"THE GROWTH CYCLE OF POLIOVIRUS IN CULTURED CELLS"

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A thesis submitted for the degree
of Doctor of Philosophy in the
Australian National University.

12 MAY 1961
151332

The first of the papers submitted in this thesis ("1. The maturation and release of virus in monolayer cultures.") was largely based on work carried out in collaboration with Dr. Magdelana Reissig, under the general direction of Dr. J.L. Melnick, at the Section of Preventive Medicine, Yale University School of Medicine. This study was completed at the Institute of Medical and Veterinary Science, Adelaide, South Australia. This work was designed to correlate the maturation and release of virus with the sequence of changes in morphology which the host cell suffers as a result of infection. A description of the changes in morphology of the host cell was published separately, (Reissig, Howes and Melnick, 1956, J. Exptl.Med., 104, 289), and this work has not been included in this thesis. The remaining papers were based on independent studies carried out at the Institute of Medical and Veterinary Science, and the Department of Microbiology, Australian National University, and are my own original work.

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I. INTRODUCTION

Although Landsteiner and Popper first isolated poliovirus in 1909, a long time elapsed before adequate techniques were developed to enable studies such as those described in this thesis to be made.

The possibility that animal cells cultured in vitro might be used as an alternative to laboratory animals for growing animal viruses was recognised many years ago when Steinhardt et al (1913) demonstrated survival and probable multiplication of vaccinia virus in tissue fragments. Multiplication of this virus was not shown unequivocally however, until 1925, when Parker and Nye carried it through many passages in rabbit testicular tissue. The first practical application of such techniques in the assay of virus was that of Huang (1943), who used the capacity of western equine encephalomyelitis virus to prevent the growth of cells as the basis of a method for the assay of virus. Although this method of assay was not widely used, it demonstrated the potential value of cell culture methods in the field of virology.

At this time polioviruses could be assayed only in monkeys, except for a few strains which could be assayed in mice or cotton rats. In 1949, however, Enders and his co-workers found that human cells of non-neural origin not only supported the growth of polioviruses, but were destroyed at the same time. These studies were of twofold importance, for they provided a simple host cell system for the growth and assay of virus, and contradicted the view that polioviruses were strict neurotropes.

These findings were rapidly confirmed by many workers using cultures of primate cells, and over the next few years cell culture techniques were quickly adapted to the needs of animal virologists. They were used initially for the intensive study of the pathogenesis and epidemiology of poliomyelitis, but were extended rapidly to other known viruses.

The assays were still of the quantal type however, and the increase in accuracy due to reduced "host" variability was only slight. The precision of assay was still insufficient for many detailed quantitative studies to be undertaken.

This problem was finally overcome in 1952 by Dulbecco, who used monolayers of animal cells grown in Petri dishes, and showed that when a solid nutrient overlay was used in place of liquid medium, western equine encephalomyelitis virus produced readily recognisable plaques in the cell layer. The technique was adapted for use with polioviruses shortly afterwards (Dulbecco and Vogt, 1954), and has since been applied to many other animal viruses.

Many viruses could now, for the first time, be assayed at a level of precision which was adequate for detailed studies of their physico-chemical and biological properties to be undertaken.

At the present time a large proportion of the research in animal virology centres around the growth of viruses in their host cells. Although such studies have as their ultimate goal the recognition of all the steps involved in the production and release of viral progeny from the infected cell, the logical first step is to define the duration of the cycle in terms of infectious or mature virus. This study of the initial disappearance of infecting virus, and the final maturation and release of the progeny of this virus, provides the framework for the analysis of the

replication of "vegetative" virus during the intervening period.

The events or steps in replication must be considered in relation to the physico-chemical nature of the virus involved. In recent years it has been shown that different animal viruses vary in important respects in their physico-chemical composition and biological properties. It is important to determine how these differences are related to differences in the nature of replication of "vegetative" virus. The study of a single "model" virus is therefore insufficient, and it is necessary to select at least one representative of each group of viruses for study. The papers which are presented in this thesis describe results of studies of various aspects of the growth of a representative of the poliovirus group.

The first paper (Howes and Melnick, 1957) is a detailed account of that part of the study which deals with the production and release of mature virus. The observations on the sequence of changes in host cell morphology, and its correlation with maturation and release of virus have been published separately

(Reissig, Howes and Melnick, 1956). The experiments described in the first paper were carried out under conditions favouring readorption of released virus to cells, and the delayed release of virus was not demonstrated unequivocally. To overcome this objection further experiments were carried out under more suitable conditions, and were reported in the second paper. The third paper is concerned with the analysis of the growth cycle seen in cell populations in terms of the growth cycle in individual cells. The relation of these papers to each other and to other studies of the growth cycles of animal viruses is discussed in greater detail in following sections.

II. PUBLISHED PAPERS PRESENTED

Howes, D.W. and Melnick J.L. (1957).

"The growth cycle of poliovirus in monkey kidney cells. I. Maturation and release of virus in monolayer cultures". Virology 4, 97-108.

Howes, D.W. (1959a).

"The growth cycle of poliovirus in cultured cells. II. Maturation and release of virus in suspended cell populations." Virology 9, 96-109.

Howes, D.W. (1959b).

"The growth cycle of poliovirus in cultured cells. III. The asynchronous response of HeLa cells multiply infected with Type 1 poliovirus." Virology 9, 110-126.

The Growth Cycle of Poliovirus in Monkey Kidney Cells

I. Maturation and Release of Virus in Monolayer Cultures¹

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Accepted May 1, 1957

Monolayers of monkey kidney cells were infected with type 1 (Brunhilde) poliovirus and observed over a single cycle of virus growth. At intervals after infection assays of the amount of virus in the cell monolayer ("cell-associated" virus) and in the medium surrounding the cells ("free" virus) were carried out using the plaque technique.

Fifty per cent of the total amount of virus produced appeared within 4.8 to 6.2 hours.

At no time did the free virus constitute more than a small proportion of the total virus and even at 11 hours was less than 20%. This suggested that the virus was released at a slow rate. It was argued that mechanical retention (readsorption) of virus in the monolayer could account for only a portion of this apparent delay.

Whatever interpretation is put on these results they show that in this and similar virus systems it is necessary to assay total virus (cell-associated plus free 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 free virus may be misleading. This is in contrast to other systems (for example WEE—chick embryo fibroblasts) where virus is released very rapidly and free virus concentration may be considered a reasonable approximation of "total" virus.

INTRODUCTION

The application of tissue culture procedures to the study of the poliomyelitis group of viruses by Enders *et al.* (1949) and the refinement of

¹ This investigation was aided by a grant from The National Foundation for Infantile Paralysis and in part by a research contract (SA43-PH720) with the Division of Biologics Standards of the National Institutes of Health, United States Public Health Service.

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these techniques by Dulbecco and Vogt (1954) to provide an accurate assay method, make the interrelationship of virus and cell far more amenable to critical study. In studies correlating virus growth with biochemical or cytological changes in the cell, it is necessary to define the kinetics of virus growth within, and release from, infected cells under various conditions of cell growth and manipulation. Such studies in most cases provide unequivocal results only when the conditions of the one-step growth experiment, first defined for bacteriophage by Ellis and Delbruck (1939), are satisfied. These conditions may to a large extent be duplicated for the poliovirus-monkey kidney cell system but for technical reasons studies of morphological changes occurring in the cells as a result of infection are best studied in monolayers.

In this paper experiments are described in which monolayers of monkey kidney cells were infected with an excess of type 1 (Brunhilde) poliovirus. At intervals after infection the amounts of virus present in the cell monolayer ("cell-associated" virus) and in the medium above the cells ("free" virus) were assayed.

Reissig *et al.* (1956) have described results of a parallel study of the morphological changes in monkey kidney cells following infection with type 1 poliovirus.

MATERIALS AND METHODS

The general procedure adopted for these experiments is outlined in the accompanying Fig. 1. Details of materials and methods are given below.

Media and Solutions

a. Growth medium (Melnick, 1955). Monolayers used in assays and in the actual growth curve experiments were grown in a medium consisting of 0.5 % lactalbumin hydrolyzate, 2 % calf serum, and 97.5 % Hanks' salt solution. The same medium was used for the maintenance of cells after infection.

b. Phosphate buffered saline (PBS), as described by Dulbecco and Vogt (1954).

c. PBS minus calcium and magnesium ions as described by Lwoff *et al.* (1955).

d. Versenate (0.02 %) as described by Lwoff *et al.* (1955).

e. Agar overlay: (1) threefold concentrated Earle's solution containing 30 % monkey serum, 300 units of penicillin/ml and 300 μ g streptomycin/

FIG. 1. General outline of procedure in growth curve experiments

ml; (2) 1:10,000 neutral red solution in water; (3) 2.7 % agar in water (Noble agar, Difco).

Equal volumes of (1) and (2) were mixed, and CO₂ bubbled through the mixture until deep red. The temperature of the mixture was raised to 43–45°.

After liquefying the agar by boiling and subsequently cooling to 43–45°, 2 volumes of the (1) plus the (2) mixture were added to 1 volume of agar (3). To each 6-cm plate, 4.5 to 5 ml of the overlay were added.

Incubation of Cultures

Cultures prior to and after use were incubated at 37° in an incubator gassed with a mixture of 5 to 10 % CO₂ in air.

Preparation of Monolayer Cultures for Use in Growth Cycle Experiments or for Virus Assays

Monkey kidney cells which had been grown for 7–10 days in growth medium in large Roux bottles (Melnick, 1955) were suspended by incubating for up to 30 minutes with 0.02 % sodium Versenate solution. After low speed centrifugation, the deposited cells were suspended in growth medium. An aliquot was diluted in a 0.1 % crystal violet solution in 0.1 % citric acid, and counted in a hemocytometer. The cell concentration was adjusted to 4×10^5 cells/ml. To each 6-cm petri dish, 5 ml of this suspension were added, and the cultures incubated for 2 to 3 days.

Infection of Monolayers

Infection of a high proportion of cells was assured by use of high input multiplicities of 4 or more (0.5 ml of tissue culture fluid containing between 10^7 and 10^8 plaque-forming units/ml). During the adsorption period of 1 hour at 37°, the cultures were agitated gently every 5 minutes to redistribute the inoculum. At the end of the adsorption period, the 0.5-ml inoculum was removed and the plates washed five times with 5 ml cold PBS. Finally, 3 ml growth medium at 37° was added.

Multiplicity of Infection

At the same time that the growth cycle experiments were carried out, a parallel series of cultures was examined for morphological changes (Reissig *et al.*, 1956). Between 200 and 1000 cells were examined at intervals after infection, and the percentage of cells showing changes characteristic of infection was recorded (Fig. 2). Using this percentage,

FIG. 2. Proportion of cells with virus-induced changes with regard to time after inoculation. Broken line: altered cells, all changes. Solid line: altered cells, excluding those with intranuclear inclusions only.

the average multiplicity of infection (m) was calculated from the following equation:

$$\text{Per cent infected} = 100 (1 - e^{-m}),$$

on the assumption that the Poisson distribution is applicable to the adsorption of virus by cells.

Since some infected cells became rounded and detached from the glass surface late in the cycle, the observed percentage of infected cells was underestimated. Hence the value obtained for the multiplicity of infection was a minimum. This procedure was followed for three of the five experiments described in this paper, when values for minimal multiplicities of 1.0, 2.1, and 3.2 were obtained.

Method of Harvesting Free Virus and Cells

At each time interval the supernatant fluid from one culture was removed. Those supernatants removed 5 hours or more after infection, were centrifuged at an average value of 200 g for 10 minutes in the horizontal head of an International refrigerated centrifuge to remove any

FIG. 3. Disruption of cells by sonic vibrations (A) or by shaking with Alundum (B).

infected cells which may have left the glass as a result of infection. To the cell monolayer, 3 ml freshly iced growth medium was added and the cells were scraped off the glass with a rubber "policeman." These cell-associated and free virus fractions were then stored frozen at -10° until assayed.

Disruption of Cells to Obtain "Cell-Associated" Virus

*a. Mickle shaker.** Initially, shaking at 120 cps for 15 minutes in a Mickle shaker with Alundum was used to disrupt infected cells. Four grams of Alundum powder to 10 ml fluid in each cup was found to produce disruption of over 95% of cells within 15 minutes at an amplitude of approximately 1 cm (Fig. 3).

*b. Raytheon Magnetostrictor.** For convenience in treating large numbers of samples, sonic vibration at 9000 cycles/sec was used in most

* Kindly made available by the Department of Microbiology.

TABLE 1
ACTION OF SONIC VIBRATION AND SHAKING WITH ALUNDUM ON TYPE 1
POLIOMYELITIS VIRUS (BRUNHILDE)

Experiment number	Treatment	Plaque counts	Total count	Mean count
1	No treatment	22, 28, 13, 20, 15, 15	113	18.8
	Sonic vibration, (9000 cps) for 3 minutes	17, 15, 18, 30, 25, 11	116	19.3
2	No treatment	16, 15, 17	48	16.0
	Shaking with Alundum for 5 minutes	33, 28, 30	91	30.3
	10 minutes	8, 11, 14	33	11.0
	60 minutes	41, 29	70	35.0
3	No treatment	23, 15, 12	50	16.7
	Shaking with Alundum for 30 minutes	10, 17, 13	40	13.3

experiments. A light plastic adaptor fitting snugly within the Raytheon head was constructed to take lusteroid tubes of 1.6 cm in diameter. Tubes were sealed with a rubber stopper and the lower portion of the tube containing 1.5 ml of cell suspension was surrounded by 10 ml of water to provide effective transmission of vibrations from the baseplate to the contents of the tube. As shown in Fig. 3, this method produced breakdown of over 99% of cells within 1 minute. Nuclei were disrupted more slowly than the cytoplasm, although even nuclei were reduced to less than 1% of the initial cell count within 3 minutes. After the 2- to 3-minute treatment was given to the samples which were to be assayed, the cellular debris was very finely dispersed.

Neither treatment in the Mickle shaker with Alundum, nor treatment with sonic vibrations at approximately 9,000 cps resulted in any significant reduction of the plaque count of a suspension of type 1 poliovirus (Table 1).

Virus assays

These were carried out using the plaque technique of Dulbecco and Vogt (1954). Primary monkey kidney cultures 5 to 7 days old, or secondary cultures 2 to 3 days old, were washed with PBS and drained, and then 0.3 ml of virus dilution was added. After 30 minutes incubation at 37° in a CO₂-gassed incubator, 5 ml of overlay at 43–45° was added and after it had set, the plates were incubated for 3 days. Plaques first

FIG. 4. "One-step" growth curves of type 1 poliovirus in monkey kidney monolayers. Data shown for Exp. I and II.

appeared under these conditions within 48 hours at which time they were marked. Plaques were counted on the third day and the results were expressed as plaque-forming units (PFU) per culture. For each sample, assays were made in duplicate cultures.

RESULTS

The results of two growth cycle experiments are shown in Fig. 4. No increase in the amount of cell-associated virus was detected until after 3 hours, when the virus began to increase rapidly. Fifty per cent of the virus produced was detected within 2 to 3 hours of the initial increase in cell-associated virus, and maturation was virtually complete within 3 more hours, i.e., 8 to 9 hours after infection of the cells. The average yields of virus per infected cell were 138 and 93 plaque-forming units for Experiments I and II of Fig. 4. In a third experiment a value of approximately 70 PFU per infected cell was obtained, giving an average value over three experiments of 100 PFU per infected cell.

To characterize the growth cycle with respect to the appearance of mature virus, we have made use of the term "average maturation time," defined as the time elapsing between inoculation and the appearance of 50% of the total mature (infectious) virus produced by the cells. Estimates of average maturation time made graphically from results of four growth cycle experiments involving different batches of cells were 5.7, 4.8, 5.3, and 6.2 hours giving an average value of 5.4 hours.

The curves for free virus in the experiments described in Fig. 4 show that increase in free virus is less rapid than increase in mature virus in monolayer, and that at the time 50% of mature virus had been produced by the cells, only a very small proportion of the total virus present had appeared as free virus. In the two experiments described, free virus amounted to 0.11 and 0.15% respectively of the total virus present at that time, while in two further experiments, values of 0.06 and 0.04% were observed. Increase in free virus could first be detected in Experiment II at the same time as increase in cell-associated virus was observed. In Experiment I however, increase in free virus was not detected until 1 to 1½ hours after cell-associated virus had begun to increase.

Once initiated, increase in free virus continued throughout the period during which the cycle was studied, but even after 11 hours (in Experiment I), less than 20% of the total virus produced had appeared in free form. The marked quantitative differences between cell-associated and free virus described above have been consistently observed in a total of five separate experiments in which these fractions have been followed for periods of from 8 to 11 hours.

DISCUSSION

In the experiments described in this paper, we found the average maturation time of type 1 poliovirus grown in monkey kidney cells to be between 4.8 and 6.2 hours, with a mean value of 5.4 hours. This may be compared with an average maturation time of approximately 4 hours for Western equine encephalomyelitis (WEE) virus determined from the data of Rubin *et al.* (1955), or an "average liberation time" of influenza virus from cells in the allantoic cavity of embryonated eggs, of 7½–9 hours for the first cycle and 4½–7 hours for subsequent cycles (Fazekas de St. Groth and Cairns, 1952).

We have attempted to characterize the growth cycle more fully by making use of the term average maturation time, defined as the time elapsing between inoculation and the appearance of 50% of the *total*

mature (infectious) virus produced by the cells. This parameter is more acceptable than the time at which new virus is first detected, as the latter is characteristic of the small proportion of cells which first produce mature virus in detectable amounts, and does not indicate the average behavior of the population as a whole.

Free virus was found to increase more slowly than cell-associated virus and while maturation was proceeding, free virus was always only a small proportion of total virus. Thus, when 50 % of mature virus had appeared in the monolayer culture, between 0.04 and 0.15 % had appeared in a free state. Even after 11 hours, free virus was less than 20 % of the maximum level of total virus observed. Proportionate differences between the curves for free virus in the experiments shown in Fig. 4 were most pronounced when the number of virus units released was small in relation to the number of infected cells present. This portion of the curve is determined by the first cells to release virus, and characterizes the extreme and not the most frequent cell type. The differences were less marked in the later stages of virus release when presumably a much higher proportion of the cell populations had contributed to the free virus levels observed. Since the initiation of the processes of virus maturation and release may be separated in time and may proceed at different rates, a growth cycle would be more completely described by determining both the average maturation time defined here, and the average liberation time of Cairns (1952). This was unfortunately not possible in these experiments since free virus increase was delayed sufficiently to prevent the 50 % liberation time being reached within the time covered by our experiments.

Growth cycles determined with cell monolayers, although assuring undamaged cells, have the disadvantage that virus released from cells may be retained mechanically in the monolayer in an intercellular location, while readsorption of released virus to cells is favored. Mechanical retention of released virus as "intercellular" virus cannot occur at the free surface of the cell, except as an inherent characteristic of the release process itself. Assuming random release on the average over the total cell surface, the proportion of virus released directly into the medium will be determined by cell density in the monolayer. The upper limit for the ratio of free to total cell surface is 50 % for a monolayer of perfectly flat cells. For cubical cells the ratio is 16.7 %, and for cells $4 \times 2 \times 1$ and $4 \times 1 \times 2$ for length, breadth, and depth, 29 and 14.3 % respectively. It seems reasonable to expect from the appearance of cells in monolayers

that between 20 and 40% of virus will be released directly into the medium where it may remain free or be reabsorbed. Mechanical retention therefore does not account for more than a small proportion of the differences observed between amounts of cell-associated and free virus which, at certain times, differed by a hundredfold or more.

Unfortunately the proportion of virus adsorbed at the surface of cells themselves producing the virus is not known, and could account for a large proportion of the quantitative differences between the two virus fractions. On the other hand, there is also a possibility that there is an appreciable delay in the release of virus from the cell. That a prolonged delay in release may occur, has since been adequately confirmed by studies of the growth cycle in suspended cells under carefully controlled conditions (Howes, data to be published). A similar delay in release may be inferred for HeLa cells in suspension (Girardi *et al.*, 1956), especially when held at 25° (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 monkey kidney cells, and that of such viruses as Western equine encephalomyelitis virus in chick fibroblasts where release is very rapid (Rubin *et al.*, 1955). The delay in virus release might lie in a physicochemical barrier to release associated with a structure normally present in the cell under these conditions, but perhaps 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, or it may in the initial stages partially or completely inhibit release of virus, and then by a process akin to lysis release it over a short time interval. In studies of release of poliovirus from single cells reported by Lwoff *et al.* (1955), release of the major part of the virus produced by a cell was found to occur over a fairly short time interval, and the authors suggested that release accompanies partial lysis of the cell.

In view of the probability of reabsorption and mechanical retention of released virus to the monolayer we have referred to the two virus fractions studied as cell-associated and free virus, rather than use the possibly misleading terms "intracellular" and "extracellular."

The results described above are of some importance in relation to studies aimed at correlating the biochemical activities of the cell, or morphological changes, with virus synthesis. With virus-cell systems in which virus release is rapid, and its initiation in no way delayed, free

virus is probably a reasonable approximation to total virus and may be used as a simple means of following virus synthesis. With the polio-virus-monkey kidney cell system, and perhaps other systems, assay of free virus is insufficient. As shown in this paper, this is particularly true of experiments involving cell monolayers in which maturation is virtually complete several hours before even half the virus appears in the supernatant fluid, and changes associated with dissolution of the cell may well be linked erroneously with virus synthesis. Where any substantial delay in virus release occurs, total virus should be assayed.

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VIROLOGY 9, 96-109 (1959)

The Growth Cycle of Poliovirus in Cultured Cells

II. Maturation and Release of Virus in Suspended Cell Populations¹

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Accepted June 10, 1959

The growth cycle of type 1 poliovirus in suspended monkey kidney cell populations is characterized by approximately linear increase in infectious virus, and delayed release. In multiply infected cells virus reaches peak levels within 10-12 hours of infection. Although some virus is released in the early stages of the maturation phase, most accumulates in association with cells, to be released after maturation has ceased. This cell-associated virus is intracellular, as was shown by its resistance to treatment with specific antibody.

INTRODUCTION

In a previous publication (Howes and Melnick, 1957), studies of the growth cycle of type 1 poliovirus (Brunhilde) in monolayers of monkey kidney cells were described. During production of infectious virus, most of the detectable virus remained associated with the cells ("cell-associated" virus), and little was released into the medium ("free" virus). Similar results for cell monolayers have since been reported for type 1 poliovirus strains by Darnell (1958), who used cells derived from the S3 clone of HeLa cells, and by Roizman *et al.* (1958), who used three cell strains of human origin.

From the total infectious virus present at any time during the growth cycle, the proportion which may be recovered in the medium will be governed primarily by (1) the delay in release of virus from the parent

¹ A part of this work was carried out under a grant from the Australian National Health and Medical Research Council.

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cell, (2) the extent to which released virus is mechanically trapped within the cell monolayer, (3) the extent of readsorption of released virus, and (4) possible differences in the rates of inactivation of free and cell-associated virus.

To obtain more precise information about the release of poliovirus from monkey kidney cells, further experiments were carried out using dilute cell suspensions, in which mechanical trapping and readsorption of free virus are negligible. The experiments described demonstrate that delayed release is a prominent feature of the growth cycle of poliovirus.

MATERIALS AND METHODS

Cell Cultures

Monkey kidney cells. Suspensions of kidney cells were prepared from young monkeys (*M. iris*) by the continuous trypsinization technique described by Rappaport (1956). Monolayer cultures were prepared in petri dishes of 50 mm internal diameter, using a growth medium consisting of 95 parts Hanks' solution containing 0.5 % lactalbumin hydrolyzate, penicillin (200 units per milliliter), and streptomycin (200 μ g per milliliter); and 5 parts calf serum. Confluent growth of cells was usually attained after 5-7 days' incubation.

ERK (Westwood et al., 1957) and HeLa Cells. These were passaged serially in Eagle's medium plus 15 % calf serum. Infected cell suspensions were prepared from monolayers of these cells grown in petri dishes.

Virus

High titer stocks of the Brunhilde strain of poliovirus were prepared in monolayers of monkey kidney cells. Initially growth medium containing 5 % calf serum was used to maintain the cells after infection. In later experiments virus stocks were prepared from cells maintained after infection in Earle's saline containing 5 mmoles glucose and 2 mmole glutamine (Darnell, 1958). Supernatant fluids were harvested 24 hours after inoculation, frozen and thawed once, and then centrifuged to remove cell debris.

Virus Assays

Plaque assays were carried out using monkey kidney monolayer cultures. Virus samples were diluted in the phosphate-buffered saline (PBS) of Dulbecco and Vogt (1954), and 0.3 or 0.5 ml of the dilutions

added to cultures washed once with 5 ml PBS. The virus was allowed to adsorb for 30 minutes at 37°, and then 5 ml agar overlay (see below) at 43° to 45° was added to each culture. The cultures were incubated at 37° in an atmosphere of 5 % CO₂ and air, and the plaques marked and counted after 2, 3, and 4 days.

The agar overlay contained 1 % agar, Earle's salt solution modified to contain 0.2 % glucose, neutral red 1:30,000, 5 % serum, and antibiotics. In early experiments calf serum was used in the overlay, but this was replaced by noninhibitory rabbit serum when some batches of calf serum were found to contain virus inhibitors. Before use, the nutrient solution was gassed with CO₂ until it had become a deep-red color.

Experimental Procedures Used in Growth Cycle Experiments

To prepare suspensions of infected cells, cultures washed once with PBS were seeded with 0.5 ml of stock virus diluted in PBS to give the required multiplicity of infection. The virus was allowed to adsorb for 30 minutes at 37°, and the cultures were then washed three or four times with PBS and once with PBS from which the calcium and magnesium salts had been omitted. Cells were then removed from the glass by incubation at 37° with a 0.02 % sodium Versenate solution (Lwoff *et al.*, 1955), or with a 0.25 % trypsin solution in Ca- and Mg-free PBS. Sometimes incubation of up to 40 minutes with these suspending agents was necessary to remove cells from the glass surface. This resulted in a marked reduction in the yield of "fertile" cells, a deficiency which was overcome in later experiments by using 0.025 % trypsin and 0.01 % Versene in Ca- and Mg-free PBS, which detached cells within 10 minutes and gave high yields of fertile cells (up to 80 %).

The suspensions were centrifuged lightly to deposit the cells, and these were then suspended in medium, pipetted gently to break up clumps, counted in a hemocytometer, and then diluted to a concentration of between 1 and 5×10^4 cells per milliliter. All solutions used in these procedures were prewarmed to 37°.

The cell suspension was finally placed in an Erlenmeyer flask treated with silicone³ to discourage attachment of cells to the glass, the air displaced by a 5 % CO₂ air mixture, and the flask incubated at 37°. The cells were kept in suspension by gentle stirring with a suspended magnet.

³ Silicone fluid M441, Nobel Division, I.C.I. Ltd., Glasgow, or "Siliclad", Clay-Adams Inc., New York, New York.

At intervals samples of the suspension were removed from the flask, and one portion of each sample was frozen immediately in an alcohol-dry ice bath. The other portion was centrifuged at 1500 rpm for 3 minutes in a bench centrifuge, and part of the supernatant was removed and frozen. These two portions were then assayed to give estimates of total virus and free virus levels, respectively, at each sampling time.

Release of Cell-associated Virus from Cells

In preliminary experiments the amount of virus released from monkey kidney or ERK cells by one or two cycles of freezing and thawing, was compared with that released by ultrasonic vibration at 20 kilocycles for 30 seconds (Mullard Drill Generator). At the time when the cells were harvested, most of the virus was still associated with the cells.

Results shown in Table 1 suggest that ultrasonic vibration may give a slightly larger virus yield than a single cycle of freezing and thawing. However, the technical difficulties involved in treating large numbers of samples by the former method outweighed the advantage of a slightly better virus yield. The much simpler single cycle of freezing and thawing was therefore used in these experiments to release cell-associated virus. Free virus was also subjected to the same treatment although, as

TABLE 1

COMPARISON OF RELEASE OF CELL-ASSOCIATED VIRUS BY FREEZING AND THAWING, AND BY TREATMENT WITH AN ULTRASONIC GENERATOR^a

Expt. No.	Cell type	Virus strain	Treatment	Virus yield (PFU/ml)
1	Monkey kidney	Brunhilde	Frozen and thawed once	16.2×10^5
			Frozen and thawed twice	12.0×10^5
			"Sonicated" 30 seconds (1)	18.4×10^5
			"Sonicated" 30 seconds (2)	19.4×10^5
2	ERK	Mahoney	Frozen and thawed once	6.5×10^5
			Frozen and thawed twice	7.4×10^5
			"Sonicated" 30 seconds	6.5×10^5
3	No cells (free virus)	Brunhilde	Frozen and thawed once	580
			Frozen and thawed twice	390
			Not treated	460

^a Cell suspensions were harvested at the end of the maturation phase when most virus was still associated with cells. Cells in aliquots of the suspension were disrupted by freezing and thawing, or by treating in an ultrasonic generator for 30 seconds. The virus concentration of each aliquot was then determined.

is shown in Table 1, such treatment did not reduce the amount of virus in this fraction.

Estimation of Multiplicity

The average number of infectious virus particles which were adsorbed per cell was determined by inoculating two or more cell cultures with 0.5 ml of the stock virus diluted in PBS so as to give a countable number of plaques. After a 30-minute adsorption period at 37°, the cultures were washed in the same way as the cultures used to prepare the cell suspensions. To each of these cultures 5 ml overlay was added, and the plaques subsequently produced were counted after 2, 3, and 4 days' incubation. From the 4-day counts and the dilution factors, the number of plaque-forming units (PFU) adsorbed by the cultures inoculated with concentrated virus was determined.

From the number of PFU adsorbed per culture and the number of cells present per culture (determined during preparation of the infected-cell suspension), the average multiplicity of infection (adsorbed PFU) was estimated.

Estimation of "Fertile" Cells Present in Suspensions

In most experiments estimates of the number of virus-yielding (fertile) cells in the cell suspensions were obtained by determining the number of plaque-forming cells present. The cell suspensions were diluted in growth medium, and 0.5-ml volumes of these dilutions were added to groups of two to four cultures. One milliliter of overlay was then added to each culture, mixed with the inoculum, and allowed to set. A nutrient layer of 4 ml overlay was then added. In this way the cells to be assayed were held within a short distance of the "indicator" cell layer. Occasionally an alternative technique was used, in which a number of tubes were seeded with diluted cell suspension so that each tube contained a few cells. Total virus yields of these tubes were determined 10–12 hours after infection. The average number of fertile cells per tube, m , may be calculated from the proportion of tubes which yielded virus, p , from the relationship $p = 1 - e^{-m}$.

EXPERIMENTAL RESULTS

I. Growth Cycle of Brunhilde Virus in Monkey Kidney Cells Suspended in Calf Serum Medium

Initially the growth cycle was studied in cell suspensions maintained in the 10 % calf serum medium described in Materials and Methods.

FIG. 1. The growth cycle of type 1 poliovirus in monkey kidney cells suspended in media composed of 10% calf serum in Hanks' solution plus 0.5% lactalbumin hydrolyzate (curve A); and 10% monkey serum, 20% chick embryo extract in Earle's solution (curve B). Maturation of virus and its subsequent inactivation are shown by the changes in total virus concentration. The progress of virus release is shown by changes in the concentration of free virus. In both experiments cells were treated with Versene after infection, suspended at a concentration of 10^4 cells per milliliter. The average adsorbed multiplicities of infection were A, 4.9; B, 10.

These and subsequent experiments were carried out using multiply infected cells, unless otherwise stated. Results of a typical experiment shown in Fig. 1 (curve A), are plotted on a linear (percentage) scale, as well as the more conventional logarithmic scale.

Production of mature (infectious) virus. Mature virus increased for a period of up to 8 hours following the end of the eclipse period which was of approximately 4 hours' duration (range 3.5–4.7 hours in seven experiments). During production of the major portion of the virus yield, virus increase was approximately linear. Similar results were obtained for singly infected cells (Fig. 2). Once peak levels were reached, a fall in concentration followed. Virus yields (peak titers) varied between 100 and 200 PFU per fertile cell.

Virus release. In both singly and multiply infected cells (Figs. 1 and 2), the latent period was 30–60 minutes longer than the eclipse period. However, although some virus was released during the maturation

Fig. 2. Maturation and release of poliovirus within suspensions of singly infected cells. Monkey kidney cells infected at average adsorbed multiplicities of 1.1 and 0.08 were suspended in 10% calf serum medium, and virus maturation and release were followed by assaying total and free virus levels, respectively.

phase, most of it accumulated in association with the cells. At the time when total virus was at its maximum level, in multiply infected cells less than 30 % was in the free state. Over the next 10 hours, as total virus concentration declined, that fraction present as free virus increased, a change consistent with continued release of virus.

II. The Effect of Medium Composition on Virus Release

Single monkey kidney cells studied by Lwoff *et al.* (1955) released their poliovirus yields within 10 hours of infection, whereas the cell populations studied in the preceding section had released only small fractions of their yields within the same period. This discrepancy could result from differences in composition of the media used in the two series of experiments. However, suspension of cells in the medium of Lwoff *et al.* did not lead to more rapid virus release (Fig. 1, curve B).

The discrepancy might also be partly explained in terms of a selective inactivation of free virus by inhibitors present in the medium. Although calf sera used in the experiments described in the previous section were free of readily detectable amounts of virus inhibitor, the presence of traces of inhibitor was not excluded. Confirmation of results obtained with serum-containing media was therefore necessary.

Inhibitors present in some calf sera were found to be precipitable by

1:3 saturation with ammonium sulfate, a finding in agreement with the results of Klein (1958), and a medium presumably free of inhibitors may be obtained if those fractions of serum which contain inhibitors are omitted. Preliminary experiments were carried out in which virus yields of three types of cells in serum-containing medium were compared with yields in media prepared by adding crystalline bovine serum albumin and glutamine to Hanks' balanced salt solution. Glutamine was included because of its capacity to enhance virus yields (Eagle and Habel, 1956). Yields were essentially the same in media containing serum + lactalbumin hydrolyzate or 1% albumin + 2 mmoles glutamine, but reduced in medium containing 0.1% albumin, and negligible in protein-free medium. Omission of glutamine reduced the virus yield only slightly (Table 2).

A detailed growth cycle experiment was then carried out using the 1% albumin + 2 mmoles glutamine medium, and results of this experiment (Fig. 3) showed the same features as previous experiments: essentially linear increase in total virus followed by a decline in total virus levels, release of some virus during the early stages of the maturation phase, and a marked delay in the release of the majority of the virus yield.

TABLE 2

COMPARISON OF VIRUS YIELDS OF THREE CELL TYPES INFECTED WITH TYPE 1 POLIOVIRUS (BRUNHILDE) AND SUSPENDED IN MEDIA OF DIFFERENT COMPOSITIONS^a

Medium	Cell Type		
	Monkey kidney	ERK	HeLa (S3)
Hanks' solution	<0.2 ^b	≈0.2	≈0.2
Hanks' soln. + 0.1% cryst. bovine albumin	—	1.12	0.70
Hanks' soln. + 1% cryst. bovine albumin	7.4	9.6	9.6
Hanks' soln. + 1% cryst. bovine albumin + 2 mmoles glutamine	—	13.8	13.6
Hanks' soln. + 10% rabbit serum + 0.5% lactalbumin hydrolyzate	7.0	10.6	14.8

^a Total virus concentration determined at 11.3 hours for monkey kidney cells, and 15 hours for ERK and HeLa cells.

^b PFU/ml $\times 10^4$.

FIG. 3. The growth cycle of type 1 poliovirus in monkey kidney cells suspended in a medium containing 1% crystalline bovine albumin and 2 mmoles glutamine in Hanks' solution. Maturation and release of virus are shown by changes in total and free virus concentration, respectively. Cells were infected at an average adsorbed multiplicity of 4.1 and were suspended at a concentration of 1.2×10^4 /ml.

The experiments described in this section show, therefore, that the delayed virus release observed in previous experiments is not due to the presence of virus inhibitors in the medium and that maturation and release of virus follows essentially the same course when nutritionally simple or complex media are used.

III. Location of Cell-associated Virus

In an attempt to determine the location of cell-associated virus, infected cells were treated with hyperimmune specific antiserum at different times during the cycle.

Cell monolayers were washed to remove free virus, and were treated at 37° for 10 minutes with excess antibody. The antibody was then removed by washing several times with PBS. To exclude possible general or local depletion of antibody, both the proportion of cells infected and the antiserum concentration were varied. Virus yields of monolayers treated with antiserum were compared with monolayers subjected to the same procedures, but with PBS substituted for antiserum.

TABLE 3
INACCESSIBILITY OF CELL-ASSOCIATED VIRUS TO SPECIFIC
ANTIBODY. EXPERIMENTS WITH MONOLAYER CULTURES^a

Time	Treatment	Plaque counts, cell-associated virus from dupli- cate cultures	Total count
7.6 Hours	PBS (Control)	53, 60	113
	Antiserum 1:4	48, 44	92
	Antiserum 1:20	52, 62	114
	Antiserum 1:100	39, 50	89
9.5 Hours	PBS (Control)	75, 61	136
	Antiserum 1:4	49, 66	115
	Antiserum 1:20	78, 88	166
	Antiserum 1:100	60, 76	136

^a Antiserum control: Stock virus mixed with an equal volume of 1:50 antiserum, incubated for 5 minutes at 37° and assayed after dilution to 10⁻⁴, produced 11 plaques per culture.

Virus mixed with an equal volume of PBS, and incubated for 5 minutes at 37°, produced 25 plaques per culture at a 10^{-5.7} dilution.

At the stated times pairs of cell monolayer cultures infected at an average adsorbed multiplicity of 0.08 were washed, treated with PBS or dilute antiserum for 5 minutes, and were then washed again to remove antibody. The virus content of the cells was then determined.

No reduction in virus content of the cells occurred as a result of treatment with antiserum (Table 3).

Cell suspensions were treated with diluted antiserum for 10 minutes at 37°, and were then diluted 1:100 in PBS prior to disruption of the cells by freezing and thawing. Residual virus was assayed and compared with a second aliquot of the cell suspension treated in the same way but with the antiserum replaced by PBS. To show that the antiserum concentration was sufficient to neutralize the virus associated with the cells, a third aliquot of the suspension was frozen and thawed to release virus, before addition of the antiserum.

Results typical of these experiments are shown in Table 4.

With cell monolayers from which free virus had been removed by washing, no reduction in virus content occurred as a result of treatment with antiserum. With cell suspensions from which free virus had not been removed, the proportion of virus neutralized was essentially the

TABLE 4

INACCESSIBILITY OF CELL-ASSOCIATED VIRUS TO SPECIFIC ANTIBODY. EXPERIMENTS WITH SUSPENDED CELLS^a

	Treatment prior to assay	Final dilution at assay	PFU/ml $\times 10^4$ Time	
			11.1 Hours	12.1 Hours
Antibody-resistant virus in suspension (A)	1. Treated with anti-serum 2. Diluted 10^{-2} 3. Frozen and thawed	10^{-4}	92	87
Antibody-resistant virus in frozen and thawed suspension (B)	1. Frozen and thawed 2. Treated with anti-serum	3.3×10^{-4}	5.7	4.5
Total virus content of suspension (C)	Frozen and thawed	10^{-4}	100	119

^a Cells infected at an average adsorbed multiplicity of 0.9 were incubated in suspension, and at the indicated times, antiserum to a final dilution of 1:2,000 was added to one aliquot of the infected cell suspension. After incubation for 10 minutes at 37° , the suspension was diluted 1:100, frozen and thawed to release cell-associated virus, and assayed to give the amount of antibody-resistant virus in the suspension (A). A second aliquot was frozen and thawed to release cell-associated virus 10 minutes later, treated with antiserum, and assayed after further dilution (B). A third aliquot was treated as for the first and at the same time, but with PBS in place of antiserum, and assayed to give the original virus content of the suspension (C).

same as the proportion of total virus present as free virus at this time. The presence of adequate amounts of antibody was shown by the neutralization of over 90 % of the virus produced by the cells, provided it was first released from the cells by a cycle of freezing and thawing.

In both types of experiment, therefore, the results show that cell-associated virus is inaccessible or resistant to antibody and is therefore intracellular.

DISCUSSION

In experiments on the growth cycle of type 1 poliovirus (Brunhilde) in monkey kidney cells, inactivation of portion of the virus yield was observed during the later stages of the cycle. This inactivation cannot

be attributed to readsorption of released virus, since it occurred in dilute cell suspensions, nor to the presence of virus inhibitors in the medium, since it occurred in a simple medium containing albumin and glutamine in place of more complex nutrients. It may be entirely due to the inherent thermolability of virus, or may be partly the result of some virus-inactivating property of cells, within which virus is retained for some time before release occurs. The occurrence of this inactivation prevents the analysis of the processes of virus maturation and release in strictly quantitative terms, for it must be assumed that inactivation occurs throughout the cycle. Nevertheless, the dissociation of maturation and release is clearly shown by the experiments described.

The growth cycle in cell populations may be briefly described as follows. The maturation phase begins approximately 4 hours after addition of virus to cells, and in multiply infected cells lasts 6-8 hours. During the appearance of most of the virus yield, maturation proceeds at an approximately constant rate. Virus release begins within 30-60 minutes of the start of maturation, but the rate of release is much less than the rate of maturation. As a result, a large proportion of the virus yield accumulates within cells as it matures. Virus which is still within cells at the end of the maturation phase is released slowly during a succeeding interval of several hours. The release phase of the cycle thus begins shortly after the start of the maturation phase, but is of much longer duration.

With cell monolayers, the amount of free virus which appears during the maturation phase (Howes and Melnick, 1957), is relatively much less than that seen in cell suspensions. This difference may be largely due to the mechanical trapping and readsorption of released virus, which might be expected to occur in cell monolayers, but not in dilute cell suspensions. In other respects the growth cycles in monolayers and suspensions are essentially the same. In both, virus accumulates in association with the cells and to determine its location the experimental approach adopted was similar to that used by Rubin *et al.* (1957) to demonstrate that cell-associated Newcastle disease virus is located at the surface of the cells. For both monolayers and suspensions, cell-associated poliovirus, unlike NDV, was found to be resistant or inaccessible to specific antibody; this indicates that the virus is intracellular.

The delay in release of poliovirus must play an important part in determining the rate at which poliovirus infection spreads through a

population of cells *in vitro*. Whether delayed release occurs *in vivo* is not known. However, should it occur, variation between virus strains in the magnitude of the delay might well be linked with differences in their virulence.

The slow release of virus from cell populations is in great contrast to the "rapid" or lysislike release of poliovirus from individual cells described by Lwoff *et al.* (1955). They found that after a latent period of from 5 to 7 hours, a large fraction of the virus yield of a cell was released in less than 1 hour. Comparison of these results with those of growth cycle experiments on cell populations (Dulbecco and Vogt, 1955) led Lwoff and associates to suggest that they had selected late releasers for study. However, if the slow or delayed release of virus from cell populations observed in the present study is interpreted in terms of a lysislike release process, the conclusion that most cells begin to release virus much later than those described by Lwoff *et al.* is inescapable. They may, therefore, have selected for a minority of cells which release virus early. The absence of any recorded studies of cells beyond 9 or 10 hours after infection, suggests that selection might have been the result of a limitation of the time of study.

If the assumption is made that each cell releases most of its virus yield during a period of 1 hour as was shown for early-releasing cells, then the over-all slow release of virus from cell populations must result from wide variations between cells in the time at which release begins. This interpretation is supported by the results of studies of the release of type 1 poliovirus from HeLa cells (Howes, 1959).

The results of Lwoff and co-workers are of importance in determining the relation between the duration of the maturation phase in cell populations and its duration in individual cells. For one cell studied by them, release of virus had been largely completed within 7 hours of the probable time of addition of virus to the cell, and from the results of growth cycle experiments with cell populations it is evident that some cells must begin to release virus even earlier than this. However, maturation in cell populations which begins after 4 hours is not complete until 10-12 hours after the addition of virus to the cells. Maturation may therefore be completed much earlier in some cells than in others, or in some cells maturation may be terminated prematurely by cell "lysis." The relation of the growth cycle of poliovirus in cell populations to that in individual cells is discussed in greater detail in the following paper (Howes, 1959).

ACKNOWLEDGMENT

I am indebted to Miss Janet Rooney for her able technical assistance.

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The Growth Cycle of Poliovirus in Cultured Cells

III. The Asynchronous Response of HeLa Cells Multiply Infected with Type 1 Poliovirus

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Accepted June 10, 1959

HeLa cells multiply infected with type 1 poliovirus vary by as much as 2-3 hours in the time at which the first mature virus may be detected within them. The shape of the virus maturation curve in a cell population must therefore to a large extent reflect the distribution of starting times among cells. Each cell releases most of its virus yield over a period of 1 hour or less, but cells vary widely in the time at which release begins. Although in cell populations the maturation and release phases of the cycle overlap, in individual cells these phases may be regarded as being separated by an intracellular retention phase which may vary from a few minutes to many hours.

INTRODUCTION

In a previous paper (Howes, 1959) the maturation and release phases of the growth cycle of type 1 poliovirus in monkey kidney cell suspensions were found to overlap, and most infectious virus particles were found to be released only after a prolonged delay. In the experiments on HeLa cells infected with type 1 poliovirus described in this paper, the growth cycle in cell populations is analyzed in terms of the growth cycle in individual cells.

Release of virus from single cells was studied directly by determining the amount of virus released from each cell during successive hourly intervals. Virus maturation within a single cell cannot be studied in this way, for in common with bacteriophage and other animal viruses such as vaccinia or herpes simplex, poliovirus accumulates within the cell as it matures and the virus content of a cell may be determined only once. The experiments on poliovirus maturation in single cells were therefore indirect and were limited largely to an examination of cells for evidence of asynchrony in the virus maturation process.

MATERIALS AND METHODS

Virus

High titer stocks of the type 1 Brunhilde strain of poliovirus were prepared as described previously (Howes, 1959).

Cell Cultures

ERK cells (Westwood *et al.*, 1957) were passaged serially in Eagle's medium plus 15 % calf serum. Monolayer cultures for experimental use were prepared in small petri dishes of 5 cm internal diameter.

HeLa cells of a cloned line developed by two successive clonal isolations and designated H4/1, were passaged in 20 % human adult serum in Hanks' solution containing 0.5 % lactalbumin hydrolyzate. Cultures for experimental use were prepared in small petri dishes.

Monkey kidney cell cultures were prepared in small petri dishes as described previously (Howes, 1959).

Assay of Virus and of Plaque-forming Cells

Details of the procedures used in the assay of virus and plaque-forming cells in monkey kidney cell monolayers have been described previously (Howes, 1959). The composition of the overlay medium used previously was modified to give an earlier appearance of plaques and higher plaque counts for Brunhilde virus preparations. The agar overlay was prepared, using equal volumes of a 2 % agar solution and twofold concentrated nutrient medium. Earle's salt solution used in the nutrient medium was modified to contain 0.0001 *M* CaCl_2 , a reduction necessary to prevent precipitation of phosphates, and 0.002 *M* MgSO_4 . The concentrated medium contained noninhibitory rabbit serum 2 %, 0.04 *M* sodium phosphate buffer pH 7.4, neutral red 1:15,000, and penicillin and streptomycin at concentrations of 400 units and 400 μg per milliliter, respectively. The concentration of the modified Earle's solution was adjusted to make the final overlay isotonic. Four milliliters of the overlay was added to each assay culture.

Media Used for the Maintenance of Infected Cells

Bicarbonate-buffered medium (BBM) contained 9 parts Hanks' salt solution containing 0.5 % lactalbumin hydrolyzate, 1 part normal monkey serum. Penicillin and streptomycin were added to give a final concentration of 200 units and 200 μg per milliliter, respectively.

Phosphate-buffered medium (PBM). The basal salt solution used contained NaCl 0.137 *M*; KCl 0.0054 *M*; CaCl₂ 0.0001 *M*; MgSO₄ 0.002 *M*; and phenol red 0.002 %. This was prepared as a double-strength solution and autoclaved.

The complete medium was prepared by adding 1 part normal monkey serum to 9 parts of single strength salt solution containing 0.02 *M* sodium phosphate buffer pH 7.4, glucose 0.1 %, and lactalbumin hydrolyzate 0.5 %. Penicillin and streptomycin were included at the concentrations used in BBM.

Preparation of Infected Cell Suspensions and Estimation of Multiplicity of Infection

Infected cell suspensions were prepared, and the average adsorbed multiplicity of infection determined by the same procedures as were used for monkey kidney cells (Howes, 1959). The agar overlay used for HeLa cell monolayers was that described by Takemori *et al.* (1958), in which noninhibitory calf serum was replaced by noninhibitory rabbit serum.

Microdrop Technique

Single cells were distributed freehand to 1–2-mm diameter microdrops of medium under paraffin oil¹ contained in small petri dishes, using micropipettes prepared as described by Lwoff *et al.* (1955). Manipulations were carried out at 100 × magnification under a dissecting microscope enclosed in a Perspex box maintained at 37°. The box was flushed continuously with humidified air when phosphate-buffered medium was used, or with a humidified 5 % CO₂-air mixture when bicarbonate-buffered medium was used. More detailed aspects of the technique are discussed in later sections.

EXPERIMENTAL RESULTS

I. The Asynchrony of Virus Maturation in Multiply Infected Cells

The course of virus maturation within a cell may be defined by the time at which maturation begins (maturation starting time), the rate at which it proceeds, and the time at which it ceases. Intercell variation in any one of these factors will lead to variation in the virus content of cells harvested at the same instant. In cells harvested when maturation

¹ W150 oil, Atlantic Union Oil Co. Ltd.

in a cell population is approaching completion, this may be due to variations in any of the three factors. However, in cells harvested shortly after maturation begins in the cell population, it might be expected to result largely from variation in maturation starting times or maturation rates in cells, since few cells will yet have ceased maturation.

Variation in the virus content of cells may be determined directly by using single cells, or indirectly by analyzing the variation in virus content of tubes to which cells have been randomly distributed. For convenience these experiments are designated respectively "single cell" and "statistical" experiments.

In assessing the extent of the variation in virus content of cells from results of the "statistical" experiments, the mean and variance of the distribution of virus among tubes must be compared with those expected for cells which have identical maturation starting times and maturation rates. If such cells are randomly distributed among tubes with a mean of $\bar{c}$ and hence a variance of $\bar{c}$, and each cell at the time of harvest contains v PFU of virus, the distribution of virus in tubes will have a mean of $v\bar{c}$ and variance $v^2\bar{c}$. The theoretical variance may be calculated from the mean number of cells per tube and the mean virus content of tubes. Variation in virus content of cells will lead to an observed variance in excess of the theoretical.

In the following experiment, maturation and release of poliovirus in a suspension of multiply infected HeLa cells was followed for approximately 13 hours, and "single cell" and "statistical" experiments were carried out on cells drawn from this suspension.

HeLa cells were infected at an average adsorbed multiplicity of 8.6, and were suspended at a concentration of 8.4×10^3 per milliliter in PBM. An aliquot of diluted suspension was found to contain 80 single cells and 2 pairs.

The single-cell experiment was set up by transferring 10–15 cells from the parent culture to a microdrop of approximately 2–3 mm diameter, using a micropipette. Immediately the cells had settled out, a large part of the fluid of the drop was removed, and with a new pipette charged with PBM, single cells were transferred to tubes containing 0.5 ml PBM. In the final stage of transfer a relatively large volume of fluid was ejected from the pipette to ensure the cell was discharged from the pipette. When the cells in the first drop had been distributed to tubes, the whole process was repeated on a fresh aliquot of cells. By working with small numbers of cells freshly drawn from the parent culture, and distributing

FIG. 1. The growth cycle of type 1 poliovirus in a suspension of HeLa cells. Cells infected at an average adsorbed multiplicity of 8.6 were suspended in PBM at a concentration of 8.4×10^8 per milliliter (4.3×10^8 plaque-forming cells per milliliter). Maturation and release of virus are shown by changes in total and free virus concentrations, respectively. To show the early stages of maturation and release more clearly, results are plotted on a logarithmic scale (upper curves) as well as a linear scale (lower curves). Harvest times in the "single cell" and "statistical" experiments, carried out using cells from the above suspension, are shown by arrows labeled SC and S, respectively.

all the cells contained in a drop, an effort was made to reduce inadvertent selection for one cell type or another to a low level.

For the statistical experiment, a second series of tubes was seeded with 0.5 ml of a 1:100 dilution of the parent culture in PBM. A 1-ml graduated pipette was used to do this, as an automatic syringe had been found to distribute cells in a nonrandom fashion. The diluted cell suspension was stirred during distribution with a bar magnet. To confirm the random distribution of cells to tubes, a series of monkey kidney monolayer cultures were seeded with 0.5-ml volumes of the same suspension. One milliliter agar overlay was added to each culture, and after this had solidified, a further 3 ml overlay was added. Plaques were counted after 2 and 3 days.

All tubes, and the parent culture, were incubated in the same water bath at 36.3° to 37° . Groups of tubes were removed from the bath at the appropriate times, frozen rapidly in an alcohol dry-ice bath, and then stored at -65° until assayed. Samples from the parent culture were treated in the same way.

The virus content of each tube was determined by adding 0.1 ml of its frozen and thawed contents to an assay culture containing 0.4 ml PBM. The remaining 0.4 ml was tipped onto another culture, and the tube rinsed with 0.1 ml fresh medium. The volume of the inoculum was thus kept constant at 0.5 ml. Samples from the parent culture were diluted in PBM, and 0.5-ml inocula were added to assay cultures. The remainder of the assay procedures have been described in Materials and Methods.

Yields of tubes harvested early in the maturation phase (4.25 and 4.8 hours) and at its end (11.1 hours) are arranged in descending order in Table 1. Counts of plaque-forming cells in aliquots of the cell suspension given in the first column, show that cells were distributed to tubes with a mean of 21.4 and variance 17.2. These values are consistent with a random distribution of cells over tubes. From the mean number of cells per tube and the mean virus yield per tube, the average virus yield per cell and the variance expected for cells with identical maturation starting times and rates of maturation were determined for each harvest time.

At 4.25 and 4.8 hours, the distributions of virus in tubes were marked by variances far in excess of those calculated for identical cells. Seven of the tubes harvested at 4.25 hours, and two of those harvested at 4.8 hours yielded fewer than 10 plaque-forming units (PFU) of virus. This was obviously not due to the presence in those tubes of very small numbers of cells, but to the presence in those tubes of many cells which had not yet produced detectable amounts of virus. Thus the excessive variation in virus content of cells is due at least partly to differences in maturation starting time, and virus maturation within cells is clearly asynchronous.

As a result of this variation in maturation starting time, variation in the virus content of cells harvested at the end of the maturation phase, when maturation is complete in all cells, should be less than the variation evident at earlier times, when some cells may contain a full virus yield and others only part of their yield or no virus. This was found to be true of the cell population studied, for variation in the virus content

TABLE 1
THE ASYNCHRONOUS MATURATION OF POLIOVIRUS IN MULTIPLY INFECTED
HELA CELLS, AS SHOWN BY EXCESSIVE VARIATION IN VIRUS CONTENT
OF TUBES TO WHICH CELLS WERE RANDOMLY DISTRIBUTED^a

Number of plaque-forming cells in 0.5-ml aliquots of cell suspension	Virus yields of tubes containing 0.5-ml aliquots of cell suspension, and harvested at stated times				
	4.25 Hours		4.8 Hours		11.1 Hours (1:80 of yield)
28	405	45	850	195	77
27	395	40	775	185	60
27	220	40	690	165	56
26	210	35	520	160	55
24	195	30	480	160	53
24	185	30	410	150	38
23	170	28	370	95	36
23	170	28	355	90	35
23	155	19	325	82	
22	100	17	305	75	
21	91	15	285	73	
20	90	12	280	65	
20	77	11	275	48	
20	74	9	255	34	
20	65	8	250	32	
19	61	8	240	29	
17	60	6	225	27	
17	56	4	205	5	
14	52	4	205	4	
13	47	2	195		
Total count	428	3,269	9,169	410	
No. of samples	20	40	39	8	
Mean count	21.4	81.7	235.1	51.25	
Observed variance	17.2	9,439	41,721	207.4	
Theoretical variance	21.4	311.9	2,582	122.7	
Ratio $\frac{\text{observed}}{\text{theoretical}}$	0.80	30.3	16.2	1.7	

^a A diluted suspension of infected cells was distributed in 0.5-ml amounts to tubes, and at 4.25, 4.8, and 11.1 hours groups of tubes were frozen and thawed to release intracellular virus, which was then assayed. The variance of the distribution of virus in tubes was calculated for each time, and this observed variance was compared with the theoretical variance for cells with identical maturation starting times and maturation rates. The expected random distribution of plaque-forming cells to tubes was checked by assaying a series of 0.5-ml aliquots of the suspension. The close approximation of mean and variance of the distribution agrees with this expectation.

of tubes harvested at 11.1 hours was much less than that in tubes harvested at 4.25 and 4.8 hours.

The conclusions drawn from the statistical experiment were supported by results obtained with single cells. The results presented in Table 2 show that, between 6 and 7.2 hours, there was an increase in the number of cells which had produced virus, and since maturation was first detected at 4 hours in the cell population (Fig. 1), cells must vary in maturation starting time by as much as 2-3.2 hours.

Maturation in the cell population lasted approximately 5 hours, and therefore maturation in late-starting cells may occupy a much smaller interval than this. In some cells it may last 1.2 hours or less, for many single cells which contained large amounts of virus at 7.2 hours must have contained little or none at 6 hours (Table 2).

TABLE 2

THE ASYNCHRONOUS MATURATION OF POLIOVIRUS IN MULTIPLY INFECTED HELA CELLS, AS SHOWN BY VIRUS YIELDS OF CELLS HARVESTED AT INTERVALS DURING THE MATURATION PHASE^a

Time of harvest	6.0 Hours	7.2 Hours	8.2 Hours	11.1 Hours
Total virus yield of fertile cells (PFU)	80	600	530	310
	61	515	510	265
	14	500	480	265
		415	405	235
		335	305	210
		275	295	190
		240	245	180
		180	225	170
		60	220	160
		27	80	105
		8	26	103
		1 ^b	7	72
		1 ^b		61
		1 ^b		56
No. of cells examined	30	29	30	30
No. of cells fertile	3	11	12	14

^a Within 3 hours of infection single cells were distributed to a series of tubes containing 0.5 ml PBM, and groups of tubes were frozen and thawed to release virus from the cells at the stated times. The virus yield of each cell was divided, and one-fifth was assayed in one culture, four-fifths in another.

^b Since this virus might be "background" virus, these cells were not scored as fertile.

II. Release of Poliovirus from Cell Populations

The delayed release of type 1 poliovirus from suspensions of HeLa cells which occurs following the completion of virus maturation is shown in Fig. 2. Multiply infected HeLa cells were suspended in phosphate-buffered medium at a concentration of 6.6×10^3 cells per milliliter. The siliconed flask containing the suspension was incubated at 36.3° – 37° in a water bath, the suspension being gently stirred by means of a suspended magnet. Five samples were taken from the culture between 11 and 23 hours after infection, and total and free virus content of each sample was determined. These estimates are plotted in the figure together with their 5 % confidence intervals.

Although a small fraction of the virus yield was released during the

FIG. 2. Release of type 1 poliovirus from a suspension of HeLa cells. Cells infected at an average adsorbed multiplicity of approximately 3 were suspended in PBM at a concentration of 6.6×10^3 cells per milliliter and incubated at 37° . Five samples were assayed for total and free virus content between 11 and 23 hours after infection. Results are shown in the figure together with the 5% confidence interval for each point. Changes in total and free virus concentrations, respectively, result from inactivation of portion of the virus yield and release of virus from cells. The progress of release might be more aptly pictured in terms of the increase in the ratio of free to total virus concentrations (F/T).

maturation phase (Fig. 1) most of the increase in free-virus concentration took place over a period of several hours following the end of this phase (Fig. 2). As is shown in the next section, the amount of free virus present at any time is related to the fraction of cells which have released their virus yield by that time. However, approximately 50 % of the virus yield was inactivated following attainment of maximum total virus concentration, so that only half the peak virus yield appeared as free virus. Therefore, the fraction of cells which have released cannot be assessed directly from the free virus curve of the growth cycle. This fraction might be estimated, however, by determining at each time the ratio of free to total virus concentration (Fig. 2), if inactivation is assumed to affect intracellular and free virus equally.

III. The Mode of Release of Virus from Single Cells

Release of virus from single cells was studied in detail by replacing the fluid surrounding cells in microdrops at hourly intervals. In three experiments, multiply infected ERK or HeLa cells were distributed in microdrops, and incubated for 10–13 hours at 37°C. The medium in each microdrop was then removed, the cell washed with fresh medium, and the drop finally replenished with fresh medium. The original medium and washings were pooled and assayed for virus content (free virus). This procedure was repeated at hourly intervals, and after the final harvest of medium, the cell itself was harvested, frozen and thawed once, and assayed for cell-associated virus. Results of these experiments are shown in Fig. 3, in which the times of first and final harvests for each cell are indicated by arrows.

The first two experiments were carried out using bicarbonate-buffered medium. In the first, the two ERK cells (*E1* and *E2*) which produced virus failed to release it during successive replacements of the medium. In the second, two HeLa cells (*H1* and *H2*) released virus toward the end of the period of observation, the yield of cell *H2* being unusually low.

The third experiment was carried out using HeLa cells in phosphate-buffered medium. Virus release from 13 cells was followed over successive hourly intervals for 7 or 8 hours, and results obtained from the 8 cells which produced virus are arranged in order according to the time at which virus release began (cells *H3* to *H10*). Two cells failed to release virus during the period of study, although they had produced normal yields.

The pattern of release shown by these cells was in general agreement with that observed by Lwoff *et al.* (1955) for early-releasing cells. The bulk of the virus yield was released during the first hour following the start of release, and successively smaller amounts were released during succeeding hourly intervals. Although some virus was retained by cells for prolonged periods following apparent cessation of release, the amount retained was small and the efficiency of the release process, once initiated, was therefore high. The time at which release started was very variable, and the gradual release of virus from cell populations is evidently the result of this variation in the starting time of release.

Mature virus in populations of multiply infected cells reaches maximal

FIG. 3. The mode of release of type 1 poliovirus from single cells. Virus released during successive hourly intervals, and the virus remaining in the cell at the end of each experiment, was determined for 2 ERK cells (*E1* and *E2*) and 10 HeLa cells (*H1-H10*). Cells *H3* to *H10* were all drawn from the same suspension. Arrows indicate the times at which the first and last samples were taken. The number of virus PFU in each sample are shown for each cell.

TABLE 3

CELL-ASSOCIATED AND FREE VIRUS YIELDS OF MULTIPLY INFECTED SINGLE CELLS IN MICRODROPS^a

Cell type	Ratio No. of cells fertile No. of cells examined	Time of harvest (hours)	Virus yields ^b of fertile cells	
			Cell-associated virus	Free virus
HeLa (clone H4/1)	14:16	12.6-13.3	C ^c	7
			C	3
			C	1
			C	1
			C	0
			C	0
			C	0
			C	0
			C	0
			C	0
			C	0
			147	0
			97	0
ERK	8:14	13.5-14	(181) ^d	0
			(141)	0
			44	0
			14	34
		14.5-15	73	0
			51	(145)
			6	64
			85	1

^a Multiply infected HeLa and embryonic rabbit kidney cells were distributed to microdrops of BBM and incubated at 37°. Free virus in each microdrop was obtained by removing the original medium and washing out the drop with fresh medium. The cell itself was then harvested and its virus content determined.

^b Three-day plaque counts.

^c C = confluent lysis or uncountable numbers of plaques after 2 days. (The mean yield of cells in the parent culture harvested at this time was 260 PFU per cell.)

^d Parentheses indicate assay cultures which were semiconfluent after 3 days. Probable 3-day counts were estimated by multiplying the 2-day counts by the factor by which plaque counts increased on less crowded cultures during the third day.

levels within 10–12 hours of infection, and release from most cells evidently begins some time after this. The maturation and release phases of the growth cycle of poliovirus in single cells may therefore be represented as being separated by an “intracellular retention phase” which may last several hours. This conclusion involves the assumption that when the total virus content of a cell suspension is maximal, the virus content of most of its constituent fertile cells is also maximal. The results of an experiment in which multiply infected HeLa cells were examined for their free and cell-associated virus yields shortly after the end of the maturation phase supports the above assumption and shows that for most cells virus release follows and does not accompany virus maturation (Table 3). Of 16 cells examined, 14 had produced large amounts of mature virus, 2 of these had released small amounts of virus but 10 had released none, and 2 had 1 PFU free and possibly “background” virus associated with them.

Virus release from cell populations begins early in the maturation phase, which suggests that although in most cells the intracellular retention phase lasts for an hour or more, in a few it may last only a few minutes, or be absent. If all cells release most of their virus yield within 1 hour, as was found for the majority, then cells which release their mature virus yield early must also have produced it early, and over a very short time interval. Whether or not early-releasing cells produce a normal virus yield has not been determined.

The maturation and release of type 1 poliovirus from cell populations follows essentially the same pattern for monkey kidney, HeLa, and ERK cells. In a limited study, single ERK cells harvested some hours after completion of virus maturation were found to have released either no virus or most of their yield (Table 3). This suggests that these cells release virus in much the same way as do monkey kidney and HeLa cells, and the conclusions reached from a study of HeLa cells may also be true for ERK cells.

DISCUSSION

Asynchrony in the virus maturation response of cells has been shown for Western equine encephalomyelitis and influenza viruses by Dulbecco and Vogt (1953) and Cairns (1957), respectively. The present study has shown that for HeLa cells multiply infected with type 1 poliovirus, both virus maturation and virus release are asynchronous.

The starting times of maturation within individual cells varied by as

much as 2-3 hours, while the maturation phase in the cell population lasted approximately 5 hours. The maturation phase in late-starting cells may therefore last for much less than 5 hours, and a study of single cells suggested that this may be true of most cells in a population, irrespective of starting time.

Intercell variation in maturation starting time cannot be explained in terms of variation in the time of adsorption of the first or all virus particles to each cell. However, it may be explained in terms of the variation in the time taken by virus particles to begin replication, as was shown for influenza virus by Cairns (1957), by pauses in development dictated by the cell itself, and by variation in the number of infecting virus particles which contribute to mature virus production. Variation in the latter may be much greater than that expected from a random distribution of virus to cells, since the number of virus particles adsorbed to a cell may be a function of its size (Dunnebacke and Reaume, 1958), and the number of virus particles which participate in virus production in a cell may be a function of its physiological state and its genetically determined resistance to infection (Vogt and Dulbecco, 1958). Identification of the major source of variation in starting time is thus impossible at present, although further detailed experiments at various infection multiplicities in cells whose growth is synchronized may provide more information.

Asynchrony in release of virus from cells is much more pronounced than that observed for the maturation process. Populations of HeLa cells multiply infected with type 1 poliovirus collectively release a large fraction of their virus yield over a period of several hours following the apparent completion of virus maturation. However, detailed experiments on single cells in microdrops showed that each cell releases a large part of its virus yield within 1 hour of the start of virus release from it. The time at which release begins varies widely from cell to cell, and the slow increase in free virus in cell populations may be explained in terms of a slow increase with time in the number of cells which have released their virus yield.

Many cells begin to release virus several hours after total mature virus in the cell population has reached its maximum level. This suggests that virus maturation is largely completed in those cells several hours before virus release begins. This cannot be proved directly for any given cell, since the determination of the virus content of a cell precludes any subsequent study of virus release from it. However, a ma-

jority of the single cells examined for cell-associated and free virus yields 11 and 13 hours after infection were found to have produced large amounts of virus, yet most of these had released none. This supports the above conclusion and clearly establishes that in most cells release of the virus yield follows, and does not accompany, its maturation.

In earlier studies of virus release from single monkey kidney cells, Lwoff *et al.* (1955) showed that each cell releases a large proportion of its virus yield within 1 hour of the start of release. However, although HeLa cells release virus in a similar manner, the cells studied by Lwoff *et al.* released virus much earlier than the HeLa cells described in the present study. The suggestion has been made (Howes, 1959) that Lwoff *et al.* selected for early-releasing cells. Limited experiments on single ERK cells infected with type 1 (Mahoney) poliovirus described in this paper, suggest that release of virus from these cells is accomplished by a similar process to that found for monkey kidney and HeLa cells. This type of release process may therefore prove to be a general feature of the growth cycle of poliovirus in cultured cells.

Lwoff *et al.* (1955) suggested that virus release occurs by a process akin to lysis. More recently Ackermann (1958) has suggested that virus release occurs by a process designated "cytotransudation," which is pictured as involving a contraction of the cytoplasm. There is no convincing evidence that cytoplasmic contraction occurs, and the data at present available on release of virus from single cells are adequately explained in terms of a simple lytic-type process, involving leakage of virus from the cell following a sudden change in permeability of the cell membrane.

Analysis of the growth cycle of poliovirus in cell populations in terms of the growth cycle in individual cells has shown that although the maturation and release phases overlap in cell populations, in individual cells maturation is essentially complete before release begins, and the maturation and release phases are in effect separated by an intracellular retention phase of variable duration. This relation is shown in Fig. 4 in which, for simplicity, the effects of virus inactivation and the adsorption and penetration phases are not shown.

The growth cycle of poliovirus thus resembles those of viruses such as bacteriophage or vaccinia, and clearly differs from those of influenza and Western equine encephalomyelitis virus, for with both of these viruses maturation and release of virus may proceed simultaneously in the same cell (Cairns and Mason, 1953; Rubin *et al.*, 1955). If, as seems likely, release of poliovirus is accomplished by a lytic type of process,

FIG. 4. The relation of the growth cycle of poliovirus in a cell population to that in a single cell.

onset of release may automatically terminate the maturation process and release of virus early in the maturation phase in cell populations may mean that some cells produce a normal yield over a very short time interval, or that maturation in some cells is terminated prematurely by the onset of release. There are apparently no theoretical objections to the premature cessation of virus maturation, since Ackermann *et al.* (1954) have shown that cytopathic changes characteristic of infection may occur in cells in which virus maturation has been prevented.

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with such particles. In more recent studies of particle size, the diameters of virus particles of the three immunological types ranged from 27.0 to 27.3 m μ , (Schwerdt, 1957) a variation which was not significant. Studies of the inactivation of virus infectivity by ionizing radiation agree with these estimates of particle size. (Pollard and Kraft, 1955; Benyesh et al, 1958).

In 1954, Crick and Watson suggested that the smaller viruses would be found to contain identical protein sub-units arranged in regular fashion in the virus particle. Their suggestion was based on the fact that almost all small plant or animal viruses are either rods or spheres and that an ordered arrangement of sub-units had already been demonstrated for tobacco mosaic and bushy stunt viruses by X-ray diffraction studies (Bernal and Fankuchen, 1941; Watson, 1954; Franklin, 1955; and Caspar, 1956). Crick and Watson reasoned that to achieve such an ordered structure most economically, the number of identical sub-units in a spherical particle would be a multiple of twelve. This suggestion was reinforced subsequently by a detailed consideration of virus structure in terms of the known size and chemical composition of some

smaller viruses, and in terms of the direction of viral protein synthesis by viral RNA (Crick and Watson, 1957). Their main argument was that if the sequence of amino acid residues in a protein molecule is determined by the sequence of nucleotides in the nucleic acid which controls its synthesis, then the high ratio of amino acid to nucleotide residues calculated from the known chemical composition of such viruses, demands the existence of repeating protein sub-units.

Recent X-ray diffraction studies of poliovirus crystals by Finch and Klug (1959), agree with Crick and Watson's predictions. Their evidence indicates that the virus particle is built up of sixty identical or structurally very similar asymmetric protein sub-units arranged with icosahedral symmetry. More recent electron microscope studies by Horne and Nagington (1960) of fragments of infected cells embedded in phosphotungstic acid, have demonstrated the existence of a sub-unit structure more directly. The arrangement of the sub-units observed was consistent with that deduced from X-ray diffraction studies.

The arguments concerning the structure of viruses which were so successfully advanced by Watson and Crick are of interest in relation to claims which have been made for the existence of smaller (24 m μ) particles, designated the "minor" component. These claims are based on sedimentation rates of infectious particles, their rates of diffusion in agar, and on the separation of the two components by selective passage techniques (Selzer and Polson, 1954; Polson, 1956; Stanley et al, 1956 a and b; and Polson and Selzer, 1957). The behaviour of the minor and major components, when isolated and resedimented, leaves little doubt that two types of particle were present (Selzer and Polson, 1954), while the studies of Stanley et al (1956 a, b and c) indicate that the two types of particle differ also in their sensitivity to ultra-violet light and in their biological properties. However, the immunological relationship of the two particles to each other has not been studied in sufficient detail, and as a consequence, the possibility that the minor component is a contaminant virus

unrelated to poliovirus has not been excluded. On the theoretical grounds advanced by Watson and Crick, a difference in particle size of the order found implies radical differences in the size and composition of the viral RNA or protein sub-units, or both. These radical differences would almost certainly reflect equally radical differences in genotype which argues against a close relationship between the two components. The possibility that the minor component is a contaminant virus should be considered seriously.

Although the analysis of the antigenic composition of polioviruses might be expected to yield information relevant to the relationship of the protein sub-units to each other, or to the structure of the sub-units themselves, the studies which have been made have not been of sufficient detail to provide this information.

Virus preparations derived from cultured cells, and fractionated by density gradient sedimentation, yield two main fractions which are associated with two distinct antigens. The heavier fraction SDG^D fails to react, or reacts

poorly, with human sera taken during the acute phase of poliomyelitis, but reacts with convalescent sera, while the lighter fraction SDG^{C} reacts with sera from both acute and convalescent cases of poliomyelitis (Mayer and Rapp, 1956; Mayer, 1957; Roizman et al, 1958 a and b, and Le Bouvier, 1959a). While the antigens predominating in the SDG^{D} and SDG^{C} fractions have been designated D and C antigens respectively, the correlation between the physical and biological properties of the particles and their antigenic nature is not complete.

Roizman et al (1958b) showed that while C antigen predominates in the SDG^{C} fraction, it is accompanied by small amounts of D antigen. The particles of this fraction are non-infectious and owe their low density, and presumably their lack of infectivity, to the presence of little or no RNA within them (Schwerdt, 1957a, Le Bouvier et al, 1957). The SDG^{D} fraction normally contains only D antigen, but only a fraction of the particles score as infectious, despite their apparent possession of a normal complement of RNA (Roizman et al, 1958b). This is probably due in part to the presence within

this fraction of particles which have lost their infectivity, for inactivation of the infectious virus yield of a cell population begins immediately following the end of the maturation phase of the growth cycle, and probably occurs during this phase (Howes, 1959a). However, it is also possible that many potentially infectious particles fail to score as infectious because each such particle has a low likelihood of successfully initiating infection.

In more recent studies of the C and D antigens, it has been shown that C antigen may be derived from D antigen by heating or by intense ultra-violet irradiation (Roizman et al, 1958b; Le Bouvier, 1959b). Heating does not result in a reduction of density (Roizman et al, 1958b) and it therefore appears that the C antigen of fraction of SDG^C is not derived from the D antigen of fraction SDG^D.

The conversion of D antigen to C antigen by heating or ultra-violet irradiation is consistent with the existence of only one type of antigenic "sub-unit" within the poliovirus particle, but does not exclude the presence of more than one. Studies

of the inactivation of the complement fixing antigen by ionizing radiation indicate that the antigen occurs as a unit of 7 m μ diameter (Benyesh et al, 1958), a value which is close to that estimated for the protein sub-unit in X-ray diffraction studies, 6 to 6.5 m μ , (Finch and Klug, 1959). This suggests that the two units are identical.

A seeming discrepancy is the difference in the sensitivities of the complement fixing and immunogenic capacities of virus to ionizing radiation (Benyesh et al, 1958). The immunogenic capacity of the virus possesses a radiation sensitive volume which approaches that of the whole virus particle. However, the C antigen is neither capable of combining with neutralising antibodies (Hummeler and Hamparian, 1958), nor able to stimulate their production (Le Bouvier, 1959a). It is possible therefore, that a single ionization within the virus particle is sufficient to convert all antigenic sub-units from the D to the C form, with an accompanying loss of the capacity of the particle to stimulate the production of neutralising antibodies. The small radiation sensitive volume of the C antigen suggests that subsequent destruction of the C reactive antigenicity of the protein sub-units requires an

ionization within each.

Although the presence of small antigenic sub-units in the whole virus particle is consistent with the presence of "soluble" antigens in virus preparations, such antigens were not found in tissue culture fluids (Mayer et al, 1957). However, soluble antigens have been described in mouse brain preparations by Rustigian et al, (1954) and Selzer and Polson (1954). The former authors found this antigen irregularly, while the latter found it in virus preparations from suckling mouse brains but not in preparations derived from adult mouse brains. The suckling mouse antigen failed to react with anti-sera from adult mice, and was not immunogenic. These results are not in accord with a close relationship between the soluble antigen and the virus particle, and it has been suggested that the antigen is produced as a specific response to infection with poliovirus, but that it is not a component of the virus particle (Kipps et al, 1957).

The evidence discussed above is thus consistent with the presence within the virus particle of a single type of antigenic sub-unit which may be altered by various treatments. It is clear however, that studies of the physico-chemical and antigenic

properties of the isolated sub-units will be necessary for this to be established conclusively.

The other main component of the virus particle, the nucleic acid, has been identified unequivocally as RNA by direct chemical methods, (Schwerdt and Schaffer, 1955, 1956) and by studies of the inactivation of virus by nitrogen mustard (Dulbecco and Vogt, 1955). The RNA content of the virus has been estimated to be 24 or 30 per cent depending on the method of chemical analysis, (Schwerdt and Schaffer, 1955). However, the high density of the particle (1.6) suggests that the RNA content might be as high as 50 per cent or perhaps that there are present in the virus particle other dense components. Schaffer and Schwerdt (1959) considered that this discrepancy may be due to the lack of precision of the methods employed, particularly in the estimation of density. Although preliminary analysis of the base composition of Type 1 and Type 2 virus strains suggested a difference in the base ratios (Schwerdt, 1957), more precise analyses revealed no significant differences between the three virus types (Schaffer and Schwerdt, 1959).

Ideas concerning the functions of virus components have been clarified by studies of the biological properties of viral RNA isolated from crude or partially purified virus preparations. The infectivity of poliovirus RNA preparations prepared by the method of Gierer and Schramm (1956) was demonstrated by Colter et al, 1957, and confirmed by Alexander et al (1958). The evidence for the infectivity of viral RNA is the low sedimentation rate of such infectivity (Colter et al, 1957), its sensitivity to ribonuclease, and its insensitivity to antibodies (Colter et al, 1957; Alexander et al, 1958). Further evidence which excludes contaminating whole virus as a source of infectivity is provided by the ability of such preparations to infect host cells normally resistant to infection by whole virus (Holland et al, 1959 a and b; De Somer et al, 1959; Mountain and Alexander, 1959).

It seems reasonable therefore to assign to viral protein the roles of protection of viral RNA in the extracellular environment, and perhaps during early stages of the infection of the host cell by virus; and that of specific attachment to and penetration of the host cell

membrane, by the virus particle. Although the above evidence indicates that the synthesis of viral components within the host cell is accomplished under the direction of viral RNA, the possibility that small amounts of other materials are also required, and are present in viral RNA preparations has not been excluded. The functions of viral RNA and protein are discussed in greater detail in later sections.

2. The initiation of infection of the host cell and the maturation and release of viral progeny

There is general agreement that, as with bacteriophages and other animal viruses which have been studied, attachment of poliovirus to the host cell surface is the first step in the infection of the host cell and this is followed by the penetration of the host cell membrane by at least part of the infecting virus. However, detailed studies of these steps have been of limited scope and have involved the use of a number of approaches differing in techniques and experimental conditions. It is not surprising therefore, that much of the evidence obtained is difficult to interpret and has led to contradictory conclusions.

The attachment of virus to the host cells may be studied by either following the disappearance of virus from the fluid surrounding the cells, or by removing unattached virus and determining the number or fraction of cells which have been infected. The former method is preferable since it does not also measure the ability of cells to respond to the virus which they have taken up. The latter method not only suffers from this defect but is also probably

sensitive to slight variations in technique which might result in removal of attached virus.

The most satisfactory study in this regard was that of Bachtold et al (1957), who sedimented cells from virus-cell suspensions at various times after mixing, and assayed the virus remaining in the supernatant. They found that attachment followed pseudo-first order kinetics, the attachment rate constant having approximately the same value as that found for the attachment of Newcastle Disease virus to chick embryo cells (Levine and Sagick, 1956). Changes in temperature within the range 0-37°C only slightly affected the process, which was found to be dependent on the concentration of divalent cations (Ca^{++} or Mg^{++}) present in the medium. They concluded from these data that the initial binding of virus by the host cell is mediated through electrostatic forces. Study of the effect of pH on adsorption was undertaken by Youngner (1956), who found that adsorption was not affected by variations in pH within the range 6 to 8, a finding consistent with electrostatic binding.

Youngner (1956) and Holland and McLaren (1959) followed the "adsorption" of virus to cell monolayers and interpreted their results to mean that attachment is temperature sensitive, thereby excluding the type of attachment postulated by Bachtold et al (1957). However, their experiments involved extensive washing of the cell monolayers to which virus had been added, with subsequent examination for residual plaque forming virus. It is probable that their experiments were no more than demonstrations of the temperature sensitive nature of reactions subsequent to attachment, for it might be expected that virus adsorbed at 37°C, and which penetrates the cell rapidly, (Payne et al, 1958; Holland and McLaren, 1959) would resist removal during washing, whereas virus adsorbed at low temperatures when penetration or other temperature sensitive reactions are inhibited, would remain at the cell surface from which it may be dislodged readily. This possibility was admitted by Holland and McLaren when they confirmed Bachtold et al's findings for cell suspensions.

Reversibility of the attachment of polio-virus to cells has not been studied, but might be expected if electrostatic forces are involved. If Bachtold et al's conclusions are accepted as valid, it follows that the studies of Youngner (1956), and Holland and McLaren (1959), which were discussed above, provide circumstantial evidence for the reversibility of attachment. There is thus at present no reason to suspect that the initial attachment of poliovirus to host cells differs in any essential way from the attachment of other viruses to their host cells.

As discussed in Section III/1, there is strong evidence that poliovirus consists of a ribonucleic acid core surrounded by protein sub-units. The initial attachment may therefore, be considered to involve interaction of protein sub-units with sites on the cell surface. The evidence available agrees with this, for the heating of virus, which alters the immunological specificity of the protein component, also destroys the capacity of the virus to attach to cells (Graham, 1959). On the other hand, low intensity ultra-violet irradiation of the virus, while destroying infectivity through its activity on the RNA component, does not destroy the capacity of virus to

attach (Drake, 1958; Taylor cited by Graham, 1959). Similar results have been obtained by Baluda (1957) for Newcastle disease virus.

Antiviral antibodies are able to prevent infection of the host cell by virus, but do not necessarily prevent attachment of virus to the cell. This was demonstrated by Mandel (1959), who found that by reducing the pH of the medium surrounding the cells, the infectivity of virus-antibody complexes attached to the cells could be restored, presumably through dissociation of antibody from the attached virus. He suggested that "a virus particle that has reacted with a number of molecules of antibody can adsorb to the cell via the same site to which the antibody molecule is attached". This referred to a virus particle which was pictured as possessing a single attachment site. However, since the virus particle probably has sixty such sites, as discussed in Section III/1, explanation in terms of a single site is no longer necessary, for a virus particle may combine with several molecules of antibody and still possess many sites capable of attaching to the surface of the host cell. With an increase in the average number of antibody molecules per virus particle, a decrease in the rate of attachment would be expected, since a decreasing

fraction of collisions between virus and cell might be expected to result in attachment. This change in rate was observed by Mandel (1959) and the above picture is undoubtedly more appropriate to our present knowledge of virus structure.

It appears also that cells possess a number of sites capable of receiving virus, since Fogh (1955) found the "adsorption" rate constant to be independent of multiplicity over a wide range.

Information concerning the nature of the components or virus receptors of the host cell surface which adsorb poliovirus, has been obtained in a number of ways. The capacity of a cell to adsorb polio-virus has been correlated with its susceptibility to infection. Thus, Kaplan (1955) found that in contrast to susceptible cells, poliovirus resistant cells from capuchin monkey kidney failed to adsorb significant amounts of this virus. This correlation was studied in greater detail by McLaren et al (1959), who found that insusceptible cultured cells derived from rabbit, guinea-pig, mouse, dog, cat, pig, calf or chicken tissues, removed only small amounts of virus from overlying fluid under conditions in which over 90 per cent of the virus was removed by susceptible cells derived from human, monkey or rabbit (E.R.K.-1) tissues.

It is clear from these studies that "adsorption" of polioviruses is not a general property of cell membranes, and suggests that the virus receptor possesses a specific physico-chemical configuration.

In an attempt to determine the nature of virus receptors by direct means, Holland and McLaren (1959) studied the inactivation of virus by debris from cells disrupted by freezing and thawing, and found that while debris from susceptible cells inactivated virus, debris from insusceptible cells did not. This paralleled the capacities of the whole cells to adsorb virus. The capacity of active cell debris to inactivate virus was destroyed by heating at 56°C for 15 minutes, or at 37°C for 24 hours. It was also destroyed by lipid solvents, by trypsin and by sonic vibration. It was resistant to lipase and also to periodate and receptor destroying enzyme. Holland and McLaren concluded that the poliovirus receptors are associated with lipoprotein.

Further information concerning the nature of receptors has come from the study of the effects of anti-cellular antibodies on the infection of cells by polioviruses. These have been shown to protect cells against the cytopathic effects of polioviruses

and to interfere with their growth (Habel et al 1958; Quersin-Thiry, 1958,1959; Holland and McLaren, 1959). These antibodies reduce the rate adsorption of virus to cells (Holland and McLaren, 1959), which indicates that they act at the surface of the host cell. In an antigenic analysis of cell lines and primary cell cultures of diverse origin, Quersin-Thiry (1959) studied the capacity of homologous and heterologous sera to agglutinate or lyse cells, or to protect them against the effects of viruses. Her results could be explained in terms of two cellular antigens, one common to all cells of human or simian origin, and another common to all cells sensitive to poliovirus. Thus, virus receptors and an antigen concerned in the protection of cells against poliovirus infection by its corresponding antibody have a common distribution among various cell types. This suggests that the receptor and the antigen may be identical or closely associated.

The above suggestions concerning the nature of polio-virus receptors will require confirmation by more extensive and detailed studies. The characterisation of receptors which is important to

the understanding of the early stages of infection, also has important bearing on the relationship of polioviruses to other members of the enterovirus group. In detailed studies of another member of this group, the GD VII strain of Theiler's mouse encephalomyelitis virus (Theiler and Gard, 1940) has been shown to attach to and agglutinate human O red blood cells at 4°C, and to elute from these cells at 37°C (Lahelle and Horsfall, 1949). The virus receptors on the red cells are resistant to periodate and RDE treatment and are therefore, distinct from influenza virus receptors (Fastier, 1950).

Virus inhibitors have been found in lipid-free extracts of normal brain and other tissues of the mouse, in normal serum from several rodents, (Fastier 1950, 1951) and in human urine and chick allantoic fluid. Mandel and Racker (1953) isolated from intestinal tissues of adult mice a mucopolysaccharide inhibitor which combined in a readily reversible manner with the virus. Dissociation of the virus-inhibitor complex yielded active inhibitor and virus (Mandel, 1957). Thus although poliovirus and G.D. VII are included in the

same broad group of enteroviruses by virtue of a similar intestinal habitat and similarities in the nature of the disease they produce in their natural hosts, the available evidence indicates that they bind to their host cells by chemically dissimilar receptors. This suggests a more distant relationship than had previously been held to exist and casts some doubt on the criteria at present used to determine such relationships.

It is generally accepted that following attachment of virus to the cell surface, at least part of the virus particle must enter the cell before virus growth can begin. Although the entry of viral RNA may be all that is required to initiate virus production, (Colter et al, 1957; Alexander et al, 1958) it is probable that in the infection of the host cell by whole virus, the entire virus particle enters the cell. The mechanism of penetration is not known, although once specific attachment has occurred, penetration of the virus particle might be accomplished by the non-specific mechanism of phagocytosis. This was suggested by Fazekas (1948), who used the term "viropexis" to describe the phagocytosis of virus

particles. This may be an over simplification for a small fraction of the virus particles which attach to susceptible cells fails to penetrate, and remains detectable for long periods as "background" virus. Insusceptible cells exposed to virus under similar conditions, bind a similar amount of such "background" virus, and Holland and McLaren (1959) suggested that this virus is non-specifically bound. However, if viropexis is non-specific such virus should penetrate the cells. Its failure to do so suggests that penetration, like the attachment leading to host-cell infection, is specific. It is possible that penetration can only occur by the successive interaction of a number of the protein sub-units of the virus with specific receptors of the host cell surface. This would result in a progressive envelopment of the virus particle by the host cell membrane, and at the same time its entry into the cell. This mechanism differs from Fazekas' (1948) viropexis only in its specificity.

The time required for virus to penetrate the cell has been studied by adding virus specific antiserum to virus infected cells at various times after infection. Virus taken up by cells is

initially sensitive to antibody, but rapidly becomes insensitive. Antibody sensitive virus has a half-life of approximately 30 minutes (Payne et al, 1958; Holland and McLaren, 1959). This estimate is consistent with the rapid change in the sensitivity of the virus-cell complex to ultra-violet irradiation which occurs shortly after infection of the cell, (Dulbecco and Vogt, 1955). This indicates that a pronounced change in the physical state of the virus occurs at this time.

While initiating infection of a host cell, poliovirus, like other animal viruses which have been adequately studied, loses its infectivity for other cells. This has been adequately demonstrated for Type 1 strains of virus by several workers (Howes and Melnick, 1957; Darnell, 1958; McLaren et al, 1959). It has been suggested that the small fraction of virus which retains its infectivity for long periods is non-specifically bound to cells, and is non-productive virus (McLaren et al, 1959).

Studies of the transforming principles of bacteria, of the mechanism of infection of host cells by bacteriophage and more recently of the biological activities of the components of animal and plant viruses have shown that nucleic acids play

a dominant role in the maintenance and transfer of genetic information. In addition, for those viruses which have been studied in sufficient detail, the nucleic acid component has been shown to occupy a central or near central position within the particle, and unmasking of this nucleic acid must be a necessary pre-requisite for it to become functional. Gierer and Schramm (1956) and Fraenkel-Conrat (1956), showed that unmasking of the RNA of tobacco mosaic virus leads to a marked loss of infectivity of the virus. The infectivity which remains was found to be associated with the RNA fraction. Similar findings have since been made for several RNA containing animal viruses. The loss of infectivity which accompanies the in vitro "unmasking" of tobacco mosaic virus RNA was shown to be due to its presumably low capacity to infect host cells under the conditions of assay and not be due to destruction of RNA, for a large part of the original infectivity could be recovered by reconstituting virus from its components (Fraenkel-Conrat and Williams, 1955, Fraenkel-Conrat, 1957; Fraenkel-Conrat and Singer, 1958).

There has been a tendency to generalise

from this and to equate the loss of infectivity which animal viruses suffer during the infection of the host cell with the unmasking process (e.g. Isaacs, 1959). This may be the mechanism by which plant viruses, which are thought to enter their host cells as a result of injury to the cell, lose their infectivity. However, more elaborate mechanisms are involved in the entry of animal viruses into their host cells and virus may lose its infectivity for other cells before unmasking occurs. Such a loss of infectivity might follow firm union of virus with receptors on the cell surface. That this may occur is shown by the inactivation of virus by debris from susceptible cells (Holland and McLaren, 1959) and by the similarities between this reaction and the neutralisation of virus by antibodies. Both are temperature sensitive reactions which are partially reversible by sonication. Since reasonable alternative mechanisms for the loss of virus infectivity may be advanced, the generalisation, that infecting virus loses its infectivity when it is unmasked is not well-founded, and may lead to false conclusions.

Thus Holland and McLaren interpreted their observation that cell debris can inactivate virus to mean that the entry of poliovirus RNA into

the host cell is accomplished by means of an injection mechanism.

They assumed that poliovirus loses its infectivity as a result of the unmasking process and argued that since host cell debris can inactivate virus, the unmasking cannot involve removal of all the viral protein from the virus particle, since this would render the RNA susceptible to the action of ribonuclease and they had shown that this does not occur. They therefore proposed that penetration of the host cell membrane, the unmasking of viral RNA, and the loss of infectivity occur simultaneously and are the result of the injection of viral RNA into the host cell. As discussed above, their initial assumption is unjustified and in addition, such a mechanism is not compatible with more recent studies of virus structure, which provide no evidence for the existence of the special structures which would almost certainly be necessary for injection to be accomplished.

Following the infection of cells by poliovirus an interval of from 3 to 4 hours elapses before mature progeny virus becomes detectable in cell cultures maintained under optimal conditions (Howes and

Melnick, 1957; Maassab et al, 1957; Darnell, 1958; Howes, 1959a). Minor differences between estimates of the duration of this eclipse period are probably due, in part, to differences in the techniques used by various workers to infect cells.

The initial increase in mature virus concentration in cell populations infected with Type 1 poliovirus is approximately exponential. However, this mode of increase is short-lived for, during the appearance of a large part of the virus yield, maturation proceeds at a constant rate (Howes, 1959a; Lwoff and Lwoff, 1959b; Lwoff, 1959; Darnell and Levintow, 1960). The duration of the maturation phase of the growth cycle cannot be determined exactly since there is a gradual diminution in maturation rate at the end of the phase. In addition, a continued slow maturation of virus which may occur after the maximum level of infectious virus has been reached may be obscured by a more rapid loss of infectious virus due to thermal inactivation (Howes, 1959a).

For practical purposes the duration of the maturation phase might be defined as the time interval between the first appearance of progeny virus, and the attainment of the maximum infectious virus concentration. Although this has been

estimated to be 5 to 7 hours, for several types of host cell (Howes and Melnick, 1957; Darnell, 1958; Howes, 1959a, Darnell and Levitow, 1960), Lwoff and Lwoff (1959b) have found that virus strains of the same immunological type, but of different biological properties, differ widely in their maturation rates and in the duration of the maturation phase.

For all strains of virus studied in sufficient detail, virus maturation has been found to proceed at a constant rate during production of the majority of the virus yield. No conclusions concerning the kinetics of maturation per se can be drawn from this finding however, for the shape of the virus maturation curve must be affected to a marked extent by the asynchronous response of cells to infection (Howes, 1959b). It seems likely therefore, that information concerning the mode of increase of mature virus within the host cell will have to be derived by indirect means.

In early studies of virus release from cells infected and maintained under conditions restricting virus growth to a single cycle, Dulbecco and Vogt (1954) found that, following a

latent period of from 3 to 4 hours, free virus concentration increases rapidly. In detailed studies of single infected cells in microdrops, Lwoff et al (1955) showed that each cell releases a major part of its virus yield within the first hour following the start of release. They designated release as "rapid" and suggested that it occurs through partial lysis of the host cell. Designation of release as "rapid" has apparently led to the view that the release of a virus particle follows its maturation with little delay, for Kallman et al (1958) regarded virus maturation and release as processes which can proceed simultaneously within the same cell.

A different picture of virus release has been developed from studies of the growth of Type 1 virus in cell monolayers, in which virus growth may be restricted to a single cycle by exposing cells to virus concentrations sufficiently high to ensure infection of a large fraction of the cells. (Howes and Melnick, 1957; Maassab et al, 1957; Darnell, 1958; Riozman et al, 1959). During the maturation phase of the cycle only a small fraction of the total virus present is found

to be present in the medium above the cell layer. Although readsorption of released virus to cells, or the mechanical trapping of extracellular virus within the cell monolayer may be expected to occur under the conditions of these experiments, it was suggested that these factors could not account for the observed discrepancy between free and total virus concentrations and that this discrepancy was partly due to the delayed release of virus (Howes and Melnick, 1957).

This interpretation was supported by results of an earlier study by Girardi et al (1956) of the growth of virus strains of all three types in cell suspensions. They found that at all times, except in the last harvest, the cells contained more virus than the medium. Despite their use of the relatively inaccurate quantal technique for virus assay, these differences were clearly significant. However, the significance of this finding, that release of poliovirus is delayed, was not discussed.

Unequivocal evidence of a pronounced delay in the release of mature Type 1 poliovirus from cells has recently been obtained by the study

of the virus growth cycle in dilute cell suspensions in which mechanical trapping and re-adsorption of released virus are negligible (Howes, 1959a). Although some virus was found to be released during the maturation phase, the majority accumulates in association with cells, and is released over a period of many hours following the apparent completion of virus maturation. Virus accumulates within the cell and not at its surface, for cell-associated virus is resistant to neutralising antibody.

The relation of the growth cycle in cell populations to that in individual cells was studied using techniques similar to those used by Lwoff et al (1955) (Howes, 1959b). It was shown that at the end of the maturation phase, when a large fraction of the virus yield of a cell population is intracellular, most cells contain large amounts of virus but have released none. This established that in most cells virus release follows and does not accompany maturation, and that the virus released during the maturation phase must be derived from only a small fraction of the cells. Release of virus from most cells

occurs during a period of many hours following the completion of virus maturation, but each cell releases its virus yield over a short time interval as was found for early releasing cells by Lwoff et al (1955). The slow release of virus from cell populations was shown to be due to wide inter-cell variation in the time at which release begins. Thus in individual cells, the maturation and release phases of the growth cycles are separated by a period during which virus is retained within an apparently quiescent cell. This was designated the "intracellular retention phase".

The kinetics of release can be adequately explained in terms of the lytic mechanism proposed by Lwoff et al (1955). They do not require explanation in terms of special mechanisms, although such mechanisms have been suggested. Since they have been based largely on studies of morphological changes in the host cell they are discussed in the following section.

3. Changes in the morphology of the infected host cell

Before cell cultures were used for the study of polioviruses, changes in the morphology of infected nerve cells had been studied extensively by histopathologists (Bodian, 1948; Howe, 1952). While it is not possible in such studies to determine directly the sequence of the changes which occur, the sequence has been deduced by comparing and grading cells according to the severity of the changes which they have suffered. Although no fine distinctions can be made, this sequence agrees in general with that observed in cultured cells examined under more favourable conditions.

The first change seen in the infected neurone in the intact animal is a reduction in the size and staining of the Nissl bodies. Chromatolysis may be complete, or may involve all but marginal areas of the cytoplasm in cells which have not been affected lethally. Changes in the nucleus follow the appearance of changes in the

Nissl bodies and take the form of nuclear shrinkage, massing of chromatin, and the occasional displacement of the nucleus from its central position. In many cells small acidophilic inclusions appear within the nucleus. These inclusions are seen in frankly necrotic cells, but are also seen occasionally in cells which appear to be recovering and within which other nuclear changes are absent or minimal. Bodian (1948) considered severe nuclear changes to be incompatible with recovery. Although no direct evidence for such recovery can be obtained, the quantitative studies of Bodian (1948) provide convincing circumstantial evidence of its occurrence. Recovery of infected cultured cells has not been described.

A major disadvantage of studies of sections of tissue taken from the host animal has been the absence of any means for determining either the duration of the sequence of changes which occur in the host cell, or the time at which the sequence begins relative to the time of infection of the cell. This difficulty is far less pronounced in studies of cultured cells, but the extent to which conclusions drawn from the study of cultured cells apply to the cells of the intact host has still to be determined.

Although nerve cells cultured in vitro would provide the best material for an initial comparison of in vitro and in vivo responses, they have been studied only briefly. Hogue et al (1955) found that neurones, astrocytes, oligodendroglia and macrophages cultured from human brain tissue, were susceptible to a Type 1 strain of virus (Mahoney). Neurones reacted more rapidly than other types of cells and cells from foetal brains reacted more rapidly than cells from adult brains. However, examination was limited to the brightfield microscopy of living cells, and the changes observed were the retraction of neurites and the rounding up of the cells. More detailed studies of fixed and stained preparations are clearly necessary.

Responses of several cell types of non-neural origin, cultured in vitro, have been studied by the use of histological techniques, by phase contrast or brightfield microscopy of living cells and more recently by electron microscopy. While histological techniques are more suitable for the detailed examination of large numbers of cells, and reveal changes in morphology before these are evident in the living cell, direct determination of the sequence

of changes is impossible. However, this sequence may be determined directly by the continuous observation of single living cells, although in less detail.

Many histological studies have been of cells fixed after several cycles of virus growth have occurred, despite the ease with which virtually simultaneous infection of all cells may be achieved. As a result, difficulties in the interpretation of the results of such studies have been no less than those forced on students of the cytopathology of the host cell.

However, even studies in which the growth of virus has been limited to a single cycle have not allowed an unequivocal ordering of the changes in morphology by which the host-cell responds to infection. This failure is the result of a great variation in the time at which changes appear in individual cells (Reissig et al, 1956; Harding et al 1956; and Dunnebacke, 1956 a and b), a variation which parallels the asynchronous response of cells observed in studies of the maturation and release of virus (Howes, 1959b).

In monolayers of monkey kidney cells infected with Type 1 virus, changes in morphology

appear in some cells during the fourth hour after infection, and the number of cells affected increases for at least a further three hours, when cell counts become inaccurate due to loss of many cells from the glass surface to which they had been attached (Reissig et al, 1956). Harding et al (1956), using Type 2 virus (MEF₁), found that changes appear in HeLa cells at about the same time, but Dunnebacke, (1956a), found no changes in either HeLa or monkey kidney cells until between six and eight hours after infection. This much slower response may be due to the use of different virus strains, or to technical factors.

In all fixed and stained cells which have been studied, the first change is seen in the nucleus, although the nature of this change differs from one cell type to another. In monkey kidney cells, it takes the form of small acidophilic inclusions similar to those described in neurones of infected animals (Reissig et al 1956; Beale et al, 1956). No inclusions were seen in HeLa cells fixed and stained by similar techniques (Harding et al, 1956), so that intra-

nuclear inclusions do not appear to be an invariable feature of the infection of cells by poliovirus. In human amnion cells, the first sign of infection was stated by Dunnebacke (1956b) to be a rapid and progressive diminution in the staining capacity of the nucleolus, which eventually becomes undetectable. Bernkopf and Rosin (1957) were not able to confirm this finding in their studies of infected amnion cells. Changes in the nucleolus have not been observed with other cell types and the nucleoli of monkey kidney, HeLa, and human foetal cells were found to be present in an apparently unchanged state even after marked alterations in cell morphology had occurred (Reissig et al, 1956; Dunnebacke, 1956 a and b).

In monkey kidney cells (Reissig et al 1956), the appearance of intranuclear inclusions is followed by the loss of chromatin from the centre of the nucleus and condensation of chromatin-like material about the nuclear membrane. A parallel change in the distribution of Feulgen positive material occurs. The nuclear membrane then becomes wrinkled and nuclear changes progress

to pyknosis and fragmentation of the chromatin. Shortly after wrinkling of the nuclear membrane becomes evident, a paranuclear mass is seen. This mass is acidophilic and Feulgen negative, and displaces the nucleus from its central position. Concurrently there is a great increase in the basophilia of the peripheral zone of the cytoplasm, and in some cells basophilic granules may be found there. Rounding of the cell does not occur until these changes are well advanced.

Similar changes have been observed in many types of human cells, including HeLa, conjunctival, kidney, liver and intestinal cells and fibroblasts. (Ackermann et al, 1954; Harding et al 1956; Barski et al, 1955; and Buckley, 1957). The changes in human amnion cells described by Dunnebacke (1956b), differ from the above description, as she stressed the vacuolation and nodulation of the cytoplasm which is seen in only a fraction of the cells and neglected to mention the frequent nuclear pyknosis evident in her photomicrographs. In their studies of human amnion cells, Bernkopf and Rosin found that the changes produced by poliovirus were similar to those described for monkey kidney cells by Reissig et al, (1956).

The vacuolation and nodule formation observed in many amnion cells have also been observed infrequently in cultures of other types of cells (Klone, 1955; Reissig et al, 1956; Harding et al, 1956; and Dunnebacke (1956a). Whether vacuolation and nodule formation are transient changes detectable at any instant in only a fraction of cells, or never affect more than a fraction of the cells, cannot be decided.

Studies of living cells have confirmed the later stages of the sequence of changes which have been deduced from studies of fixed and stained cells. Barski et al (1955), used phase contrast microscopy combined with time lapse cinematography to study infected HeLa cells. They found that after an initial "thickening" of the perinuclear area, a granular mass appears alongside the nucleus. In contrast to the surrounding cytoplasm which shows active movement, the cytoplasmic mass is motionless, suggesting a greater density and rigidity. As this mass increases in size, it displaces the now altered nucleus from its central position. The nucleus becomes irregular and appears less distinct.

Up to this stage the cell retains its mobility, but subsequently a rapid rhythmic appearance and disappearance of cytoplasmic protrusions which resembles the "bubbling" seen in cells in telophase, is observed. The cell then becomes immobile, when characteristic vacuolization of the outer layer of the cytoplasm occurs and there is a gradual disintegration of the cellular outline. Bits of cytoplasm may become detached at this stage.

These changes were first observed twenty hours after infection. This delayed response is partly due to the incubation of cells at 30-31°C, but may also be due partly to the use of small amounts of virus to infect the cells. The few cells studied may have been infected at the end of the first cycle of virus growth rather than at the beginning of the experiment. Once the first changes had been detected the remainder of the sequence occupied a further six to thirteen hours. Less detailed studies by Harding et al (1956), and Reissig et al (1956) have confirmed the results of Barski et al.

Kallman et al (1958) have used the electron microscope to study changes produced in cultured cells by poliovirus. A large fraction of their cells were infected over a short time in order to synchronise the response of cells as much as possible and to restrict virus growth to a single cycle. Samples of cells were fixed at intervals up to nine hours after infection. Changes in morphology were classified into three stages. In cells in the first stage, the nucleus has been displaced from the centre of the cell by cytoplasm of increased density. The outline of the nucleus is irregular and dense material is seen to have accumulated at its margin. Such cells were abundant at three and four hours after adsorption of virus had been completed. It was suggested that the numerous small vacuoles seen in circumscribed areas of the cytoplasm in some cells may represent a still earlier stage of the response to infection.

Stage II was marked by accentuation of changes present in stage I and by the presence of discrete bodies, designated U-bodies, in the cytoplasm.

These were distributed throughout the cytoplasm in some cells, but occurred as small accumulations or large masses in others. Their size was variable, but they were most commonly about 120-130 m μ in diameter. Since they were occasionally surrounded by a membrane, Kallman et al suggested that they may represent dense material which forms in or invades a canalicular system in the cell. Stage II cells were most common in samples taken at five and seven hours.

Stage III cells were most commonly seen at 7½ and 9 hours. Changes in nuclei were more severe and probably correspond to pyknotic degeneration. Mitochondria were swollen and distorted, and the cytoplasm was filled with coarse and fine vacuolar elements. Recognisable U bodies were absent.

The most striking features of this study were the recognition of U bodies which have not been described previously and the failure to detect particles resembling virus in the cells. It is probable that this failure was due to technical factors since Stuart and Fogh (1959) have found crystalline arrays and aggregates of virus-like particles in the cytoplasm of infected FL cells.

In addition, Horne and Nagington (1959) have recently applied the phosphotungstate negative staining technique to the study of fragments of poliovirus infected HeLa cells. Within these fragments they found bodies of from 30m μ to greater than 1 μ diameter, which were surrounded by a membrane, and which contained particles closely resembling virus. Complete and incomplete particles which possessed a sub-unit structure were found within the bodies, and the arrangement of the sub-units within these particles was consistent with the 532 symmetry found in X-ray diffraction studies of virus crystals. It seems likely that the U-bodies of Kallman et al (1958) and the bodies described by Horne and Nagington are identical.

Attempts have been made to correlate the maturation and release of virus with particular changes in cell morphology. Barski et al (1955) suggested that release is associated with the vacuolisation and bubbling of the cytoplasm which occurs at a late stage in the response of HeLa cells to infection, while Dunnebacke (1956 b) suggested that the nodules seen in amnion cells are eruptions of cellular material containing virus,

and that release of virus from these cells is accomplished by this process. In her studies of monkey kidney cells (Dunnebacke 1956 a), she defined four stages in the response of cells to infection and concluded that virus was released between stages III and IV. Since, by definition, stage III cells become stage IV cells at the instant the cells become detached from the glass of the culture vessel, it follows that virus is released at this instant. Dunnebacke undoubtedly did not mean this, but what she did mean is not apparent. More recently Ackermann (1958) has suggested that release is accomplished by a process he calls cytotransudation. This he identifies with cytoplasmic retraction which in turn he pictures as cytoplasmic contraction (Figure 7 of his review). The highly speculative nature of the above suggestions is evident from the variety of different mechanisms proposed and the uncritical way in which the evidence has been considered, and it is perhaps not surprising that the authors have sought specific and specialised mechanisms for virus release. It is difficult to conceive that any of the proposed mechanisms would allow release of any more than a fraction of the virus contained by cells.

The studies of Lwoff et al (1955) were more realistic both in execution and interpretation. The amount of virus released from single cells during successive time intervals was determined and release was correlated directly with changes in the morphology of the cell. Lwoff et al attributed both the changes which occur in the peripheral hyaline zone of the cell, and virus release, to partial lysis of the cell. However, since their studies were almost certainly restricted to a minority of cells which release virus early, (Howes, Paper II) it does not necessarily follow that all cells behave in the same manner, or that release is always correlated with the changes in morphology they observed at this time.

However, more recent studies in which care was taken to avoid selection of cells, indicate that all cells release virus in the same manner, irrespective of the time at which this begins (Howes, Paper III). No attempt was made in these studies to correlate release with the appearance of the cells, and it is clear that if characteristic changes accompany release, further studies will be needed to detect them. At present however, there is no feature of virus release which demands

explanation in terms of a special mechanism. The bubbling or nodule formation, disappearance of the hyaline zone of the cytoplasm, and vacuolization are late stages in the response of cells, and occur just prior to the cessation of visible activity of the cell (Barski et al, 1955). If they are correlated with release of virus, this correlation may be fortuitous, in that these changes signal the impending death of the cell. At this time the cell membrane might be expected to lose its integrity and allow intracellular particles to diffuse from the cell. In this regard the studies of Girardi et al 1956, are of interest, for they suggest that the quantity of virus released and the fraction of cells which had become freely permeable to trypan blue may be correlated. Thus at present, there appears to be no need for explanations of release in terms of special mechanisms, and all data available are adequately accounted for in terms of release by a partial lysis following the death of the cell.

Attempts have also been made to identify the site of the first detectable change within the cell with the site of virus "production", or the site of the primary action of the virus.

These changes appear relatively late in the cycle however, and may occur as an indirect result of changes which occur elsewhere in the cell but which are not detectable by ordinary histological techniques. Although changes in morphology revealed in this way may eventually be explained in terms of events which occur during the synthesis of viral components, the nature of these events is at present largely a matter for speculation.

Although ordinary histological techniques allow no firm conclusions to be drawn about the sites of synthesis of viral components, the use of the fluorescent antibody technique has allowed the appearance of viral antigen within the cell to be studied. Buckley (1956) used this technique and found diffuse granular staining of the cytoplasm, and in some rounded cells, staining of the nucleus. However, the value of her experiments was reduced by her use of cultures infected with small amounts of virus, and examined after several cycles of virus growth had occurred. More careful studies by Oddo (1957) strongly suggest that large amounts of antigen appear in the cytoplasm before any appears in the nucleus. Fine granular staining of the

cytoplasm appears initially but this becomes diffuse as the amount of antigen increases. The fluorescent cytoplasmic granules seen in some severely affected cells may result from aggregation of antigen, or may reflect the synthesis of antigen in regions from which its diffusion cannot occur.

It is clear from the above discussion that although the study of cells by ordinary histological techniques has amply demonstrated that severe changes in the morphology of the host cell normally follows its infection by poliovirus, it has not led to any understanding of the nature of the synthesis of viral components. Study by means of the electron microscope or the fluorescent antibody technique have been more rewarding, and have allowed the probable site of viral antigen synthesis and virus maturation to be identified as the cytoplasm.

4. The production of virus components
by the infected cell

Colter et al (1957) and Alexander et al (1958) have shown that the RNA of poliovirus can infect cells, and it is now generally accepted that it carries the information necessary for the infected cell to produce the components of virus. These studies were carried out with susceptible cells, but it has since been shown that cells which are insusceptible to whole virus can also be infected with viral RNA. (Holland et al, 1959 a and b; De Somer et al, 1959; Mountain et al 1959). This suggests that the steps of the virus-cell interaction which follow the unmasking of viral RNA are much less specific than those which precede it, and it might be argued on these grounds that it is the nature of the substances synthesised by the cell and not the nature of the synthetic apparatus of the cell which determines the capacity of the cell to produce viral components.

However, it has been found that the genetic constitution of virus affects the capacity of the infected host cell to produce infectious virus under different environmental conditions. Thus Vogt et al

(1957) found that virus mutants may differ from wild type virus in their capacity to produce plaques in cells maintained under an acid overlay. Although wild type virus possessed a high efficiency of plating (e.o.p) under acid overlay the mutants showed a greatly reduced e.o.p. They were designated "d" or delayed mutants. Vogt et al followed the "growth" of d and d+ viruses in cells maintained in liquid medium and found that there were no demonstrable differences between the two virus types in the production of free virus under acid conditions. However, they found that if cells are maintained under an acid agar overlay, after an initial delay of approximately 12 to 24 hours duration, the rate of increase of free d virus decreased to a level below that of d⁺ virus. Lwoff and his co-workers (Lwoff, 1959) were able to demonstrate a marked effect of pH on virus growth, measured in terms of the total virus yields, in liquid media. They showed that the effect is important only below a critical pH of approximately 6.9, and that lowering of the pH even as late as three hours after infection results in a marked reduction in virus yield. The effects of environmental temperature on virus growth are similarly dependent

upon the genetic constitution of the infecting virus (Dubes and Chapin, 1956; Dubes and Wenner, 1957; Lwoff, 1959), and again are demonstrable several hours after infection (Lwoff, 1959). It is apparent therefore that environmental pH and temperature changes influence relatively late stages in the virus growth cycle, and that the interactions between virus and host cell which occur at this time show some degree of specificity.

In the studies considered above, the effects of environmental factors were determined solely in terms of the infectious virus produced by cells, and it is not known whether these effects are established through a general inhibition or perversion of viral synthetic processes, or through action on a specific process.

Although the viral protein and RNA which are produced by the host cell constitute only a very small fraction of the total protein and RNA content of the infected cell, Maasab et al (1957) and Ackerman et al (1959) have reported that the infection of HeLa cells by poliovirus stimulates the net synthesis of cytoplasmic RNA and protein, and increases the rate of turnover of both RNA

and DNA. These results could not be confirmed by Salzman et al (1959) who followed the incorporation of cytidine-2-C¹⁴ into RNA and DNA and studied the synthesis of RNA, DNA and protein by direct chemical methods. They found that the net synthesis of all three fractions is inhibited within the infected cell, and that this effect becomes evident within the first few hours after infection. It is not known how this inhibition is accomplished. Salzman et al suggested that the apparent stimulation of RNA synthesis by poliovirus which was claimed by Ackerman and his co-workers, was due to the presence, in their RNA fractions, of impurities which also incorporated P³². Studies of the nutritional requirements of infected cells agree with the results of Salzman et al (1959) for they show that these requirements can be met from within the cell. Thus Eagle and Habel (1956) and Darnell and Eagle (1958) found that cell monolayers are able to produce maximum yields of virus in media containing only glucose, glutamine and salts.

Darnell et al (1959) found that the nutritional requirements for the synthesis of virus by suspended cells are greater for dilute than concentrated suspensions and this was attributed to the rapid depletion of the free amino acid pool of the cell which occurs under these

conditions. Darnell et al concluded that poliovirus protein is synthesised de novo from the amino acids of this pool, a conclusion which has been confirmed by the study of viral protein synthesis using isotopically labelled l-valine (Darnell and Levintow, 1960).

Similar studies of the source of the nucleotides used in the synthesis of viral RNA have not yet been reported, although since Salzman et al have reported that an increase in the size of the nucleotide pool of the infected cell is apparent within 3 hours of infection, it is possible that the nucleotides used in the synthesis of viral RNA are derived partly from the RNA of the host cell.

If both viral protein and viral RNA are derived from such pools, it is possible that the specificity which exists in late stages of the virus-host cell interaction will be found to be associated with those enzymes and ancillary substances within the cell which are responsible for the ordered assembly of amino acids and nucleotides to form proteins and nucleic acids. An initial step towards the resolution of this problem may be the study of viral antigen production.

A few years ago it was customary to speak of the site of virus multiplication within the host cell,

and attempts were made to identify this site by determining the distribution of mature virus in the various fractions which could be isolated by the centrifugation of cell homogenates. In such studies, Schwerdt and Pardee (1952) found that the great bulk of virus infectivity could be isolated in the "microsome" fraction obtained from infected brain tissue of cotton rats. Similar results were obtained by Kaplan and Melnick (1953) who studied infected mouse brain preparations. However, free virus particles might be expected to sediment with the microsome fraction, and these studies therefore, failed to demonstrate any unequivocal and meaningful association of mature virus with any cell structure. More recently it has been realised that different components of animal viruses may be produced in different parts of the cell.

It is currently believed that cellular RNA, cytoplasmic or nuclear, is synthesised in the nucleus, and since in cultured cells infected with poliovirus, nuclear changes are seen early in the morphological response of the host cell, the synthesis of viral RNA may occur in the nucleus also. The study of the site or sites of synthesis of viral RNA may be approached

in a number of possible ways, but at present little information has been obtained.

Tenenbaum (1956) studied the changes which occur in cellular nucleic acids following infection, by means of fluorescence microscopy of cells stained with acriflavine. She observed an increase in the green fluorescence characteristic of DNA in the nucleus 4 hours after infection, and an increase in the red (RNA) fluorescence of the cytoplasm at the same time. At 5 hours she found that the bright green nuclear fluorescence had disappeared in over 50 per cent of the cells, while a bright green spherical body was found in the cytoplasm at the centre of the cell. These changes occurred at about the same time that changes detectable by other techniques become apparent. However, since there is no increase in the amounts of either RNA or DNA in the infected cell (Salzman et al, 1959), the increases in green and red fluorescence of cells stained with acriflavine presumably represents a redistribution or re-orientation of existing nucleic acids. How these changes are related to the production of virus components is not apparent. Autoradiography of infected cells exposed to labelled precursors of RNA is also unlikely to reveal the site of viral RNA

production since, although the amount of label incorporated into viral RNA may be appreciable, the turnover of cellular RNA would tend to obscure this.

Although the site of viral RNA synthesis has not yet been identified, the results of studies of infected cells by means of the fluorescent antibody technique strongly suggest that viral protein is produced in the cytoplasm. As discussed in Section III/3 this was first suggested by the results of Buckley (1956), although due to inadequacies of experimental design this was not demonstrated unequivocally. However, the studies of Oddo (1957) were carried out more carefully and showed that antigen appears in the cytoplasm of a few cells as early as 3.5 hours after infection. Although it first appears in the vicinity of the nucleus, where it has a fine granular distribution, it is later found distributed diffusely throughout the cytoplasm.

Oddo's results suggested that antigen is produced in detectable amounts only a short time before virus maturation begins. This has been firmly established only recently by Darnell and Levintow (1960), who followed the incorporation of l-valine- C^{14} added to cell cultures at intervals following infection of

the cells, into the mature virus harvested after the completion of the growth cycle. They found that viral protein is synthesised only a short time before it is incorporated into virus particles.

Since viral antigen is produced in the cytoplasm, where it accumulates, it might be expected that the assembly of mature virus occurs here also. This expectation is supported by the results of electron microscopic studies of infected cells, within whose cytoplasm virus-like particles can be detected (Stuart and Fogh, 1959, Horne and Nagington, 1960).

It is evident from the preceding discussion that at present there is insufficient experimental evidence available to allow an ordered picture of events within the infected cell to be built up, even in the simplest terms. However, there are several aspects of the interactions which occur within cells infected with two virus types whose study may at least define some of the problems which must be considered for such a picture to be constructed, and may provide indirect information concerning the production of viral components.

In recent studies of the virus produced by cell

suspensions infected with more than one type of virus, it has been found that many virus particles produced by the cells can be neutralised by antisera against either type of parent virus. This phenotypic mixing is evident even at average multiplicities of infection less than 1 (Ledinko, cited by Hirst, 1959). This mixing was more readily demonstrated in virus harvested early in the growth cycle, and Ledinko and Hirst proposed that antigen is produced in zones which overlap. They suggested that the higher yields of phenotypically mixed virus are found in early harvests because maturation will occur first where antigen concentration is highest, and that it therefore occurs where the two zones overlap. This argument is not sound, and results can be more readily explained in terms of the asynchronous maturation of virus in infected cells (Howes, 1959 b). If this asynchrony is due to variable delays experienced by virus particles during the initiation of virus multiplication, the first cells to respond to infection are more likely to be multiply infected than singly infected, while the reverse would hold for cells which produce their progeny late in the cycle. Hirst also proposed that virus particles which are taken up by cells and which fail to initiate the production of mature virus, may nevertheless

contribute to the pool of antigen used in the assembly of the viral progeny of successful particles. This is based on the unexpectedly high yields of phenotypically mixed virus at low multiplicities. However, accurate estimation of the average multiplicity of infection is difficult, and the assumption which is inherent in Hirst's arguments, that virus is randomly distributed amongst cells is probably not true, for it has been shown that cells may vary genetically in their susceptibility to infection. (Vogt and Dulbecco, 1958; Darnell and Sawyer, 1959). Thus the cells of any line which has been cultured for some time might be expected to vary appreciably in their susceptibilities to infection. Physiological variation in susceptibility may be superimposed on this genetic variation. Although the experimental data on phenotypic mixing is inadequate, the study of phenotypic mixing in the viral progeny of single cells infected simultaneously with different virus types may help to decide whether virus antigen is produced at many or a few sites within the cell.

A further result of the infection of a cell by more than one virus particle has recently been described. This is the appearance, amongst the viral progeny of such cells, of particles which can induce

the production of phenotypically mixed virus in new host cells (Sprunt et al, 1959). This suggests that some particles may contain both parental genotypes, or that some form of interaction between the parental virus genomes can occur within the infected cell. Since the frequency with which such particles occur amongst the viral progeny of doubly infected cells has not been established, and since recombination between virus strains of the same immunological type but carrying genetic markers has not been reported, this phenomenon requires further extensive study.

Interference between polioviruses has been described by a number of workers. Examples of interference, expressed as a protection of cells by virus strains of reduced cytopathogenicity against the action of fully cytopathogenic strains, have been provided by Sabin (1954) and Le Bouvier (1954), but the mechanism of this interference was not determined. However, Ho and Enders (1959 a and b) have recently shown that it may be mediated through a protein or polypeptide inhibitor which is produced by cells infected with the interfering virus, and which inhibits the further multiplication of both the interfering and the interfered virus. Since the inhibitor

neither reacts with virus in vitro, nor prevents its adsorption to cells, its site of action is presumably intracellular. Although the inhibitor was produced in response to infection, and could not be demonstrated in uninfected cell cultures, it could not be neutralised by antisera against the virus. In addition it showed a lack of specificity in its capacity to interfere with the spread of infection in cell cultures inoculated with unrelated viruses. Although the mode of action of this inhibitor is unknown, results of further studies should provide information with bearing on the nature of the steps involved in the production of virus by the infected cell.

Interference between fully cytopathogenic virus strains of different types has been studied in terms of the effects on mature virus production by doubly infected cells. In early studies Ledinko and Melnick (1954) determined the effects of infection by one virus type on the multiplication of a second type introduced at intervals after the first. Although the yields of virus of the second type were found to be reduced, the design of the experiments was such that this was probably largely due to the destruction

of cells by the first virus prior to the introduction of the second. In more recent studies, Drake (1958) studied interference during a single cycle of virus growth by determining whether cells infected with one virus type, could produce virus of a second type introduced after the first. This was done by determining the capacity of cells to produce plaques in the presence of antiserum against the first or interfering virus. Although he found that the number of cells producing virus of the superinfecting type was reduced, in retrospect this could be explained as a manifestation of the phenotypic mixing which can occur in mixedly infected cells. Although a cell might produce both genotypes, if all the viral progeny possess antigen of both types, such a cell would appear to have produced no virus of the superinfecting genotype under the conditions of Drake's experiments. Thus, even though reasonable arguments might be advanced for the occurrence of interference of this type, Drake's results do not provide unequivocal evidence of its occurrence.

It is possible that interference may also occur within cells infected simultaneously with two virus types. Since virus maturation in

individual cells of a population is asynchronous (Howes, 1959b), it is possible that infecting particles suffer variable delays during the initiation of virus growth within the host cell. If this is so, the progeny of the first particle to achieve this initiation within a cell may predominate in the virus yield of that cell, and the progeny of the next particle to "start" would be correspondingly reduced. This would score as interference within that cell, even though in the cell population as a whole, neither virus appeared to have a growth advantage over the other. These arguments are also germane to the possible genetic interaction between virus particles within the mixedly infected cell, since it is possible that in a cell infected with only one particle of each type, the discrepancy in starting times might be so great that the cell produces only one genotype, and genetic interaction is precluded. The role of asynchrony in interference and in genetic interactions between polioviruses should be considered carefully.

5. A general discussion of the growth of animal viruses

It has long been accepted that DNA controls the synthesis of both RNA and protein within the cell, and is capable of self replication. More recently it has been shown that the RNA which is incorporated into ribosomes in the nucleus is responsible for the synthesis of the variety of specific proteins found within the cell, and it is through this RNA that DNA is able to dictate the range of synthetic activities which characterise a particular type of cell.

Animal viruses have been found to fall into two broad classes on the basis of the type of nucleic acid which they contain, and it is important to determine the extent to which various members of these two classes utilize existing host cell mechanisms for the synthesis of their components.

Within each class there is considerable variation in the complexity of the physico-chemical compositions of member viruses but, in general, the RNA containing viruses appear to be of simpler composition than the DNA containing viruses. Despite this, the development of a picture of the way in

which a virus induces the synthesis of viral components is more difficult for RNA than for DNA viruses.

The information which is contained in the DNA of poxviruses might be expected to be expressed in essentially the same way as that contained in the DNA of the host cell. Cairns (1960) has recently shown that the DNA of vaccinia virus is synthesised in the cytoplasm of the host cell, and that both DNA and viral antigen synthesis become detectable at the same time and within the same circumscribed region of the cytoplasm.

Joklik and Rodrick (1958) found that there is an increased turnover of "microsomal" RNA during the first 12 hours after the infection of cells with vaccinia, which agrees with the expectation that, for the cell to produce viral antigen, the corresponding ribosomes must first be produced. Were these produced in the nucleus of the host cell it might be expected that the antigen produced by them would be synthesised at many sites, and would therefore be diffusely distributed in the cytoplasm. Its appearance in close association with the site of viral DNA synthesis suggests that

if ribosomes are necessary for antigen synthesis they are also produced at the site of viral DNA synthesis. The poxviruses may therefore carry out within the cytoplasm all those functions which are necessary for the final expression of the information contained in DNA and which are normally carried out in the host cell nucleus.

In contrast to the poxviruses, two other groups of viruses which are thought to contain DNA, the herpes and adenovirus groups, stimulate the synthesis of DNA within the host cell nucleus (Newton and Stoker, 1958; Boyer et al, 1957, Block et al 1957, Ginsberg and Dixon, 1959, Green, 1959). It seems likely that the components of adenoviruses are wholly synthesised and assembled within the nucleus (Boyer et al, 1959; Morgan et al, 1956a, Langermalm et al, 1957), but although herpes virus antigen appears initially within the nucleus (Lebrun, 1956), and the virus is probably partly assembled there, electron microscopic studies suggest that the final stages of assembly occur in the cytoplasm (Morgan et al, 1954; Gaylord, 1960).

It is necessary to ask why some DNA viruses are produced in the cytoplasm, and others

in the nucleus. The simplest explanation might be that the viral DNA initiates the synthesis of viral components at whatever site the DNA of the infecting virus is unmasked. Alternatively, the information carried by herpes and adenoviruses may relate solely to the components of mature virus, whereas the pox-viruses carry, in addition, the information necessary for the synthesis of those enzymes which are involved in the synthesis of DNA and which are normally found only in the cell nucleus. Until these questions are answered, no satisfactory generalisations about the growth of DNA viruses can be made, except at a superficial level. The problems are of a different kind to those which must be dealt with in the study of the growth of RNA viruses, and it seems that they will contribute to the study of RNA viruses largely through their contributions towards the understanding of the synthesis and function of nucleic acids in general.

There is ample evidence that viral RNA carries the information necessary for the replication of itself and for the synthesis of viral antigens (Colter et al, 1957; Wecker and Schafer, 1957; Alexander et al, 1958; Huppert and Sanders, 1958), but the way in which the viral RNA is synthesised has not been demonstrated. Nor can it be readily

visualised in terms of current theories concerning the control of RNA and protein synthesis by DNA, since the host cell presumably does not contain the necessary information in its own DNA, and there is no known counterpart of viral RNA within the host cell which possesses the dual capacity to control its own replication and to control the synthesis of a specific protein. In myxoviruses, the viral RNA is present in the form of a ribonucleoprotein, whose protein portion is antigenic. This antigen is produced in the nucleus of cells (Breitenfeld and Schafer, 1957) and it is therefore possible that the viral RNA is produced there also. The haemagglutinin, whose production is also presumably under the control of viral RNA, is produced in the cytoplasm, but since it appears later than the ribonucleoprotein antigen, its production may occur only after RNA has migrated to the cytoplasm. With polioviruses there is at present no information with direct bearing on the site of viral RNA synthesis, and no readily apparent way in which such information might be obtained. Encephalomyelitis virus may prove the most satisfactory virus for the study of viral RNA

synthesis for Huppert and Sanders (1958) and Sanders (1960) have shown that infectious RNA may be obtained from EMC infected cells by a technique which fails to extract it from the mature virus particle. The component from which they obtain infectious RNA increases in infected cells during the first 5 hours after infection, fails to sediment in the ultracentrifuge under conditions which sediment mature virus, and is possibly the primary product of the replication of viral RNA. It seems possible that the fractionation of cells harvested during the eclipse phase, and the examination of the resulting fractions for the presence of infectious RNA extracted with cold phenol, may allow the site of synthesis of this component to be determined. Similar studies should be possible with arbor viruses, for Wecker (1959) has shown that infectious RNA can be obtained from cells infected with western equine encephalomyelitis virus by extracting them with cold phenol, whereas to obtain it from virus particles, hot phenol is required.

Indirect evidence that viral RNA synthesis precedes antigen synthesis on cells infected with encephalomyocarditis virus has been obtained by Sanders (1960). In adequate quantitative studies he has shown that euflavine, added to cells at varying times after infection, continues to inhibit

haemagglutinin and infectious virus production after it has ceased to have any effect on RNA production. However, the time at which 5-fluorouracil ceases to be inhibitory is the same for all three, which suggests that RNA synthesis is a necessary prerequisite for the production of haemagglutinin and infectious virus. Less detailed studies of the effects of benzimidazole derivatives on influenza and poliovirus multiplication suggest that the synthesis of the RNA of these viruses occurs during the first half of the latent period (Tamm et al, 1953; Tamm and Tyrrell, 1954; Nemes and Tamm, 1958). Many other derivatives of imidazole or amino acids have been shown to affect the growth of animal viruses (Horsfall, 1959), but these have not been studied in sufficient detail to provide information which is of use in ordering the early events which occur in the infected cell.

The antigens necessary for the maturation of RNA viruses appear within cells only a short time before the maturation of virus begins. This has been shown for myxoviruses by means of the fluorescent antibody technique (Breitenfeld and Schafer, 1957; Franklin, 1958) and by following the production of haemagglutinin (Ledinko et al, 1957). Similar results

have been obtained in studies of enteroviruses by the fluorescent antibody technique (Oddo, 1957; Henry and Franklin, 1959), by following the production of complement fixing antigen (Roizman et al, 1958a) and more recently by following the incorporation of labelled amino acids into mature virus (Darnell and Levintow, 1960). The assembly and maturation of enteroviruses evidently occurs in the cytoplasm, since mature virus has been shown to accumulate in the cell (Howes and Melnick, 1957, Henry and Franklin, 1959, Howes, 1959a, and Somerville et al, 1958), and aggregates or crystalline arrays of virus-like particles have been observed in the cytoplasm of cells examined with the electron microscope. (Morgan et al, 1959; Fogh and Stuart, 1959; Stuart and Fogh, 1959; Horne and Nagington, 1960).

The evidence available therefore, suggests that viral RNA is synthesised early in the eclipse period, and the antigenic sub-units of the protein coat are synthesised towards the end of this period. This is in agreement with what is expected on theoretical grounds.

The synthesis of the components of virus culminates in the assembly and maturation of progeny virus. The time required for this to reach completion

is similar for those RNA viruses which have been studied quantitatively. The minor differences between unrelated viruses in the duration of the eclipse period and the duration of the maturation phase of the growth cycle do not appear to be any greater than the differences which have been observed between different strains of the same virus (Lwoff, 1959). The kinetics of virus maturation per se cannot be determined by the study of cell populations, since the response of cells to infection is asynchronous (Dulbecco and Vogt, 1953; Cairns, 1957; Howes, 1959b), and it is probable that the initial exponential increase in mature virus which is commonly observed in the study of the maturation of many viruses, is largely a reflection of this asynchrony. Where the quantitative study of virus maturation has been sufficiently detailed, a linear increase in mature virus has been observed during the production of the major part of the virus yield (Dulbecco and Vogt, 1953; Howes, 1959a; Lwoff, 1959).

In theory, the only satisfactory way to determine the kinetics of virus maturation within the host cell is to study maturation in a single cell. This is impossible with viruses whose growth cycles are characterised by the accumulation of

mature virus within the cell. However, with viruses which become extracellular at the same time as they mature, studies of the release of virus from single cells would allow the kinetics of maturation to be defined. Cairns' (1952) study of the release of influenza virus from single cells is of interest in this regard, for in his experiments he used RDE to prevent the readsorption of released virus. It was subsequently shown by Cairns and Mason (1953) that the treatment of cells with RDE prevents the retention of mature virus at the cell surface and mature virus is therefore released from cells with virtually no delay. Cairns earlier data therefore also describe the kinetics of maturation within a single cell. The rate of maturation reaches a maximum shortly after it begins, and thereafter declines, at first rapidly, and then more gradually. Dulbecco and Vogt (1953) analysed the yields of free virus which had been produced by single cells infected with western equine encephalomyelitis at various times after infection, and concluded that the amount of virus released by a cell increases exponentially with time. Rubin et al (1955) subsequently showed that the maturation and release of this virus occur almost simultaneously,

so that Dulbecco and Vogt's results also describe the kinetics of maturation. It should be possible to confirm that maturation within a cell follows exponential kinetics during its early stages, by following the release of virus from single cells.

As was indicated above, the enteroviruses on one hand, and the arbor and myxoviruses on the other, differ in the way in which they are released from cells. The enteroviruses accumulate within cells as they mature, and are subsequently released by a process likened to lysis (Lwoff et al, 1955, Howes, 1959b). The arbor and myxoviruses become extra-cellular as they mature, and there is good electron microscopic evidence (Hotz and Schafer, 1955; Morgan et al, 1956b) to support the conclusion that this maturation occurs at the cell surface.

These differences in behaviour are correlated with differences in the chemical composition of the viruses, and Franklin (1958) has suggested that all viruses which contain lipids as essential components are assembled from sub-units at or near the cell surface and acquire their lipid component and infectivity as they pass through the cell surface.

In recent studies of the haemadsorption phenomenon as applied to cells infected with influenza virus, Hotchin et al (1958) found that erythrocytes could attach to the infected cell through virus filaments projecting from its surface, or could become more intimately attached over large areas of cell membranes which lacked filaments. This suggests that the surface of the infected cell acquires similar properties to the surface of the virus particle as a result of infection. In these terms the acquisition of infectivity by virus as it passes through the cell membrane is readily understood.

In the above discussion the growth cycles of animal viruses have been compared and discussed in general terms so that the general pattern of virus growth could be defined. Many features of the virus growth cycle which reveal the underlying complexity of the virus-host cell interactions were not considered, and their detailed consideration is beyond the scope of this discussion. That genetic interactions can occur between different virus particles which infect the same cell has been adequately demonstrated for myxoviruses (Burnet, 1959; 1960), and poxviruses (Fenner and Comben, 1958; Gemmel and Cairns, 1959;

Fenner, 1959; Gemmel and Fenner, 1959). However, the interpretation of these results in genetic terms is not yet clear, and the stage in the replication of nucleic acid at which recombination occurs is not known. The quantitative study of recombination is rendered more difficult by the size of the host cell, which may allow different virus particles to multiply at different sites within the same cell. Recombination may not be possible for this reason until replication of the parental viral genomes has already been in progress for some time. Although asynchrony is not likely to prove a major problem in the study of pox-viruses (Cairns, 1960) it may well be important in the study of recombination between RNA viruses, and may preclude recombination except under special circumstances.

The phenotypic mixing which may be observed in cells infected with related viruses (Sprunt et al, 1955, 1959; Hirst, 1959, Granoff, 1959) may reflect a mixing of the components of virus prior to their assembly, or may reflect the dispersal of the "information" necessary for the synthesis of viral protein from the site of replication of the viral nucleic acid.

Interference between viruses has long been recognised, but the mechanisms of interference are still not known. Many examples of interference may be due to an abnormal response of the host cell to infection which results in the production of materials other than the components of virus which are capable of interfering with the growth of virus in other cells (Isaacs et al, 1957; Lindenmann et al, 1957; Ho and Enders, 1959a and b). In the absence of such an abnormal response, interference may be due to the exclusion of one virus by another, or to simple competition in which asynchrony, the site at which replication is initiated, and virus controlled differences in virus production rates may all assume importance.

It seems probable that many of the finer details of the biosynthesis of DNA, RNA and protein, will be resolved through the study of the interactions which occur between viruses, or between viruses and the abnormal products of infection. It also seems probable that one of the criteria by which general theories on the biosynthesis and function of DNA, RNA, and protein will be judged, will be their capacity to explain these interactions.

Acknowledgements

I am indebted to Professor F.J. Fenner and Dr. B. Morris of the Departments of Microbiology and Experimental Pathology, Australian National University, for criticising sections of the General Discussion. I am also indebted to Dr. S. Fazekas de St. Groth and Dr. H.J.F. Cairns of the Department of Microbiology, Australian National University, for their criticism of the manuscripts of the second and third papers presented in this thesis.

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