CELLULAR ASPECTS OF THE CELL-VIRUS RELATIONSHIP[1]

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I. ORIENTATION

At the moment in virology, there is an air of optimism. This results from recent successes in the expression of the problems of this field in terms of general unknowns which cut across all biology. While in isolated instances this may encourage excessive reification, in the broad view it fosters our capacity to follow actively the findings of diverse disciplines and to interpret these in relation to virology.

Understanding in virology has advanced so in the last decade, that repeatedly we must pause and question what are the outstanding problems of today. Much research in the area of biologic interest seems to arrange itself orderly in three categories: The origin of large molecules (which involves some special synthetic mechanism), the relation of fine structure to function, and the mechanism of integrating competing synthetic reactions. Clearly, these are pertinent problems to all biology.

In addition, there are specific problems unique to the viral infection. One of these is the mechanism of cell-virus integration. At times the virologist seems almost preoccupied with this single aspect. Indeed, one might adopt an attitude that this is the chief aspect and that once the virus has entered the cell, the problem is over, because replication must follow the course of normal nucleoproteins, and that from this stage on the responsibility for understanding is shared with biologists of other disciplines.

However, this may be an undesirable limitation, particularly if we define our interest by asking "What of the cell?" and not about the virus. We may choose to ask why viral replication in some cases is not integrated with the synthesis of other cellular nucleoproteins. Why does the synthesis of a relatively small amount of virus destroy the cell? Or if this seems quite reasonable, then why does considerable viral synthesis in other cells produce no lysis? In short, what is the basis of the diverse responses of a cell to interaction with different viruses?

[1] Based upon the Eli Lilly and Company Award Address in Bacteriology and Immunology. Presented at the 58th Annual Meeting of the Society of American Bacteriologists in Chicago, Illinois, on May 1, 1958. Many of these studies were aided by a grant from the National Foundation for Infantile Paralysis.

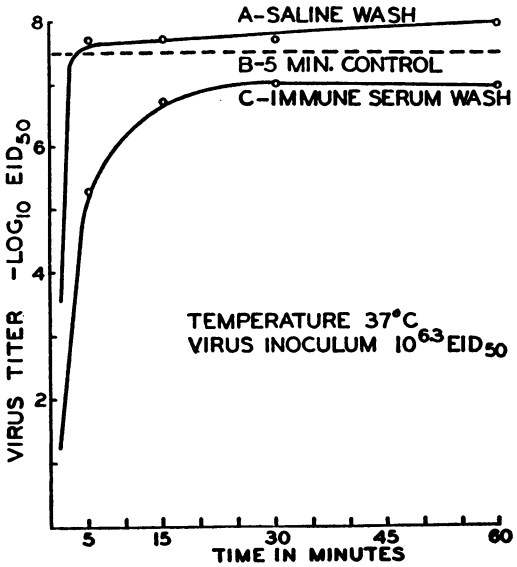

Figure 1. Differentiation of cell-virus reactions. *Curve A:* 0.4 mg portions of tissue were exposed to the virus at a concentration of $10^{6.3}$ EID_{50} per 0.1 ml. At 5, 15, 30, or 60 min, replicate tissues were removed, washed in three 50-ml portions of buffered saline, and returned to fresh medium. After 12 and 24 hr, the medium was titrated for viral infectivity. The 24-hr data are recorded here. *Curves B and C:* 5 replicate tissues were exposed to virus for 5 min and then washed to remove virus as described under curve *A*, above. Four of these were later removed from the growth medium and washed again at 5, 15, 30, or 60 min with immune rabbit serum for 3 min, and then with three 50-ml portions of buffered saline, after which they were returned to growth medium. At 12 and 24 hr, the fluids were titrated for virus. The 24-hr data are recorded above.

While we know something of the comings and goings of the virus and considerably less about the cell, the emphasis in this essay will be upon the latter aspect. The effort will not be in the form of a comprehensive annotated bibliography. Specific comments will be taken mainly from observations with poliovirus which in most cells produces a destructive reaction. Interspersed will be comparative references to influenza virus. The formulation of a time schedule of events will be attempted, correlating the metabolic activity, the gross morphologic alterations, and viral synthesis. The interpretation will be at the cellular level. However, this story will be prefaced with certain fundamentals which are necessary to justify the experimental system and give proper weight to the report of material which has not been described before this time.

II. INITIATION OF INFECTION

Let us begin with the primary interaction of cell and virus. This study has been pressed not only because it is strictly in the domain of virology but also for practical considerations. If we are to observe multicellular cultures and draw conclusions at the cellular level, then the activities of the various cells must be synchronized. The whole must be a reflection of the parts. This is best done by infecting all cells in a reasonably short period, thus preventing secondary spread of infection and reducing the individual variation. Confidence in this matter can be had only by knowing something of the kinetics of the reactions involved.

The pattern for influenza virus is one in which virus reacts with the cell by two recognizable successive stages. The product of the first reaction is a cell-virus complex stable to dilution, which is neutralizable by specific antiviral serum and from which the virus in transmissible form cannot be recovered by extraction. The reaction proceeds rapidly at 4 C. However, the second reaction is favored by higher temperatures and yields a product which is insensitive to the neutralizing action of specific immune serum (34, 35).

The differentiation of the two reactions is illustrated in figure 1, wherein the relative number of virus-yielding cells which results after varying intervals of cell-virus contact was determined by a measurement of the viral yields. Curve *A* represents the rate of the first reaction between cell and virus, *i.e.*, the formation of a cell-virus complex which is stable to simple dilution and ultimately, upon further incubation, is capable of giving rise to a viral yield. The rate of the second reaction is expressed by curve *C*. It represents the formation of a cell-virus complex which is stable to dilution and in addition whose potential for virus production is resistant to the action of specific antiviral serum. At the various times indicated, the complex was treated with antiviral serum and the yield of virus obtained from those cell-virus complexes resistant to serum action at that time was determined after a further incubation period. Curve *B* represents the amount of the first complex present at 5 min in the experiment

where the formation of the second complex was studied, *i.e.*, curve *C*. It is evident that the second reaction is appreciably slower than the first. Perhaps it is not surprising that these observations, made first with influenza virus (34, 35), have been confirmed by Rubin *et al.* with Newcastle disease virus, which is another myxovirus (52, 53). However, more recently in our laboratory we have extended the observation to the reaction of poliovirus with HeLa cells (50), where the cell receptors are quite different. In each of these three instances, initiation of infection has been found to proceed in a two stage manner, distinguishable by the action of immune serum.

III. MECHANISM OF NEUTRALIZATION BY ANTIBODY

This interesting phenomenon, in which virus is capable of being neutralized by specific antiviral serum after it is complexed with cells, is of some general interest. This fact demonstrates that the neutralizing effect of antiviral serum is not at all times a simple steric interference with the attachment of virus to the cell, but rather makes the virus incapable of participating in some subsequent reaction. Many evidences in the literature confirm the existence of cell-virus-antibody complexes. If phages are neutralized with antibody, they will still bind to bacteria but do not initiate viral production (13, 48, 62). The fixation of neutralized influenza virus by susceptible cells has also been described (33). Viral antibody can be adsorbed selectively by erythrocytes to which influenza virus is irreversibly bound (36). However, when virus is held reversibly in the ordinary situation, hemagglutination can be prevented by immune serum, presumably by blocking the cell-virus linkage. Likewise, the adsorption of virus to host cells can be blocked by the intervention of several molecules of antibody (52).

The mechanism of action of neutralizing antibody has been studied intensely again in recent years by applying newer, more precise methods of viral assay. The most striking claim has been a new theory of neutralization proposed by Dulbecco *et al.* (23), which offers that the virus-antibody complex is formed by an irreversible process and further that a persistent fraction of virus always exists which cannot be neutralized by antibody. They believe that this interesting material is a phenotypic variant of the viral population and propose that it originates by a random assembly of building units during the viral synthetic process (21). Despite the fact that these claims made from the study of poliovirus and Western equine encephalomyelitis virus have been reiterated more recently by Rubin and Franklin (52), the older theory of Burnet *et al.* (13) of the reversible virus-antibody reaction cannot be readily disregarded at this time.

Virus in combination with antibody can be regenerated by displacement with inactivated virus (33, 38). Further, there is strong evidence that the cell-virus-antibody complex can dissociate to produce an active center of infection. The reactivation of the cell-virus-antibody complex has been carefully examined by Mandel (46) and found to be sensitive to a variety of influences, such as salt and antibody concentration and pH. The non-neutralizable, so-called persistent virus of Dulbecco (21) would seem to be a phenomenon of reactivation occurring in the assay procedure rather than a particular material.

Neutralization from its definition must be measured in terms of the capacity of virus to interact with some cell system. The problem in the study of mechanism is to interpret the degree and quality of the virus-antibody interaction as it occurs in the test tube by the capacity to initiate infection subsequently in the cell system. Because of the interaction of virus-antibody complexes with cells and the subsequent reactivation of the triple complex under various environmental influences whose quantitative effects are not readily predicted, the quantitative value of the plaque assay is limited in this situation. The technique is ambiguous in separating the measurement of virus-antibody interaction as it occurs in the test tube from those phenomena subsequently occurring in the cell culture. In these instances the validity of deductions made exclusively from quantitative considerations is highly suspect, particularly when they contradict other lines of evidence. For a broader and more exacting evaluation of the theories of virus-antibody reaction, the reader may refer to the recent critiques of de St. Groth *et al.* (19, 20) in which are pointed up the various theoretical and systematic errors which they believe have confounded the results from which the theory of nondissociation (21) was formulated.

IV. LINKAGES OF VIRUS AND CELL

The linkage of influenza virus with its host-cell is established through at least two types of bonding at the same site. One bond involves a mucopolysacharide related to sialic acid, which

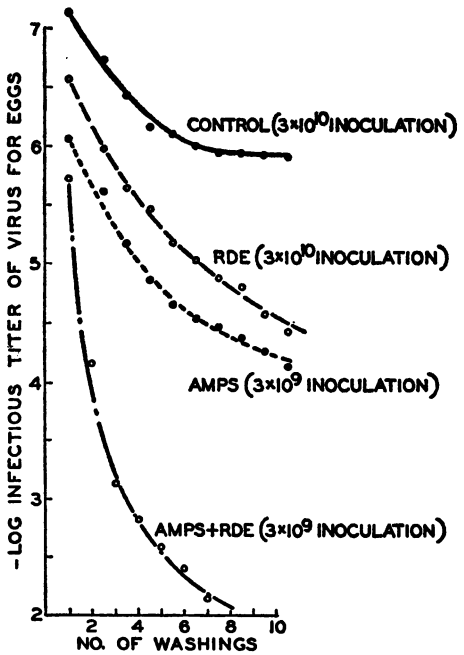

Figure 2. Washing curves of the tissue treated with AMPS (α-amino-*p*-methoxyphenyl-methanesulfonic acid) and RDE (specific enzyme of the cholera vibrio) before exposure to virus. Each tissue was treated with AMPS or RDE for 1 hr at 37 C. prior to exposure to the viral inoculum. The period of exposure was 1 hr. All tissues were washed 6 times in saline and then 9 times with shaking at 37 C in the various media used for the primary adsorption of virus. All flasks contained fluorophenylalanine to prevent viral increase. The control curve is a composite from two closely comparable experiments.

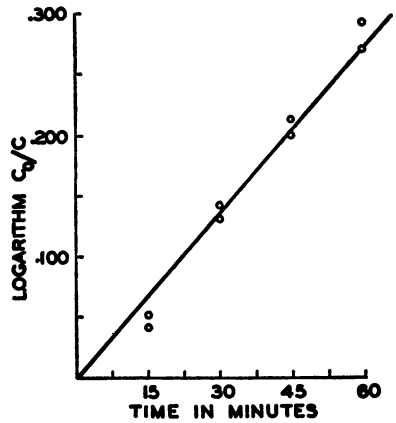

Figure 3. Rate of the first reaction between HeLa cells and poliovirus. Each culture was exposed to 5.4 PFU (plaque-forming units) of virus per cell for the interval indicated. Antiviral serum was added to each culture 90 min after termination of the exposure period and the number of surviving cells was determined at 24 hr.

can be altered with a specific enzyme (RDE) of the cholera vibrio. The chemical nature of this receptor has been studied intensely by the Australian School of virologists (29). The second type of bond is sensitive to the blocking effect of AMPS (α-amino-*p*-methoxyphenylmethanesulfonic acid) and involves potassium ions (1, 6, 7, 40).

Virus will attach to some tissue cells in all cultures but because the process of penetration is arrested the normal pattern of development does not ensue. The virus does not pass into the eclipse phase but is recoverable in transmissible form (1). Subsequently this phenomenon was confirmed in studies with another myxovirus (54). (An anomalous binding reaction of virus with red cells has also been reported (14). The relation of this situation to that of the host-cell has not been determined.) Many of the factors which influence the initiation of the productive infection also affect such binding of virus activity in the abortive infection. Some of our concepts regarding the initiation of infection are deduced from virus complexed with host-cells in this anomalous manner (1).

The ability of cells to bind influenza virus after the receptors have been modified or blocked is illustrated in figure 2. The modified cells have been exposed to virus to allow attachment and then transferred successively to fresh fluid to allow equilibrations. The release of the bound virus to the fluid phase can be followed while multiplication of virus from successfully initiated infections is prevented with a metabolic antagonist, fluorophenylalanine.

As seen in the control curve, figure 3, after the superficially held virus is removed, each successive wash removes a constant fraction—about 3 per cent of the remaining bound virus. The characteristics of the curve suggest that the bound and free virus are in equilibrium. After treatment with receptor-destroying enzyme or after blocking with AMPS, binding of virus by cells still occurs but the forces are reduced and the equilibrium shifts in favor of the free virus. About 30 per cent is removed with each equilibration. However, when both receptors are modified

simultaneously, no binding of virus will occur. The two binding effects are not simply additive, but rather the force of binding by which the majority of the virus is held is greatly affected by either treatment.

Since modification of either receptor precludes virus multiplication, binding by both receptors apparently is necessary to initiate infection. According to Levine *et al.* (40), AMPS and potassium ions have mutually antagonistic effects. In the presence of the sulfonic acid or a deficiency of potassium ions, the second of the two stages of initiation of infection does not proceed and the infectious center remains sensitive to the neutralizing action of specific immune serum.

V. RATE AND EXTENT OF INFECTION

In the presence of a high concentration of virus, the initiation of infection proceeds according to pseudo-first order kinetics (34, 35, 50). The rate of reaction is proportional to the concentration of susceptible cells remaining at any moment. Although it is possible to infect, with poliovirus, nearly every HeLa cell in a monolayer culture, clearly every cell will not be infected simultaneously. When the rate of reaction is sufficiently great, as with poliovirus or influenza virus, a high proportion of cells may be infected in an interval of 10 to 15 min depending upon the concentration of virus employed. The relationship for poliovirus is expressed in figure 3, where a linear relationship between log C_0/C and time is followed until 70 per cent of cells are infected. The number of remaining susceptible cells is expressed by C and the original number by C_0.

The second stage of the interaction of HeLa cells and poliovirus also obeys first-order kinetics. The rate of development of cell-virus complexes which are resistant to antiviral serum action is proportionate to the fraction of sensitive complexes present at any given time. The reaction is illustrated in figure 4. The uniformity of infection in a high proportion of the cells in a culture can be confirmed by visual observation of the developing cytopathology and by the nature of the viral growth curve. If one adds receptor-destroying enzyme or immune serum at a proper interval after initiation of infection it is possible to limit the viral development to a single cycle (50). The proper interval is after viral penetration has occurred and before viral progeny have appeared.

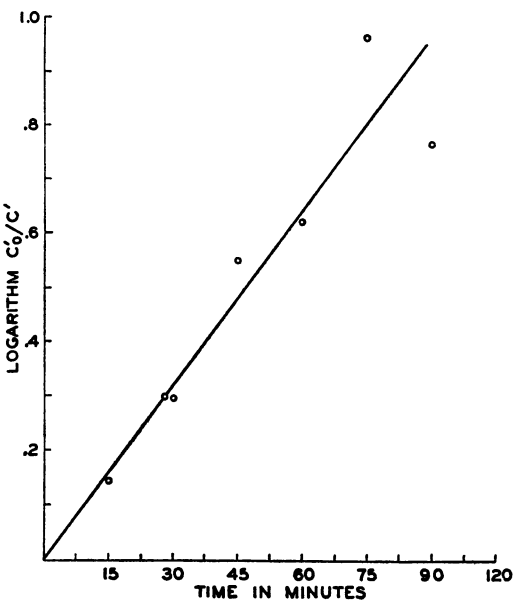

Figure 4. Rate of development of resistance to immune serum. The fraction of cells sensitive to the action of antiviral serum at the end of the period of exposure to virus, $C_0' = C_{30} - C_{180}$, and the fraction sensitive to serum at any time t, designated C', is $C_t' = C_t - C_{180}$ where C_{180} = the fraction of cells surviving after 30 min exposure to virus and 150 min incubation before addition of antiviral serum and C_{30} = the fraction of cells surviving in cultures exposed to virus for 30 min and immediately supplemented with antiviral serum. Each point represents an average of values obtained from two cultures. Each culture was exposed to 3.3 PFU (plaque-forming units) of virus per cell for 30 min and washed; 600 units of antibody were added at appropriate intervals.

With the enzyme, the susceptible cells remaining after exposure to virus are modified, at least temporarily. In contrast, the specific immune serum acts upon the viral progeny to prevent reinfection. In either case, the completeness of infection can be quantitated by counting the cells which survive after the viral productive cycle is completed. With this technique the data in figures 3 and 4 were obtained (50). A direct count of the number of infected cells can also be made. During the early part of the infectious cycle, a monodispersion of the cell layer can be prepared and plated on an intact monolayer culture. The extent of infection can be determined from the ratio of visibly countable cells to plaque-forming

units. The site of plating of each infected cell will result in a single plaque. From these four lines of evidence, one has some assurance that it is possible to produce a single cycle of infection in HeLa cells with poliovirus.

VI. THE ECLIPSE PHASE

If shortly after the initiation of infection, the washed infected cells in a suspension are mechanically disrupted, the plaque-forming capacity of the suspension may be reduced by 95 per cent (50). This is the counterpart in the animal virus system of the eclipse-phase phenomenon first described for bacteriophages. There is convincing evidence for a nontransmissible phase in the life cycle of the viruses of Western equine encephalomyelitis, poliomyelitis, influenza, fowl plague, and vaccinia. The recent experiments of Hoyle and Frisch-Niggemeyer (32), using influenza virus labeled with radioactive phosphorus, and similar experiments of Wecker (*personal communication*, from Max-Plank Institut. für Virusforschung, Tübingen) with fowl plague virus offer some understanding of this phenomenon. Their data indicate that during the process of integration of cell and virus there is an actual physical disintegration of the virus particle.

We can speculate, perhaps with some oversimplification, that the virus becomes synthetically active only when the nucleic acid is released from the protein components. Acting upon an intracellular environment, the active RNA (ribonucleic acid) conditions certain loci of the cell to synthetic activity, replication of all nucleic acid templates in the loci proceeds and in time gives rise, among other products, to specific viral protein. Indeed, several attempts already have been made to test with animal viruses (viruses of poliomyelitis, West Nile encephalitis, and Mengo encephalitis) the general significance of the function defined for certain nucleic acids of plant viruses (17, 18). The RNA of tobacco mosaic virus is reported to have a low order of infectivity but once in the cell it gives rise to the complete virus (27).

VII. NATURE OF THE PRECURSORS

In addition to the dual stages in the initiation of infection and the eclipse phase, there is yet another observation which may have general significance. In contrast to the complex requirements for cell growth, the exogenous requirements for viral synthesis are very simple. A wide range of metabolic intermediates and nutritional factors which are essential for or stimulate the growth of the normal cell, do not stimulate the production of virus by the cell-virus complex. For example, maximal yields of influenza virus are produced by allantoic cells in the presence of inorganic salts, glucose, and oxygen (2, 3). Yet, despite this simplicity, a few interesting variations have been reported even among strains of influenza virus. The synthesis of the WS strain can be supported by either glucose or pyruvate, whereas the neurotropic variant of the same strain, *i.e.*, the NWS strain, cannot develop when the sole source of energy is pyruvate; it must have glucose. This observation has been described in detail by Levine and Rouse (41). Another specific example was reported by Eagle (24), who showed that the production of poliovirus in HeLa cells was enhanced by glucose and glutamine but not by other supplements.

Thus, nearly all requirements for viral synthesis are supplied by the intracellular milieu, and necessarily our knowledge of the endogenous viral precursors comes largely from the interpretation of the action of specific metabolic antagonists and the use of radioactive tracer techniques. Without elaboration, the state of affairs can be summarized with the statement: there is no clear evidence of precursors of proteins larger than amino acids. At present, no basis exists for believing that the synthesis of viral protein or nucleic acid differs from that of other organisms, or that the over-all host-virus system is more elemental than other living systems. Exactly how in time the new protein and nucleic acid arise and the effects of these syntheses upon the cell will be considered in a subsequent section.

VIII. MORPHOLOGY OF INFECTION

Considerable effort has been expended in several laboratories to catalogue in the sequence of their occurrence the visible morphologic alterations of cells which follow infection with poliovirus (4, 10–12, 22, 23, 25, 30, 37, 51, 61). These are essentially cellular responses but result from the interplay of intrinsic properties of both cell and virus. The responses upon infection of cells from monkey kidney, human fetus, human tonsillar fibroblasts, and cell strain HeLa, as described, are quite similar, but not so with cells

of human amnion (23). Without qualification one cannot write of the poliovirus infection, much less generalize to other animal viruses. This level of observation is derived from fixed and stained preparations, from phase-contrast cinematophotomicrography of living cells, and from the use of fluorescent antibody. The materials described were not comparable in all cases and the degree of synchrony of infection in parallel cultures was not at all times known. However, if one avoids fine detail, a morphology of infection can be formulated which will serve as a reference point from which to consider the biochemistry of infection.

It was known from the earliest studies of fixed and stained HeLa cells that upon infection with poliovirus both the nucleus and cytoplasm were involved; staining with Giemsa revealed a marked increase in cytoplasmic basophilia and an irregular pyknotic nucleus; by 9 hr extreme alteration of both structures and maximum virus production were effected. Late in the infection, many blue-staining cytoplasmic granules, irregular in size and shape, were seen which were sensitive to digestion with ribonuclease (4). These data suggested a profound stimulation of the nucleic acid metabolism of the cytoplasm which later was verified by biochemical studies (42, 43, 45). Similarly suggestive are the recent cytological data obtained with fluorescent microscopy (61). Repeatedly, there has been described in infected cells, a centrally located cytoplasmic eosinophilic lesion about the size of a normal nucleus (10, 11, 22, 37, 51). Reissig *et al.* (51) alone describe an early eosinophilic nuclear inclusion which precedes the development of nuclear pyknosis. The central lesion may represent a localization of cytoplasmic particulates in the juxtanuclear position. Dunnebacke (22) has described the sequence of characteristic changes as a wrinkling of the nuclear membrane, increased granularity of the cytoplasm, rounding of the cell, and development of the large central lesion. The infection of human amnion differs with changes in the nucleolus and the formation of peripheral cellular nodules (23).

The development of viral antigen in the infected cell can be visualized with the fluorescent antibody technique (12). But, as yet, data have not been obtained in a single sequence of infection which would allow correlation in time with other cytological and biochemical observations.

Most instructive is the sequence of events recorded by phase-contrast cinematophotomicrography in a single living HeLa cell infected with poliovirus. The changes we have seen in order of appearance are: increased activity of the peripheral undulating membranes, movement of granules to the nuclear area, a violent boiling-like movement of the cytoplasm occurring about the 4th hr, and at the 6th hr a retraction of the cellular processes and a rapid and dramatic loss of cytoplasmic area with retention of the cytoplasmic granules about a highly refractive nuclear residue. The process in most cells is complete by the 9th hr (Ackermann and Kurtz, *unpublished data*). Quite similar results with this technique have been described in detail by Barski *et al.* (10). In the latter instance, the effects of poliovirus on a fibroblast which originated from human tonsil were recorded photographically. The large, central cytoplasmic lesion was also visible with phase-contrast microscopy (10).

IX. INTRACELLULAR APPEARANCE OF POLIOVIRUS

Cells from parallel cultures undergoing a single cycle of infection with poliovirus have been separated at various times from the fluid and fractionated into nucleus and cytoplasm by classic methods using neutral citrate-saline. The intracellular and extracellular appearance of viral infectious activity is then readily determined (42, 45).

The boiling-like movement of the cytoplasm recorded in phase-contrast cinematophotographs occurs at about the time virus is first detectable in the cytoplasm, *i.e.*, by the 4th hr. At this time, about 7 per cent of the final yield of virus has been produced. The virus continues to accumulate at what appears to be an exponential rate until the 7th hr when 90 per cent of the ultimate viral yield is present in the cytoplasm. At this time only 1 per cent of the virus produced can be found in the extracellular state. There is a very pronounced intracellular phase (42, 45). These findings for HeLa cells are consistent with the observations of Girardi *et al.* (28) and Larson *et al.* (39). Howes and Melnick (31) have also described a similar intracellular phase which precedes release of poliovirus from monkey kidney cells. Further, application of the Coons fluorescent antibody technique in the hands of Buckley (12) gave data supporting these analyses. The first detection of viral antigen was reported to be exclusively in the cytoplasm. Only in late

stages of poliovirus infection is the antibody fixed in the nuclear area. Upon careful examination in the various stages of a single infectious sequence, at no time have we been able to find a higher concentration of virus infectivity in the nucleus than in the cytoplasmic fraction of the cell.

X. VIRAL RELEASE

The release of the virus begins at about the 7th hr when cytoplasmic retraction is observed. Release of poliovirus from HeLa cells proceeds over a period of several hr (4, 42, 45). With other cell-virus systems, the prolonged release phenomenon may be more pronounced. Release of virus may be concomitant with production and yet allow an intracellular accumulation, as in case of influenza virus (63); or, as with Western equine encephalomyelitis virus (55) and the virus of vesicular stomatitis (26), it may be so rapid as to preclude accumulation. It is highly probable that the sustained release seen in multicellular cultures is a reflection of a cellular phenomenon and that the release from a single cell proceeds over several hr without a bursting or lysis of the cell (5).

For influenza virus, this is indicated by the analysis of viral release from multicellular cultures. The constant equal release of virus over many hr would require a sustained maturation of equal numbers of cells of different latent periods, while in contrast one would expect the individual differences in cells to conform to a bell curve (63). Further, with influenza virus, release from cells occurs without the reduction of respiration and metabolic activity which occurs with mechanical rupture in simple medium (2). Supporting data have been reported by Cairns (15), who followed the release of virus from a multicellular culture in which there was a high probability that only one cell was infected.

A direct attack upon the problem was made by Lwoff *et al.* (44) by isolating a single monkey kidney cell infected with poliovirus and observing it under the microscope while following the release of virus into a microdrop of medium. Data given for four cells are essentially in agreement with the conclusions drawn from the study of multicellular cultures. Necessarily, the relation of viral production to release was not followed with this technique. Whether effects of trauma influence the kinetics of release is difficult to evaluate. Although each of these approaches has some limitations, together they are quite convincing.

XI. MECHANISM OF VIRAL RELEASE

Little is known about the actual mechanism of release of viruses from cells, *i.e.*, about their movement across the limiting membrane of the cell. Electron micrographs of cells infected with influenza virus suggest that a physical process is involved in which there is a limited extrusion of cell cytoplasm (47, 64). Interestingly, the release of influenza virus from the cells of the chorioallantoic membrane is inhibited by AMPS. This is the same substance which prevents one stage of cell-virus integration, prerequisite to the initiation of infection (7). During liberation-inhibition, virus production continues and virus accumulates in some cellular state from which it can be removed by mechanical extraction. Spontaneous release into the medium is apparently not necessary for viral maturation (6).

The release of poliovirus is also sensitive to environmental effects as reported by Larson and associates (39). Their observation that synthesis of poliovirus continues in cells at lowered temperatures but release is prevented has been confirmed in our laboratory. However, at 30 C the rate of virus synthesis is also reduced.

For poliovirus, the release from HeLa cells, monkey kidney cells, and tonsillar fibroblasts is probably related to the phenomenon of cytoplasmic retraction which as noted above occurs at the same time. We have suggested the term cytotransudation, for during the observed loss of cytoplasmic area, there is an actual loss of cytoplasmic material to the extracellular medium.

Radioactive phosphate is readily taken up by HeLa cells from the culture medium and incorporated into molecules of various sizes up to polymerized nucleic acids. Upon transfer to unlabeled medium, the cells release some of the isotope to the medium but shortly an equilibrium is established and the extracellular radioactivity is stable at a relatively low level. The cell infected with poliovirus behaves similarly until the 6th or 7th hr, when there is a dramatic outpouring or transudation of the radioactive label into the extracellular fluid. The process is sustained and selective; optically visible particulates are retained. The isotope is found chiefly in the acid-soluble fraction of the transudate and hence is

not only the virus itself. The acid-soluble fraction contains inorganic phosphate and a complex variety of nucleotides and nucleosides, whereas the virus is presumably precipitated in the acid-insoluble residue (Ackermann, Loh, and Maassab, *unpublished data*).

XII. DEFINITION OF CYCLE OF INFECTION

This brief summary of the morphologic behavior of the cell during the goings and comings of the virus tells us little of the mechanisms of viral synthesis or the biochemical nature of the viral lesion. However, it is a basis from which to proceed. It is clear that infected cells can be recognized, that nearly every cell is infected during the period of exposure of cells to virus, that within reasonable limits the cytopathology is proceeding uniformly in the population, and that even if susceptible cells did remain, a second cycle of infection could not be initiated, for the virus progeny is still intracellular during the first 7 hr. It is possible, then, to proceed to consider metabolic and chemical analyses with the assurance that observations made on the multicellular culture can be interpreted at the cellular level. In this 9 hr cycle of infection the phases of interest are: the initiation of infection, the preproductive phase, the viral production, and the phase of transudation. Biochemical studies are best confined to the first 7-hr period when the essential cell integrity remains and all newly formed virus is intracellular.

XIII. NATURE OF VIRUS AND CELL

The poliovirus, as is well known, has been purified and crystallized. It contains only protein and nucleic acid, and the base composition of the latter has been determined. This is also pertinent background information for which we are greatly indebted to the virus laboratory at Berkeley (56–58). The HeLa cell, of course, has not been so well characterized chemically. In the rounded state it is about 17μ in diameter. The nucleus is relatively large, containing almost as much RNA and protein as the cytoplasm. As expected, the DNA (deoxyribonucleic acid) is all nuclear. Such cells in sucrose or citrate-saline can be disrupted, and the particulates separated into fractions of nuclei, mitochondria, microsomes, ultramicrosomes, and cell sap. Each fraction may be analyzed by conventional means for RNA, DNA, protein, radioactive isotopes, or virus.

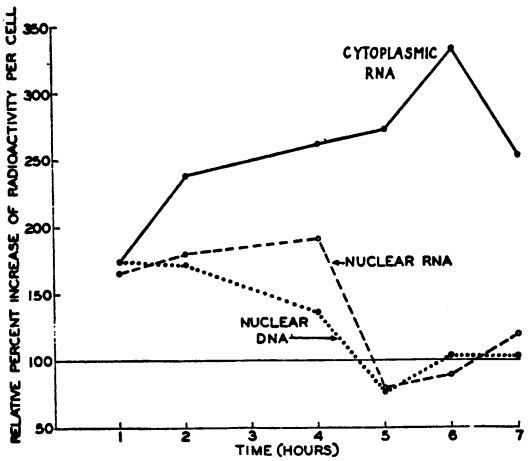

Figure 5. The rate of P^{32} incorporation into the three nucleic acid fractions measured over ½-hr periods. The cells were exposed to an undiluted inoculum containing a total of 1×10^9 PFU (plaque-forming units) of virus for a period of 1 hr. Then the culture was washed 3 times with balanced salt solution, replaced in fresh medium, and incubated at 37 C. The additions of P^{32} contained a total of 143 μc of activity. The amount of P^{32} incorporated is expressed as the per cent change related to the control. RNA and DNA, ribonucleic and deoxyribonucleic acids, respectively.

XIV. UPTAKE OF P^{32} UPON INFECTION

Assay of uptake of P^{32} upon infection has been done also with parallel infected cultures at 1-hr intervals throughout the first 7-hr period of infection (42, 43, 45). When a HeLa cell is exposed for a short interval to a culture medium containing radioactive phosphate, the isotope is rapidly incorporated into the nuclear RNA, nuclear DNA, and into the cytoplasmic RNA. However, if the cell is infected with poliovirus, the rate of incorporation is greatly enhanced. Further, whereas the rate of incorporation of isotope by the normal resting cell is the same at various intervals of incubation, it is not so with the infected cell. How this rate varies during the first 7 hr of infection is illustrated in figure 5. By the 1st hr after infection, the rate of incorporation of P^{32} into the two nuclear fractions was from 150 to 160 per cent of the control. The enhanced rate continues in the nuclear RNA for the next 3 hr until there is an abrupt decline in this nuclear activity. This is about the time the infectious activity begins to accumulate in the

TABLE 1

Amount of each nucleotide in cytoplasmic RNA (CRNA) of normal and infected HeLa cells

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

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

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

cytoplasm, *i.e.*, at the beginning of the infective phase. The incorporation of P^{32} into the nuclear DNA also shows an early acceleration but very quickly begins a steady decline and by the 5th hr it is below normal.

In both normal and infected cells the initial rate of incorporation of P^{32} is much more rapid in the nuclear RNA than in the cytoplasmic RNA. Upon infection the incorporation by cytoplasmic RNA not only begins abruptly but continues to accelerate during the first 6-hr interval after which there is a marked decline in this particular activity. Shortly after this decline, transduation begins. When the activity is maximal, it may exceed by 2.5 to 3 times that of the normal cell. In the noninfectious phase, both the nuclear and cytoplasmic RNA metabolism are affected. Upon the first appearance of virus, the activity is shifted to the cytoplasm.

This incorporation of P^{32} may be defined as nucleic acid synthesis. However, it need not represent a net synthesis such as would be reflected in an accumulation and increase in the total amount of nucleic acid polymer in the cell. It conceivably might be that the synthesis and degradation are proceeding at equal rates. These two processes could be an integral mechanism in the biologic functioning of the RNA. The following quantitative analysis of nucleic acid material clearly emphasizes this point.

XV. NUCLEIC ACID SYNTHESIS

Direct chemical analyses of various morphologic components of the cell for nucleic acids show no accumulation of RNA or DNA in the nucleus of the infected cell, but in contrast, a dramatic net synthesis of cytoplasmic RNA. The increase in the latter component may be 2.5 times that of normal. This is indicated by the classic analytical methods of Schneider and of Thannhauser and Schmidt (45). Further, the estimations of the nucleic acids by phosphorus determinations were confirmed by a quantitative analysis of the component nucleotides obtained by alkaline hydrolysis of the isolated nucleic acid polymers. Only the four usual bases of RNA were encountered. In table 1 the increase in the number of μmoles per cell of bases of the cytoplasmic RNA are given. The analyses were made 6 hr after infection (43).

The accumulation of new cytoplasmic RNA is detectable within 1 hr of infection, it increases until the 6th hr, then stops abruptly (figure 5). The rate of synthesis is not quite constant over this period. An accumulation curve is always slightly concave upward. However, it is not exponential but appears as a composite of two linear curves, one which begins shortly after infection and a second which is evident only after the 4th hr. Twenty per cent of the total newly formed RNA is a result of the additional increment of enhanced synthesis occurring between the 4th and 6th hr. This change in the rate of accumulation of RNA implies the synthesis of two species of RNA, one of which only appears at about the 4th hr. This hypothesis is supported in part by subsequent characterization of the cytoplasmic RNA in terms of its base composition.

XVI. PROTEIN SYNTHESIS

Concomitant with the induced cytoplasmic RNA production is protein synthesis. This is a net synthesis as determined by nitrogen analysis of isolated protein. There is no detectable increase in the nucleus. However, all cytoplasmic fractions, the mitochondria, microsomes, ultramicrosomes, and cell sap contain increased amounts of protein. The protein of the soluble fraction obtained after removal of the cytoplasmic particulates by centrifugation may exceed that of the normal cell by 70 to 80 per cent. The

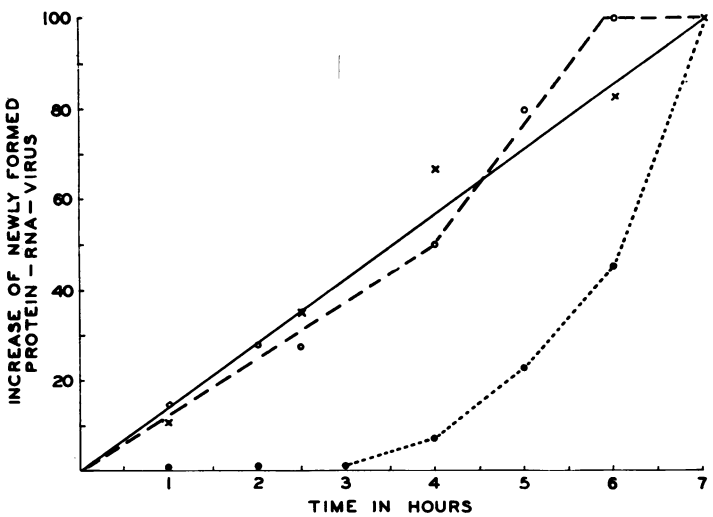

Figure 6. The increase of newly formed cytoplasmic protein, RNA (ribonucleic acid), and virus in HeLa cells at various times following infection with poliovirus. The newly formed, or Δ, material represents the difference between normal and infected cells in the amount of each material. Values plotted here are expressed as a percentage of the amount of Δ material found at 7 hr following infection. ×——× , protein of fraction III of cytoplasm; O- - -O, RNA of total cytoplasm; and ●-----●, virus infectivity of total cytoplasm.

accumulation of new cytoplasmic protein as indicated by the soluble proteins in figure 6 is detectable by the 1st hr after infection. The increase proceeds at a constant rate and has been followed until the 7th hr. The accumulative curve is strictly linear and extrapolates to zero time. This soluble protein fraction contains about 250 $\times 10^{-10}$ mg nitrogen per cell and may increase by 140 $\times 10^{-10}$ mg upon infection. This is a major portion of the cell protein.

XVII. VIRUS APPEARANCE

The first detectable increase in virus infectivity occurs when the cytoplasmic accumulation of RNA and soluble protein has reached about 50 per cent of the maximal values to be reached. This is just the time when there is a sharp increase in the rate of cytoplasmic RNA accumulation. When the increment of enhanced synthesis of RNA is plotted on the same scale to indicate rate, it precedes viral synthesis. Further, it will be noted that over 50 per cent of the virus is formed after the synthesis of RNA has ceased. Apparently the rate-limiting reaction in the acquisition of the infectious property is other than synthesis of RNA (figure 6).

XVIII. SUMMARY OF DATA

In figure 7 we see summarized diagramatically the data presented so far. Quickly upon the initiation of infection, accomplished in two stages, accumulation of protein and RNA begins in the cytoplasm. Rapid incorporation of inorganic phosphorus into nuclear RNA and DNA also occurs but there is no net synthesis.

By 4 hr the rate of synthesis of cytoplasmic RNA increases, virus appears in the intracellular state, and turnover of nuclear RNA abruptly ceases. Progressively, the rate of accumulation of virus increases. By 6 hr synthesis of cytoplasmic RNA ceases while protein and virus continue to increase. By the 7th hr release of virus begins, accompanied by an outpouring or transudation of much of the cytoplasmic contents.

XIX. NATURE OF THE INCREMENTAL RNA AND PROTEIN

The increases in cytoplasmic RNA and protein are huge to the proportions of the cell. They represent a doubling of much of the cytoplasmic material in a few hr. What is the nature of this material: is it virus, is it material once destined to be virus, is it normal or abnormal

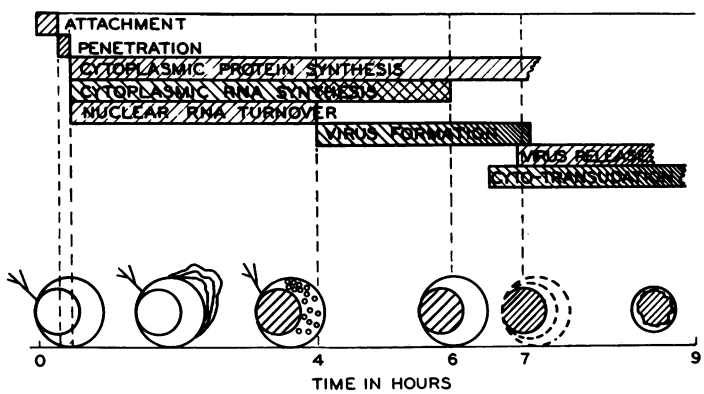

Figure 7. The biochemical and morphologic response of the HeLa cell upon infection with poliovirus. RNA, ribonucleic acid.

TABLE 2

Ratio of Δ protein-N to Δ RNA-P in the cytoplasmic components of normal and infected HeLa cells

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

Note: Cytoplasm for ultracentrifugal fractionation was obtained from HeLa cells 6 hr after initiation of infection and from normal cells treated in the same manner without exposure to virus. Δ RNA-P and Δ protein-N values were derived from data presented in table 3 and represent the differences between normal and infected cell fractions in the amount of RNA-P and protein-N. All values are on a per cell basis.

* Plaque-forming units.

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

cellular material? There are several lines of evidence which may be meaningful to consider in this connection.

First, the yield of virus seldom exceeds 1000 plaque-forming units per cell. According to Schwerdt and Fogh (59), the number of characteristic particles in a preparation which are countable with the electron microscope is not less than 30 per plaque-forming unit under the best plating conditions. They estimate the number of particles produced per kidney cell to be in the range of 50,000 to 300,000. This they estimate represents about 3 to 5 per cent of the dry weight of the cell. The increases in cell mass actually found are of a different order of magnitude. If the incremental RNA were all virus, the yield would be about 1,000,000 particles per cell and the protein would correspond to 10,000,000 per cell.

Secondly, when the cytoplasm is separated into fractions representing mitochondria, microsomes, and cell sap (table 2), 80 per cent of the virus activity is in the microsome fraction while 10 per cent of the incremental protein is found here; 70 per cent of the incremental protein is in the cell sap. Throughout, the distributions of incremental protein, RNA, and virus activity among the various fractions do not parallel one another as would be expected if all represented the same particle. The ratio of the protein nitrogen to RNA-phosphorus in the virus particle is about 2.3. Only in the microsome fraction does

TABLE 3

Nucleotide composition of cytoplasmic RNA (CRNA) in normal and infected HeLa cells

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

Note: Relative values for the amounts of nucleotides in the CRNA of normal and infected cells were derived from data presented in table 1. These values are for cells 6 hr after initiation of infection.

* Data on the nucleotide composition of poliovirus type 1 (Mahoney) are from Schwerdt (60).

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

TABLE 4

Distribution of protein-N and RNA-P in the cytoplasmic components of normal and infected HeLa cells

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

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

* Cultures containing approximately 6×10^6 cells were exposed to 125 μc of P^{32} during the last 30 min of incubation.

† Plaque-forming units.

the ratio of the incremental protein and RNA approach this with a value of 2.25. The characteristic value for normal microsomes is 11.9.

In addition, the poliovirus as studied by Schwerdt and Schaffer has a characteristic ratio of bases (table 3) (60). These are quite different from the cytoplasmic RNA of HeLa cells, being relatively low in cytidylic and guanylic acids. At present it is not possible to separate the incremental RNA or newly synthesized RNA of infected cells from the normal RNA component originally present. However, if it is assumed that the incremental RNA is viral RNA and that the increase upon infection was 120 per cent (in most experiments the increase is in the range of 150 to 200 per cent of normal), then it is a simple matter to calculate the ratio of bases that would obtain from a mixture of 120 parts of viral RNA and 100 parts of cytoplasmic RNA of the HeLa cell. The ratios of bases of such a hypothetical mixture, as shown in table 3, are sufficiently different from normal cytoplasmic RNA that they could be determined easily within the range of error of the method. When the ratios of the bases of the RNA isolated from the cytoplasm of infected cells were determined, a close similarity to the normal was found rather than to the hypothetical mixture. Thus, the major portion of the incremental RNA must be of a composition different from that of poliovirus.

XX. THEORY

From consideration of the character of the RNA as revealed by the base ratios, the distribution of incremental RNA, protein, and virus activity among the cytoplasmic fractions and the amount of material involved, we are led to conclude—the incremental protein and RNA are not all virus. It is highly probable they are not viral material.

All cytoplasmic fractions of the infected cell—mitochondria, microsomes, ultramicrosomes, cell sap—show increased amounts of protein (table 4). This is also true of the RNA. Superficially a hyperdevelopment of the whole cytoplasm has occurred. However, the cellular growth is unnatural and nuclear development does not proceed.

The findings presented here are not consistent with that concept which proposes the viral synthetic process to be a highly efficient governor suppressing all normal syntheses, redirecting the synthetic powers of the cell to produce a single new product. It is easier to believe that when the conditions of colloidal state, pH, concentration of cofactors, etc., are suitable in some area of the

cell, then all specific templates, including virus, present in the milieu will function. Thus we may expect as a result of infection many large molecules to be formed which were never destined to be virus or to function secondarily in virus production.

The simultaneous syntheses of diverse species of large molecules are possibly competitive, as suggested by the enhanced viral production obtained in cells with certain types of injury. The production per cell of vaccinia virus is at least 3 times greater in HeLa cells which have been rendered incapable of division by X-irradiation then in the untreated cell (Kurtz and Ackermann, *unpublished data*).

One must propose that virus has the power to condition certain areas of the cell to synthetic activity. Although all viruses might possess this fundamental capacity, different viruses such as those containing RNA or DNA would condition different structural and metabolic areas. Indeed, a recent observation describes a specific hyperdevelopment of DNA in cells infected with herpes virus, which in concept parallels the effects of poliovirus upon RNA metabolism (49).

The synthetic activity induced in the resting cell by the infection might not exceed that of the ordinary cell during the growth phase, particularly if we consider that the RNA and DNA of the daughter cell are probably formed only during a portion of the interphase and that the total generation time is only 18 hr. One need not assume that the virus increases the basic synthetic potential of the cell. The action evoked by the virus in the resting cell may be similar to that which stimulates a synchronous synthesis of diverse RNA molecules during the normal life cycle of the cell. Since the cell may respond in a limited number of ways, simplicity suggests that virus merely deludes the cell into believing it is in one of its mitotic or premitotic phases. More specifically, the virus would trigger or initiate some one of the stepwise phases that constitute the normal life cycle of the cell. The mechanism by which multiple templates are triggered to function might be the mere presence in the metabolic milieu of any activated RNA molecule, *i.e.*, one unassociated with large molecules of the same or different kind.

The question, why the production of a relatively small mass of virus results in cell destruction, is partially elucidated by the present data, which indicate that the viral effect upon metabolism is far more extensive than previously reckoned. The cytopathogenic effect might have its basis not in the synthesis of a small amount of foreign material but rather in an unbalanced growth of the cell. However, cytotransudation may be a result of the action of a particular substance. From this line of reasoning, the difficulty arises when the normal activity stimulated by the virus is out of phase with other normal activities of the cell. Illustrative of this concept are recent observations of the cytopathic effect of 5-fluorouracil upon the HeLa cell. In this instance the function of thymine in the synthesis of DNA is blocked. Cellular division is prevented but cell growth continues, producing a giant cell often with multiple micronuclei. The effect can be prevented by thymine, thymidine, or thymidylic acid. But once the unbalanced development has occurred, the effect is not reversible and the giant cell proceeds ultimately to death and disintegration (8). This effect of fluorouracil seems comparable to those described recently for thymidine-less mutants of bacteria, where death is linked to conditions permitting unbalanced growth (9, 16).

If this hypothesis is at all useful, it is because it proposes a single capacity of virus to account for the wide range of cytopathogenic effects that the spectrum of viruses produces, namely, the capacity to condition certain loci of the cell to synthetic activity. The extent and nature of the loci affected will vary with individual viruses. The concept readily embraces the neoplastic agents, which evoke an unnatural though balanced cellular development. Obviously the correctness of this speculation will be determined only by further application of the experimental method.

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