Advances in VIRUS RESEARCH

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VOLUME 7

ACADEMIC PRESS INC.

111 FIFTH AVENUE

NEW YORK 3, N. Y.

United Kingdom Edition

Published by

ACADEMIC PRESS INC. (LONDON) LTD.

17 OLD QUEEN STREET, LONDON S.W. 1

Library of Congress Catalog Card Number 53-11559

PRINTED IN THE UNITED STATES OF AMERICA

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I. Introduction

A. Purpose and Scope

In the past two decades there have been striking advances in our understanding of the biology of viruses and of the processes involved in their replication. These studies, which have thrown considerable light on such basic questions as the chemical nature of the determinants of heredity, and the interrelationships of nucleic acid and protein synthesis, have until recently dealt almost exclusively with bacterial and plant viruses, primarily because these could be studied with much greater precision than was possible with animal viruses. During the past few years, however, experimental systems have been developed which allow quantitative chemical, physical, and biological experiments with animal viruses and their host cells. Among the major accomplishments were the growth of poliovirus in cultured human (Enders *et al.*, 1949; Scherer *et al.*, 1953) and monkey (Syverton *et al.*, 1951; Morann and Melnick, 1953) cells, the development of techniques for the growth of single mammalian cells (Puck *et al.*, 1956), the development of a plaque assay for animal viruses (Dulbecco, 1952; Dulbecco and Vogt, 1954a), the puri-

fication and crystallization of polioviruses (Schwerdt and Schaffer, 1955, 1956; Schaffer and Schwerdt, 1955, 1959), and the delineation of the nutritional requirements of cultured animal cells suitable for the production of viruses (Eagle, 1955, 1960). With the aid of these techniques, poliovirus has probably been studied in greater detail than any other animal virus, and it is the purpose of this article to consider the recent findings with respect to its biosynthesis. Since some of the techniques, such as rapid purification of the virus, have only recently become available, experiments which will make much of our discussion incomplete are probably being done at the time this is written. It is nevertheless hoped that the evidence summarized here will point out the usefulness of poliovirus as a model for studies on the replication of RNA-containing viruses.

B. Host Cells of Choice

Results of quantitative significance concerning the biosynthesis of poliovirus have come almost exclusively from the use of dispersed mammalian cells, either in primary culture, exemplified by monkey kidney epithelium (Morann and Melnick, 1953), or in serially propagated cultures, such as the HeLa cell (Scherer *et al.*, 1953). Either of these cell lines can be used for both the production of virus and its quantitative plaque assay (Dulbecco and Vogt, 1954a; Gifford and Syverton, 1957). Both kinds of cells satisfy most of the requirements for an adequately quantitative system, as suggested by Dulbecco (1955); both permit the measurement of the adsorption rate of virus, the proportion of infected cells, and the virus yield per cell. As Dulbecco points out, however, these measurements can best be made with cell suspensions; and this may weigh the balance in favor of the HeLa cell, suspension cultures of which (McLimans *et al.*, 1957) also provide a continuing supply of rapidly growing cells. HeLa cells offer a further advantage in that cultures deriving from a single cell (Puck *et al.*, 1956), and thus presumably uniform in viral susceptibility, can be easily obtained. This consideration assumes importance in light of the wide variations in susceptibility to poliovirus among cultures derived from various primates (Kaplan and Melnick, 1955; Hsiung and Melnick, 1958), and among clonal isolates of the HeLa strain (Darnell and Sawyer, 1959). An additional safeguard against changing cellular susceptibility is provided by the fact that serially propagated cultures can be stored for many months at —70°C. (Scherer and Hoogasian, 1954; Stulberg *et al.*, 1958), assuring a reservoir of cells which behave predictably with respect to viral infection.

The question as to the host cell with the greatest susceptibility to poliovirus is still unsettled. McLaren and Syverton (1957) failed to find

any significant variation in virus susceptibility among monkey kidney cells or a number of parental and clonal human cell types. Schwerdt and Fogh (1957) and Fogh (personal communication) have found that primary human amnion cells, or a serially propagated line deriving from human amnion, the FL cell (Fogh and Lund, 1957), gave a higher plaque-titer than any other cell line tested, and five times greater than that obtained with two strains of HeLa cells. However, since clonal strains of HeLa themselves differ 10–15-fold (Darnell and Sawyer, 1959), some lines of HeLa cells may be even more susceptible than the FL strain.

Recent reports have suggested that nonprimate cells in culture may acquire the ability to produce poliovirus (Sheffield and Churcher, 1957; Mascoli *et al.*, 1958). In one instance this "adaptation" to poliovirus susceptibility was associated with the appearance in the cell of antigens which cross-reacted with antisera to HeLa cells (Melnick and Habel, 1958). This suggests that the culture may have been contaminated with HeLa cells, which thereupon outgrew the original strain. Such contamination has been shown to be responsible for at least one instance of the sudden "adaptation" of surviving monkey kidney cells to rapid and sustained growth in cell culture (Siminovitch, personal communication). Another "nonprimate" but polio-susceptible cell strain, originally derived from guinea pig spleen, now has a chromosomal complement similar to HeLa cells (Dulbecco, personal communication), again suggesting the possibility of contamination. The one type of nonprimate cell to which poliovirus does definitely "adapt" is the chick embryo fibroblast in primary culture (Roca-Garcia *et al.*, 1952; Dunham and Ewing, 1957). While this property could prove useful in the study of the genetic control of host-range variation, the chick fibroblast has no other apparent advantage over either monkey kidney cells or HeLa cells for the production of virus, or for the study of its biosynthesis.

C. Description of the Virus

1. The Growth Cycle

The initial step of adsorption of poliovirus to a susceptible cell is followed by the phenomenon of "eclipse," the loss of infectivity of the absorbed virus. During this time the virus particle presumably enters the cell and is broken down into its protein coat and its infectious component, the nucleic acid (Alexander *et al.*, 1958a,b). Three to four hours after infection (end of eclipse period), new mature virus begins to accumulate intracellularly (Howes and Melnick, 1957; Darnell, 1958). This maturation process is complete within 7–9 hours after infection. Spontaneous release of the virus (end of latent period), which begins

about 5 hours after infection, goes on at a much slower average rate than maturation (Howes and Melnick, 1957; Darnell, 1958), and is not completed until 18–24 hours after infection. The production of mature active virus is accompanied by profound chemical changes in the cell (Maassab *et al.*, 1957; Ackermann *et al.*, 1959; Salzman and Lockart, 1959; Salzman *et al.*, 1959), eventuating in its death.

2. Purified Virus

a. Physical and Chemical Characterization. The pioneer work of Schwerdt and Schaffer (1955, 1956; Schaffer and Schwerdt, 1955, 1959) on the purification of poliovirus has been followed by its physical and chemical characterization. The viral particle is a nucleoprotein sphere approximately 280 Å in diameter which crystallizes in an orthorhombic form (Finch and Klug, 1959). The best estimates of its composition show that it is 20–25% ribonucleic acid (RNA), the remainder being protein. Its mass is about 1.1×10^{-17} gm. and its density 1.33 [Dulbecco; Schaffer (personal communications)]. The latter value, determined by sedimentation in a centrifugally established CsCl density gradient (Meselson *et al.*, 1957), is lower than the earlier estimates (Schaffer and Schwerdt, 1959). Since the density in CsCl of large molecular weight RNA is approximately 2.0, while that of protein is about 1.29–1.30 (Watson, personal communication), and since the poliovirus particle is 25% RNA and 75% protein, its expected density would be approximately 1.47, rather than 1.33. However, the density of 2.0 for RNA includes the cesium ions which are bound to it. If poliovirus RNA in the intact particle were unable similarly to combine with CsCl because it is already combined, either with a nonexchangeable lighter cation, or with the excess amino groups of the basic amino acids in virus protein, this would account for the apparent discrepancy.

The protein of Type 1 virus contains all the usual amino acids, and no abnormal amino acid (Levintow and Darnell, 1960a). So far as has been determined, the nucleic acid bases are adenine, guanine, cytosine, and uracil. The base ratios and percentage compositions are the same for all three antigenic types of poliovirus (Schaffer and Schwerdt, 1959); and since there are no major differences in their biologic behavior in cell cultures, the antigenic types are not distinguished in any of the following discussion. The virus particle contains no deoxyribonucleic acid (DNA), no lipid, and no carbohydrate other than the ribose of the viral RNA (Schwerdt, 1957).

Although the particle appears spherical or polyhedral in ordinary electron micrographs, certain details of structure can be best obtained by the study of virus crystals. Finch and Klug (1959) have recently

reported the first X-ray crystallographic studies of poliovirus, which indicate that the particle has an icosahedral configuration made up of sixty identical subunits on the surface. This structure is the same as that of such spherical plant viruses as turnip yellow mosaic (Klug *et al.*, 1957a,b) and tomato bushy stunt (Casper, 1956).

According to Crick and Watson (1956, 1957), it should be the protein coat of the virus particle which dictates its icosahedral structure. The postulated sixty protein subunits per poliovirus particle would each have a molecular weight of around 80,000, using Schwerdt's (1957) estimate of 6.7×10^6 as the weight per particle, and a subunit diameter of approximately 60–65 Å (Finch and Klug, 1959). It is not known whether this basic unit is divisible into yet smaller units that may or may not be chemically identical, but according to Finch and Klug (1959) this is not ruled out by present evidence.

A relevant question is the number of antigens in the poliovirus particle. Mayer *et al.* (1957) have investigated the complement-fixing reactivity of purified virus preparations. These purified preparations have two major components, a fraction D which contains virtually all the infectious virus, and a fraction C which appears on the basis of electron micrographs, sucrose density centrifugation, and chemical measurement to contain little or no RNA. Although both fractions give equivalent complement fixation with convalescent human serum, fraction D reacts much more weakly than C with the nonspecific antibodies in acute phase serum. Finally, the reactivity of D antigen can be changed to that of C by heating, or by large doses of ultraviolet irradiation (LeBouvier, 1957). Fraction C protein may therefore be either a degradation product of poliovirus protein, a precursor, or perhaps part of a subunit which is not exposed as an antigenic stimulus when whole particles are injected. However, since crude suspensions and heated preparations are often used to produce antiserum, the conversion of D to C could have taken place prior to immunization, and there may in truth be only one protein in poliovirus. This aspect of the problem can now be attacked directly by working with protein obtained from purified virus preparations.

b. Biologic and Biochemical Behavior. A critically important finding is the fact that the number of characteristic particles visible in the electron microscope is always many times greater than the number of infectious particles (plaque-forming units), the ratio varying between 36 and 2000. This is true for both crude virus suspensions in infected culture fluid, and highly purified material (Schwerdt and Schaffer, 1955; Schwerdt and Fogh, 1957).

The use of radioactively labeled purified poliovirus preparations has provided additional information with respect to its biologic and physio-

chemical behavior. Taylor and Graham (1959; Graham, 1959), using virus labeled in its RNA with P^{32} and purified by ion exchange chromatography (Taylor and Graham, 1958), found that the adsorption of radioactivity by monkey kidney cells paralleled the adsorption of plaque-forming activity, indicating that all the particles in their preparations behaved identically with respect to adsorption. Recent experiments in this laboratory, however, using virus purified by cellulose columns and by CsCl density gradient centrifugation (Joklik and Darnell, 1960), indicate the HeLa cells in suspension may adsorb 90–95% of the infectivity, while only 20–30% of the radioactivity remains attached to the cells, whether the isotopic label is in RNA or protein. By adsorbing labeled virus for only five minutes, washing, and then studying the fate of the adsorbed radioactivity, the following points have been established:

1. At both high (1000–2000 particles per cell) and low (1–2 particles per cell) multiplicities, 50–60% of the radioactivity initially attached to the cells is rapidly and spontaneously eluted at 37°C.

2. The specific infectivity (plaque-forming units per count) of the eluted material is very low in comparison with that of the starting virus preparation.

3. The eluted fraction does not readsorb.

4. The eluted virus contains as much extractable infectious RNA as the starting virus.

5. In the nonelutable adsorbed virus, about 50% of the RNA is degraded to acid-soluble material, while most of the other 50% becomes ribonuclease-sensitive. Detailed experiments on the fate of this fraction of the viral RNA are in progress.

In summary, it would appear that the majority of the particles in a purified preparation of poliovirus attach to the cell surface, and most of these elute spontaneously. The fraction of the original virus inoculum that is permanently "adsorbed," as measured by the disappearance of radioactive material from the supernatant fluid, agrees with the fraction that fails to elute from cells which had been exposed to virus for a very short time (5 min.). The discrepancy between these results and those of Taylor and Graham (1959) and Graham (1959) may be due to differences in the method of preparation of pure virus, or in the host cells which were used for adsorption.

II. Initial Events in Infection

A. Adsorption

The initial step in poliovirus infection, as in most other virus infections, is the specific adsorption of the virus to the surface of a susceptible

host cell. Since the development of the accurate plaque assay (Dulbecco and Vogt, 1954a), many factors affecting adsorption rates of infectious virus have been quantitatively explored. (The adsorption of noninfectious particles has been discussed in the preceding section, pp. 5–6.) Bachtold *et al.* (1957) found that either calcium or magnesium was necessary for a maximum rate of attachment of virus to monkey kidney cells suspended in a phosphate-buffered saline solution containing NaCl and KCl. With optimum concentrations of Ca^{++} or Mg^{++} (10^{-3} *M*) the attachment rate constant was 2.3×10^{-8} ml./min./cell, four times that observed in their absence, and was virtually independent of temperature from 1° to 37°C. A slightly slower rate of attachment was observed with suspended HeLa cells in media containing approximately 10^{-3} *M* Ca^{++} and Mg^{++} (Drake, 1958; Darnell and Sawyer, 1960). In contrast to the findings of Bachtold *et al.* (1957), Holland and McLaren (1959) reported that Na^{+} facilitated adsorption to HeLa cells to the same degree as Ca^{++}. Although they also found no temperature effect in the adsorption of virus to suspended cells, adsorption to monolayers was markedly depressed at 0° compared to 37°C., and was not completely inhibited in the absence of any added cations.

There has been no demonstration that infectious poliovirus, once attached to the cell, can be quantitatively eluted in active form, as can myxoviruses and bacteriophages (Burnet, 1960; Garen and Kozloff, 1959; Garen and Puck, 1951). The attachment of active virus cannot therefore be divided into a reversible and irreversible stage on this basis. By analogy with other systems, it is nevertheless probable that initial attachment is a temperature-independent electrostatic phenomenon requiring cations.

B. Eclipse and Penetration

Once attachment has taken place, most of the adsorbed virus disappears as infectious material (Howes and Melnick, 1957; Darnell, 1958; McLaren *et al.*, 1959), and cannot be recovered by disrupting the cell with sonic vibration, or by freezing and thawing, even though these procedures are known to liberate newly formed intracellular mature virus. The small fraction of infectious virus which is recoverable, about 1–5%, is the same whether cells have been singly or multiply infected (Darnell, 1958). At least some of this "cell-associated virus" is insensitive to antiserum (Howes, 1959b; McLaren *et al.*, 1959), indicating either that the virus is not held at the cell surface, or that its antigenic site has been masked by its attachment to the cell.

The nature of the attachment and eclipse process can now be studied in a cell-free system. Holland and McLaren (1959) obtained cell-free

extracts from sensitive cells (HeLa) which inactivated poliovirus at a rapid rate. The activity of this extract was abolished by treatment with trypsin or with ether, but was insensitive to lipase, periodate, or receptor-destroying enzyme from cholera vibrio. They suggested that this material may represent a lipoprotein structure from the surface of the cell concerned with viral attachment. (It is significant in this connection that nonprimate cells which were resistant to virus infection by virtue of their failure to adsorb virus did not contain the virus-inactivating material.)

This thesis is strengthened by the experiments of Quersin-Thiry (1958), who demonstrated that the effect of anticellular serum in preventing infection with poliovirus is due to the fact that adsorption of virus is prevented. Presumably, the antibody reacts with and blocks the adsorptive sites on the cell surface. These data all suggest that viral attachment and eclipse may be related phenomena occurring at the cell surface.

The rate at which virus enters HeLa cells, or leaves the immediate surface of the cell, has been measured by several workers (Mandel, 1958; Holland and McLaren, 1959; Darnell and Sawyer, 1960). Cells which had been infected by exposure to virus for only a few minutes were subsequently treated at varying intervals with a potent antiserum. The number of infected cells which were unaffected by this treatment gave a measure of the proportion of cells in which penetration had already occurred. For monolayer cells at 37°C., the average time that an adsorbed virus stayed on the cell surface was 30 minutes (Holland and McLaren, 1959) or less (Mandel, 1958), while for suspended cells it was less than 12 minutes (Darnell and Sawyer, 1960). The rate of penetration could be markedly decreased by keeping the cell-virus complex at 22°C. (Mandel, 1958), and penetration was completely prevented at 1°C. (Holland and McLaren, 1959). Holland and McLaren were not able to block the "penetration" reaction with fluoride, azide, dinitrophenol, Tween 80, or cytotoxic cellular antisera. Although the entry of virus is temperature-dependent, it apparently occurs independently of at least some types of cellular metabolism.

Once inside the cell, the infecting virus is presumably broken down to protein and nucleic acid, since the viral nucleic acid alone suffices to initiate infection (Alexander *et al.*, 1958a,b). (The plaque-forming activity of these nucleic acid preparations is destroyed by ribonuclease, while gamma globulin containing poliovirus antibodies has no effect. The converse is true for whole viral particles.) The release of the infectious RNA may take place on the surface of the cell, during the process of "penetration," or it could be effected inside the cell; but in any case,

it does not happen sufficiently near the surface to permit added ribonuclease to interfere with infection (Holland and McLaren, 1959).

There is indirect evidence that the breakdown to protein and nucleic acid is effected by the cell, and is not an inherent property of the virus itself. Two strains of HeLa cells which adsorbed whole virus equally well, but which differed 15-fold in the probability of becoming infected, were equally susceptible to infection by infectious RNA (Darnell and Sawyer, 1960). The "resistant" or less susceptible cell was apparently unable to effect the release of RNA from whole virus with the same efficiency as the sensitive cell. If this interpretation is correct, the disruption of an incoming virus particle is perhaps a function of the same cellular component which Holland and McLaren (1959) have shown to be capable of "eclipsing" poliovirus in a cell-free system.

III. Intracellular Events

A. Effects on the Cell

Poliovirus infection eventually results in the death of the cell. Between the initial stages of infection and that final outcome, however, many changes have been observed. Some of these are cytologic, and poorly understood on a chemical basis. Others are chemical changes difficult to relate to any process necessary for virus replication, and may be secondary effects in a dead or dying cell which are not directly concerned with virus replication.

1. Cytologic Effects

Some of the earliest studies on the effects of poliovirus on cultured cells dealt with the microscopic changes occurring during virus production (Ackermann *et al.*, 1954; Dunnebacke, 1956a,b; Reissig *et al.*, 1956). Although there were minor differences in the several studies, the general picture was the same whether the cell line was primary monkey kidney, human amnion cells, or HeLa cells. This cytopathic effect in cell cultures was used in titrating the infectivity of virus suspensions (Enders *et al.*, 1949). Although this method is still widely used, especially in large-scale epidemiological work, it has been largely supplanted in quantitative biologic and chemical studies by the elegant plaque assay of Dulbecco (1952).

The first observable microscopic change is in the nucleus, where there is concurrently a central loss of chromatinic material and a peripheral deposition of dark-staining material. The nucleus tends to become indented and distorted, and extrudes into the cytoplasm a large acidophilic mass which has been identified as DNA on the basis of its stain-

ing reaction with a fluorescent acridine dye (Tenenbaum, 1957). The nucleolus is discernible throughout the process of infection, and the nuclear membrane also usually remains intact, despite its probably increased permeability (Tenenbaum, 1957).

Although the cytoplasm does not change as dramatically as the nucleus, definite changes have been reported. There is a generalized increase in basophilia, most noticeable after the eosinophilic mass has been extruded from the nucleus into the cytoplasm. Cytoplasmic vacuoles develop which are reported to move to the edge of the cell (Reissig *et al.*, 1956), perhaps concurrently with virus liberation (Lwoff *et al.*, 1955). Late in infection, acidophilic masses about 1μ in diameter may appear, which are eventually lost into the medium. During the period of virus release there is hyalinization of the outermost rim of cytoplasm; the surface of the cell can be seen to "bubble," and bits of cytoplasm are pinched off and lost into the medium (Lwoff *et al.*, 1955). Tenenbaum (1957) has found that this material contains large amounts of material with the staining characteristics of RNA.

As the final reaction, the cells begin to round up, detach from the glass, and float up into the medium. By the time half the cells have detached, 95% of the virus has been released (Dunnebacke, 1956a).

Until recently, the use of the electron microscope in the study of poliovirus-infected cells had not been particularly rewarding. Both Kallman *et al.* (1958) and Harford *et al.* (1959) failed to find characteristic intracellular virus particles, despite care to ensure that the majority of the observed cells had actually been infected. In possible explanation, Kallman *et al.* (1958) have calculated that one would not expect to see poliovirus in electron microscope sections if the particles were distributed randomly, and if there were no more than 10^4 particles within a cell at any given time. However, since the average yield is from 1 to 5×10^5 particles per cell (Schwerdt and Fogh, 1957) and since most of the virus is retained intracellularly for at least several hours after its formation (Howes and Melnick, 1957), viral particles might be expected to be evident even on the basis of these calculations. The electron microscope studies have borne out some of the morphologic changes observed with the light microscope, particularly with respect to the development of dense cytoplasmic masses in the infected cell. These U-bodies (unknown bodies) were not, however, regarded as virus, but as electron-dense material that had invaded a canalicular system. More recently, Stuart and Fogh (1959) have demonstrated in electron micrographs of infected cells cytoplasmic "crystallites" which appear to be comprised of whole virus particles. Horne and Nagington (1959), using the phosphotungstic acid negative staining technique of Brenner and Horne