity of these two components

appears to vary with the age of the infection. Early in the infectious sequence a major portion of the total infectious activity is sensitive to ribonuclease, but not so later.

Since the initial study of Grierer and Schramm (1956) on the isolation of infectious ribonucleic acid from tobacco mosaic virus, the infectivity of ribonucleic acid preparations from different animal viruses has been reported by Colter, Bird and Brown (1957) for Mengo encephalitis virus; Wecker and Schäfer (1957) for Eastern equine encephalitis virus; Colter, et al., (1958) and Alexander, et al., (1958) for poliovirus; and Cheng (1958) for Semliki Forest virus. That the ribonucleic acid preparation is the unit responsible for infectivity and genetic continuity was clearly demonstrated by Fraenkel-Conrat and Singer (1957) with the use of two strains of tobacco mosaic virus. They combined the nucleic acid from strain HR with the protein shell of a different strain, and observed after infection of tobacco leaves with the recombined unit, the production of virus possessing only the serological and chemical properties peculiar to the HR strain. However, it must be remembered that in the case of the animal viruses, there is yet no clear evidence that RNA is the only material which enters the cell.

Preparations of deoxyribonucleic acid from T2 bacteriophage, infectious for protoplasts, have been reported independently by Spizizen (1957) and Fraser, et al., (1957). However, a certain degree of protein has been found to be associated with the infectious DNA preparation. Removal of this protein material appears to render the preparation non-infectious.

Other lines of indirect evidence indicating the formation of a new entity after the entrance of the virus particle into a cell have been obtained through the genetic approach, for example, the recombination studies in phage and influenza virus systems (Visconti and Delbrück, 1953; and Burnet and Lind, 1954); and transformation studies on fibroma and myxoma viruses (Berry and Dedrick, 1936).

(b) The Infected Cell: Despite the loss of infectivity and structurally visible components which occurs with the entrance of the virus particle into the host cell, one can observe certain morphological and chemical changes occurring in the cell before the appearance of new viral progenies.

It may well be impossible to provide any general statement about the influence of viruses on the cells in which they multiply. We have to deal on one hand with viruses of very different size, morphology and chemical composition which may well multiply by different methods and, on the other, with the host cells of very different quality. Nevertheless, in attempting a general discussion of virus-infected cells, the only practical approach would seem to be to choose a few well studied examples and comment on biochemical, morphological and histochemical observations, as well as the distribution and development of virus particles and viral related and nonrelated structures.

Earlier investigations of changes in host cell metabolism during viral infection was at first limited to the observation of respiratory activity. Measurements involved complex biological systems such as, embryonated eggs (Parodi, et al., 1948; McLimans, et al., 1950; and Pinkerton, et al., 1950), and thus the results cannot be readily interpreted.

The respiration of several mammalian tissues, such as, the chorioallantoic membranes infected with either influenza, myxoma or vaccinia viruses (Ackermann and Francis, 1954; Kun and Smith, 1950; and Overmann and Tamm 1957), and HeLa cells with poliovirus (Ackermann and Francis, 1954), has been measured and was found to remain fixed at the preinfection rate throughout the period of viral development and release. This was also found to be true for Escherichia coli infected with either the T2 or T4 coliphages (Cohen and Anderson, 1946).

A dependence on aerobic respiration of host cells for synthesis of new virus has been shown for several animal virus-cell systems. Under anaerobic conditions, virus production was markedly reduced or abolished in cultures of influenza virus and chorioallantoic membranes (Ackermann, 1951), and feline pneumonitis virus and yolk sac (Moulder, et al., 1953). However, working with poliovirus and HeLa or monkey kidney cells, Gifford and Syverton (1957) found that even under anaerobic conditions the yield of poliovirus per destroyed cell was essentially unaltered, though the rate of production was reduced.

So far, all the results on the study of energy requirements for synthesis of viruses, have been interpreted in terms of well-known metabolic pathways. The implication of the Krebs cycle as a possible source of the energy for virus multiplication has been demonstrated by several investigators through the use of metabolic antagonists. Ackermann and Johnson (1953), and Eaton and Perry (1953), have shown that 2,4-dinitrophenol inhibits the growth of influenza virus in the chorioallantoic membrane. The action of this substance appears to be due to an uncoupling of phosphorylation from oxidation.

It has been shown by Ackermann (1951 a, b) that administration of fluoroacetate or malonate, which inhibits the oxidation of citrate, and dehydrogenation of succinate, respectively, prevents viral synthesis. Using the same inhibitors, Moulder, et al., (1953) and Zahler and Moulder (1953), obtained similar results with the pneumonitis virus, and concluded that the energy for the synthesis of pneumonitis virus is probably supplied by high energy phosphate bonds generated by the aerobic oxidation of endogenous substrates.

Ackermann (1951) has shown that an energy source such as glucose is necessary for synthesis of certain strains of influenza type A virus in chorioallantoic membranes suspended in a synthetic medium. Levine and Rouse (1957), using a non-neurotropic (WS) and a neurotropic (NWS) strains of influenza type A virus, confirmed Ackermann's findings. However, they found that pyruvate could serve as a sole source of energy for only the WS strain. The strain NWS when grown in pyruvate appeared to form a latent type of infection, for when glucose was added later, virus multiplication occurred. When other types of influenza viruses and some strains of Newcastle disease virus were tested for their ability to multiply in the presence of pyruvate, they all gave various rates of multiplication. The need for glucose as an energy source for poliovirus multiplication in HeLa or human amnion cells has been shown by Eagle and Habel (1956) and Becker, et al., (1958). The latter authors also found that inhibitors of the oxidative pathways of glucose metabolism such as, potassium cyanide or sodium azide, did not effect either cell maintenance nor viral reproduction, and assumed that glycolytic rather than oxidative processes are important for reproduction of poliovirus.

Although glucose utilization via the oxidative pathway is sharply curtailed in phage-infected Escherichia coli, the energy for phage synthesis also appears to be a product of host enzyme activity shunted from use in bacterial synthesis (Cohen and Roth, 1953; Cohen and Weed, 1954; and Lanning and Cohen, 1955). The rate of glucose uptake is not altered, but less of the metabolized glucose is degraded by the oxidative pathway in the infected cell than in the normal cell. There is a predominent switch to the anaerobic pathway in the infected cell (Cohen, 1951). Inhibitors that block the energy-yielding process of bacteria were found to prevent phage reproduction.

In the animal virus-cell system, the exogenous requirements for viral synthesis appear to be very simple. Nearly all the requirements for viral synthesis are supplied by intracellular sources. For example, Eagle (1955) and Eagle and Habel (1956) showed that the amount of poliovirus produced by HeLa cells was quantitatively unaffected by the omission of serum protein, amino acids, or vitamins from a basal medium. In such a deficient medium the protein components of the virus are necessarily built up entirely at the expense either of the host cell protein or of its amino acid and peptide pool; and if cofactors are required for that synthesis, those already in the cell, or their precursors, suffice. On the other hand the omission of both glucose and glutamine from the medium resulted in a marked decrease in virus production. The omission of each singly either had no effect or caused only partial reduction. Eagle and Habel (1956) postulated that glucose and glutamine are used either, as sources of energy, as precursors for

the synthesis of viral nucleic acid, or both. Ackermann (1951) has shown that maximal yields of influenza virus can be obtained from infected chorioallantoic membranes maintained only in the presence of inorganic salts and glucose.

The exogenous nutritional requirements for phage reproduction are also quite simple. Phage reproduction can be obtained from bacteria maintained only in the presence of inorganic salts and glucose (Cohen, 1949; and Luria, 1953). However, in bacteria that require for growth a supply of some essential building block such as an amino acid, phage synthesis requires the same supplement (Raff and Cohen, 1950; and Burton, 1955).

Many investigators have unsuccessfully sought for some change in the enzymatic activity of the infected cell that might give a clue toward explaining the influence of the virus on cell metabolism. The search for specifically altered enzymatic activities or appearance of new enzymes upon infection with animal viruses, has so far not been very successful. The early investigations involved the use of whole animals, and the changes observed were probably complicated by secondary processes, such as, inflammation. Bauer (1953) has summarized a variety of studies of the changes produced in animal tissues following virus infection. Changes in the activities of certain enzymes have been observed in poliovirus-infected HeLa or monkey kidney cell cultures (Matzelt, et al., 1958; and Kovacs, 1956), and also in chorioallantoic membranes infected by a variety of animal viruses (Kun and Smith, 1954). However, whether these changes in enzymatic activity upon infection are specifically and directly involved in the synthesis of the viral particle awaits further development.

So far changes in enzymatic activity wholly ascribable to virus infection have been found only in the Escherichia coli bacteriophage systems. In the phage system one might anticipate both qualitative and quantitative alterations in the activity of enzymes involved in nucleic acid metabolism, for host DNA is degraded for transfer to virus progeny (Putnam, 1953; Evans, 1954), and DNA containing a new kind of pyrimidine is being formed (Wyatt and Cohen, 1953).

While investigating the activity of nine bacterial enzymes in phage-infected Escherichia coli, Pardee, et al., (1953) found that only deoxyribonuclease activity was enhanced. Kozloff (1953) independently also discovered this increase in deoxyribonuclease activity, and traced this to destruction of a specific deoxyribonuclease inhibitor. The inhibitor was identified as a particular kind of RNA in the host cell. Recently, Flaks and Cohen (1958) have isolated from phage-infected bacteria an enzyme that will hydroxymethylate deoxycytidine-5-phosphate in an in vitro system. Thus the major quantitative and qualitative change in enzymatic activity upon phage infection is ascribable to release of an inhibitor and the enzymatic pathway for synthesis of hydroxymethylcytosine, respectively.

Both Monod and Wollman (1947) and Benzer (1953) have shown that adaptive enzyme formation of β-galactosidase in phage-infected Escherichia coli B cells, grown in the presence of lactose, is inhibited within a few minutes after addition of phage, that is, after allowance of time for adsorption. All further production of this enzyme is blocked. This holds true regardless of the level of activity before infection. The enzyme level is "frozen", neither increasing nor decreasing during the

subsequent growth of phage. This effect is not characteristic of all phages, for Jacob (1952) showed that infection of Pseudomonas pyocynea by a temperate phage fails to block completely bacterial growth or synthesis of glucozymase. Siminovitch and Jacob (1952) also observed the Escherichia coli K12 could still synthesize β-galactosidase during the induced development of phage λ. Although the adaptation towards oxidation of acetate, alanine and lactose was completely inhibited by infection with T2 coliphage, in marked contrast, the formic hydrogen lyase system was greatly stimulated (Joklik, 1952). The induction of the latter system occurred more rapidly in infected than uninfected cells.

Profound chemical alterations in protein, nucleic acid and carbohydrate metabolism have been observed to occur in the infected bacteria during the course of phage multiplication. Although a search for similar biochemical changes accompanying the multiplication of animal and plant viruses has not been as fruitful, certain chemical, isotopic and cytochemical changes have been reported to occur. Most attention has been focused on nucleic acid and protein synthesis and on amino acid, phosphorus and nucleotide metabolism. In most of these studies, isotopic techniques have proved to be a valuable tool.

Increase incorporation of the isotope P^{32} into the various nucleic acids of the cell has been observed in several animal and plant viral systems such as, the feline pneumonitis virus-yolk sac system (Moulder, 1954); encephalomyocarditis virus-Erhlich ascites tumor cell system (Levy, et al., 1956); poliovirus-HeLa or human amnion cell systems

(Miroff, et al., 1957; Becker, et al., 1957; and Rothstein, et al., 1958); and in the turnip yellow mosaic virus-Chinese cabbage leaf system (Matthews, 1958). In most of these studies no increases in the concentrations of the nucleic acids were observed. Both Miroff, et al., (1957) and Becker, et al., (1957) did not determine the concentrations of the nucleic acids. However, Matthews (1958) found an increase in total RNA of the infected leaves, which is entirely accounted for by the amount of viral RNA that can be isolated in combination with the virus protein antigen. Also analyses of the bases showed that when the values for the healthy leaf are subtracted from those for the virus-infected material, the ratios obtained resemble that of the virus. A similar study with tobacco mosaic virus in Nicotiana glutinosa leaves by Basler and Commoner (1956) indicates that the excess RNA which accumulated in the infected leaves before the appearance of mature virus particles, is quite similar in composition to that found in viral RNA.

In all the above studies, the infective agents used were those which belong to the RNA-containing group of viruses. Recently, Newton and Stoker (1958) observed a progressive accumulation of DNA in HeLa cells infected with herpes virus. No further characterization of the excess DNA was done, but the authors suggested that it might represent either viral nucleic acid which was not incorporated into particles, or that it was HeLa cell DNA, normal or abnormal, which was produced as an indirect manifestation of virus multiplication.

Increased incorporation of radioactive phosphorus into the various nucleotide components of the acid-soluble fraction of chorio-allantoic membranes infected with influenza virus (Johnson and

Ackermann, 1958), and also into the phospholipid fractions of the same virus-tissue system (Cohn, 1952), has been observed. The increased incorporation of the isotope into the phospholipid fractions of HeLa cells infected with poliovirus, was also described by Miroff, et al., (1957).

Cytochemical evidence appears to lend further support to the chemical and isotopic observations, that alterations in nucleic acid metabolism are occurring in the virus-infected cell. Changes in the various nucleic acids of the infected cell have been suggested in several animal virus-cell systems through the use of a variety of basophilic stains. Increasing cytoplasmic basophilia, together with nuclear chromatin changes and pyknosis, have been observed in several types of tissues infected with poliovirus (Ackermann, et al., 1954; Harding, et al., 1956; Reissig, et al., 1956; and Howes and Melnick, 1957). These changes begin before the appearance of mature particles intracellularly. With the use of the methyl green-pyronin stain, Burnet (1955) observed a deepening in pyronin staining (RNA) with time, in allantoic membrane cells infected with influenza virus. The increase in DNA content in the nuclei of HeLa cells infected with the adenovirus has been detected by Boyer, et al., (1957) by means of Feulgen microspectrophotometry. Whether the higher DNA quantities observed were due to viral DNA or due to an unusual and irregular synthesis of nuclear DNA induced by the presence of the virus could not be decided with certainty from the data available.

Changes in the free amino acid balance have been reported by Johnson, et al., (1956) in chorioallantoic membranes infected with

influenza virus, and by Commoner and Nehari (1953) in Nicotiana glutinosa leaf infected with tobacco mosaic virus. With the use of two amino acid analogues, fluorophenylalanine and methoxinine, Ackermann and Maassab (1954) and Maassab (1955), have been able to differentiate two separate and distinct stages of influenza virus development in chorioallantoic membranes, which appear to require phenylalanine and methionine respectively, as normal metabolites. Increases in protein content have been observed by Matzelt, et al., (1958) in HeLa cells infected with poliovirus, and by Nossal and de Burgh (1953) in mouse liver cells infected with ectromelia virus.

As soon as phage DNA has been injected into the bacteria, several metabolic activities occur. There is almost immediate protein synthesis. This initial requirement for protein synthesis in the step-wise development of the T-even phage was clearly demonstrated through the use of chloramphenicol, which stops both induced enzyme biosynthesis and net protein synthesis in Escherichia coli. It was found by Crawford (1957), Tomizawa, et al., (1956) and Rosenbaum and Preston (1958), that treatment of bacterial cells with chloramphenicol before T2 infection results in an inhibition of DNA synthesis. If, however, protein was allowed to be synthesized for a few minutes and then halted by use of chloramphenicol, the infected cells can still form phage DNA. Crawford (1957) in a further extension of his studies observed a difference in the effect of chloramphenicol on DNA syntheses between T-even and T-odd infected cells. Pre-treatment with chloramphenicol in T-even infected systems inhibited DNA synthesis, whereas with the T-odd systems it did not. Thus prior protein synthesis seems to be necessary for synthesis of DNA of T-even phages but not for

others. This difference the author feels can be connected to the occurrence of 5-hydroxymethylcytosine (5-HMC), which is only found in T-even phages, and the metabolism of this compound might involve formation of new enzymes. However, the same author (1958) recently found that one of the T-odd phages, T5, does require prior protein synthesis, even though it does not contain 5-HMC. Through the use of amino acid-requiring mutants of Escherichia coli, Burton (1955) has endeavored to study the separate aspects of protein and nucleic acid synthesis, and has shown that the synthesis of phage DNA is dependent on the initial synthesis of proteins.

Cohen (1948 a,b) made the striking observation that in infected cells, RNA production "ceases" abruptly, whereas DNA synthesis is stimulated about threefold. The phosphorus that would normally enter host ribose nucleic acid and host deoxyribonucleic acid, is diverted to the rapid synthesis of viral nucleic acid, that is solely of the deoxyribose type. The same author (1951), studying the metabolism of glucose-1-C^{14} in normal and phage-infected bacterial cells, observed that considerably less glucose was metabolized by the oxidative phosphogluconate pathway and more by the Embden-Meyerhof pathway. This result was consistent with the observed decrease in synthesis of ribose and the marked increase in synthesis of deoxyribose that was found in viral DNA. However, Cohen and Roth (1953) have shown that the depression in utilization of the oxidative phosphogluconate pathway as a result of infection is not caused by an inhibition of the enzymes of this pathway, for substrates such as, gluconate, arabinose and ribulose, can still be used by infected cells for the synthesis of viral DNA. It was concluded that control of glucose utilization is determined at some level apart from the enzymes that exist in the pathway themselves.

In contrast to the dynamic state of DNA metabolism within Escherichia coli infected with virulent phage, is the equiescent state of RNA. Cohen (1948) first observed the lack of turnover of RNA-phosphorus, an observation confirmed several times by Koch, et al., (1952); Manson, (1953); and Kozloff, et al., (1951). In contradiction, Hershey (1953) has reported, and it has been confirmed by Volkin and Astrachan (1956), that there is a short burst of strong metabolic activity in the host RNA immediately after infection. This increased incorporation of P^{32} into a specific RNA fraction lasts a few minutes only. This phenomenon might be connected with the synthesis of the protein, which is required for the synthesis of phage DNA. Nothing is yet known about the nature of this protein, which might possibly be the hydroxymethylating enzyme of Flaks and Cohen (1957), or a series of enzymes connected with DNA synthesis. Experiments by Jeener (see Brachet, 1957) on lysogenic bacteria treated with ultraviolet light tends to implicate the RNA in the initial phases of phage multiplication. He found that in the presence of ribonuclease, phage multiplication is inhibited. In a similar system, Hamers (1956) found that the addition of thiouracil, which is incorporated into the RNA of the bacteria, inhibited phage multiplication. The inhibition could be reversed by the addition of uracil. Recently Fraser, et al., (1957) have shown that removal of RNA from pre-infected T2 or T3 protoplasts with ribonuclease resulted in the inhibition of phage reproduction.

Several instances of the acquisition of new metabolic capabilities upon phage infection have been reported for lysogenic systems, for example, transduction (Zinder and Lederberg, 1952), and the conversion of non-toxigenic Corynebacterium diptheriae to a strain producing toxin (Groman,

1955). There is still only one known instance of the induction of a new metabolic capability in systems wherein infection leads to the inhibition of cell multiplication, and where virus synthesis becomes the dominant metabolic activity; namely, the induction of an active net synthesis of thymine and of 5-hydroxymethylcytosine (5-HMC) upon T2 infection of a thymine-requiring mutant of Escherichia coli (Barner and Cohen, 1954). Neither pyrimidine is synthesized appreciably in uninfected, thymineless organisms. The mode of synthesis of the virus specific DNA which contains 5-HMC is of great interest. This unique viral pyrimidine is present in the DNA of the T-even bacteriophages (Wyatt and Cohen, 1953), and is absent from the nucleic acids of the host bacterium Escherichia coli, which contain cytosine. It has been shown by Cohen and Weed (1954) that the pyrimidine ring of viral HMC can be derived from the cytosine of host bacterium DNA. The same authors have shown that the hydroxymethyl substituent of HMC, as well as the methyl group of thymine can be derived from the β-carbon of serine, but not from the methyl group of methionine (Green and Cohen, 1957). Cohen, et al., (1958) have shown that the formation of HMC does not occur in infected cells at the level of the free pyrimidines or their nucleosides. The formation of HMC has now been found to take place at the level of the nucleotide by Flaks and Cohen (1957). These authors have been able to isolate from phage-infected bacteria, an enzyme that will hydroxymethylate deoxycytidine-5'-phosphate (deoxy-CMP) in an in vitro system in the presence of tetrahydroxyfolic-acid and C^{14}-labeled formaldehyde. Although extracts of normal cells displayed such activity, it was in lesser amounts, and the authors were not certain that the very slight uptake of labeled

formaldehyde with deoxy-CMP in extracts of normal cells led to the formation of hydroxymethyldeoxy-CMP.

The cytological evidence of phage development appears to support the changes observed with chemical methods (Luria and Human, 1950; Murray, et al., 1950; and Murray and Whitfield, 1953). One of the first changes noticed in a bacterium after phage infection was an alteration of the morphology of the bacterial nuclei, the DNA-containing bodies. In fixed and stained preparations of bacteria infected with phages like T2, the nuclei were seen to disrupt into chromatin blocks, which move to the periphery of the cell. The nuclear disruption is followed several minutes later by appearance of new granular chromatin, which stains Feulgen positive (Beutner, et al., 1953). With phage T5 similar changes were observed, but the time interval between nuclear breakdown and the appearance of new chromatin is prolonged. During this period even though no DNA was observed through cytochemical methods, it was detectable by chemical techniques (Murray and Whitfield, 1953).

As the viral infection progresses, some antigenic particles which may be viral or non-viral in serologic character appear in the cell either before or together with the fully infectious virus particles. This has been observed to occur in the animal, plant and bacterial virus systems. These particles in the majority of instances are non-infectious and may represent intermediate products of virus synthesis, or products that were never destined to be incorporated into infective particles, or irrelevant products.

Although strong evidence for the occurrence of developmental stages of viruses in any cell has not yet been obtained, there is evidence in the case of animal viruses of the occurrence of a variety of antigens

which differ in their reaction with complement-fixing and neutralizing antibodies in immune sera. Antigens other than those in the virus particles themselves are demonstrable by complement-fixation in many virus-infected tissue suspensions. These are called soluble antigens and are obtained from infected tissues.

Several antigenic products smaller than the virus particles are seen in vaccinia-infected tissues. These include the soluble LS antigen of Craigie (1932), extensively investigated by Smadel and Hoagland (1942); the hemagglutinin investigated by Nagler (1942), Burnet (1946), and Chu (1948); and the NP antigen reported by Smadel and Hoagland (1942). These antigens are distinct from the virus, and are antigenic and the antibodies produced to them are unrelated to the neutralizing antibodies. There is evidence that both the LS and NP antigens are surface constituents of the virus. The LS antigen, which has been crystallized, is a protein of molecular weight about 240,000 and does not contain nucleic acid. It is capable of stimulating formation of antibodies which not only precipitate soluble antigen, but will agglutinate elementary body suspensions. The NP antigen is a nucleoprotein obtained from elementary bodies by extraction with dilute alkali, and antibodies made to this antigen will agglutinate elementary bodies. The hemagglutinin is associated with particles 65 mu in diameter according to Chu (1948). Indirect evidence suggests strongly that it is a lipid-protein complex, and the hemagglutinin action is due to a phospholipid (Burnet and Stone, 1946). They suggested that since the hemagglutinin is found in less abundance relative to virus in necrotic material of skin or chorioallantoic membranes than the deeper non-necrotic tissue, its formation may be an early phase in vaccinia virus multiplication.

Tissues infected with influenza virus have been found to be rich in soluble antigen (S antigen). Depending upon the strain of virus, relatively little of the S antigen is released along with virus from the infected cells. The S antigen is serologically distinct from the strain specific V antigen of the mature virus particle (Henle and Wiener, 1944; and Hoyle, 1945). The S antigen produced in tissues is type-specific in that it is identical in all strains of the same type; for example, the S antigen of influenza A virus is distinct from that of influenza B virus (Henle and Wiener, 1944; Hoyle, 1945; and Kirber and Henle, 1950). It is also serologically similar to the soluble antigen which is obtained through ether disintegration of the influenza virus particle (Hoyle, 1950, 1952). Both soluble antigens are smaller (12 to 15 mu in diameter) in size than the mature particle (Hoyle, et al., 1953). Although both were found to be nucleoproteins (Hoyle, 1952, 1954; and Ada and Perry, 1954), Ada (1957) reported that the RNA associated with that isolated from cellular debris differs both in amount and character from that present in the nucleoprotein isolated from the virus.

Although it is clearly a sub-unit of the virus, the role of the S antigen in the infectious process is still obscure. Hoyle (1950, 1952, 1953) suggested that the S antigen represents the fundamental multiplying unit during intracellular synthesis, the repository of biological continuity; however, it cannot initiate the infectious process. His view is based upon the results of treatment of elementary bodies with ether, their appearance during the growth cycle of the virus, and electron micrographs of the S antigen. This concept seems to be supported by the evidence

obtained through breakdown of seed virus labeled with P^{32} (Hoyle and Frisch-Niggemeyer, 1955) and S^{35} (Hoyle, 1957). It is also supported by the work of Hoyle (1948, 1952) and Henle and Henle (1949) on the appearance of S antigen in the infected tissue prior to the development of V antigen and hemagglutinin activity.

Particles similar to those found in the influenza virus system have been found in the fowl plague system. Disruption of the fowl plague virus by ether has been reported by Schäfer and Zillig (1954). The appearance of the soluble antigen in infected tissues has been demonstrated by Schäfer and Munk (1952) and by Breitenfeld and Schäfer (1957). The soluble antigen obtained through ether disintegration of the elementary body of fowl plague has been called the "gebundenes antigen" (g antigen) by Schäfer and Zillig (1954). As proved by physicochemical, chemical and serological methods, the S antigen found in infected tissues has many properties in common with the g antigen from elementary particles (Schäfer, 1957). The evidence obtained here, like that from the influenza virus system, suggests that the S antigen in fowl plague virus is the RNA-containing unit of the virus, which is released during infection and is replicated after infection in the host cell (Schäfer, 1957). In the same report, the author offers evidence of a serological cross relationship between the g antigen of fowl plague and the S antigen of a strain of influenza virus (FM/1 strain A', type A).

Two distinct viral antigens present in tissue culture fluid from monkey kidney cells infected with poliovirus, and which may be separated by ultracentrifugation, have been reported by Mayer, et al., (1957). These antigens were named C and D, and differ in physicochemical

characteristics, in addition to the difference in regard to immunologic specificity. Particle C is noninfectious, in complement-fixation tests it reacts predominantly with acute sera, its sedimentation constant is lower than that of the infectious particle, and it lacks nucleic acid. Particle D or fraction D reacts predominantly with convalescent sera, comprises both infectious and noninfectious particles, and has a sedimentation constant of 160 Svedberg units. In a recent paper Roizman, et al., (1958) studied the kinetics of the formation and release of C and D antigens, and mature infectious particles in three lines of cells. In all three lines of cells both antigens were found to accumulate intracellularly together with infectious virus before release into the extracellular fluid.

The production of soluble antigen has been encountered in several other animal virus systems. Wildy and Holden (1954) have demonstrated the occurrence of soluble antigen in association with herpes virus. Bradish, et al., (1952) have shown that there are two specific components in extracts of tissues infected with foot and mouth disease virus. The larger has a sedimentation constant equivalent to the infective virus and the other corresponds to the complement-fixing antigen. Kipp, et al., (1957) have studied the soluble antigens of poliovirus and rabies viruses.

Von Magnus (1951) has shown that upon serial passage of undiluted allantoic fluid, containing high titers of influenza virus inoculum, hemagglutinins are produced to high titers, while the infectivity does not develop to a corresponding extent, and may not even increase; he called the particle which also has a lower sedimentation constant an "incomplete"

virus. The evidence of production of incomplete virus in the allantoic membrane is supported by Schlesinger's (1950) work in the central nervous system of mice after injection of large doses of non-neurotropic influenza virus. Cairns et al., (1952) demonstrated that multiple infection is not a prerequisite for production of incomplete virus and suggested "incomplete" virus was produced as a result of a shortage of some factor responsible for conversion of it to fully active virus. In determining the amount of ribonucleic acid in the influenza virus, Ada and Perry (1956) found that a reduction in the ratio of infectivity to hemagglutination (I/HA) is accompanied by a decrease in nucleic acid content. From their data, they postulated the possibility of various degrees of "incompleteness" depending upon the nucleic acid content of the particle.

Various other hypotheses have been advanced to account for the formation of the "incomplete" virus. Although many of these interpretations appear very plausible, especially the concept of the "incomplete" virus as an immature stage of the fully active virus, it should be noted that conclusive proof is still lacking (von Magnus, 1954; Henle, 1956).

There is apparently a series of phage-related particles which appear during infection of Escherichia coli with the T-series of bacteriophages. De Mars et al., (1953) employing proflavine to inhibit production of mature T2 phage particles, found particles resembling empty phage heads ("doughnuts"). These particles, although antigenically (C-F test) related to the complete T2 particle, do not contain the phage antigen that combine with and give rise to phage-neutralizing antibodies. The "doughnuts" contain essentially no deoxyribonucleic acid. In a recent study the same author (1955), utilizing the same system and inhibitor, obtained

through ultrafiltration or high speed centrifugation from normal and premature lysates of T2-infected cells, noninfectious materials that can block phage-neutralizing antibodies. In normal lysates one-twentieth to one-fifth of the serum-blocking activity is in the form of non-infectious materials smaller than phage. Premature lysis of the infected cell during the latent period reveals that the noninfectious, serum-blocking materials appear several minutes before infectious phage, and accumulate in the cell. Bacteria infected with T2 in the presence of proflavine, which on lysis liberates no active phage, produce about as much serum-blocking activity as they would in the absence of proflavine. There is some evidence that the doughnut shaped antigen, and the serum-blocking antigen are similar to the head and tail respectively of the mature phage (Lanni and Lanni, 1953). Lanni (1954), demonstrated through use of complement-fixation, the appearance of a new phage-related antigen during infection of E. coli, strain B cells with T5 phage. The new phage antigen appears before infectious phage, and continues until lysis to increase at a faster rate than infectious phage. Normal phage lysates contain at least one-half of their phage antigen in noninfectious form.

The non-nucleic acid proteins that appear during plant virus infection, such as tobacco mosaic and turnip yellow mosaic viruses, have been studied extensively by Takahashi and Ishii (1952), Takahashi (1955), Commoner et al., (1953), Commoner and Yamada (1955), Jeener and Lemoine (1953) Jeener et al., (1954), Consentino et al., (1956), and Markham and Smith (1949). These authors have found that these different proteins are identifiable because they differ from one another either by size,

weight or electrophoretic mobility, and when separated from one another they have been found to differ in their infectivity and chemical constitution. Jeener et al., (1954) showed that the two crystallizable, non-infectious forms of TMV are immunologically related to the virus, but lack RNA. These are thought to be identical with components B_2 and B_6 of Commoner and Yamada (1955). From work using radioactive isotopes, Van Rysselberger and Jeener (1955) suggested that the small, noninfective particles in plants infected with TMV are precursors of larger particles, but as the ratio of small to large particles can be changed in fully infected leaves by altering the conditions under which they are kept, Bawden and Pirie (1956) feel that they can be produced by the degradation of larger particles. Bawden (1957) feels that there is no reason to conclude that all specific particles that are noninfective are necessary precursors or breakdown products of large particles; some may well be concomitant products of protein synthesis that were never destined to be incorporated into infective particles.

Markham and Smith (1949, 1953) found that the protein which accompanies TYM infections is of the same general size, appearance and immunologically related to the virus itself. The protein could not be separated from the mature particles electrophoretically, but could be differentiated by the use of the ultracentrifuge. The protein remaining in the supernatant, while the mature particle is sedimented. Fraser and Consentino (1957) found that the amino acid composition of both particles to be similar in composition. Matthews (1958) reported that the ratio of numbers of virus nucleoprotein to protein particles remained close to 2:1 under a wide range of growing conditions. This ratio did not change significantly from the earliest stage of infection at which crystalline virus

could be isolated to the time when plants contained maximal amounts of virus, and were approaching senescence.

Characteristic intracellular inclusions appearing either in the cytoplasm or the nuclei of infected cells have been observed in several different viral systems.

It has been generally accepted that the formation of intracytoplasmic inclusions is a part of the virus multiplication process. The formation of this type of inclusion has been observed among the large particle viruses such as vaccinia (Himmelweit, 1938; Bland and Robinow, 1939; and Crawford and Sanders, 1952), and the psittacosis-lymphogranuloma group of viruses (Bland and Certi, 1935; Yanamura and Meyer, 1941; and Sigel, et al., 1951). Both Himmelweit (1938) and Bland and Robinow (1939) have shown that the inclusion body of the vaccinia, developed out of a condensation of basophilic material in the perinuclear region and that in its mature form it represented an accumulation of elementary bodies in a "structureless" matrix. Elementary bodies appeared once again after a period of several hours especially around the matrices. The reappearance of elementary bodies has been confirmed through the electron-microscopical studies of Gaylord and Melnick (1953 a, b) and Morgan, et al., (1954). The particles around the matrix were found to consist of a central body enclosed by a single membrane, but those which were found near the surface of the host cell and in the extracellular spaces possessed a double membrane. The initial sites of development were confined to the cytoplasm of the host cell.

The nature of intranuclear inclusions is not as well understood as that of the cytoplasmic inclusions. Herpes simplex virus may be taken

as a good representative of this group of viruses. The development and formation of this type of inclusion in the infected cell has been followed by several investigators (Crouse, et al., 1950; Scott, et al., 1953; and Lebrun, 1956). First there is an enlargement of the nucleolus which disappears and is replaced with a great increase in basophilic material (Feulgen positive) which fills the nucleus. Gradually the basophilia disappears until finally there remains only an eosinophilic mass within the nucleus, which is devoid of nucleic acid (Feulgen negative). These observations have been further substantiated by Lebrun (1956) through the use of cytochemical and fluorescent antibody techniques. Antigen characteristic of the infection, probably viral antigen, was first detected as a tiny round spot well within the nucleus followed by development of increasing numbers of such spherical areas. Gradually a network of antigenic material appeared inside the nucleus until the nucleus contained a large homogenous area of viral antigen. This large area was basophilic and Feulgen positive. Later, specific antigenic materials appeared diffusely in the cytoplasm, increasing in amount, whereas the antigenic material in the nucleus decreased until it became small or absent. Cells at this stage of the process showed a typical esoinophilic intranuclear inclusion (Feulgen negative). On the basis of this evidence, Lebrun (1956) postulated that viral synthesis occurs in the nucleus and that the inclusion formed represented an "intranuclear scar". The electron microscopical evidence of Morgan, et al., (1954) appear to support this contention. Only particles with a single membrane were seen in the nuclei, while particles with double membranes were restricted to the cytoplasm and the extracellular environment.

Crystalline formations presumably of virus particles, have been observed in the nuclei of HeLa cells infected with adenoviruses (Harford, et al., 1956; Morgan, et al., 1956; and Tousimis and Hilleman, 1957). No evidence was found to suggest that the development of the virus occurred within the cytoplasm. In cytochemical studies these crystals have been demonstrated to be strongly Feulgen positive. However, in cells infected with type 5 adenovirus, Morgan, et al., (1957) have observed in the nuclei large crystals, readily seen with the light microscope, which are Feulgen negative. The significance and nature of this protein is yet to be determined, although the authors have likened it to the noninfectious protein of plant viruses.

Characteristic intracellular inclusions have been observed to appear among the various insect viral diseases studied thus far (Bergold, 1953). Most of the studies on the crystalline inclusions resulting from insect viral infections have been conducted with the polyhedral disease virus. The polyhedral disease virus, besides being enclosed within a crystalline matrix, is surrounded by two membranes (Bergold, 1953 a,b; and Morgan, et al., 1955). Bergold (1954) on the bases of chemical evidence has demonstrated that the membranes are different from the virus. The membranes lack deoxyribonucleic acid but possess a higher content of lipids and nonprotein components than the virus. The amino acid analyses of polyhedral protein, virus and membranes, showed that each is significantly different from the other. The virus and membranes are more similar to one another than either is to the polyhedral protein. From this the author suggests that the polyhedral protein is not a building block or precursor of the virus particles, but rather a metabolic by-product of the host induced by the virus infection. Bergold and

Friedrich-Freksa (1947) have shown also that the protein of the crystalline polyhedral matrix is not serologically related to the virus particles.

The Virus Progeny:

Most of our information concerning the origin of the molecular precursors of the virus particle have been derived from the bacteriophage system. It is in this aspect that the isotopic technique has proved most valuable and has only recently been applied to other viral systems.

The incorporation of radioactive phosphorus from the medium into certain animal viruses has been reported by Graham and McLelland (1949); Hoyle, Jolles and Mitchell, (1954); and Hoyle and Frisch-Niggemeyer, (1956) for influenza virus, and Franklin, et al., (1957) for the Newcastle disease virus. In both virus systems the isotope is incorporated into the nucleic acid and phospholipid fractions. These studies have not attempted to distinguish quantitatively the contributions of the host cell and medium toward the formation of the new particle. However, it is known from investigations on the growth requirements of influenza virus and poliovirus, that nearly all precursors are of endogenous origin, that is, contributed by the host cell and not from the medium (Ackermann and Francis, 1954; and Eagle and Habel, 1956).

In a series of experiments with P^{32} (Kozloff, 1953; Kozloff and Putnam, 1950; Putnam, et al., 1952; and Putnam, 1952), with N^{15} (Putnam, et al., 1952; Putnam, 1952; and Siddiqi, et al., 1952) with C^{14} purines (Koch, et al., 1952; Koch, 1953), and with C^{14} lysine (Siddiqi, et al., 1952), it was shown that about one-third of the P of T6 phage and about one-quarter of the N originates in components of

the host cell laid down prior to infection, whereas three-fourths of the P and N of the small virus T7 originates from the same source. These observations were confirmed by Labaw (1951) for the P of other T viruses. Through the use of bacterial cells doubly labeled with N^{15} and P^{32}, Kozloff, (1953); Putnam (1953); and Evans (1954), have reported the conversion of bacterial DNA to viral DNA. The data indicated that most of the bacterial DNA was transferred to the virus, but it appeared that prior to the synthesis of the phage DNA, host DNA was degraded to fragments such as deoxyribonucleotides. This was further confirmed from the quantitative similarity in the transfer of P, nucleotide bases and deoxyribose (Lanning and Cohen, 1954). With regard to the synthesis of viral 5-hydroxymethylcytosine (5-HMC) Cohen and Weed (1954) found that the cytosine of host DNA is converted to 5-HMC and thymine of virus DNA, whereas the thymine of host DNA is used to make viral thymine, but not 5-HMC. The complete details of this conversion are not yet known, but recently Flaks and Cohen (1957) have been able to demonstrate the presence of an enzyme in extracts of phage-infected bacteria which will hydroxymethylate deoxy-cytidylicmonophosphate in an <u>in vitro</u> system.

A series of kinetic studies have established that there is no specific obligate transfer of a moiety of host DNA to each virus particle. Weed and Cohen (1951) have infected bacteria in which the pyrimidines of the nucleic acid were labeled with C^{14}, with phage T6. They analyzed the progeny obtained after normal lysis as well as phage obtained by premature lysis of the infected cells with sodium cyanide. From comparison of the specific activity of the thymidylic and deoxcytidylic acids of the virus derived from premature lysis of the cells, with that in which

lysis was normal, Weed and Cohen (1951) deduced that the early virus particles formed contained all the pyrimidines transferred from the host. Through studies on the kinetics of P^{32} assimilation in T4 phage, Stent and Maaløe (1953) concluded that the earlier formed virus particles received a greater proportion of their P from the host than do the later ones. These studies, while they confirm transfer of host cell material to the virus, disaffirm the concept of a specific material host contribution to each virus particle. In other words all individual phages do not receive an equal measure of P or nitrogenous bases from the host cells.

The extensive studies on the synthesis of viral DNA have no counterpart in the formation of phage protein. Most of the protein of T6 is synthesized de novo from the components of the medium. Experiments with N^{15} (Kozloff et al., 1951) and with C^{14} lysine (Siddiqi et al., 1952), have shown that only about 10 per cent of the viral protein N of T6 is derived from host protein. The results with labeled lysine indicated that a small contribution to the new viral mass originates from the bacterial protein, while the major part is derived from the pool of free amino acids. Nor does bacterial protein significantly contribute N for synthesis of viral nucleic acid (Koch et al., 1952). Unlike the case with T6, almost half of the protein N of the small phage T7 is derived from host sources (Putnam et al., 1952).

In studying the nature of the parental contribution to the progeny, the isotopic marker incorporated into the virus particle has once again proved valuable. Work along these lines has been most extensive within the phage system. Research along similar lines among animal viruses has hardly been initiated. Quantitative studies of the parental contribution in phage systems have shown that from 10 to 50 per cent of the infecting

viral nucleic acid N, C, and P is contributed to progeny, but less of the N and none of the S appears in the progeny protein (Putnam and Kozloff, 1950). Maaløe and Watson (1951), French, et al., (1952), and Hershey, (1953) all have reported a transfer of about 35 per cent of the parental P to the progeny. About the same extent of C^{14} labeled adenine transfer has also been reported by Watson and Maaløe (1953). However, the values for P transfer appear to be lower for the small phage T7 (Mackal and Kozloff, 1954). All these transfers alone indicate an extensive chemical rearrangement and excludes a transfer via intact virus.

The question whether the parental nucleic acid contribution has any genetic significance or not is yet to be fully resolved. Although the subject will not be discussed in this review, mention must be made of the work of Levinthal (1956). The author through autoradiography showed that the viral DNA is made up of two fractions that differ in particle size; one consists of uniform pieces of about 50 million molecular weight units, the other of smaller pieces of undetermined size. The large pieces, each containing 40 per cent of the P from one phage particle, number one per particle. According to the author its singularity and uniformity suggest that the large piece is either germinal substance or chromosome. The fact that the DNA of T2 is composed of two fractions is further supported by the investigation of Brown and Martin (1956), who through chromatography successfully separated out two fractions, each differing slightly in purine-pyrimidine composition.

In conclusion it can be said that much of our current concept of animal virus reproduction has been drawn by analogy from what is known of the bacteriophage system. Although advances in our knowledge of phage

multiplication have acted as a definite stimulus in presenting new approaches to the problems of mammalian virus-cell interaction, the greatest caution should be exercised in accepting a priori the above mentioned analogies. Our knowledge of animal viruses is now in its "logarithmic phase of development" and as the results of the present investigation as well as other current literature would indicate - animal viruses may show marked differences from bacteriophage and even among themselves.

MATERIALS AND METHODS

Many of the methods adopted during this work are well established biochemical and virological techniques, and therefore do not necessitate detailed description.

The Virus:

The virus selected for these studies was the Mahoney strain of Type I poliovirus, originally isolated in this laboratory. It has been passaged serially in HeLa cells.

Virus pools were made by introducing a massive inoculum of virus into a 500 ml Blake bottle containing about 20 to 25 million HeLa cells grown as a monolayer. The multiplicity of exposure was about 100 to 150 plaque-forming units (PFU) per cell, thus permitting a single cycle growth of the virus. Previous to the introduction of virus, the growth medium of the cultures had been changed to maintenance solution 90 per cent and horse serum 10 per cent (MS90EqS10). After two hours of incubation at 37°C, the media of the infected cultures were removed and the cells washed three times with 20 ml Hank's balanced salt solution to reduce the residual virus to a minimum. The cultures were then overlayed with 40 ml of fresh MS90EqS10 and re-incubated for another 12 to 18 hours at which time their fluids were withdrawn and centrifuged at 581 x g for 15 minutes to remove cellular debris. The supernatants were pooled and distributed in 2 ml aliquots to screw-capped lustroid tubes, then stored frozen at minus 45°C. Upon thawing, a virus pool prepared in this manner had an infectivity

titer of 2 to 3 x 10^8 PFU per ml. Aseptic techniques were used throughout, and the pools were tested for bacterial contamination by inoculation into thioglycolate broth and beef heart infusion.

The virus pools used during the investigations of the radioactive phosphorus-labeled HeLa cells, were prepared in the same manner as above except that all viral titrations were made in tube cultures of HeLa cells. The titers were expressed as the number of tissue culture infectious doses ($TCID_{50}$) per ml of sample. A virus pool prepared in this manner had an infectivity titer of $10^{6.5}$ to $10^{7.5}$ tissue culture infectious doses. The virus pool was stored and tested for bacterial contamination in the same manner as described above.

The Cell Line:

An epithelial cell line of carcinomatous origin, strain HeLa, obtained from Dr. J. T. Syverton, was used in all the studies. It has been kept in continuous passage in the laboratory since 1953. It was grown essentially according to the method of Syverton, Scherer and Elwood (1954) in 40 per cent human serum (HS40), and 60 per cent Hank's balanced salt solution (BSS60). Only five day old cultures prepared by the method described in detail by the above authors were used.

For the labeling of HeLa cells, the cultures were grown for five days at 37°C in the presence of growth medium containing 125 to 150 microcuries of radioactive phosphorus.

In order to facilitate the comparison of results between each experiment and also permit the quantitation of these results, all data were placed on a per cell basis.

The number of cells in each preparation was determined by counting a suspension of it in a hemacytometer. To obtain the quantity of any component present on a per cell basis, the total amount of that component is divided by the number of cells present in the same preparation.

Growth and Maintenance Media:

For the growth of the cells, a medium composed of 60 per cent Hank's balanced salt solution (BSS60)(Hank, et al., 1949), and 40 per cent human serum (HS40), was used according to the method described by Syverton, Scherer and Elwood (1954).

For the propagation of virus and the study of the metabolic changes of the infected cell, a medium composing of 10 per cent equine serum (EqS10), and 90 per cent Scherer's maintenance solution (MS90) (Scherer, 1953) was used.

Procedure of Infection:

The procedure used for studies of the metabolism of HeLa cells infected with poliovirus was as follows: Before introduction of the virus, the maintenance medium which had been introduced 24 hours previously was removed, and the cell cultures were rinsed with 5 ml BSS100 twice, and replaced with 4 ml of MS90EqS10. Since a one-step growth curve of the virus was desirable, the cultures were given a massive inoculum of virus permitting the maximum number of cells to be infected simultaneously. The multiplicity of exposure is about 100 PFU per cell. The completeness of infection was verified by

maximal yields of virus obtained at 7 hours, and also by supporting studies in which the spread of infection was restricted by the use of immune serum (Payne, Kurtz and Ackermann, 1958).

After introduction of the virus, the cultures were incubated at 37°C for one hour, at which time the residual virus was removed and the cultures washed three times with 10 ml of BSS100. Seven ml of new MS90EqS10 were introduced, and the cultures were replaced in the incubator until the desired period of infection was attained.

The control cultures were treated in the same manner, but instead of the virus, they received a like quantity of MS90EqS10 medium.

Before introduction of the virus into the radioactive phosphorus labeled HeLa cells, the cultures were washed twice with BSS100, then incubated with maintenance solution at 37°C for a half hour. This latter treatment was repeated one more time. When such a procedure is followed, the same or nearly the same level of activity in the medium is re-established. If the incubation is carried on further, this loss of radioactivity soon levels off (6 to 7 hours), and an equilibrium appears to be established between the cells and the bathing medium.

After the washing sequence, fresh medium consisting of MS90EqS10 is added, and the cultures are then ready for infection.

The control cultures underwent the same treatment, but in place of the virus, they received an equal amount of MS90EqS10 medium.

Radioactive Phosphorus:

Sterile solutions of $P^{32}O_4^{=}$ in dilute HCl (specific activity 60 to 90 x 10^3 millicuries per gram) were obtained from the Oak Ridge

National Laboratories, Tennessee. The concentration of P^{32} introduced into each bottle was 125 microcuries (μc). The radioactive sample was neutralized with 0.1 N NaOH before addition to the culture.

In experiments where P^{32} was used, the isotope was introduced at the last half hour of any infectious period under study in both control and infected cultures. Radioactive samples were plated on aluminum dishes with depressions 3/4" in diameter and 1/8" in depth and dried under a heat lamp. The amount of radioactivity was determined with a Geiger tube (Tracer Laboratory, Model TGC-1), which was situated in a lead-shielded chamber (Technical Associates, Model LS6) and connected to a scaler (Nuclear Instrument and Chemical Corporation Model D181).

The amount of P^{32} incorporated into the various cellular fractions was expressed on a per cell basis.

For the determinations of the amount of radioactivity released from infected and normal cultures into the media, samples of the media from the respective cultures were plated on aluminum dishes, dried and counted according to the method described above.

Separation of Cytoplasm and Nucleus:

Both the control and infected cultures were treated in the same manner for the separation of cytoplasm and nucleus. All the work was carried out at 0 to 4°C.

At the end of any period of study the cultures were examined for any changes in cellular morphology due to infection. In certain experiments where the appearance of extracellular virus was determined, samples of the media were saved. In other instances the media were discarded.

The cultures were then washed three times with 15 ml of buffered saline at room temperature. The washings were intended to reduce to a minimum the contaminating P^{32}, virus and maintenance medium. The cells were then removed with a rubber policeman into an ice-cold saline-citrate mixture and homogenized three times with a Potter-Elvehjem homogenizer (Teflon pestle).

The homogenates were centrifuged in the refrigerated International centrifuge at 581 x g for 10 minutes, and the supernatants after each centrifugation were pooled. The pooled supernatants represented the cytoplasmic fraction and the sediment the nuclei. A sample of the nuclei was stained with methyl green and examined under high power for the completeness of the separation. Only 10 to 20 per cent of the cells were still intact.

Differentiation Into Sub-cellular Components:

In certain experiments the cytoplasmic fraction was further differentiated into its sub-cellular components by high speed differential centrifugation in a refrigerated Spinco Model L centrifuge using rotor S40. The speeds employed were those recommended by Hogeboom and Schneider (1955) and were as follows: 6590 x g for 20 minutes for Fraction I (mitochondrion), 41,190 x g for one hour for Fraction II (microsome); the resulting supernatant from the latter centrifugation being Fraction III (cell sap). No further efforts were made to determine the efficiency of such a fractionation.

Virus Assays:

Virus assays were made to determine the efficiency of the infection and the appearance and distribution of intracellular virus.

The plaque technique of Dulbecco and Vogt (1954), modified for use with the HeLa cell system by Payne, Kurtz and Ackermann (1958), was used. All the titers determined in this manner are expressed as plaque-forming units (PFU) of virus per cell.

In the series of experiments using HeLa cells labeled with radioactive phosphorus, the amount of virus was estimated by determining the final limiting dilution which would initiate infection in 50 per cent of the tube cultures of HeLa cells inoculated. The method is essentially that described by Ackermann, Rabson and Kurtz (1954). The titers are expressed as the number of tissue culture infectious doses ($TCID_{50}$) per ml of sample. The 50 per cent endpoints were calculated by the method of Reed and Muench (1938).

Extraction of Nucleic Acids:

The isolation of cytoplasmic ribonucleic acid (cRNA) and the ribonucleic acid (nRNA) and deoxyribonucleic acid (nDNA) of the nucleus was accomplished by using a combination of several published methods. The method of Schneider (1945) employing cold and hot 5 per cent trichloroacetic acid was used to obtain the cRNA fraction. The nRNA and nDNA of the nucleus were separated and determined according to the method of Schmidt and Thannhauser (1945).

To determine the efficiency of the separation into cytoplasmic and nuclear nucleic acids, the cytoplasmic fractions after RNA extraction from both infected and normal cultures, were tested for the presence of

DNA by the diphenylamine reagent as described by Dische (1955). No contamination of the cytoplasmic fraction with DNA was observed in any of the experiments.

Phosphorus Determination:

The phosphorus content of the nucleic acid fractions was determined according to the method of Fiske and Subbarow (1925). The data are expressed on a per cell basis.

Protein-Nitrogen Determination:

The nitrogen content of the washed protein residue obtained after acid precipitation and extraction of lipid and nucleic acid, was determined according to the method of Koch and MecMeekin (1924). The results are expressed on a per cell basis.

Base Analyses of RNA Preparations:

Base analyses were made in certain experiments on the isolated cytoplasmic ribonucleic acids from infected and normal HeLa cell cultures. The nucleic acids were hydrolyzed to a mixture of mononucleotides according to the method of Volkin and Carter (1951), and separated by anion-exchange chromatography on Dowex-1 (formate) columns according to the method of Hurlbert, et al., (1956).

The eluate, containing the nucleotides, was collected in 5 ml fractions by a G. M. E. Volumetric Fraction Collector (Gilson Medical Electronics, Madison, Wisconsin), and the 260 mu optimal density of each of the fractions was measured in a Beckman Spectrophotometer, Model DU.

From the specific extinctions of the nucleotides, the amount of each nucleotide was calculated. The extinction coefficients used here were: at pH 2 Adenylic acid E_{260} = 14.2; Cytidylic acid E_{260} = 6.2; Uridylic acid E_{260} = 10; Guanylic acid E_{260} = 11.8 (Beaven, Holiday and Johnson, 1955).

In order to facilitate the comparison of the composition of different RNA preparations, the results are expressed as the molar ratios of the nucleotides relative to adenine as 10.

Immune Serum:

Type specific hyperimmune monkey sera, prepared against HeLa cell propagated poliovirus Type I, (Mahoney strain) was obtained from Dr. F. E. Payne of our laboratory. It was titrated in stationary tube cultures of HeLa cells and neutralized 100 $TCID_{50}$ of virus at dilutions from 1:3200 to 1:6400. The serum was pre-heated at 56°C for 30 minutes before use.

In all neutralization experiments, the serum-virus mixture and the virus controls were pre-incubated for one hour at 37°C before inoculation into the HeLa cell cultures.

Reagent Solutions:

(a) Phosphate-Buffered Saline (PBS): The 0.01M phosphate-buffered physiological saline was taken from the general laboratory supply. The pH was adjusted to 7.2.

(b) Citrate-Saline Solution: This extraction medium is neutral with regard to hydrogen ion concentration and is made up of 0.1M sodium chloride containing 0.05M sodium citrate as described by Crampton, Lipshitz and Chargaff, (1954).

EXPERIMENTS AND RESULTS

I. METABOLISM OF PHOSPHORUS-BEARING COMPOUNDS FROM NORMAL AND INFECTED HELA CELLS LABELED WITH RADIOACTIVE PHOSPHORUS (P^{32})

Radioactive phosphate is readily taken up by HeLa cells and contributes to the endogenous pool of phosphate where it is available for incorporation into a number of compounds of varying complexities; for example, the phosphorproteins, phospholipids, nucleic acids ... etc. Thus it is possible to pre-label many of the phosphorus-containing structures of the cell prior to infection, and to follow the fate of these compounds after infection, and during the synthesis of virus and its subsequent release. The stability and integrity of the cell structure can thus be followed during the period of interest. This is accomplished simply by determining the loss or transfer of labeled structures from the cell to the extracellular medium.

If HeLa cells are grown in growth medium (BSS60HS40) containing radioactive inorganic phosphate, the isotope is readily incorporated into the various phosphorus-bearing compounds of the cell. The procedure for labeling cells and the preparation of them for infection is described in detail in the previous section under Materials and Methods.

(a) The Release of P^{32}-Labeled Compounds from Normal Cells:

A five-day monolayer culture containing approximately 4 to 5 x 10^6 cells labeled with P^{32} and containing 8 ml of unlabeled maintenance medium (MS90EqS10), was allowed to incubate at 37°C for a period of 12 hours. At hourly intervals 1.5 ml aliquots of the medium were removed and their amount of radioactivity determined. The aliquots were replaced each time with the same volume of fresh maintenance medium.

The data which were obtained by this experiment are recorded as the concentration of radioactivity found in the medium at various times, and are expressed as counts per minute per ml (Figure 1). The data have been corrected for the concentration of radioactivity contained in those samples removed at the various times, and replaced with fresh unlabeled maintenance medium.

It will be noted that there was an appearance of radioactivity within the first hour and that the concentration increased with time at a decreasing rate as the cells carried on their normal metabolic activities, and adjusted to the changed medium. Ultimately, by the sixth or eighth hour, the rate of accumulation approached zero and the concentration of radioactivity remained constant.

It is clear since the only source of radioactivity is the cell, that these data indicate an initial release or loss of cellular material to the medium which in time approaches zero. This is probably due to the introduction of fresh medium, which causes the cells to undergo a period of reconditioning, during which an equilibrium is being attained between the endogenous supply of P^{32} and that of the medium. However, the specific activities of the medium at termination of this release and that of the endogenous pool of phosphate were not measured.

(b) The Release of P^{32}-Labeled Compounds from HeLa Cells Infected with Poliovirus: A five-day old monolayer culture which contained approximately 4 to 5 x 10^6 cells labeled with P^{32} was prepared for experimentation (Cf. section on Materials and Methods). The growth medium had been replaced with 7.2 ml of unlabeled maintenance medium and a sufficient amount of poliovirus (0.8 ml) was introduced to produce a

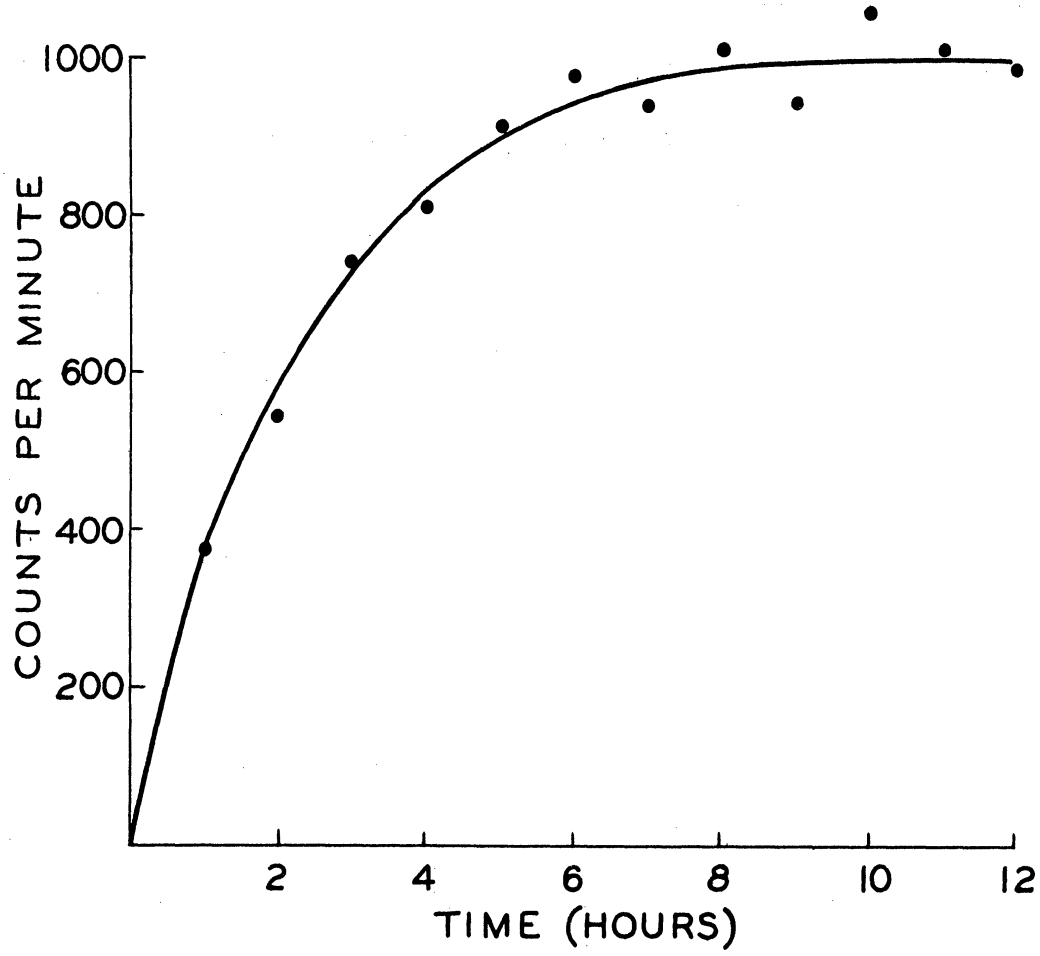

Figure 1. Release of Radioactivity from Uninfected HeLa Cells Labeled with Radiocative Phosphorus: The cells were grown in the presence of growth medium containing 125 μc of P^{32}. Then the culture was washed and reincubated at 37°C in fresh isotope-free MS90EqS10 and reincubated at 37°C. At hourly intervals samples were taken and the amount of radioactivity released into the medium was determined. The results are expressed as counts per minute per ml.

final concentration of $10^{6.5}$ tissue culture infectious doses per ml. In a similar manner a replicate culture without poliovirus was prepared as a control. Both cultures were incubated at 37°C for a period of 12 hours. At hourly intervals 1.5 ml aliquots of medium were removed for determination of radioactivity. The aliquots were replaced each time with an equal volume of fresh medium. During the terminal hours of the incubation period, the samples were centrifuged lightly to remove any intact cells or large cellular debris, which may have been separated from the cell layer.

The data obtained are recorded in Figure 2 where the concentration of radioactivity found in the extracellular fluid is plotted as a function of time. Since the volume of the culture is essentially constant, the curve obtained represents the rate of the release. The rates for both the control and infected cultures are initially quite similar and by the sixth hour both approach zero. However, after the sixth hour, they diverge sharply as the release by the infected culture begins to accelerate. The curve is concave upward indicating a continuous increase in the rate even until the twelfth hour when the experiment was terminated.

(c) The Release of P^{32}-Labeled Compounds from HeLa Cells Infected with Varying Concentrations of Poliovirus: In order to establish whether the release of compounds from the cell into the medium is due to viral action, the following experiments were formulated.

It has been shown that in a given culture of HeLa cells infected with poliovirus, the number of cells undergoing cytopathology is related to the exposure multiplicity of infection (Payne, Kurtz and

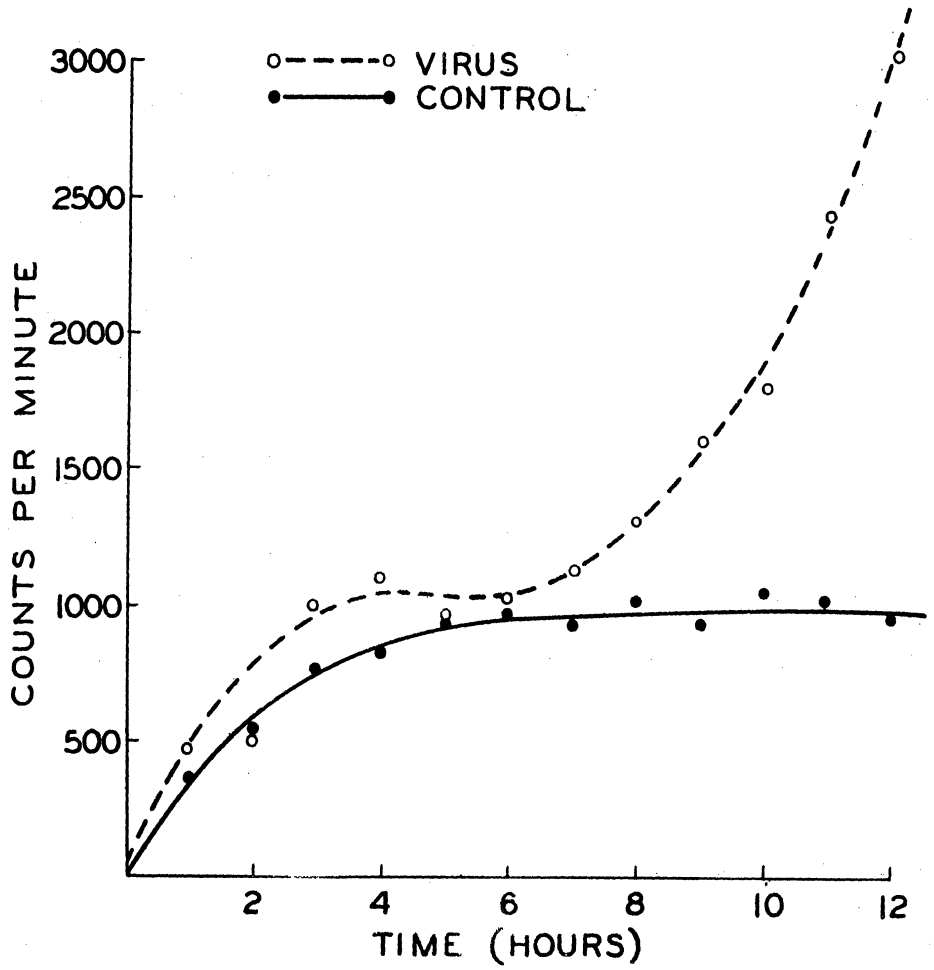

Figure 2. The Release of Radioactivity from Uninfected and Infected HeLa Cells Labeled with Radioactive Phosphorus: The amount of poliovirus introduced was sufficient to produce a final concentration of $10^{6.5}$ $TCID_{50}$ per ml. At hourly intervals, samples were taken from each culture, and the amount of radioactivity released determined. The results are expressed as counts per minute per ml.

Ackermann, 1958). One might, therefore, expect that the release of labeled compounds from the infected cells would bear a relationship to the multiplicity of exposure. The next experiment was principally designed to determine this relationship.

Comparable cultures of HeLa cells, prepared as detailed in the previous section, were exposed to a range of viral concentrations. Various cultures were infected with virus ranging in concentration from 2.5×10^5 to 30×10^5 tissue culture infectious doses per ml for each bottle culture. To another comparable culture, which served as the control, an equivalent amount of maintenance medium was added. All of these cultures were incubated at 37°C for a period of 12 hours. The procedures for collecting samples and determining the radioactivity were the same as described in the previous section.

The results of this experiment can be seen in Figure 3 where the radioactivity released from the sixth to the twelfth hour following infection by the various virus concentrations is plotted against time. These values have been corrected for the dilution factor caused by the replacement of the samples taken with unlabeled medium and also for the normal release of radioactivity by the uninfected culture. The latter correction is made by subtracting the amount of radioactivity released at any time by uninfected cultures from that by infected ones. Along the abscissa is plotted the various viral concentrations. The largest concentration of virus used is assigned the value of 100, the next concentration 50, the next 25, then 12.5, and lastly, the smallest concentration 8.3.

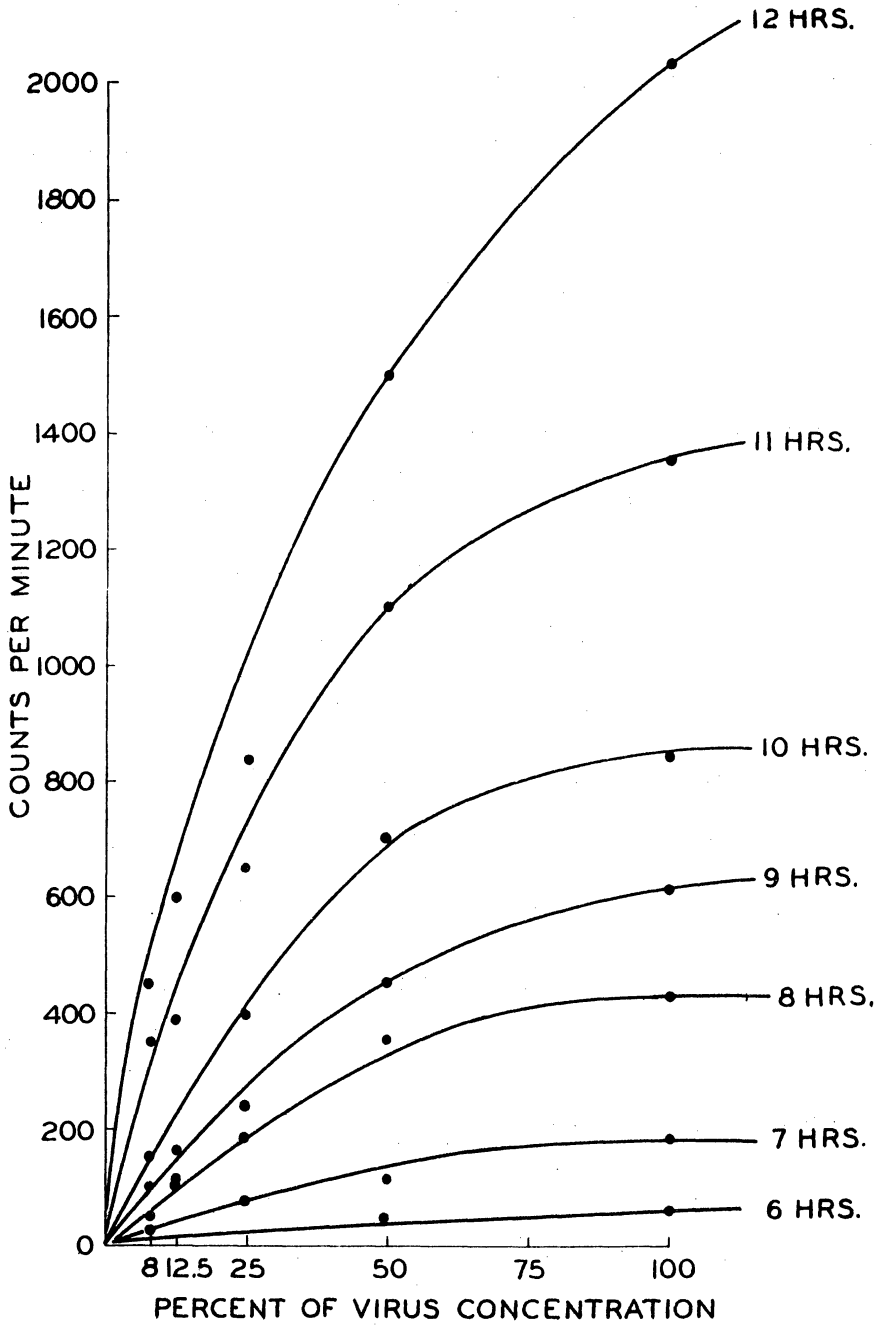

Figure 3. The Release of Radioactivity in Response to Graded Doses of Virus: The concentrations employed were 30×10^5, 15×10^5, 8×10^5, 4×10^5 and 2.5×10^5 $TCID_{50}$ per ml of culture medium. The largest concentration of virus is assigned the value of 100, the next 50, then 25, 12.5 and lastly 8.3. At hourly intervals samples were taken from each cultures, and the amount of radioactivity released determined. The results are expressed as counts per minute per ml. These values have been corrected for the release of radioactivity from uninfected cells.

The results show that with increasing virus concentrations, there is an increase in the rate of P^{32}-labeled compounds released. However, the rate of release does not have a proportional relationship with the viral concentration. There are a number of possible explanations for this phenomenon. It may be due to the fact that when a small concentration of virus is used, only a few cells become infected, and only these cells are producing virus and releasing labeled cellular constituents. However, even with a massive concentration of virus, not all of the cells become infected, for in a line of heterogenous cells their susceptibility to virus infection, their metabolic and physiologic activities are not all uniform. There also exists a constant fraction of cells which remains resistant to the virus (Ackermann, 1957). Thus the efficiency of infection decreases with high concentrations of virus to a greater degree than might be expected by a multiplicity of infection which obeyed a Poisson distribution.

Also the production of virus is affected by the size of the viral concentration used. With small multiplicities of virus the latent period, that is the time from adsorption to intracellular appearance of new virus, is prolonged; whereas with larger multiplicities this period is considerably shortened (Dulbecco and Vogt, 1954; Dunnebacke, 1956; and Darnell, 1958). On the basis of the preceding observation one can then suggest that if there exists more than one site of viral replication in these cells, then in a singly infected cell, the virus would only activate a single site before other sites become activated; while in the case of a multiply infected cell several sites would be activated simultaneously. If this is so then the cells that are multiply infected will

be releasing larger amounts of labeled constituents at certain times than those that are singly infected.

(d) The Action of Virus Neutralized with Antiviral Serum on the Release of P^{32}-Labeled Compounds from HeLa Cells: To further establish this relationship between release of cellular compounds and virus action, an experiment was designed to study the effect of virus neutralized with antiviral serum on the release of compounds labeled with P^{32} from HeLa cells.

A viral inoculum containing $10^{7.5}$ tissue culture infectious doses was neutralized by the addition of specific hyperimmune monkey serum and the mixture was incubated for one hour at 37°C. The neutralized mixture (0.8 ml) was added to a culture of HeLa cells which contained 7.2 ml of unlabeled maintenance medium. The bottle culture contained approximately 4 to 5 x 10^6 cells labeled with radioactive phosphorus, and was prepared for experimentation as described in the preceding sections.

To one of two other cultures prepared in a similar manner, an equivalent amount of maintenance medium was added, and to the other, an equal amount of virus. All these cultures were incubated at 37°C for a period of 12 hours. The procedures for collecting samples and determination of radioactivity were the same as those described in the preceding sections.

The data obtained are plotted in Figure 4. It will be noted that the pattern of release displayed by the cultures inoculated with the neutralized mixture is similar to that of the uninfected control. The patterns of release of radioactivity by the three cultures are quite

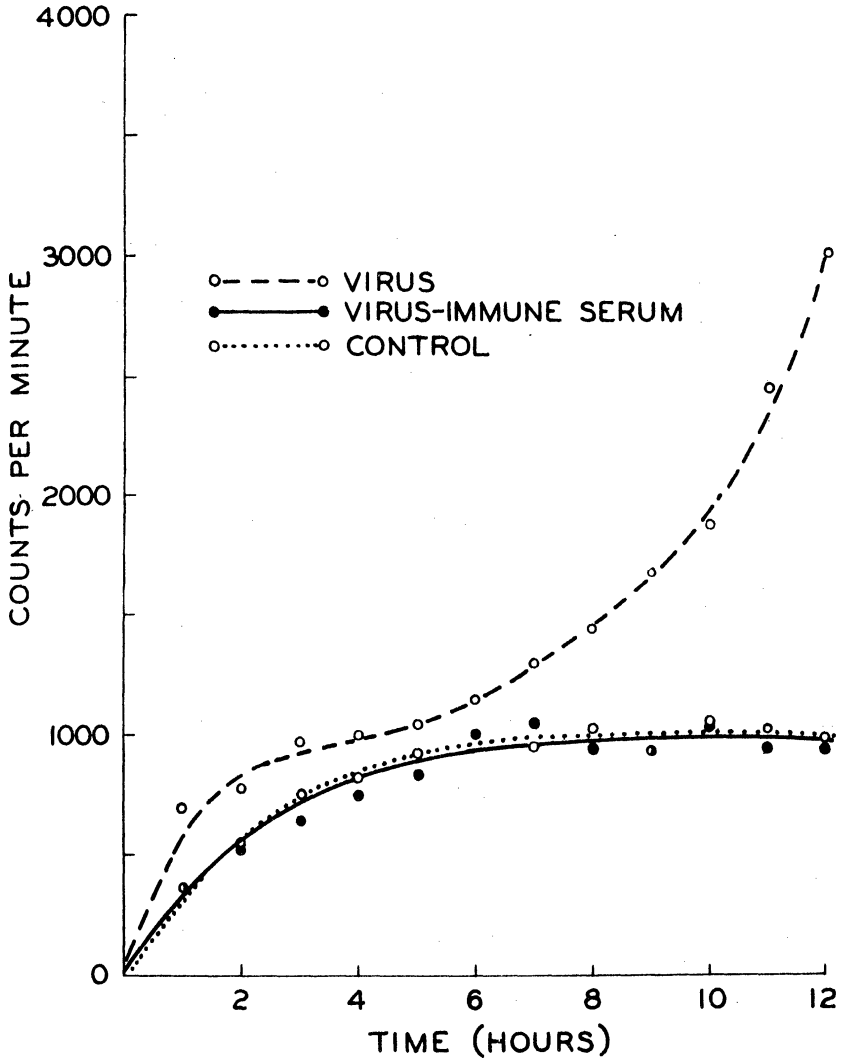

Figure 4. The Action of Virus Neutralized with Antiviral Serum on the Release of Radioactivity From HeLa Cells Labeled With Radioactive Phosphorus: A viral inoculum containing $10^{7.5}$ $TCID_{50}$ was neutralized with an equal amount of undiluted antiviral serum, and the mixture incubated for one hour at 37°C. The neutralized mixture was then added to a culture of HeLa Cells. To a second culture an equal amount of unneutralized poliovirus was introduced. The uninfected control received an equivalent amount of maintenance medium. At hourly intervals samples were taken from each culture and the amount of radioactivity released determined. The results are expressed as counts per minute per ml.

similar during the early hours (1 to 5 hours). However, after 6 to 8 hours, and even up to the twelfth hour when the experiment was terminated, increasingly greater radioactivity is released by the infected culture; whereas, that released by the culture which received the neutralized viral mixture is as low as that of the uninfected control.

The experiments up to the present suggest that the increased rate of release of labeled compounds from infected cells is a direct result of viral action. This conclusion is based upon the following observations:

a. the increase in rate of release is seen only in virus-infected cultures;

b. the response to graded concentrations of viral inocula is varied; and

c. the increase in rate of release is not seen when a neutralized mixture of virus and specific immune serum is added.

While each of these experiments in themselves may not be regarded as absolutely conclusive, together they strongly suggest that this phenomenon is due to viral action.

(e) The Relation of Release of Radioactivity to Virus Production: Since the increase in release of labeled compounds has been observed to accompany viral action, an obvious question arises as to its relative position in the virus multiplication process. With that purpose in mind the following experiment was set up to determine the temporal relation of virus production to the release of labeled compounds.

Of three replicate cultures of HeLa cells, two were inoculated with virus to give a final concentration of $10^{6.5}$ tissue culture infectious doses per ml, and one was inoculated with an equivalent amount of maintenance medium as a control. One of the infected cultures was used to measure virus production. After an hour exposure to the virus at 37°C, the residual inoculum was removed from this bottle, and the culture washed three times with balanced salt solution. The washing procedure was to reduce the amount of residual virus that might otherwise have masked the beginning of viral release. After washing, the culture received fresh maintenance medium. All cultures were then incubated at 37°C for a period of 12 hours. At two hour intervals, samples for virus determination were taken and titered in tube cultures of HeLa cells (Cf. section on Materials and Methods). The procedures for collecting samples and determination of radioactivity were the same as those described in the previous sections.

The results can be seen in Figure 5. The amount of radioactivity released and the amount of virus released into the extracellular fluid phase are plotted against time. The values portray only that radioactivity which is in excess to that released from normal cells. After a constant period of approximately 5 to 6 hours, during which the release of radioactivity is similar to that of the control, there is in the infected culture an increase in release of radioactivity. The beginning of this release appears to accompany the release of the virus, which occurs at about 5 to 7 hours.

Comparable cultures were examined for morphologic changes through time-lapse cinematography by Dr. W. W. Ackermann of the laboratory.

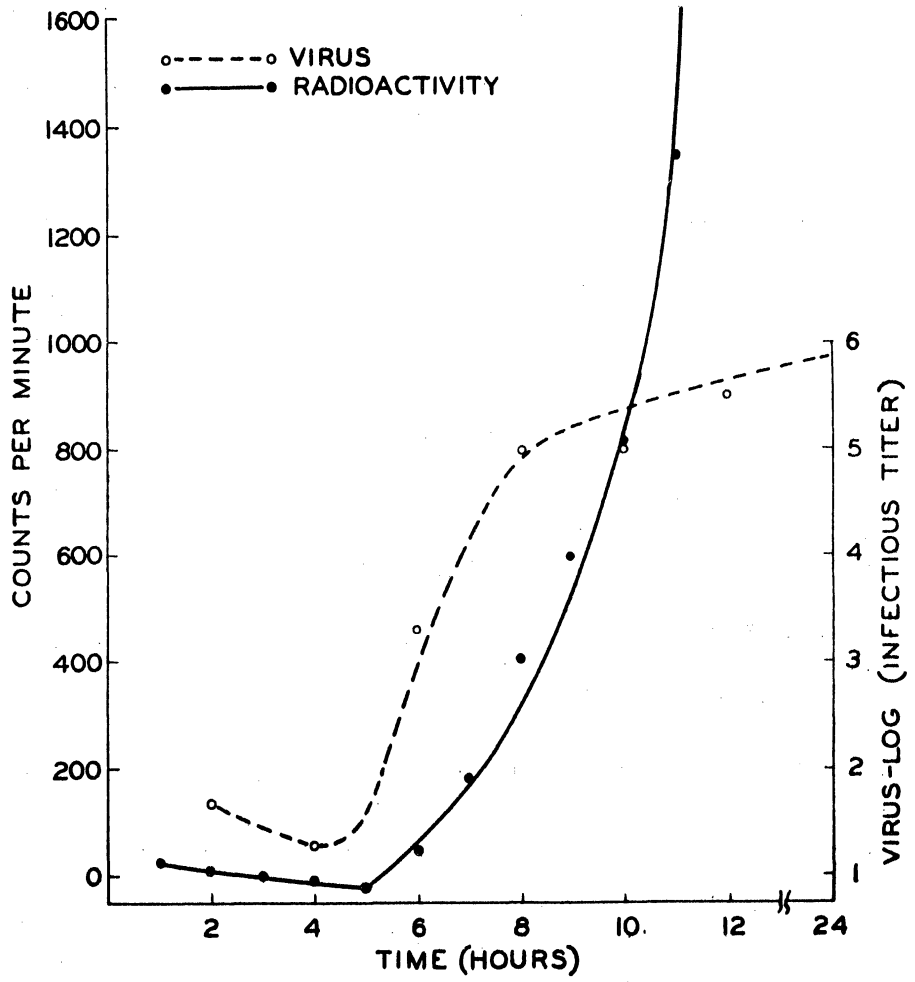

Figure 5. The Relation of Release of Radioactive Phosphorus to Virus Production: The amount of poliovirus introduced was sufficient to produce a final concentration of $10^{6.5}$ $TCID_{50}$ per ml. Extracellular virus samples were taken at two hour intervals, and titered in tube cultures of HeLa cells. The titers were expressed as the number of $TCID_{50}$ per ml of sample. At hourly intervals, samples were taken from each culture, and the amount of radioactivity released determined. The results are expressed as counts per minute per ml. These values have been corrected for the release of radioactivity from uninfected cells.

When release of radioactivity from infected cells is compared with the morphological changes that are seen in time-lapse cinematography, one observes a retraction of the cytoplasm, which appears to accompany the beginning of release of labeled compounds and also of virus. Similar morphologic changes have been observed through time-lapse cinematography techniques by Barski, et al.,(1955) and also by Harding et al., (1956) in multicellular cultures, and in single cell cultures by Lwoff, et al, (1955). In all instances cytoplasmic retraction is observed to begin at about the time virus is released.

Lwoff, et al., (1955) had observed in single monkey kidney cell cultures infected with poliovirus a release pattern which together with the morphologic data suggested that viral release occurred through partial lysis of the cell. They found that the bulk of the virus is released within thirty minutes, and practically all the virus is released in less than one hour. However, in interpreting their results on single cells, the fragility of isolated cells cannot be disregarded. Also the present evidence and that which will follow later appears to indicate otherwise. Furthermore, evidence presented by other investigators using multicellular systems (Howes and Melnick, 1957; and Roizman, et al., 1958) appear contrary to Lwoff's suggestion. However, in multicellular systems, only the average release pattern is observed, for it is difficult to know if the infection is synchronized.

In spite of the limitations of the present technique, some justifiable conclusions can be derived. It can be said that two processes appear to accompany the release of new virus particles into the extracellular medium. The two processes are: (1) cytoplasmic retraction,

and (2) increased release of labeled cellular constituents into the extracellular medium. Further, these data appear mandatory to defining the period during which a chemical study of virus synthesis is feasible.

II. THE NUCLEIC ACID METABOLISM OF HELA CELLS DURING VIRAL DEVELOPMENT

In the previous series of experiments it was shown that when radioactive phosphorus labeled HeLa cells were infected with poliovirus, increased release of labeled cellular material into the extracellular medium occurred at about the time of viral release. However, it did not reveal the early changes that might occur during the process of viral multiplication.

It has been found by cytochemical and histological methods that infection of a cell with poliovirus produces clearly discernible changes in the cytoplasm and nucleus of the cell. Chief among these detectable by fixation and staining are the early alterations of the chromatin pattern of the nucleus, nuclear pyknosis, and the progressively increased basophilia of the cytoplasm (Ackermann, et al., 1954; Reissig, et al., 1956; Howes and Melnick, 1956; and Harding, et al., 1956). Thus an examination of the various chemical constituents, particularly the nucleic acids, of the cell early during infection may prove to be fruitful. The previous series of experiments have shown, that if such a biochemical study is to be made, the period studied should not extend further than seven hours, for after this period the infected cell undergoes chemical and morphological disintegration. The next phase in the present investigation is an examination of the changes in nucleic acid

metabolism of the infected cell through chemical and isotopic methods. Nucleic acids of the various morphologic components of the cell were isolated and determined throughout the growth cycle of the virus.

All the data presented in the next series of studies were derived through the same experimental design described in detail in the section under Materials and Methods. Briefly the design was as follows: In a typical experiment a series of bottle cultures containing 4 to 6 x 10^6 cells in a monolayer, previously incubated for 18 hours with maintenance medium, were rinsed twice with 5 ml of balanced salt solution and 4 ml of maintenance medium were added. Two ml of undiluted tissue culture fluid (MS90EqS10) containing poliovirus were introduced to give a final concentration of 1 x 10^8 PFU per ml. The cultures were incubated at 37°C for one hour, washed three times with 10 ml of BSS to remove the residual virus, and overlayed with 7 ml of MS90EqS10. The cells were then replaced in the incubator until the desired period of infection was achieved. In certain experiments where radioactive phosphorus was used, the isotope was introduced at the last half hour of any infectious period under study. The amount of P^{32} introduced into each bottle was 125 microcuries.

At the end of any particular period of infection, the cultures were washed three times with 15 ml buffered saline at room temperature and well drained. The cells were then removed with the aid of a rubber policeman into an ice-cold citrate-saline solution. Cell fractionation, isolation of sub-cellular components and subsequent chemical analyses were carried out as described in the section under Materials and Methods.

During the period of exposure to virus nearly all cells were infected. The completeness of infection was verified by maximal yields of virus obtained by seven hours, and also by corollary experiments of Payne and his collaborators (1958) in which the spread of infection was restricted by the use of antiviral immune serum. Further, there is a pronounced intracellular phase in the virus development, as will be seen later in this study, wherein extracellular virus does not appear until the seventh hour. Thus, in these biochemical studies which are limited to the first seven hours of infection, the opportunity of successive cycles of infection being produced by secondary spread of infection to any cells not initially involved is improbable. The observations recorded in the following experiments concern a single cycle of infection.

(a) The Nucleic Acids and Radioactive Phosphorus Incorporation of Normal HeLa Cells: When ordinary HeLa cells, kept in MS90EqS10, were fractionated into cytoplasm and nucleus and analysed, the total RNA was found to be approximately 2.5 times greater than the DNA. Although the amount of nuclear RNA is similar to the cytoplasmic RNA, both are larger than the nuclear DNA. The high content of RNA is in agreement with the view that actively growing tissues, such as tumor cells, or embryonic cells, and those possessing high metabolic activities, such as organ tissues, are rich in RNA content (Smellie, 1955).

In the second column of Table I, the actual amounts of the nucleic acids per cell fraction are given. They are expressed as milligrams of nucleic acid phosphorus per cell.

TABLE I

THE PHOSPHORUS AND P^{32} DISTRIBUTION IN THE HeLa CELL

Fractions*	CONTROL		
	Phosphorus mgm x 10^{-10}/cell	Counts+ x 10^{-10}/cell	Relative Specific Activity counts/cell / mgm P/cell
cRNA-P	24.11 ± 1.85	2.65 ± 1.17	109.91
nRNA-P	27.37 ± 3.90	40.75 ± 1.93	1488.85
nDNA-P	19.81 ± 1.00	1.28 ± 0.43	64.61

* The amount of nucleic acid is expressed as milligrams of phosphorus in each fraction. The cRNA-P, nRNA-P and nDNA-P fractions correspond respectively to cytoplasmic ribonucleic acid phosphorus, nuclear ribonucleic acid phosphorus, and nuclear deoxyribonucleic acid phosphorus. The values recorded here are averages from ten experiments.

+ The rate of uptake of radioactive phosphorus (P^{32}) was determined over a half hour period. The P^{32} added contained a total of 125 μc of activity.

The uptake of P^{32} into the various nucleic acid fractions is tabulated in the third column of the same Table. HeLa cell cultures were exposed to P^{32} as inorganic phosphate in the fluid medium during the last half hour of a 6 hours incubation period, and the uptake is expressed as counts per three minutes per fraction of a single cell. The pattern of uptake generally follows that of cells from several species as studied by other investigators (Marshak, 1948; Jeener and

Szafarz, 1950; and Smellie, et al., 1953). The highest rate of incorporation of P^{32} is seen in the nuclear RNA, followed by the cytoplasmic RNA and nuclear DNA. The amount of P^{32} incorporated into the total RNA is about 34 times greater than that in the DNA fraction. This phenomenon has generally been found true for resting cells (Smellie, 1955), for the ratio of the amount of P^{32} in RNA to that in DNA is smaller only in tissues, such as regenerating liver or embryonic tissues, where the metabolic activity of the DNA is greatly increased. Although the amounts of RNA are similar in the cytoplasm and the nucleus, the latter fraction displays the highest incorporation of P^{32} within a definite period of exposure.

In column 4 is given the specific activity of each fraction calculated by dividing the number of counts per cell fraction by the milligrams of nucleic acid phosphorus in that fraction. The relative incorporation is seen to be greatest in the nuclear RNA and least in nuclear DNA.

Since the experimental period studied extended from zero to 7 hours, normal HeLa cell cultures, which underwent the same preparative procedures as the infected ones, but only received maintenance medium, were incubated at 37°C. At various intervals of time these cultures were taken out and analysed for the nucleic acids of the various fractions. These results can be seen in Table II, which contains data from four representative experiments wherein the cell number, composition with regard to DNA and RNA, and radioactive phosphorus incorporation into each of the nucleic acid fractions were determined at two time intervals of the 7 hour experimental period. The amounts

TABLE II

CELL NUMBER AND NUCLEIC ACID COMPOSITION OF UNINFECTED CONTROL CULTURES AT VARIOUS TIMES DURING INDIVDUAL EXPERIMENTS

Exps.*	Time** Hours	Cell Count x 10^6	cRNA-P		nRNA-P		nDNA-P	
			mgm x 10^{-10}/cell	Counts x 10^{-4}/cell	mgm x 10^{-10}/cell	Counts x 10^{-4}/cell	mgm x 10^{-10}	Counts x 10^{-4}/cell
A	2	40.64	23.56	3.05	25.78	42.30	20.23	1.24
	4	44.16	22.50	3.20	26.71	41.13	18.95	1.30
B	2	51.87	26.46	2.82	32.70	40.66	18.70	1.33
	4	49.56	25.51	2.72	31.80	42.63	21.35	1.31
C	1	50.81	23.82	2.84	28.30	42.31	19.51	1.09
	6	47.47	22.36	2.63	24.81	39.89	20.30	1.06
D	1	39.60	22.50	2.56	30.10	40.72	19.81	1.31
	6	37.57	23.12	2.82	25.91	41.37	20.43	1.40

* These cultures were prepared and treated with maintenance medium in parallel with virus infected culture (see Materials and Methods). The amount of P^{32} added per bottle culture is 125 μc. The isotope was added during the last half hour of any incubation period.

** Time is measured from the inoculation of the replicate cultures with virus.

of nucleic acids and P^{32} incorporated by the various fractions did not vary significantly within the experimental period studied. The invariance of the normal cultures allows for the use of a single control culture at any period in an experiment. It is also of interest to note that although there was no net increase in the various nucleic acid fractions, the respective fractions readily incorporated the isotope. The incorporation of exogenous inorganic radioactive phosphorus into the different nucleic acid fractions of resting cells of animals and in tissue culture systems has been demonstrated by several investigators (Brown and Roll, 1955; Smellie, 1955; Miroff, et al., 1957; Rothstein, et al., 1958; and Becker, et al., 1958).

(b) Incorporation of P^{32} Into Nucleic Acids of Infected HeLa Cells: The data of Figure 6 show the amount of radioactive phosphorus incorporated into the various nucleic acid fractions of the infected cell. These data represent the uptake of P^{32} over a short period of time at various intervals of the infectious cycle, and hence they are an expression of rate. Since the pattern of incorporation of the isotope of the uninfected controls did not vary significantly throughout the period studied, the experimental values are recorded as per cent changes relative to the control.

By the first hour after infection, the rate of incorporation of P^{32} into the two nuclear fractions was from 150 to 160 per cent of the control. This enhanced rate continued for the next 3 hours, after which there was an abrupt decline in this nuclear activity. Nuclear morphologic changes have been observed by Reissig, et al., (1956); Harding, et al., (1956); and Howes and Melnick, (1957), as early as

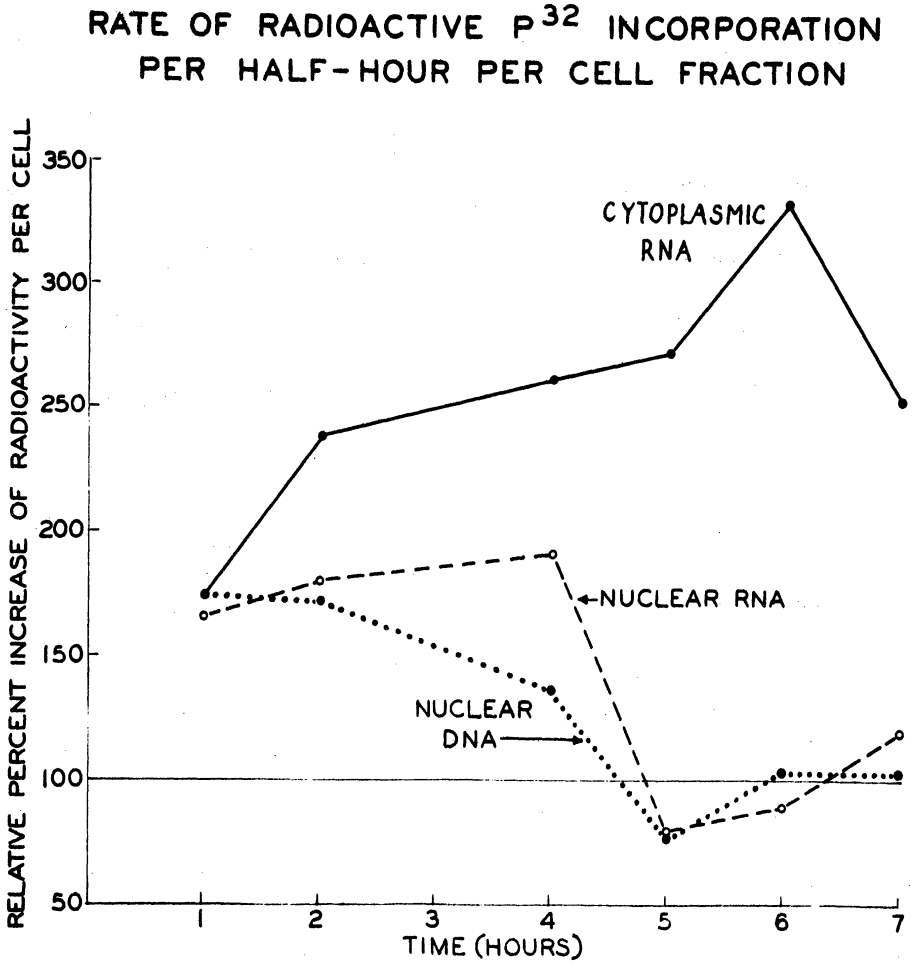

Figure 6. The rate of P^{32} Incorporation Per Half-Hour in Nucleic Acids: The cells were exposed to a final concentration of 1 x 10^8 PFU per ml of virus for a period of one hour. Then the culture was washed three times with BSS, replaced with fresh maintenance medium and incubated at 37°C. The additions of P^{32} contained a total of 125 μc per culture. The amount of P^{32} incorporated is expressed as the per cent change relative to the control.

3 to 4 hours after infection with poliovirus. There is alteration of the chromatin pattern and appearance of Type B acidophilic intranuclear inclusions followed later by the occurrence of nuclear pyknosis. Tenenbaum (1957) by means of fluorescence microscopy found in poliovirus infected monkey kidney cells a modification in the staining pattern of nuclear DNA. This change appeared to be an early consequence of the infection. Changes in cytoplasmic RNA appeared a little later.

The uptake of P^{32} into the cytoplasmic RNA was also enhanced by the first hour, and the rate increased until it was maximal at the sixth hour. This maximal rate might be 250 to 300 per cent greater than that of the uninfected cell. After the sixth hour the activity of the cytoplasmic fraction declined sharply.

The increased incorporation of exogenous inorganic radioactive phosphorus into the ribonucleic acids of cells infected with poliovirus, has been observed by several investigators (Miroff, et al., 1957; Becker, et al., 1958; and Rothstein, et al., 1958). However, they have not reported on the later decline in the incorporation of the isotope. This may be due to the fact that these investigators have in some instances utilized a different cell line, a different type of poliovirus or a different set of experimental conditions; but it is more likely that these results were obtained because an incomplete sequence of infection was studied.

(c) Changes in Nucleic Acids Following Infection: When cultures of HeLa cells were analysed for nucleic acids after various periods of exposure to poliovirus, they were found to undergo progressive

changes during a 7 hour period. Since the nucleic acids of ordinary cells were found not to differ significantly during the 7 hour period studied, the resulting data seen in Figure 7, were recorded as the per cent change in the infected cell relative to the uninfected control. By the first hour after infection, there appeared a small increase in nuclear RNA and DNA. These increases remained somewhat constant until the fourth hour, after which they declined. This pattern appears to be related to the increased incorporation of P^{32} into these two fractions, and as will be seen later, to the appearance of intracellular virus which occurs at the end of the fourth hour. It indicates an enhanced metabolic activity rather than net synthesis of nucleic acids. Early changes occurring in the nuclei of poliovirus-infected cells, as observed by several investigators, have been mentioned in the previous section.

Usually by the first hour after infection, an increase in cytoplasmic RNA was detectable. The actual increase varied from one experiment to another. It might be from 110 to 150 per cent of the control. The increase in cytoplasmic RNA continues progressively until the sixth hour at which time the RNA might have increased by 250 per cent. However, between the sixth and seventh hour no further accumulation occurred.

This accumulation, when taken together with the increased incorporation of P^{32}, undoubtedly reflects an actual synthesis of nucleic acid. It is suggestive that virus material most probably is being synthesized in the cytoplasm. Buckley (1956), using the fluorescent antibody technique, has been able to detect traces of antigen in

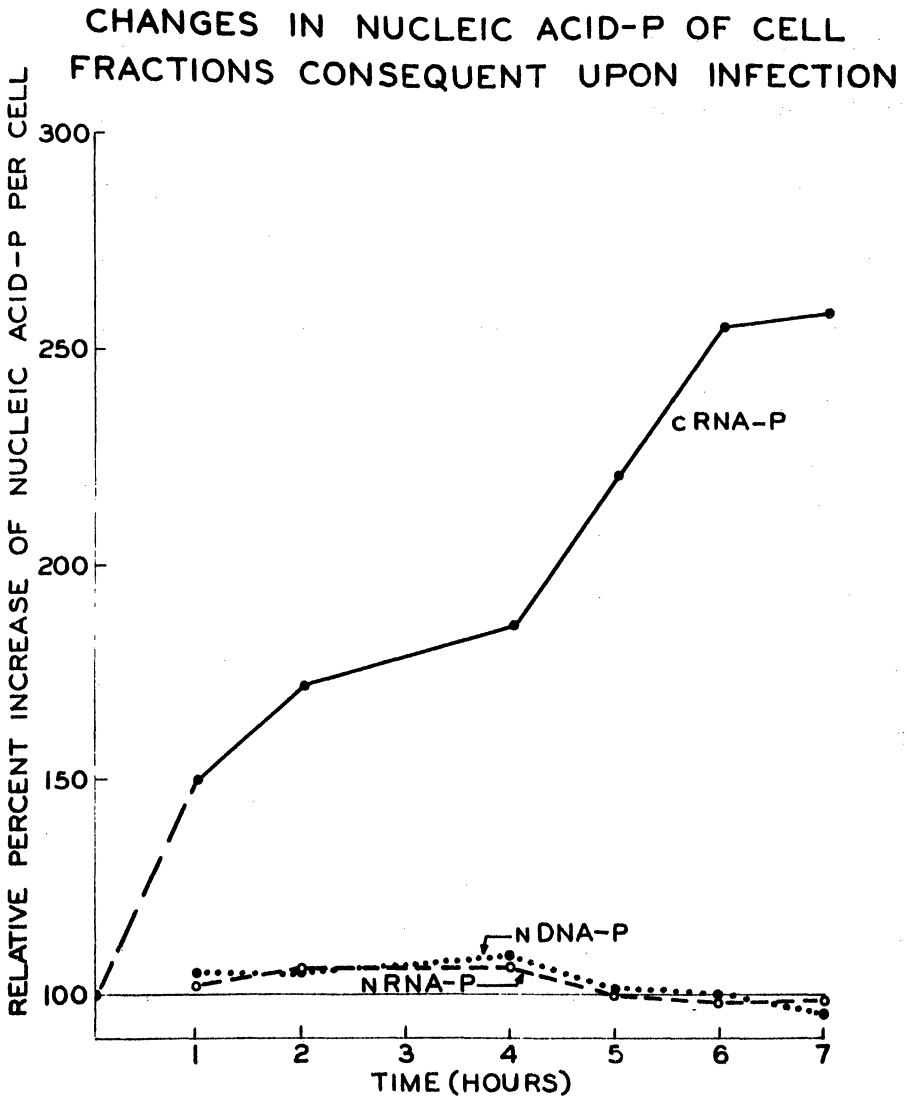

Figure 7. Changes in the Nucleic Acid-Phosphorus of Cell Fractions Consequent Upon Infection: The cells were exposed to a final concentration of 1 x 10^8 PFU per ml of virus for a period of one hour. Then the culture was washed three times with BSS, replaced with fresh maintenance medium and incubated at 37°C. Nucleic acid was determined as nucleic acid-phosphorus, and expressed as the per cent change relative to the control.

the cytoplasm as early as 3 to 5 hours following inoculation of poliovirus. The changes observed here are congruous to those obtained by tinctorial methods as reported by other investigators (Ackermann, et al, 1954; Reissig, et al., 1956; Howes and Melnick, 1957). They observed an increase in cytoplasmic basophilia, which progressed further with advancement of the infection. Harding, et al., (1956) with the use of ribonuclease and cytological techniques, showed that the surrounding cytoplasm of infected cells appeared to be rich in ribonucleic acid.

(d) Intracellular Appearance of Viral Activity: When poliovirus-infected HeLa cells at various periods of infection are homogenized and separated into their nuclear and cytoplasmic fractions, the preponderance of virus was found in the latter fraction. The intracellular appearance of the virus can be seen in Figure 8. The results are presented as the number of plaque-forming units (PFU) of virus per cell. The first increase in viral activity was not detectable until the third hour after infection. The virus continues to increase and accumulate in the cytoplasm until the seventh hour. Extracellular virus does not appear until the sixth to seventh hour after infection. It must be noted that at the seventh hour, when 99 per cent of the total viral yield had been formed, only 1 per cent of the new virus is in the extracellular state. These data clearly indicate an intracellular phase in the development of poliovirus as has been reported by other investigators (Reissig, et al., 1956; Buckley, 1956; Howes and Melnick, 1957; Roizman, et al., 1958; and Darnell, 1958). They have observed that at no time during the period of infection studied did the free virus constitute more than a small proportion of the total virus

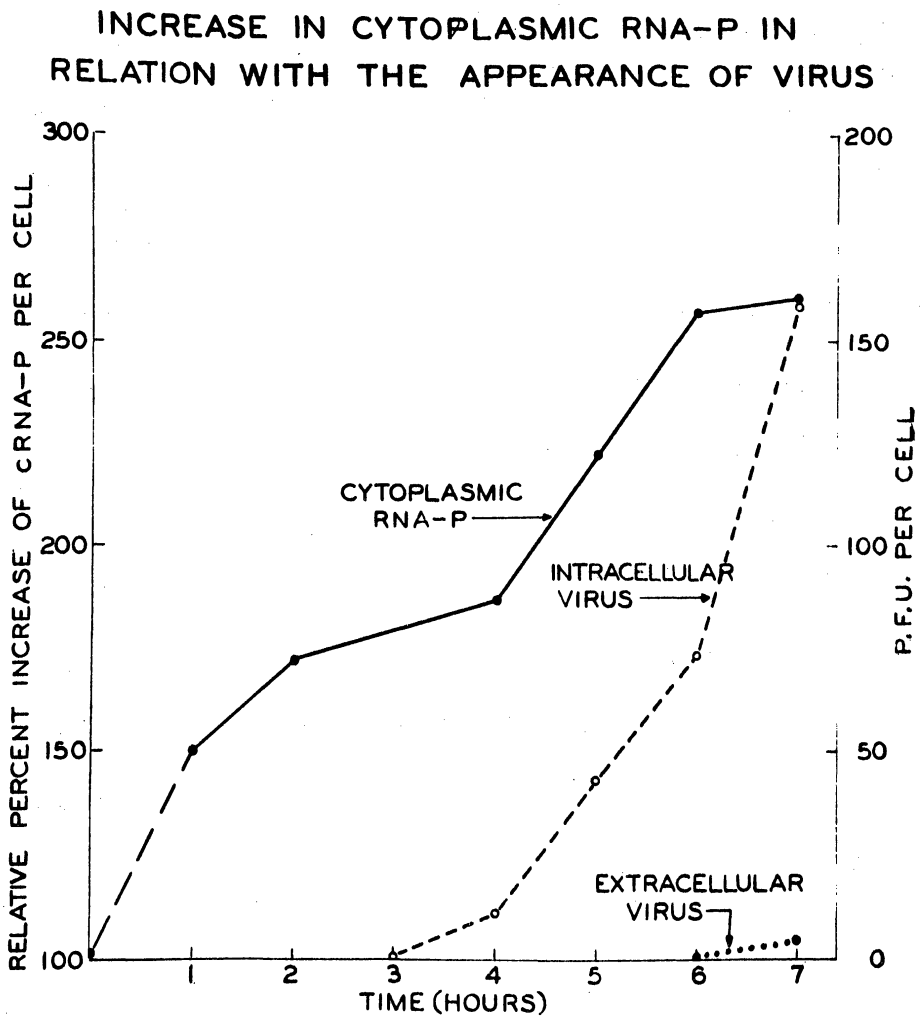

Figure 8. Increase in the Cytoplasmic RNA-Phosphorus in Relation to the Appearance of Virus: The cells were exposed to a final concentration of 1 x 10^8 PFU per ml of virus for a period of one hour. Then the cultures were washed three times with BSS, replaced with fresh maintenance medium and incubated at 37°C. Both intracellular and extracellular virus samples were taken at hourly intervals, and titered according to the plaque technique. Virus titers are expressed as PFU per cell. The amount of cytoplasmic RNA-phosphorus is expressed as the per cent change relative to the control.

(less than 20 per cent). That a prolonged delay in release may occur, has been shown by Girardi, et al., (1956) for HeLa cells in suspension, especially when held at 25°C (Larson, et al., 1957). This delay in release of poliovirus from cells suggests a possible fundamental difference between the growth cycle of this virus in HeLa cells, and that of such viruses as Western equine encephalomyelitis virus in chick fibroblasts or vesicular stomatitis in monkey kidney or chick embryo cells, where release is very rapid (about one minute)(Rubin, et al., 1955; and Franklin, 1958).

Howes and Melnick (1957) have postulated that the delay in viral release might lie in a physicochemical barrier to release associated with a structure normally present in the cell but perhaps under these conditions modified by infection, or with abnormal structures present only in the infected cell (Reissig, et al., 1956). Such a barrier may permit release at a restricted rate so that mature virus accumulates in the cell during maturation.

As has been discussed in the previous section, the release of virus appears to be accompanied by a release of P^{32}-labeled compounds into the medium; this process occurring in a continuous progressive manner for a period of several hours.

The results obtained above show that in this and similar virus systems, it is necessary to assay total virus (free virus plus intracellular virus) in order to obtain an accurate picture of virus maturation. In studies concerned with the temporal relationship between virus synthesis and biochemical activities or morphological changes in the cells, assay of only the free virus may be misleading.

In the same Figure 8 the increase in cytoplasmic RNA synthesis during the same period of infection is also illustrated. It can be seen that virus appears in the cytoplasm after the synthesis of RNA has begun. The virus increases concomitantly with the RNA synthesis and continues for some time (between the sixth and seventh hour) after the maximal synthesis of RNA has been reached.

When the changes in cytoplasmic RNA are correlated with the intracellular growth pattern of the virus, they appear to agree with the cytological data found by other investigators (Ackermann, et al., 1954; Harding, et al., 1956; and Reissig, et al., 1956). They observed an early increase in cytoplasmic basophilia which progressed further with advancement of the infection. Initiation of all such changes in the infected cell appears to precede the appearance of virus with the cytoplasm.

The evidence obtained here is once again suggestive that virus material is most probably being synthesized in the cytoplasm. Buckley (1956) using the fluorescent antibody technique, observed traces of poliovirus antigen in the cytoplasm as early as 3 to 5 hours following inoculation of the virus, and concluded the possibility of virus antigen being synthesized in the cytoplasm.

Thus far the investigation has revealed, among the biochemical changes that occur during the course of poliovirus infection in the HeLa cell, an increase in cytoplasmic RNA synthesis. It is apparent that the next phase of this investigation would be: a) a substantiation of the results utilizing another method for the determination of the increase in cytoplasmic RNA; and b) a characterization of the incremental cytoplasmic RNA.

(e) Amounts of Nucleotides in Cytoplasmic RNA of Normal and Infected HeLa Cells: To substantiate the increase seen in cytoplasmic RNA, as determined by the Schneider method and phosphorous analysis, the cytoplasmic ribonucleic acids obtained from both infected (6 hours after infection) and normal HeLa cells were hydrolyzed to a mixture of nucleotides and chromatographically separated. The methods employed are described in the previous section under Materials and Methods.

Upon chromatographic separation of the hydrolyzed nucleic acids, only the usual four nucleotides of RNA were obtained (Table III). The number of micromoles of each nucleotide was determined. When infected and normal cells were compared, the former was found to contain approximately 150 per cent more of each of the nucleotides.

(f) Nucleotide Composition of Normal and Infected HeLa Cells:

In order to facilitate the comparison of the composition of normal and infected RNA preparations, the results were expressed as the molar ratios of the nucleotides relative to adenine as 10. It is in this manner that the composition of RNA preparations from normal and infected cultures is presented in Table IV. These values likely represent the average composition of several species of molecules of RNA which comprise the cytoplasmic fraction.

The nucleotide composition of the RNA isolated from the cytoplasmic fraction of normal HeLa cells is very similar to that reported for RNA isolated from other mammalian tissues, in that it is rich in guanylic and cytidylic acids and poor in adenylic and uridylic acids. The ratio of purines to pyrimidines is generally not far from unity. However, the molar ratio of the bases of the cytoplasmic RNA does appear

TABLE III

AMOUNT OF EACH NUCLEOTIDE IN CYTOPLASMIC RNA (cRNA) OF NORMAL AND INFECTED HeLa CELLS

Nucleotide	μM of Nucleotide x 10^{-9}/cell		
	Normal	Infected	ΔNucleotide*
Cytidylic acid	11.26 ± 3.4	28.75 ± 8.6	17.49
Adenylic acid	7.4 ± 1.9	19.05 ± 6.1	11.65
Guanylic acid	16.05 ± 3.1	38.49 ± 8.8	22.44
Uridylic acid	8.41 ± 1.7	21.03 ± 7.54	12.62

NOTE: Samples for analysis were obtained from HeLa cells 6 hours after initiation of infection with poliovirus and from normal HeLa cells treated in the same manner without exposure to virus. Values recorded here are averages of data from five experiments.

*Δ Nucleotides = the difference between normal and infected cells in the amount of each nucleotide in the cRNA.

to be different from those of other animals and from that of a variety of human tissues (Smellie, 1953; Chargaff, 1955).

The nucleotide composition of the RNA isolated from purified poliovirus (Mahoney strain) Type I is recorded in column 5 (Schwerdt, 1957). The viral RNA is quite different in composition from that of the uninfected HeLa cell. The values for the virus are lower, especially for cytidylic and guanylic acids. If a minimal value of 120 per cent is taken for the increase in cytoplasmic RNA which results from infection,

TABLE IV

NUCLEOTIDE COMPOSITION OF CYTOPLASMIC RNA (cRNA) IN NORMAL AND INFECTED HeLa CELLS

Nucleotide	Normal	Infected		Virus**
		Observed	Calculated*	
Cytidylic acid	15.4 ± 0.41	15.3 ± 1.14	9.2	6.3
Adenytic acid	10.00	10.00	10.00	10.0
Guanylic acid	20.2 ± 1.14	19.8 ± 1.13	12.0	8.4
Uridylic acid	10.2 ± 0.95	10.9 ± 0.51	8.8	8.1

NOTE: Relative values for the amounts of nucleotides in the cRNA of normal and infected cells were derived from data presented in Table III. These values are for cells 6 hours after initiation of infection.

* The nucleotide composition of cRNA of infected cells was calculated assuming that a conservative minimal increase of 120 per cent above normal would be attributable to the RNA of poliovirus.

** Data on the nucleotide composition of poliovirus type 1 (Mahoney) is from Schwerdt (1957).

and if the incremental RNA is assumed to be viral RNA, the ratio of bases that would be obtained from such a mixture of 120 parts of viral RNA and 100 parts of ordinary cytoplasmic RNA can be calculated. The calculated ratio of bases in such a theoretical composite is given in column 4 of Table IV. The hypothetical composite is shown to differ significantly from that of the normal cell and also that of the virus.

However, when the nucleotide composition of the RNA from the cytoplasm of the infected cell which contains the increment of newly formed RNA was determined experimentally, it was found to differ significantly from the hypothetical composite, but not from that of the normal cell (column 3, Table IV). While viral RNA must be synthesized in the infected cell, the major portion of the newly formed RNA induced by virus infection is not of the virus type, resembling more closely that of the ordinary cell cytoplasm.

(g) Analysis of the Sub-Cellular Components of Normal and Infected HeLa Cell Cytoplasms: Since the cytoplasmic RNA is a composite of several species of ribonucleic acids existing in the cytoplasm (Smellie, 1955), fractionation by differential centrifugation procedures of the cytoplasmic RNA into its sub-cellular components would thus indicate where such an increase exists in the infected cytoplasm. Further, the elimination of excess normal RNA would facilitate detection of viral nucleic acid, if it were localized, and also indicate the distribution of viral activity among the various sub-cellular components. If the chemically detectable protein and RNA constitutes virus, then their distribution among the various sub-cellular elements of the cytoplasm should parallel that of viral activity. The cytoplasmic fraction from infected (sixth hour after infection) and ordinary HeLa cells were obtained and then further fractionated into three sub-cellular components according to the methods described in the section under Materials and Methods. The amount of P^{32}, RNA-P, protein-N and virus present in each fraction was determined and recorded in Table V.

TABLE V

DISTRIBUTION OF PROTEIN-N AND RNA-P IN THE CYTOPLASMIC COMPONENTS OF NORMAL AND INFECTED HeLa CELLS

Cytoplasmic Fraction	Normal			Infected			Virus PFU
	RNA-P			RNA-P			
	Counts* x 10^{-4}	mgm x 10^{-10}	mgm N x 10^{-10}	Counts x 10^{-4}	mgm x 10^{-10}	mgm N x 10^{-10}	
I	1.45	1.95	59.74	2.73	3.21	91.52	15.5
II	1.25	4.55	54.37	2.88	13.26	73.05	313.5
III	4.45	20.38	248.19	6.54	25.51	379.87	54.5

NOTE: Cytoplasm for ultracentrifugal fractionation was obtained from HeLa cells 6 hours after initiation of infection with poliovirus and from normal HeLa cells treated in the same manner without exposure to virus. All data are on a per cell basis.

* Cultures containing approximately 6 x 10^6 cells were exposed to 125 microcuries of P^{32} during the last 30 minutes of incubation.

The rate of incorporation of radioactive isotope (P^{32}) for a half hour period of exposure varied with the individual sub-cellular components of the normal cytoplasm (Table V). There is no significant difference in the amount of P^{32} incorporated into the ribonucleic acids of the two particulate fractions. However, the activity of the RNA of Fraction III was considerably higher than that of the particulate fractions. This was found also by Barnum and Huseby (1950, 1953) for the cytoplasmic fractions of mouse mammary carcinoma, mouse liver tissues and for cells of other species by Jeener and Szafarz (1950) and Smellie, et al., (1953). In general, the activity of the microsomal RNA proved

to be higher than that of the mitochondria, although in certain instances such as, normal, regenerating and weanling rat liver, normal and embryonic mouse liver, and mouse mammary carcinoma, no significant differences in uptake of P^{32} between the two particulate fractions were observed (Barnum and Huseby, 1950, 1953; and Smellie, 1955).

After infection the incorporation of P^{32} into all fractions increase with that of Fraction II (130.4 per cent) being somewhat greater than Fractions I (88.3 per cent) and III (46.9 per cent). It should be noted that while the rate of incorporation of P^{32} by the infected cell is about twice that of the normal, the net synthesis of RNA is considerable, whereas in the instance of the normal it is not detectable.

The distribution of total RNA-P follows the same pattern as seen with the radioactivity data. In general tumor cells have been reported to have a relatively high percentage of their total cellular RNA in the cell sap fraction, which is represented here in these experiments by Fraction III (Stevens, et al., 1953; Howatson and Hamm, 1955; and Jardetsky and Barnum, 1957). After infection there is an increase in all fractions, but by the sixth hour the greatest percentage accumulation is in Fraction II (191.4 per cent), followed by Fractions I (64.6 per cent) and III (25.1 per cent) respectively. Likewise, all fractions show an increase in protein content. The largest accumulation of protein is in Fraction III, although the per cent increases in Fractions I and III are the same and greater than in Fraction II. The virus activity is found predominantly in Fraction II.

These data are more easily interpreted if it is assumed that there is essentially no degradation of the original components of the

cell after infection, and that the increment of RNA-P and protein measured represents all of the newly formed material. The data for each fraction are expressed in this form in Table VI under the headings RNA-P and protein-N which represent the amounts of newly formed materials. It will be noted that the virus distribution only approximately parallels the RNA-P and does not correspond at all to the protein-N. Only 10 per cent of the incremental protein is in Fraction II, while 81 per cent of the virus and 57.7 per cent of the RNA-P are there.

Further, there is a characteristic ratio of protein-N to RNA-P for each of the components of the uninfected cell cytoplasm and also for the purified virus. (The latter is taken from data of Schaffer, 1957.) Such a ratio can also be calculated for the material in each fraction newly formed after infection. A comparison of these values allows one to determine if the newly formed materials resemble the composition of the normal component with which it is associated, that of the virus, or if it is anomalous. Only in Fraction II are these minimal restrictions of composition such that they do not rule out the possibility that the incremental material is all virus. Only in this case does it not resemble the composition of the normal material of Fraction II. The incremental materials of Fractions I and III more closely resemble the normal components than the virus. However, the variation between the increment of Fraction III, and the normal component is beyond the error of the analytical method.

(h) Kinetics of Net Synthesis of RNA, Protein and Virus: In the previous sections the biochemical situation in the cell at the sixth hour of infection has been described. The following is concerned with

TABLE VI

THE RATIO OF ΔPROTEIN-N TO ΔRNA-P IN THE CYTOPLASMIC COMPONENTS OF NORMAL AND INFECTED HeLa CELLS

Cytoplasmic Fraction	Infected						Normal	
	Virus		ΔRNA-P		ΔProtein-N		ΔProtein-N / ΔRNA-P	Protein-N / RNA-P
	PFU	%	mgm x 10^{-10}	%	mgm x 10^{-10}	%		
I	15.5	4.04	1.26	8.3	31.78	17.3	25.14	30.63
II	313.5	81.74	8.71	57.7	19.68	10.7	2.25	11.94
III	54.5	14.21	5.13	33.9	131.68	71.9	25.76	12.17
Virus							2.33*	

NOTE: Cytoplasm for ultracentrifugal fractionation was obtained from HeLa cells 6 hours after initiation of infection and from normal cells treated in the same manner without exposure to virus. ΔRNA-P and ΔProtein-N values were derived from data presented in Table V and represent the difference between normal and infected cell fractions in the amount of RNA-P and Protein-N. All values are on a per cell basis.

* This ratio was calculated on the basis that poliovirus is 30 per cent RNA and 70 per cent protein.

the temporal sequence by which the situation developed. In attempting to characterize the pattern of increase of cytoplasmic RNA, protein-N of Fraction III and virus, replicate cultures of cells were analysed at various times in the interval from the initiation of infection to the seventh hour. So that the characteristics of development of these materials which differ in absolute amount may be compared, the amount of each at the seventh hour was assigned the value of 100 and the amount at other times some proportion of 100. In this form, data concerning the newly formed protein of Fraction III of the cytoplasm, the total cytoplasmic RNA and virus infectivity are plotted in Figure 9. The synthesis of protein appears to proceed at a constant linear rate from the first hour until the seventh. It must begin almost immediately since the curve extrapolates to zero time. The synthesis of cytoplasmic RNA also begins close upon initiation of infection and appears to be a composite of two phases. The earlier phase, occurring between zero and the fourth hour, closely parallels that of the protein, but proceeds at a slightly slower rate than the second. The latter phase begins at the fourth hour and plateaus after the sixth hour. It is also of interest to note that 50 per cent of the protein and RNA are formed before the appearance of any viral activity. Further, 50 per cent of the virus appears after the synthesis of RNA has halted. With the appearance of virus at the fourth hour, there is an increase in the rate of synthesis of cytoplasmic RNA, and a marked decline in the rate of P^{32} incorporation in the nuclear RNA, which has been described in the previous section (see Figure 6).

It is possible that the increased rate of RNA synthesis which occurs between the fourth and sixth hour of infection represents the synthesis of viral RNA which is superimposed upon the synthesis of

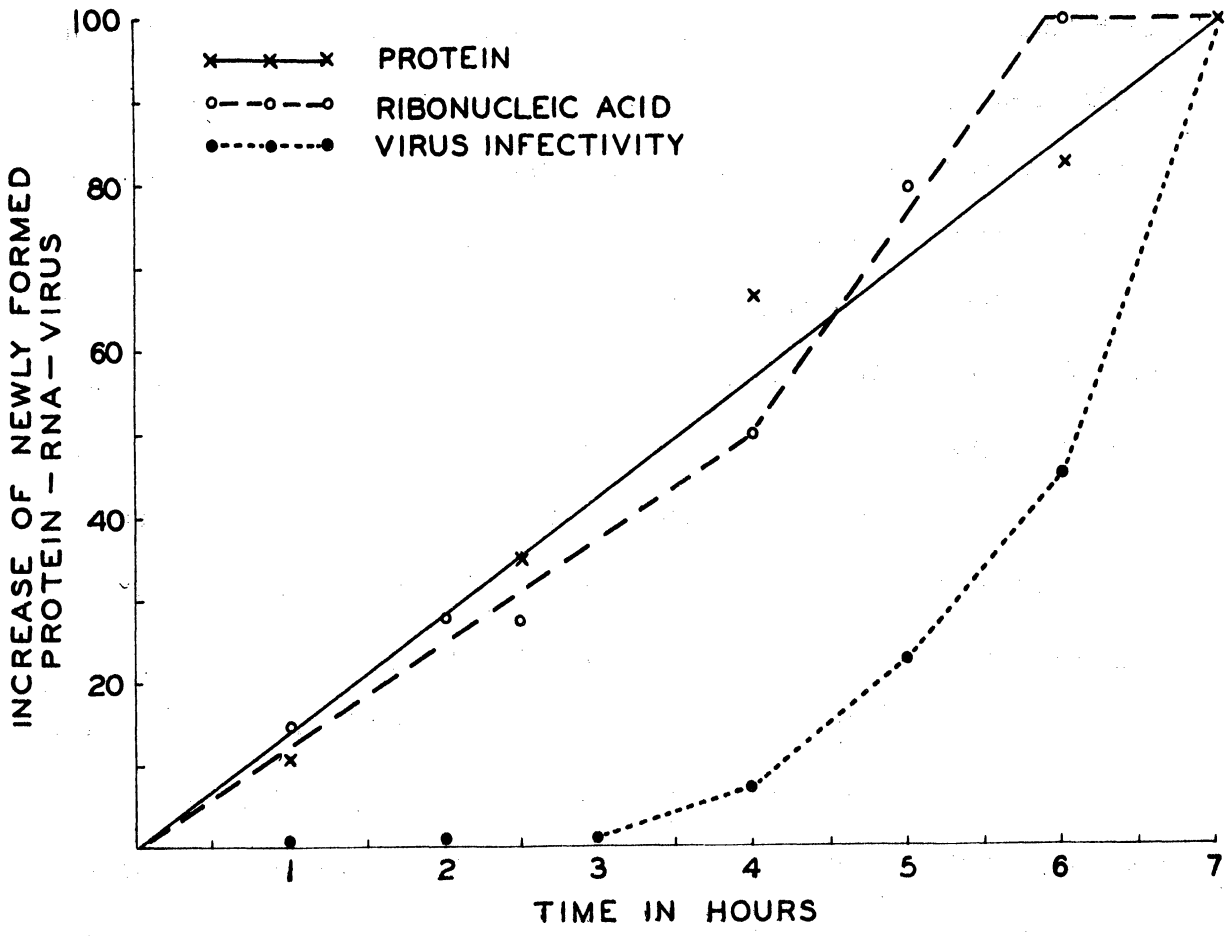

Figure 9. The Increase of Newly Formed Cytoplasmic Protein, RNA and Virus in HeLa Cells at Various Times Following Infection with Poliovirus: The newly formed, or Δ, material represents the difference between normal and infected cells in the amount of each material. Values plotted here are expressed as a percentage of the amount of Δ material found at 7 hours following infection.

KEY: x___x___x Protein of Fraction III of cytoplasm.

o___o___o Ribonucleic acid of total cytoplasm.

•___•___• Virus infectivity of total cytoplasm.

non-viral RNA, which began by the first hour and continues through the sixth hour. Goldfine, et al., (1958) on the basis of incorporation of C^{14}-labeled cytidine into HeLa cells infected with poliovirus, have suggested that the synthesis of viral nucleic acid may occur at about the fifth hour after infection.

The incremental soluble protein fraction may correspond in part to the C and D antigens obtained after ultracentrifugation, and found by Mayer, et al., (1957) and Roizman, et al., (1958) to appear in the soluble fraction of homogenates of poliovirus-infected HeLa cells. Matzelt, et al., (1958) have also found a significant increase in protein content of HeLa cells infected with poliovirus. They have also demonstrated, in a study of six glycolytic enzymes of the cell, that there was a significant increase in enzymic activity of these enzymes in infected cells. This increase in glycolytic activity of cells infected with poliovirus has also been observed by Becker, et al., (1958) in infected human amnion cells. It is a well recognized fact that a major portion of these glycolytic enzymes normally reside in the cell sap or soluble fraction of the cell cytoplasm (Hogeboom and Schneider, 1955).

DISCUSSION

The following picture of the intracellular changes that occur during poliovirus Type I infection of HeLa cells has now emerged from an interpretation of the results of these studies. In Figure 10 is presented a schematic summary of the infection of the HeLa cell with poliovirus. In this diagram the synthesis of large molecules (RNA, DNA, protein and virus) is related in space to the morphologic sub-units of the cell, and in time to the developing infectious sequence and to the gross cytopathogenic effects.

After attachment and penetration of the virus into the cell, there begins almost immediately an accumulation of protein and ribonucleic acid in the cytoplasm. Rapid incorporation of radioactive inorganic phosphate into the nuclear RNA and DNA also occurs, but there is no detectable net increase exhibited by these two fractions. During this phase which lasts for several hours and is generally known as the "eclipse period", no virus is detectable. However, the sequence of events as shown by phase-contrast cinematography in a single cell show the following changes: increased activity of the peripheral undulating membranes and movement of granules to the nuclear area (Barski, et al., 1955; Harding, et al., 1956; and Ackermann, 1957).

By four hours the rate of synthesis of cytoplasmic RNA increases, virus appears in the intracellular state, and incorporation of P^{32} into the nuclear RNA and DNA fractions ceases abruptly. Progressively the rate of accumulation of virus increases. By six hours, synthesis of cytoplasmic

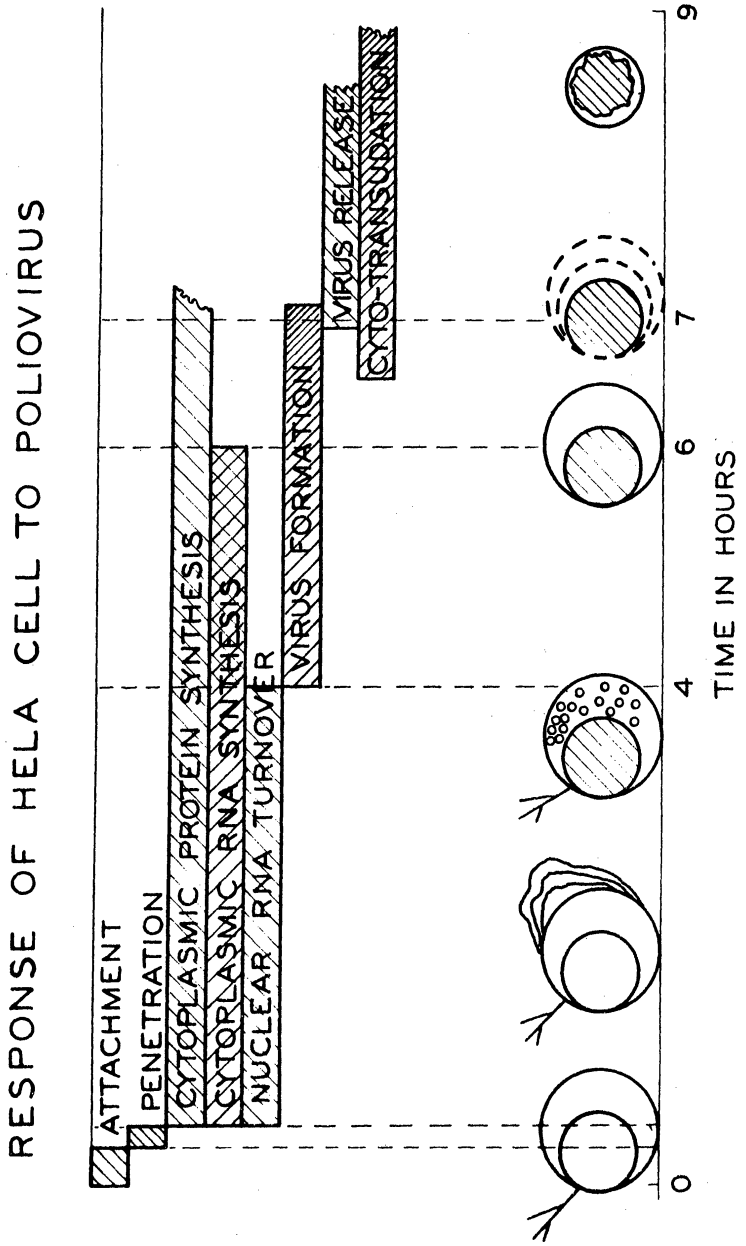

Figure 10. Response of HeLa Cell to Poliovirus.

RNA ceases and the incorporation of P^{32} begins to decline, while protein and virus continue to increase. At the seventh hour release of virus begins accompanied by a release of other cellular material.

During this period the changes observed by phase-contrast cinematography are as follows: a violent boiling-like movement of the cytoplasm occurring about the fourth hour, and at the sixth hour a retraction of the cellular processes and a rapid and dramatic loss of cytoplasmic area with retention of the cytoplasmic granules about a highly refracting nuclear residue.

It appears from these results that HeLa cells producing poliovirus are also synthesizing cytoplasmic RNA and protein in excess of what is normally present in the cell cytoplasm. This synthesis represents a doubling of the cytoplasmic material in a few hours. What is the nature of this material: is it virus, is it material destined to be virus, is it normal or anomalous cellular material? There are several lines of evidence which may be meaningful to consider in this connection.

Firstly, the yield of virus seldom exceeds a thousand plaque-forming units per cell. According to Schwerdt and Fogh (1957) the number of characteristic particles in a preparation which are countable with the electron microscope is approximately 30 per plaque-forming unit under the best plating conditions. They estimated the number of particles produced per monkey kidney cell to be in the range of 50 to 300 thousand, which they claim actually constitutes only a small fraction (1 to 5 per cent) of the total solid mass of a spherical epithelial cell of 10 μ diameter. In the present investigation the increases in cell mass actually found are of a different order of magnitude. The amount of virus which could

be produced by a single cell, can be calculated from the information that poliovirus is composed of 30 per cent RNA, 70 per cent protein and has a mass of 1×10^{-17} grams. If the incremental RNA and protein were all destined to be virus, then the calculated yield would be about one million per cell based upon the surplus RNA, and about ten million from the protein. Under the present experimental conditions the greatest number of particles produced by a cell was found to be 30 thousand. This would suggest, either that most of the excess RNA was viral nucleic acid which was not incorporated into particles, or that it was HeLa cell RNA, normal or abnormal, which was produced as an indirect manifestation of virus multiplication.

Secondly, when the base ratios of cytoplasmic RNA from normal and infected HeLa cells were compared, no significant difference in their composition was observed. The characteristic base ratios of Type I (Mahoney strain) poliovirus have been reported already by Schwerdt (1957). These values are quite different from that of the cytoplasmic RNA from infected and normal HeLa cells, being relatively low in cytidylic and guanylic acids. Unfortunately, it is not possible at present to separate the incremental RNA of infected cells from the normal RNA component originally present.

However, assuming that all of the incremental RNA in the infected cell is attributable to viral nucleic acid, the nucleotide composition of the infected cells cytoplasmic RNA was calculated and found to show no similarity to the composition of the ribonucleic acids from either the infected or normal HeLa cells, nor to the virus (Table IV). Thus the major portion of the incremental RNA must be of a composition different from that of poliovirus.

In addition, when the cytoplasm is separated into its various subcellular components (Table VI), 80 per cent of the virus activity was found in Fraction II, along with 58 per cent of the incremental RNA and 10 per cent of the incremental protein. Fraction III with 70 per cent of the incremental protein contained only about 14 per cent of the virus. Throughout, the distribution of incremental protein, RNA and virus activity amongst the various fractions does not correspond one to another as they would if all represented the same particle. The ratio of the protein nitrogen to RNA-phosphorus in the virus particle is about 2.3. Only in Fraction II does the ratio of the incremental protein and RNA approach this with a value of 2.25. The characteristic value for Fraction II obtained from normal cells is 11.9.

From these considerations of the nucleotide composition of the RNA, the distribution of incremental RNA, protein and virus activity amongst the cytoplasmic fractions and the amount of material involved, one is led to conclude that the accumulated protein and RNA does not constitute poliovirus and at least in the case of RNA does not represent material of the viral type.

It is possible that a small portion of the incremental RNA may represent viral nucleic acid, which thus far is not separable and therefore not detectable. The soluble protein fraction may also correspond in part to the viral C and D antigens which were found in the soluble fractions of poliovirus-infected HeLa cell homogenates after ultracentrifugation (Mayer, et al., 1957; and Roizman, et al., 1958). However, the amount of C and D antigens produced would represent a small fraction of the incremental protein found here. On the basis of the amounts of

soluble antigens produced, Roizman, et al., (1958) have estimated that approximately one hundred thousand to five hundred thousand virus particles can be formed. However, the calculated yield of virus from the incremental protein was found to be approximately ten million. In addition, at least a part of the incremental protein must be normal since there is found an increased activity of a series of glycolytic enzymes in HeLa cells infected with poliovirus (Matzelt, et al., 1958). If the above statements are true, then they would lend further support to the suggestion that viral nucleic acid and viral protein are produced separately in different areas of the cell, and then later combine (Luria, 1953; Bawden, 1957; Breitenfeld and Schäfer, 1957).

So far each of the sub-cellular fractions of the cytoplasm examined show increases in protein and RNA. The data suggests that a hyperdevelopment of the cytoplasm without corresponding nuclear development is occurring. Perhaps it is this unbalanced development which precipitates the cellular instability. An analogous situation can be seen in the studies of Barner and Cohen (1954) of the "unbalanced growth" phenomenon seen in thymidine-requiring mutants of Escherichia coli. This effect is ascribed to a continuing cell synthesis in the absence of a balanced synthesis of normal DNA. The same authors have obtained evidences that suggest that many types of bactericidal treatments produce their effects in this way (Barner and Cohen, 1956) and more particularly have demonstrated that some lethal effects of ultraviolet irradiations are consequences of unbalanced growth (Cohen and Barner, 1954). More recently Ackermann, et al., (1958) have demonstrated the occurrence of an unbalanced development in HeLa cell cultures grown

in the presence of 5-fluorouracil, an antagonist of thymine synthesis. Chemical analyses of these cells grown in the presence of the fluorinated pyrimidine analogue indicate that while cellular DNA synthesis is inhibited, protein and RNA syntheses are continuing. However, the inhibition of multiplication can be prevented by the early addition of thymidylic acid, but once unbalanced cellular growth has occurred, the effect of the inhibitor is not reversed and the inhibited cell proceeds to form giant cells and ultimately dies.

Thus, one can propose that the virus, after having entered the cell, has in some way the ability to condition certain areas of the cell to synthetic activity. Different viruses such as those containing RNA or DNA would condition different structural and metabolic areas. This proposed generality was supported in part recently by Newton and Stoker (1958) who have shown an increase in DNA synthesis occurring in herpes virus-infected HeLa cells. No increase in RNA synthesis was detected, and unfortunately no protein determinations were made. It is generally accepted that the herpes virus belong to the DNA group of viruses.

The findings here are also not consistent with the concept which proposes that in viral reproduction, all of the synthetic powers of the cell are redirected to produce a single new product. Rather, it is proposed that if the conditions in any certain area of the cell are suitable, then all specific templates including that of the virus present may be expected to function. Thus one can expect as a result of infection many large molecules to be formed which were never destined to be virus or to function secondarily in virus production.

Since the cell may respond in a limited number of ways, the virus might trigger or initiate some one of the step-wise phases that constitute the normal life cycle of the cell. The cell now appears to be out of phase in certain of the critical areas involved in the normal growth process, and thus cellular death or unceasingly stimulated mitosis as in malignancy results.

This hypothesis is of particular interest in that it provides a single explanation of the development of cytopathology by animal viruses. Its general validity awaits further experimental observations on other mammalian host cell-virus systems.

SUMMARY

A biochemical and a biological investigation of poliovirus multiplication in HeLa cells has been made. When HeLa cells labeled with radioactive phosphorus are exposed to poliovirus, there occurs after several hours an enhanced release of labeled materials into the extracellular fluid. This transudation begins at about the sixth hour following infection, and appears to coincide with the first release of virus and the first morphological changes as observed through time lapse cinematography. The enhanced release of radioactivity is related to the size of the viral inoculum and is not seen when virus, pre-neutralized with antiviral serum, is used. These data emphasize the importance of defining the period during which a chemical study of virus synthesis is feasible.

A chemical and isotopic analysis of sub-cellular fractions of HeLa cells at various times during a single sequence of infection revealed the following changes: Within one hour after the initiation of infection, there is a detectable accumulation in the cytoplasm of newly synthesized protein and RNA (ribonucleic acid). Also at this time there is an enhanced incorporation of P^{32} by nuclear RNA and DNA (deoxyribonucleic acid) without a net increase in either nuclear nucleic acid. The synthesis of cytoplasmic protein continues at a constant rate until the seventh hour of infection. RNA of the cytoplasm is formed at a constant rate until the fourth hour, at which time the rate is markedly enhanced. The fourth hour is also marked by a sharp decline in the incorporation of P^{32} into both the nuclear RNA and DNA, and the appearance

of the first virus, as infectious activity, in the cytoplasm. The accumulation of cytoplasmic RNA ceases at the sixth hour, but virus accumulates at an increasing rate in the cytoplasm from the fourth to the seventh hour, when 99 per cent of the virus formed is present in the intracellular state. From the amounts of nucleic acid and protein produced, their distribution relative to virus among various sub-cellular fractions, and from the necleotide composition of the RNA, it was concluded that the major portion of the newly formed materials was not virus and at least in the case of the RNA was not destined to be virus. From consideration of these observations, a hypothesis is presented, which suggests that "unbalanced growth" is responsible for cytopathology.

BIBLIOGRAPHY

Ackermann, W. W. (1951a) Concerning the Relation of the Krebs Cycle to Virus Propagation. J. Biol. Chem. 189:421-428.

Ackermann, W. W. (1951b) The Relation of the Krebs Cycle to Viral Synthesis. I. The Effect of Sodium Fluoroacetate on the Propagation of Influenza Virus in Mice. J. Exptl. Med. 93: 635-642.

Ackermann, W. W. (1957) Unpublished observations.

Ackermann, W. W., and Francis, T., Jr. (1954) Characteristics of Viral Development in isolated Animal Tissues. pp. 81-108. In Advances in Virus Research. Vol. II. Edited by Smith, K.M., and Lauffer, M. A. Academic Press, Inc., New York, New York.

Ackermann, W. W., and Johnson, B. R. (1953) Some Enery Relations in a Host-Virus System. J. Exptl. Med. 96:151-277.

Ackermann, W. W., and Kurtz, H. (1952) The Relation of Herpes Virus to Host Cell Mitochondria. J. Exptl. Med. 96:151-157.

Ackermann, W. W., Loh, P. C., and Payne, F. E. (1958) Effects of 5-Fluorouracil Upon Nucleic Acid Synthesis of Ordinary and Viral Infected HeLa Cells. Bacteriol. Proceedings. 58th. pp. 39.

Ackermann, W. W., and Maassab, H. F. (1954) Growth Characteristics of Influenza Virus: Biochemical Differentiation of Stages of Development. J. Exptl. Med. 100:329-339.

Ackermann, W. W., Rabson, A., and Kurtz, H. (1954) Growth Characteristics of Poliomyelitis Virus in HeLa Cell Cultures: Lack of Parallelism in Cellular Injury and Virus Increase. J. Exptl. Med. 100: 437-450.

Ada, G. L. (1957) Ribonucleic Acid in Influenza Virus. pp. 104-115. In The Nature of Viruses. Ciba Foundation Symposium. Little, Brown and Company, Boston, Massachusetts.

Ada, G. L., and Perry, B. T. (1954) Studies on the Soluble Complement Fixing Antigens of Influenza Virus. III. The Nature of the Antigens. Aust. J. Exptl. Biol. Med. Sci. 32:177-186.

Ada, G. L., and Perry, B. T. (1955) Infectivity and Nucleic Acid Content of Influenza Virus. Nature 175:209-211.

Ada, G. L., and Perry, B. T. (1956) Influenza Virus Nucleic Acid: Relationship Between Biological Characteristics of the Virus Particle and Properties of the Nucleic Acid. J. Gen. Microbiol. 14:623-633.

Bang, F. B. (1950) Cellular Changes in the Chick Chorio-Allantoic Membranes Infected with Herpes Simplex and Vaccinia. A Study With Thin Sections for the Electron Microscope. Bull. Johns Hopkins Hospt. 87:511-518.

Bang, F. B., and Isaacs, A. (1957) Cell Morphology in Myxovirus Infections. In The Nature of Viruses. Ciba Foundation Symposium. Little, Brown and Company, Boston, Massachusetts.

Barner, H. D., and Cohen, S. S. (1954) The Induction of Thymine Synthesis by T2 Infection of a Thymine-requiring Mutant of Escherichia coli. J. Bacteriol. 68:80-88.

Barner, H. D., and Cohen, S. S. (1956) The Relation of Growth to the Lethal Damage Induced by Ultraviolet Irradiation in Escherichia coli. J. Bacteriol. 71:149-157.

Barnum, C. P., and Huseby, R. A. (1950) The Intracellular Heterogeneity of Pentose Nucleic Acid as Evidenced by the Incorporation of Radiophosphorus. Arch. Biochem. 29:7-26.

Barnum, C. P., Huseby, R. A., and Vermund, H. (1953) A Time Study of the Incorporation of Radiophosphorus into the Nucleic Acids and Other Compounds of a Transplanted Mouse Mammary Carcinoma. Cancer Research 13:880-889.

Barski, G., Robineaux, R., and Endo, M. (1955) Phase Contrast Cinematography of Cellular Lesion Produced by Poliomyelitis Virus in vitro. Proc. Soc. Exptl. Biol. Med. 88:57-59.

Basler, E., and Commoner, B. (1956) The Effect of Tobacco Mosaic Virus Biosynthesis on the Nucleic Acid Content of Tobacco Leaf. Virology 2:13-28.

Bauer, D. J. (1953) Metabolic Aspects of Virus Multiplication. pp 46-78. In The Nature of Virus Multiplication. Edited by Fildes, P., and Heyningen, W. E. van. Cambridge University Press, Cambridge, England.

Bawden, F. C. (1957) The Multiplication of Plant Viruses. pp. 170-181. In The Nature of Viruses. Ciba Foundation Symposium. Little, Brown and Company, Boston, Massachusetts.

Bawden, F. C., and Pirie, N. W. (1956) Observations on the Anomalous Proteins Occurring in Extracts From Plants Infected with Strains of Tobacco Mosaic Virus. J. Gen. Microbiol. 14:460-477.

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955) Optical Properties of Nucleic Acids and Their Components. pp. 493-553. In The Nucleic Acids. Vol. I. Edited by Chargaff, E., and Davidson, J.N. Academic Press, Inc., New York, New York.

Becker, Y., Crossowicz, N., and Bernkopf, H. (1958) Metabolism of Human Amnion Cell Cultures Infected with Poliomyelitis Virus. I. Glucose Metabolism During Virus Synthesis. Proc. Soc. Exptl. Biol. Med. 97:77-82.

Bedson, S. P., and Bland, J. O. W. (1932) A Morphological Study of Psittacosis Virus, with the Description of a Developmental Cycle. Brit. J. Exptl. Path. 13:461-473.

Benzer, S. (1952) Resistance to Ultraviolet Light as an Index to Reproduction of Bacteriophage. J. Bacteriol. 63:59-72.

Benzer, S. (1953) Induced Synthesis of Enzymes in Bacteria Analysed at the Cellular Level. Biochim. et Biophys. Acta 11:383-395.

Bergold, G. H. (1947) Die Isolierung des Polyedervirus and die Natur der Polyder. Z. Naturforsch. 2b:122-143.

Bergold, G. H. (1950) The Multiplication of Insect Viruses as Organisms. Can. J. Research E28:5-11.

Bergold, G. H. (1953a) Insect Viruses. pp. 91-139. In Advances in Virus Research. Vol. I. Edited by Smith, K, M., and Lauffer, M. A. Academic Press, Inc., New York, New York.

Bergold, G. H., (1953b) The Multiplication of Insect Viruses. pp. 276-283. In The Nature of Virus Multiplication. Edited by Fildes, P., and Heyningen, W. E. van , Cambridge University Press, Cambridge, England.

Bergold, G. H., and Friedrich-Freksa, H. (1947) Zur Grösse" and Serologie des Bombyxmori-Polyedervirus. Z. Naturforsch. 3b:406-410.

Bergold, G. H., and Wellington, E. F. (1954) Isolation and Chemical Composition of the Membranes of an Insect Virus and Their Relation to the Virus and Polyhedral Bodies. J. Bacteriol. 67:210-216.

Berry, G. P., and Dedrick, H. M. (1936) Further Observations of the Transformation of the Virus of Rabbit Fibroma (Shope) into that of Infectious Myxomatosis (Sanarelli). J. Bacteriol. 32:356-361.

Beutner, E. H., Hartman, P. E., Mudd, S., and Hillier, J. (1953) Light and Electron Microscopic Studies of Escherichio coli-coliphage Interactions. I. Preparative Method. Comparative Light and Electron Microscopic Cytology of the E. coli B-T2 System. Biochim. et Biophys. Acta 10:143-152.

Bielig, H. J., Kausche, G. A., and Haardick, H. (1949) Uber" den Nachweis von Reduktionsorten in Bakterien. Z. Naturforsch. 4B:80-91.

Bird, F. T. (1957) On the Development of Insect Viruses. Virology 3:237-240.

Bland, J. O. W., and Robinow, C. F. (1939) The Inclusion Bodies of Vaccinia and Their Relationship to the Elementary Bodies, Studied in Cultures of the Rabbit Cornea. J. Path. Bacteriol. 48:381-394.

Boyd, J. S. K. (1949) Development of Bacteriophage in Escherichia coli B. Nature 164:874-877.

Boyer, G. S., Leuchtenberger, C., and Ginsberg, H. S. (1957) Cytological and Cytochemical Studies of HeLa Cells Infected with Adenoviruses. J. Exptl. Med. 105:195-216.

Bradish, C. J., Brooksby, J. B., Dillon, J. F., and Norambuena, M. (1952) Ultracentrifugal Studies of the Infective and Complement-Fixing Components in the Virus System of Foot-and-Mouth Disease. Proc. Roy. Soc. (London) B140:107-118.

Breitenfeld, P. M., and Schafer, W. (1957) The Formation of Fowl Plague Virus Antigen in Infected Cells, as Studied with Fluorescent Antibodies. Virology 4:328-345.

Brown, G. L., and Martin, A. W. (1956) Fractionation of the Deoxyribonucleic Acid of T2 Bacteriophage. Nature 176:971-973.

Buckley, S. M. (1956) Visualization of Poliomyelitis Virus by Fluorescent Antibody. Arch. ges. Virusforsch. 6:388-399.

Burnet, F. M. (1955) Morphological and Functional Changes in Cells Infected with Viruses. pp. 157-176. In Principles of Animal Virology. Academic Press, Inc., New York, New York.

Burnet, F. M. (1946) Vaccinia Hemagglutinin. Nature 158:119-121.

Burnet, F. M., and Stone, J. D. (1946) The Hemagglutinins of Vaccinia and Ectromelia Viruses. Aust. J. Exptl. Biol. Med. Sci. 24:1-11.

Burnet, F. M., and Lind, P. E. (1954) Reactivation of Heat Inactivated Influenza Virus by Recombination. Aust. J. Exptl. Biol. 32:133-144.

Burton, K. (1955) The Relation Between the Synthesis of Deoxyribonucleic Acid and the Synthesis of Protein in the Multiplication of Bacteriophage T2. Biochem. J. 61:473-483.

Cairns, H. J. F., Fazekas de St. Groth, and Edney, M. (1952) Quantitative Aspects of Influenza Virus Multiplication. I. Production of Incomplete Virus. II. Heterologous Interference. III. Average Liberation Time. IV. Definition of Constants and General Discussion. J. Immunol. 69:155-181.

Chargaff, E. (1955) Isolation and Composition of the Deoxypentose Nucleic Acids and of the Corresponding Nucleoproteins. pp. 308-371. In The Nucleic Acids. Vol. I. Edited by Chargoff, E., and Davidson, J. N. Academic Press. Inc., New York, New York.

Cheng, Ping-Yao (1958) Infectivity of Ribonucleic Acid from Mouse Brains Infected with Semliki Forest Virus. Nature 181: 1800-1801.

Chu, C. M. (1948) Studies on Vaccinia Hemagglutinin. II. Some Immunological Properties. J. Hyg., Camb. 46:49-55.

Cochran, G. W., and Chidester, J. L. (1958) Infectious Nucleic Acid in Plants with Tobacco Mosaic. Virology 4:390-391.

Cohen, S. S. (1948a) The Synthesis of Bacterial Viruses. I. The Synthesis of Nucleic Acid and Protein in Escherichia coli B Infected with T2r$^+$ Bacteriophage. J. Biol. Chem. 174:281-293.

Cohen, S. S. (1948b) The Synthesis of Bacterial Viruses. II. The Origin of the Phosphorus Found in the Deoxyribonucleic Acids of the T2 and T4 Bacteriophages. J. Biol. Chem. 174:295-303.

Cohen, S. S. (1949) Growth Requirements of Bacterial Viruses. Bacteriol. Rev. 13:1-24.

Cohen, S. S. (1951) Utilization of Gluconate and Glucose in Growing and Virus-Infected Escherichia coli. Nature 168:746-747.

Cohen, S. S. (1951) The Synthesis of Nucleic Acid by Virus-Infected Bacteria. Bacteriol. Revs. 15:131-140.

Cohen, S. S., and Anderson, T. F. (1946) Chemical Studies on Host-Virus Interations. I. The Effect of Bacteriophage Adsorption on the Multiplication of Its Host Escherichia coli B. Brit. J. Exptl. Med. 84:511-523.

Cohen, S. S., and Barner, H. D. (1954) Studies on Unbalanced Growth in Escherichia coli. Proc. Natl. Acad. Sci. 40:885-893.

Cohen, S. S., Lichstein, J., Barner, H. D., and Green, M. (1958) J. Biol. Chem. in press.

Cohen, S. S., and Roth, L. (1953) The Phosphogluconate Pathway of Carbohydrate Metabolism in the Multiplication of Bacterial Viruses. J. Bacteriol. 65:490-495.

Cohen, S. S., and Weed, L. L. (1954) Some Precursors of the 5-Hydroxy-Methylcytosine of T6r$^+$ Bacteriophage. J. Biol. Chem. 209:789-794.

Cohn, Z. A. (1952) Quantitative Distribution of Phosphorus in Chorio-Allantoic Membranes Infected with Influenza Virus PR8. Proc. Soc. Exptl. Biol. Med. 79:566-568.

Commoner, B., and Mercer, F. L. (1952) The Effect of Thiouracil on the Rate of Tobacco Mosaic Virus Biosynthesis. Arch. Biochem. 35:278:289.

Commoner, B., and Nehari, V. (1953) The Effects of Tobacco Mosaic Virus Synthesis on the Free Amino Acid and Amide Composition of the Host. J. Gen. Physiol. 36:791-805.

Commoner, B., and Rodenberg, S. D. (1955) Relationships Between Tobacco Mosaic Virus and the Non-Virus Proteins. J. Gen. Physiol. 38:475-482.

Commoner, B., Yamada, M., Rodenberg, S. D., Wang, T. Y., and Basler, E. (1953) The Proteins Synthesized in Tissue Infected with Tobacco Mosaic Virus. Science 118:529-534.

Consentino, V., Paigen, K., and Steere, R. L. (1956) Electron Microscopy of Turnip Yellow Mosaic Virus and the Associated Abnormal Protein. Virology 2:139-148

Craigie, J. (1932) The Nature of the Vaccinia Flocculation Reaction and Observations on the Elementary Bodies of Vaccinia. Brit. J. Exptl. Path. 13:259-268.

Crampton, C. F., Lipshitz, R., and Chargaff, E. (1954) Studies on Nucleoproteins. I. Dissociation and Reassociation of the Deoxyribonucleohistone of Calf Thymus. J. Biol. Chem. 206: 499-510.

Crawford, L. V. (1957). DNA Synthesis in Bacteriophage-Infected Escherichia coli. Biochem. J. 65:17p.

Crawford, L. V. (1958) Deoxyribonucleic Acid Synthesis in Phage-Infected Bacillus megatherium KM. Biochim. et Biophys. Acta 28:208-209.

Crawford, G. N. C., and Sanders, F. K. (1952) The multiplication of Vaccinia Virus in Tissue Cultures of Adult Rabbit Skin. Quart. J. Micro. Sci. 93:110-116.

Crouse, H. V., Corielli, L. L., Blank, H., and Scott, T. F. McN. (1950) Cytochemical Studies on the Intranuclear Inclusion of Herpes Simplex. J. Immunol. 65:119-128.

Darnell, J. E., Jr. (1958) Adsorption and Maturation of Poliovirus in Singly and M ltiply Infected HeLa Cells. J. Exptl. Med. 107:633-641.

De Mars, R. I. (1955) The Production of Phage-Related Materials When Bacteriophage Development is Interrupted by Proflavine. Virology 1:83-99.

De Mars, R. I., Luria, S. E., Fisher, H., and Levintal, C. (1953) The Production of Incomplete Bacteriophage Particles by the Action of Proflavine and the Properties of the Incomplete Particles. Ann. Inst. Pasteur 84:113-128.

Dische, Z. (1955) Color Reactions of Nucleic Acid Components pp. 285-305. In The Nucleic Acids. Vol. I. Edited by Chargaff, E., and Davidson, J. N. Academic Press Inc., New York, New York.

Doerman, A. H. (1952) The Intracellular Growth of Bacteriophages. I. Liberation of Intracellular Bacteriophage T4 by Premature lysis with Another Phage or with Cyanide. J. Gen. Physiol. 35:645-654.

Donald, H. B., and Isaacs, A. (1954) Some Properties of Influenza Virus Filaments Shown by Electron Microscopic Particle Counts. J. Gen. Microbiol. 11:325-331.

Dulbecco, R., and Vogt, M. (1954) One-Step Growth Curve of Western Equine Encephalomyelitis Virus on Chick Embryo Cells Grown in vitro and Analysis of Virus Yields From Single Cells. J. Exptl. Med. 99:183-194

Dulbecco, R., and Vogt, M. (1955) Biological Properties of Poliomyelitis Virus as Studied by the Plaque Technique. Ann. N. Y. Acad. Sci. 61:790-800.

Dunnebacke, T. H. (1956) Correlation of the Stage of Cytopathic Change with the Release of Poliomyelitis Virus. Virology 2:399-410.

Dunnebacke, T. H. (1956) Cytopathic Changes Associated with Poliomyelitis Infections in Human Amnion Cells. Virology 2:811-819.

Eagle, H. (1955) Nutrition Needs of Mammalian Cells in Tissue Culture. Science 122:501-504.

Eagle, H., and Habel, K. (1956) The Nutritional Requirements for the Propagation of Poliomyelitis Virus by the HeLa Cell. J. Exptl. Med. 104:271-287.

Eaton, M. D., and Perry, M. E. (1953) Further Observations on the Effect of 2,4-dinitrophenol on the Growth of Influenza Virus, J. Infect. Dis. 93:269-277.

Eisenberg-Merling, K. B. (1940) Observations on Living Vaccinia Virus in the Corneal Cells of the Rabbit. J. Path. Bacteriol. 50: 279-288.

Evans, E. A. (1954) Bacterial Viruses (with Particular Reference to the Synthesis of) Ann. Rev. Microbiol. 8:237-256.

Franklin, R. (1958) Studies on the Growth of Vesicular Stomatitis Virus in Tissue Culture. Virology 5:408-424.

Fraser, D., and Consentino, V. (1957) The Amino Acid Composition of Turnip Yellow Mosaic Virus and the Associated Abnormal Protein. Virology 5:192-205.

Fraser, D., Mahler, H. R., Shug, A. L., and Thomas, C. A., Jr. (1957) The Infection of Sub-Cellular Escherichia coli, Strain B, with a DNA Preparation From T2 Bacteriophage. Proc. Natl. Acad. Sci. 43:939-947.

Fiske, C. H., and Subbarow, Y. J. (1925) The Colorimetric Determination of Phosphorus. J. Biol. Chem. 66:375-400.

Flaks, J. G., and Cohen, S. S. (1957) The Enzymic Synthesis of 5-hydroxymethyl-deoxycytidylic Acid. Biochim. et Biophys. Acta 25: 667-668.

Flewett, T. H., and Challice, C. E. (1951) The Intracellular Growth of Fowl-Plague Virus: A Phase-Contrast and Electron Microscopical Study of Infected Tissue Cultures. J. Gen. Microbiol. 5:279-290.

Fraenkel-Conrat, H., and Singer, B. (1957) Virus Reconstitution. II. Combination of Protein and Nucleic Acid From Different Strains. Biochim. et Biophys. Acta 24:540-548.

French, R. C. (1954) The Contribution of Protein From Parent to Progeny in T2 Coliphage. J. Bacteriol. 67:45-49.

French, R. C., Graham, A. F., Lesley, S. M., and Rooyen, C. E. van. (1952) The Contribution of Phosphorus From $T2r^+$ Bacteriophage to Progeny. J. Bacteriol. 64:597-607.

Gard, S., Magnus, P., von, Svedmyr, A., and Burch-Andersen, A. (1952) Studies on the Sedimentation of Influenza Virus. Arch. f. d. gesamte Virusforsch. 4:591-611.

Gaylord, W. H., and Melnick, J. L. (1953a) Development of Forms of Vaccinia Virus. Science 117:10-12.

Gaylord, W. H., and Melnick, J. L. (1953b) Intracellular Forms of Pox Viruses as Shown by the Electron Microscope (Vaccinia, Ectromelia, Molluscum Contagiosum). J. Exptl. Med. 98: 157-169.

Gifford, G. E., and Syverton, J. T. (1957) Replication of Poliovirus in Primate Cell Cultures Maintained Under Anaerobic Conditions. Virology 4:216-223.

Girardi, A. J., Allen, E. G., and Sigel, M. M. (1952) Studies on the Psittacosis-lympho-granuloma Group. II. A Non-Infectious Phase in Virus Development Following Adsorption to Host Tissue. J. Exptl. Med. 96:233-241.

Gostling, J. V. T. (1956) Intracellular Site of Developing Herpes Virus. Nature 178:1238-1239.

Granoff, A. (1955) Noninfectious Forms of Newcastle Disease and Influenza Viruses. Studies on Noninfectious Virus Occurring Within Cells That Are Producing Fully Infectious Virus. Virology 1:516-531.

Granoff, A., Liu, O. C., and Henle, W. (1950) A Small Hemagglutinating Component in Preparations of Newcastle Disease Virus. Proc. Soc. Exptl. Biol. Med. 75:684-691.

Gray, A., and Scott, T. F. McN. (1954) Some Observations on the Intra-Cellular Localization of the Virus of Herpes Simplex in the Chick Embryo Liver. J. Exptl. Med. 100:473-484.

Green, M., and Cohen, S. S. (1957) Studies on the Biosynthesis of Bacterial and Viral Pyrimidines. I. Tracer Studies. J. Biol. Chem. 225:387-396.

Grierer, A., and Schramm, G. (1956) Infectivity of Ribonucleic Acid from Tobacco Mosaic Virus. Nature 177:702-703.

Groman, N. B. (1955) Evidence for the Active Role of Bacteriophage in the Conversion of Non-Toxigenic Corynebacterium diptheriae to Toxin Production. J. Bacteriol. 69:9-15.

Hamers, R. (1956) Incorporation of S^{35}-Thiouracil in Bacillus megatherium. Biochim. et Biophys. Acta 21:170-178.

Hamers-Castermann, C., and Jeener, C. A. (1957) The Initial Ribonuclease-Sensitive Phase in the Multiplication of Tobacco Mosaic Virus. Virology 3:197-206.

Harding, C. V., Harding, D., McLimans, W. F., and Rake, G. (1956) Cytological Changes Accompanying the Growth of Poliomyelitis Virus in Cells of Human Origin. Virology 2:109-125.

Harford, C. G., Hamlin, A., Parker, E., and Ravenswaay, T., van. (1956) Electron Microscopy of HeLa Cells Infected with Adenoviruses. J. Exptl. Med. 104:443-454.

Hartman, P. E., Payne, J. I., and Mudd, S. (1955) Cytological Analysis of Ultraviolet Irradiated Escherichia coli. J. Bacteriol. 70:531-539.

Henle, W. (1956) The Use of Phosphorus-32 in the Study of Incomplete Forms of Influenza Virus. Trans. N. Y. Acad. Sci. 18:255-260.

Henle, W., and Henle, G. (1949) Studies on Host-Virus Interations in the Chick Embryo-Influenza Virus System. III. Development of Infectivity, Hemagglutination and Complement Fixation Activities During the First Infectious Cycle. J. Exptl. Med. 90:23-37.

Henle, W., and Wiener, M. (1944) Complement Fixation Antigens of Influenza Viruses Type A and B. Proc. Soc. Exptl. Biol. Med. 57:176-179.

Hershey, A. D. (1953) Nucleic Acid Economy in Bacteria Infected of with Bacteriophage T2. II. Phage Precursor Nucleic Acid. J. Gen. Physiol. 37:1-23.

Hershey, A. D. (1955) An Upper Limit to the Protein Content of the Germinal Substance of Bacteriophage T2. Virology 1:108-127.

Hershey, A. D. (1957) Experimental Problems Concerning the Role of Deoxyribonucleic Acid in Growth of Bacteriophage T2. pp. 251-257. In Cellular Biology Nucleic Acids and Viruses. New York Acad. Sci., New York, New York.

Hershey, A. D., and Chase, M. (1952) Independent Functions of Viral Protein and Nucleic Acid in Growth of Bacteriophage. J. Gen. Physiol. 36:39-56.

Himmelweit, F. (1938) Observations on Living Vaccinia and Ectromelia Viruses by High Power Microscopy. Brit. J. Exptl. Path. 19:108-116.

Hogeboom, G. H., and Schneider, W. C. (1955) The Cytoplasm. pp. 199-246. In The Nucleic Acids. Vol. II. Edited by Chargoff, E., and Davidson, J. N. Academic Press, Inc., New York, New York.

Howatson, A. F., and Ham, A. W. (1955) Electron Microscope Study of the Sections of Two Rat Liver Tumors. Cancer Research 15:62-69.

Howes, D. W., and Melnick, J. W. (1957) The Growth Cycle of Poliovirus in Monkey Kidney Cells. I. Maturation and Release of Virus in Monolayer Cultures. Virology 4:97-108.

Hoyle, L. (1945) An Analysis of the Complement-Fixation Reaction in Influenza. J. Hyg., Camb. 44:170-175.

Hoyle, L. (1948) The Growth Cycle of Influenza Virus A. A Study of the Relations Between Virus, Soluble Antigen and Host Cell In Fertile Eggs Inoculated With Influenza Virus. Brit. J. Exptl. Path. 29:390-399.

Hoyle, L. (1950) The multiplication of Influenza Viruses in the Fertile Egg. J. Hyg., Camb. 48:277-245.

Hoyle, L. (1952) Structure of the Influenza Virus. The Relation Between Biological Activity and Chemical Structure of Virus Fractions. J. Hyg., Camb. 50:229-245.

Hoyle, L. (1957) The Use of Radioactive Influenza Virus to Determine the Fate of the Infecting Particle on Entry into the Host Cell. pp. 211-218. In The Nature of Viruses. Ciba Foundation Symposium. Little, Brown and Company, Boston, Massachusetts.

Hoyle, L., and Frisch-Niggemeyer, W. (1955) The Disintegration of Influenza Virus Particles on Entry Into the Host Cell. Studies with Virus Labelled With Radiophosphorus. J. Hyg., Camb. 52:119-130.

Hoyle, L., Jolles, B., and Mitchell, R. G. (1954) Electron Microscopic Studies of the Structure of the Influenza Virus. Nature 171:256.

Hurlbert, R. B., Schmitz, H., Bruum, A. F., and Potter, V. R. (1954) Nucleotide Metabolism. II. Chromatographic Separation of Acid-Soluble Nucleotides. J. Biol. Chem. 209:23-39.

Jacob, F. (1952) Effects de la carence en aliment carbone sur l' induction d'un Pseudomonas pyocyanea lysogéne. Ann. Inst. Pasteur 82:578-587.

Jardetsky, C., and Barnum, C. P. (1957) Metabolic Activity and Intracellular Distribution of Nucleic Acid Phosphorus in Regenerating Liver. Arch. Biochem. 67:350-365.

Jeener, R. (1954) A Preliminary Study of the Incorporation in Growing Turnip Yellow Mosaic Virus and Its Related Noninfective Antigen of Labelled Amino Acids. Biochim. et Biophys. Acta 13:307-308.

Jeener, R. (1957) see Brachet, J. (1957) Nucleic Acids in Heredity and Protein Synthesis. pp. 226-295. In Biochemical Cytology. Academic Press, Inc., New York, New York.

Jeener, R., and Lemoine, P. (1953) Occurrence in Plants Infected with Tobacco Mosaic Virus of a Crystalline Antigen Devoid of Ribonucleic Acid. Nature 171:935-938.

Jeener, R., Lemoine, P., and Lavand'Homme, C. (1954) Detection et proprietes de formes du virus de la mosaique du tabac deprouvue d'acide ribonucleique et non infectieuses. Biochim. et Biophys. Acta 14:321-334.

Jeener, R., and Szafarz, D. (1950) Relations Between Rate of Renewal and Intracellular Localization of Ribonucleic Acid. Arch. Biochem. 26:54-67.

Johnson, B. R., and Ackermann, W. W. (1958) Unpublished data.

Johnson, C. A., Kempf, J. E., and Bergeim, O. (1956) Levels of Certain Free Amino Acids of Chorioallantois During Influenza Virus Infection. J. Bacteriol. 71:496-497.

Joklik, W. K. (1952) The Effect of Phage Infection on the Metabolic Activity of the Host Cell. Brit. J. Exptl. Med. 33:368-379.

Kaplan, A. S., and Melnick, J. L. (1953) The Intracellular Localization of Poliomyelitis Virus. J. Exptl. Med. 97:91-104.

Kirber, M. W., and Henle, W. (1950) A Comparison of Influenza Complement Fixation Antigens Derived from Allantoic Fluids and Membranes. J. Immunol. 65:229-244.

Kipps, A., Naude, W., duT., Polson, A., Selzer, G., and Ende, M., van den. (1957) The Size Distribution of Specific Antigens in Virus-Infected Tissues and Their Significance. pp. 224-244. Ciba Foundation Symposium. Little, Brown and Company, Boston, Massachusetts.

Koch, A. L. (1953) Biochemical Studies of Virus Reproduction. XI. Acid-soluble Purine Metabolism. J. Biol. Chem. 203:227-240.

Koch, F. C., and McMeekin, T. L. (1924) A New Direct Nesslerization Micro-Kjeldahl Method and a Modification of the Nessler-Folin Reagent for Ammonia. J. Am. Chem. Soc. 46:2066-2069.

Koch, A. L., Putman, F. W., and Evans, E. A., Jr. (1952) Biochemical Studies of Virus Reproduction. VIII. Purine Metabolism. J. Biol. Chem. 197:113-120.

Kovacs, E. (1956) Comparative Biochemical Studies on Normal and Poliomyelitis Infected Tissue Cultures. IV. Enzyme-Changes In Host Cells. Proc. Soc. Exptl. Biol. Med. 92:183-187.

Kozloff, L. M. (1953) Origin and Fate of Bacteriophage Material. Cold Spring Harbor Symposia Quant. Biol. 18:209-220.

Kozloff, L, M., and Putman, F. W. (1950) Biochemical Studies of Virus Reproduction. III. The Origin of Virus Phosphorus in the Escherichia coli T6 Bacteriophage, J. Biol. Chem. 182:229-250.

Kozloff, L. M., Knowlton, K., Putnam, F. W., and Evans, E. A., Jr. (1951) Biochemical Studies of Virus Reproduction. V. The Origin of Bacteriophage Nitrogen. J. Biol. Chem. 188:101-116.

Kun, E., and Smith, M. H. D. (1954) Morphological and Biochemical Studies on the Choria-Allantois of the Chick Embryo Following Infection with Certain Viruses. Brit. J. Exptl. Path. 24:1-10.

Labaw, L. W. (1951) The Origin of Phosphorus in Escherichia coli Bacteriophage. J. Bacteriol. 62:169-173.

Lanni, Y. T. (1954) Infection by Bacteriophage T5 and its Intracellular Growth -- A Study by Complement Fixation. J. Bacteriol. 67:640-649.

Lanni, F., and Lanni, Y. T. (1953) Antigenic Structure of Bacteriophage. Cold Spring Harbor Symposia Quant. Biol. 18:159-167

Lanning, M. C., and Cohen, S. S. (1954) The Mechanism of Ribose Formation in Escherichia coli. J. Biol. Chem. 207:193-199.

Lanning, M. C., and Cohen, S. S. (1955) On the Origin of Deoxyribose in Escherichia coli and T6r$^+$ Bacteriophage. J. Biol. Chem. 216:413-423.

Larsen, E., McLimans, W. F., Rake, G. W., and Harding, C. V. (1957) Effect of Environmental Temperature on the Host Cell (HeLa) Poliovirus Interaction. Fed. Proceedings 16:421.

Latarjet, R. (1953) The Multiplication of Bacterial Viruses Studied by Radiobiological Methods. pp. 175-193. In The Nature of Virus Multiplication. Edited by Fildes, P., and Heyningen, W. E. van. Cambridge University Press, Cambridge, England.

Lebrun, J. (1956) Cellular Localization of Herpes Simplex Virus by Means of Fluorescent Antibody. Virology 2:496-510.

Le Clerc, J. (1956) Action of Ribonuclease on the Multiplication of the Influenza Virus. Nature 177:578-579

Lepine, P., and Sautter, V. (1946) Etude histochimique des lesions dues aux ultravirus les acides nucleiques. Ann. Inst. Pasteur 72:174-183.

Levine, A. S., and Rouse, H. C. (1957) Further Studies of the Effect of Pyruvate on Viral Multiplication in Tissue Culture. Bacteriol. Proceedings pp. 77.

Levinthal, C. (1956) The Mechanism of DNA Replication and Genetic Recombination in Phage. Proc. Natl. Acad. Sci. 42:394-404.

Levinthal, C., and Fisher, H. W. (1952) The Structural Development of a Bacterial Virus. Biochim. et Biophys. Acta 9:419-429.

Levinthal, C., and Fisher, H. W. (1953) Maturation of Phage and the Evidence of Phage Precursors. Cold Spring Harbor Symposia Quant. Biol. 18:29-34.

Levy, H. B., and Snellbar, L. F. (1956) Effects of Animal Viruses on Host Cell Metabolism. I. Effect of Encephalomyocarditis on Nucleic Acid and Phosphorus Fractions of Erhlich ascites tumor cells. J. Inf. Dis. 98:270-276.

Lief, F. S., and Henle, W. (1956a) Studies on the Soluble Antigen of Influenza Virus. I. The Release of S Antigen From Elementary Bodies by Treatment with Ether. Virology 2:753-771.

Lief, F. S., and Henle, W. (1956b) Studies on the Soluble Antigen of Influenza Virus. II. A Comparison of the Effects of Sonic Vibration and Ether Treatment of Elementary Bodies. Virology 2:772-781.

Lief, F. S., and Henle, W. (1956c) Studies on the Soluble Antigen of Influenza Virus. III. The Decreased Incorporation of S Antigen Into Elementary Bodies of Increasing Incompleteness. Virology 2:782-797.

Liu, C. (1955) Studies on Influenza Infection in Ferrets by Means of Fluorescein-Labelled Antibody. II. The Role of Soluble Antigen In Nuclear Fluorescence and Cross-Reactions. J. Exptl. MEd. 101:665-677.

Luria, S. E. (1953) An Analysis of Bacteriophage Multiplication. pp. 99-118. In The Nature of Virus Multiplication. Edited by Fildes, P., and Heyningen, W. E. van. Cambridge University Press, Cambridge, England.

Luria, S. E., and Human, M. L. (1950) Chromatin Staining of Bacteria During Bacteriophage Infection. J. Bacteriol. 59:551-560.

Lwoff, A., Dubecco, R., Vogt, M., and Lwoff, M. (1955) Kinetics of the Release of Poliomyelitis Virus from Single Cells. Virology 1:128-139.

Maaløe, O, and Symonds, N. (1953) Radioactive Sulfur Tracer Studies on the Reproduction of T4 Bacteriophage. J. Bacteriol. 65: 177-182.

Maaløe, O., and Watson, J. D. (1951) The Transfer of Radioactive Phosphorus from Parental to Progeny Phage. Proc. Natl. Acad. Sci. 37:507-513.

Maassab, H. F. (1955) Approaches to the Biochemical Analysis of Stages of Viral Development. Thesis. University of Michigan, Ann Arbor, Michigan.

Mackal, R. P., and Kozloff, L. M. (1954) Biochemical Studies of Virus Reproduction. XII. The Fate of Bacteriophage T7. J. Biol. Chem. 209:83-90.

Magnus, P. Von. (1951) Propagation of the PR8 Strain of Influenza A Virus in Chick Embryo. II. The Formation of "Incomplete" Virus Following Incubation of Large Doses of Seed Virus. Acta. Path. Microbiol. Scand. 28:278-292.

Magnus, P. von. (1954) Incomplete Forms of Influenza Virus. pp. 59-78. In Advances in Virus Research. Vol. II. Edited by Smith, K, M., and Lauffer, M. A. Academic Press, Inc., New York, New York.

Manson, L. A. (1953) The Metabolism of Ribonucleic Acid in Normal and Bacteriophage-Infected Escherichia coli. J. Bacteriol. 66:703-711.

Markham, R. (1953) Nucleic Acids in Virus Multiplication. pp. 85-98. In The Nature of Virus Multiplication. Edited by Fildes, P., and Heyningen, W. E. van. Cambridge University Press, Cambridge, England.

Markham, R., and Smith, K. M. (1949) Studies on the Virus of Turnip Yellow Masaic. Parasitology 39:330-338.

Marshak, A. (1948) Evidence for a Nuclear Precursor of Ribo- and Desoxyribonucleic Acids. J. Cellular Comp. Physiol. 32:381-406.

Matthews, R. E. F. (1953) Chemotherapy and Plant Viruses. J. Gen. Microbiol. 8:277-288.

Matthews, R. E. F. (1958) Studies on the Relation Between Protein and Nucleoprotein Particles in Turnip Yellow Mosaic Virus Infection. Virology 5:192-205.

Matzelt, D., Homann, J., and Lennartz, H. (1958) Das Verhalten Glykolytischer enzymaktivitaten in gewebekulturen vor und nach beimfung mit virus. II. Messungen an gewebekulturen, die mit poliomyelitisvirus, type I, infiziert wurden, im vergleich zu "hormalen" gewebekulturen. Biochem. Z. 330:260-268.

Mayer, M. M., Rapp. H. J., Roizman, B., Klein, S. W., Cowan, K. M. Lukens, D., Schwerdt, C. E., Schaffer, F. L., and Charney, J. (1957) The Purification of Poliomyelitis Virus as Studied by Complement Fixation. J. Immunol. 78:435:455.

McLimans, W. F., Siem, R. A., Mark, B. C., and Pinksa, E. (1950) A Physiological Study of Virus Parasitism. II. The Effect of Environmental Temperature on the Rates of Oxygen Consumption of Normal Eggs and Eggs Infected with Newcastle Disease Virus. J. Immunol. 64:475-488.

Meneghini, M., and Delwiche, C. C. (1951) The Multiplication of Tobacco Mosaic Virus in the Host Tobacco Plant. J. Biol. Chem. 189: 177-186.

Miroff, G., Cornatzer, W. E., and Tischer, R. G. (1957) The Effect of Poliomyelitis Virus Type I (Mahoney Strain) on the Phosphorus Metabolism of the HeLa Cell. J. Biol. Chem. 228:255-263.

Monod, J., and Wollman, E. (1947) L'inhibition de la croissance et de l'adaptation enzymatique chez les bacteries infectees per le bacteriophage. Ann. Inst. Pasteur 73:937-949.

Morgan, C., Bergold, G. H., Moore, D. H., and Rose, H. M. (1955) The Macromolecular Paracrystalline Lattice of Insect Viral Polyhedral Bodies Demonstrated in Ultrathin Sections Examined in the Electron Microscope. J. Biophys. Biochem. Cytol. 1:187-190.

Morgan, C., Ellison, S. A., Rose, H. M., and Moore, D. H. (1954) Structure and Development of Viruses as Observed in the Electron Microscope. I. Herpes Simplex Virus. J. Exptl. Med. 100: 195-202.

Morgan, C., Ellison, S. A., Rose, H. M., and Moore, D. H. (1954) Structure and Development of Viruses Observed in the Electron Microscope II. Vaccinia. J. Exptl. Med. 100:301-310.

Morgan, C., Howe, C., Rose, H. M., and Moore, D. H. (1956) Structure and Development of Viruses Observed in the Electron Microscope IV. Viruses of the RI-APC Group. J. Biophys. Biochem, Cytol, 2:351-360.

Morgan, C., Godman, G. C., Rose, H. M., and Huang, J. S. (1957) Electron Microscopic and Histochemical Studies of an Unusual Crystalline Protein Occurring in Cells Infected by Type 5 Adenovirous. Preliminary Observations. J. Biophys. Biochem. Cytol. 3:505-508.

Moulder, J. W. (1954) Biochemical Aspects of the Growth of Feline Pneumonitis Virus in the Chick Embryo. Bacteriol. Revs. 18: 170-177.

Moulder, J. W., McCormack, B. R. S., and Itatani, M. K. (1953) Energy Requirements for Synthesis of Feline Pneumonitis Virus in Isolated Chick Embryo Yolk Sac. J. Inf. Dis. 93:140-149.

Murphy, J. S., and Bang, F. B. (1952) Observations with the Electron Microscope on Cells of the Chorio-Allantoic Membrane Infected With Influenza Virus. J. Exptl. Med. 95:259-268.

Murray, R. G. E., Gillen, D. H., and Heagy, F. C. (1950) Cytological Changes in Escherichia coli Produced by Infection with Phage T2. J. Bacteriol. 59:603-615.

Murray, R. G. E., and Whitfield, J. F. (1953) Cytological Effects of Infection with T5 and Some Related Phage. J. Bacteriol. 65: 715-726.

Nagler, F. P. O. (1942) Application of Hirst's Phenomenon to the Titration of Vaccinia Virus and Vaccinia Immune Serum. Aust. J. Med. 1:281-283.

Nossal, G. J. V., and Burgh, P. de. (1953) Growth Cycle of Ectromelia Virus in Mouse Liver, Nature 172:671-672.

Newton, A., and Stoker, M. G. P. (1958) Changes in Nucleic Acid Content of HeLa Cells Infected with Herpes Virus. Virology 5:549-560.

Overman, J. R., and Tamm, I. (1957) Multiplication of Vaccinia Virus in the Chorioallantoic Membrane in vitro. Virology 3:173-184.

Pardee, A., and Williams, I. (1953) Enzymatic Activity and Bacteriophage Infection. III. Increase of Deoxyribonuclease. Ann. Inst. Pasteur 84:265-272.

Parodi, A. S., Lajmanovich, S., Pennimpede, F., and Mettelman, N. (1948) Changes in Embryonated Egges Inoculated with Influenza Virus J. Immunol. 58:109-117.

Paucker, K., Lief, F. S., and Henle, W. (1956) Studies on the Soluble Antigen of Influenza Virus. IV. Fractionation of Elementary Bodies Labeled with Radioactive Phosphorus. Virology 2:798-810.

Payne, F. E., Kurtz, H., and Ackermann, W. W. (1958) Initial Stages of the Interaction of HeLa Cells with Poliovirus. Arch. f. d. gesamte Virusforsch. 8:1-15.

Pinkerton, H., Greiff, D., Blumenthal, H. T., and Hensley, R. (1950) Influenza. II. The Effect of Influenza Virus Multiplication on the Oxygen Consumption Curves of Embryonated Egges Incubated at Different Temperatures. J. Exptl. Med. 91:331-342.

Putnam, F. W. (1952) The Origin of Bacteriophage Nitrogen, Carbon, and Phosphorus. Exptl. Cell. Research, Suppl. 2:345-366.

Putnam, F. W. (1953) Bacteriophages: Nature and Reproduction. In Advances in Protein Chemistry. 8L175-284

Putnam, F. W., and Kozloff, L. M. (1950) Biochemical Studies of Virus Reproduction. IV. The Fate of the Infecting Particle. J. Biol. Chem. 182:243-250.

Putnam, F. W., Miller, D., Palm, L., and Evans, E. A., Jr. (1952) Biochemical Studies of Virus Reproduction. X. Precursors of Bacteriophage T7. J. Biol. Chem. 199:177-191.

Raff, N., and Cohen, S. S. (1950) The Effect of Virus Infection on the Utilization of Tryptophan by Escherichia coli. J. Bacteriol. 60:69-80.

Reed, L. J., and Muench, H. (1938) A Simple Method for Estimating Fifty Per Cent Endpoints. Am. J. Hyg. 27:493-497.

Reissig, M., Howes, D. W., and Melnick, J. L. (1957) The Growth Cycle of Poliovirus in Monkey Kidngy Cells. I. Maturation and Release of Virus in Monolayer Cultures. Virology 4:97-108.

Reissig, M., and Melnick, J. L. (1955) The Cellular Changes Produced in Tissue Cultures by Herpes B Virus Correlated With the Concurrent Multiplication of the Virus. J. Exptl. Med. 101: 341-351.

Robinow, C. F. (1950) A note on stalked forms of viruses. J. Gen. Microbiol. 4:242-243.

Roizman, B., Hopkin, W., and Mayer, M. M. (1958) Immunological Studies of Poliovirus. II. Kinetics of the Formation of Infectious and Noninfectious Type I Poliovirus in Three Cell Strains of Human Derivation. J. Immunol. 80:386-395.

Rosenbaum, M., and Preston, W. S. (1958) Protein and Nucleic Acid Relationships in Escherichia coli Infected with Phage T2. J. Bacteriol. 76:155-159.

Rothstein, E. L., Colter, J. S., and Manson, L. A. (1958) Nucleic Acids of Polio-Infected HeLa Cells. Fed. Proc. 17:533.

Rubin, H., Baluda, M., and Hotchin, J. E. (1955) The Maturation of Western Equine Encephalomyelitis Virus and Its Release from Chick Embryo Cells in Suspension. J. Exptl. Med. 101:205-212.

Rysselberger, C. van, and Jeener, R. (1955) The Role of the Soluble Antigens in the Multiplication of the Tobacco Mosaic Virus. Biochim. et Biophys. Acta 17:158-165.

Schäfer, W. (1957) Units Isolated After Splitting Fowl Plague Virus. pp. 91-103. In The Nature of Viruses. Ciba Foundation Symposium. Little, Brown and Company, Boston, Massachusetts.

Schäfer, W., and Munk, K. (1952) Eigenschaften tierischer Virusarten untersucht an den Geflugelpestviren als Modell. IV. Z. Naturforsch. 7b:608.

Schäfer, W., and Zillig, W. (1954) Über den Aufbau des Virus-Element arteilchens der klassischen Geflugelpest. I. Gewinnung physikalisch-chemische und biologische Eigenschaften einiger Spaltprodukte. Z. Naturforsch. 9b:779-788.

Schäfer, W., Zillig, W., and Munk, K. (1954) Isolierung und Characterisierung hamagglutinierender, nicht infektioser Einheiten bei klassischer Geflugelpest. Z. Naturforsch. 9b:329-336.

Scherer, W. F. (1953) The Utilization of a Pure Strain of Mammalian Cells (Earle) for the Cultivation of Viruses in vitro. I. Multiplication of Pseudorabies and Herpes Simplex Viruses. Am. J. Path. 29:113-126.

Schlesinger, W. R. (1950) Incomplete Growth Cycle of Influenza Virus In Mouse Brain. Proc. Soc. Exptl. Biol. Med. 74:541-548.

Schwerdt, C. E. (1957) Physical and Chemical Characteristics of Poliomyelitis Virus. pp. 157-172. In Cellular Biology Nucleic Acids and Viruses. Vol. V. Special Publication of the New York Academby of Sciences, New York.

Schwerdt, C. E., and Fogh, J. (1957) The Ratio of Physical Particles per Infectious Unit Observed for Poliomyelitis Viruses. Virology 4:41-52.

Schwerdt, C. E., and Schaffer, F. L. (1955) Some Physical and Chemical Properties of Purified Poliomyelitis Virus Preparations. Ann. New York Acad. Sci. 61:740-750.

Scott, T. F. McN., Burgoon, C. F., Corielli, L. L., and Blank, H. (1953) The Growth Curve of the Virus Herpes Simplex in Rabbit Corneal Cells Grown in Tissue Culture with Parallel Observations on the Development of the Intranuclear Inclusion Body. J. Immunol. 71:385-396.

Siddiqi, M. S. H., Kozloff, L. M., Putnam, F. W., and Evans, E. A., Jr. (1952) Biochemical Studies of Virus Reproduction. IX. Nature of the Host Cell Contributions. J. Biol. Chem. 199: 165-176.

Sigel, M. M., Girardi, A. J., and Allen, E. G. (1951) Studies on the Psittacosis-lymphogranuloma Group. I. The pattern of multiplication of meningopneumonitis virus in the allantois of the chick embryo. J. Expt. Med. 94:401-411.

Siminovitch, L., and Jacob, F. (1952) Biosynthèse induite d'un enzyme pendant le developpement des bactériophages chez Escherichia coli K12. Ann. Inst. Pasteur 83:745-754.

Smadel, J. E., and Hoagland, C. L. (1942) Elementary Bodies of Vaccinia. Bacteriol. Revs. 6:79-98.

Smellie, R. M. S. (1955) The Metabolism of the Nucleic Acids. pp. 393-434. In The Nucleic Acids. Vol. II. Edited by Chargaff, E., and Davidson, J. N. Academic Press, Inc., New York, N. Y.

Smellie, R. M. S., McIndoe, W. M., Logan, R., Davidson, J. N., and Dawson, I. M. (1953) Phosphorus Compounds in the Cell. IV. The Incorporation of Radioactive Phosphorus into Liver Cell Fractions. Biochem. J. 54:280-286.

Smith, K. M., and Xeros, N. (1954) A comparative Study of Different Types of Viruses and Their Capsules in Polyhedroses and Granduloses of Insects. Parasitology 44:400-406.

Spizizen, J. (1957) The Effect of Virus Infection on Pyruvate Metabolism. Biochim. et Biophys. Acta 23:333-341.

Spizizen, J. (1957) Infection of Protoplasts by Disrupted T2 Virus. Proc. Natl. Acad. Sci. 43:694-701.

Stent, G. S., and Maaløe, O. (1953) Radioactive phosphorus Tracer Studies on the Reproduction of T4 Bacteriophage. II. Kinetics of Phosphorus Assimilation. Biochim. et Biophys. Acta 10:55-69.

Stevens, C. E., Daoust, R., and Leblond, C. P. (1953) Rate of Synthesis of Desoxyribonucleic Acid and Mitotic Rate in Liver and Intestine. J. Biol. Chem. 202:177-186.

Takahashi, W. N. (1955) Anomalous Proteins Associated with Three Strains of Tobacco Mosaic Virus. Virology 1:393-396.

Takahashi, W. N., and Ishii, M (1952) An Abnormal Protein Associated With Tobacco Mosaic Virus Infection. Nature 169:419-420.

Tenenbaum, E. (1957) Changes in Cellular Nucleic Acids During Infection With Poliomyelitis Virus as Studied by Fluorescence Microscopy. Nature 150:1044-1052.

Tomizawa, J., and Sunakawa, S. (1956) Effect of Chloramphenicol on DNA Synthesis and the Development of UV Resistance in E. coli Infected with T2. J. Gen. Physiol. 39:553-565.

Tousimis, A. J., and Hilleman, M. R. (1957) Electron Microscopy of Type 4 Adenovirus Strain RI-67. Virology 4:499-508.

Uhlendorf, B. W. (1957) The Intracellular Synthesis and Maturation of a Viral Precursor. Thesis. University of Michigan, Ann Arbor, Michigan.

Uhler, M., and Gard, S. (1954) Lipid Content of "Standard" and "Incomplete" Influenza A Virus. Nature 173:1041-1043.

Visconti, N., and Delbrück, M. (1953) The Mechanism of Genetic Recombination in Phage. Genetics 38:5-21.

Volkin, E., and Carter, C. E. (1951) The Preparation and Properties of Mammalian Ribonucleic Acids. J. Am. Chem. Soc. 73:1516-1530.

Volkin, E., and Astrachan, L. (1956) Phosphorus Incorporation in Escherichia coli Ribonucleic Acid After Infection with Bacteriophage T2. Virology 2:149-161.

Watson, B. K., and Coons, A. H. (1954) Studies on Influenza Virus Infection in the Chick Embryo Using Fluorescent Antibody. J. Exptl. Med. 99:419-428.

Watson, J. D., and Maaløe, O. (1953) Nucleic Acid Transfer from Parental to Progeny Bacteriophage. Biochim. et Biophys. Acta 10:432-442.

Wecker, von E., and Schäfer, W. (1957) Eine infektiöse Komponente von Ribonucleinsäure Pferde-Encephalomyelitis (Typ Ost) Z. Naturforsch. 12b:415-417.

Weed, L. L., and Cohen, S. S. (1951) The Utilization of Host Pyrimidines In the Synthesis of Bacterial Viruses. J. Biol. Chem. 192: 693-699.

Wildman, S. G., Cheo, C. C., and Bonner, J. (1949) The Proteins of Green Leaves. III. Evidence of the Formation of Tobacco Mosaic Virus Protein at the Expense of a Main Protein Component in Tobacco Leaf Cytoplasm. J. Biol. Chem. 180:985-1001.

Wildy, P., and Holden, H F. (1954) The Complement Fixing Antigen of Herpes Simplex Virus. Aust. J. Exptl. Biol. 32:702-710.

Wyatt, G. R., and Cohen, S. S. (1953) The Bases of the Nucleic Acids of Some Bacterial and Animal Viruses: The Occurrence of 5-hydroxymethylcytosine. Biochem. J. 55:774-784.

Wyckoff, R. W. G. (1948) The Electron Microscopy of Developing Bacteriophage. Biochim et Biophys. Acta 2:27-39.

Wyckoff, R. W. G. (1953) Formation of the Particle of Influenza Virus. J. Immunol. 70:187-196.

Yanamura, Y. H., and Meyer, K. J. (1941) Studies on the Virus of Psittacosis Cultivated in vitro. J. Inf. Dis. 68:1-11.

Zinder, N. D., and Lederberg, J. (1952) Genetic Exchange in Salmonella. J. Bacteriol. 69:9-15.