# GROWTH CHARACTERISTICS OF POLIOVIRUS IN HeLa CELLS: NUCLEIC ACID METABOLISM*

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Direct chemical analysis and histochemical studies of cells infected with herpes virus or influenza virus have shown a remarkable retention of cellular structure and a constantness of composition which persist throughout the productive period of the infection (1, 2). In contrast the infection of HeLa cells with poliovirus produces clearly discernible changes in the cytoplasm of the cell. Chief among these detectable by fixation and staining is the early development of an intense basophilia (3). The implication is that profound changes in nucleic acid metabolism accompany the development of virus. This speculation is fully supported by the present investigation in which direct chemical analysis of the various morphologic components of the HeLa cell were made at intervals throughout the growth cycle. The rates of incorporation of radioactive phosphorus into the various nucleic acid fractions and the changes in net synthesis of the ribose and deoxyribose nucleic acids are described at the various phases of infection.

## Materials and Methods

*Virus.*—The virus chosen for these studies was the Mahoney strain of type 1 polio-virus. It is passaged as routine in HeLa cells; the last eight passages were in cultures of HeLa cells adapted to grow in equine serum.

*Cell Culture.*—The line of HeLa cells used was obtained from Dr. J. T. Syverton. As routine cultures were implanted with $1.5 \times 10^6$ cells and incubated with 8 ml. of growth medium consisting of 40 per cent human serum and 60 per cent Hanks's balanced salt solution. At the 4th day, the growth medium was discarded and a new overlay consisting of 80 per cent Eagle's basal medium (4) with 20 per cent equine serum was added. The cultures were used 5 days after implantation.

*Maintenance Medium.*—For the propagation of virus and the study of the synthesis of nucleic acid, a medium was used composed of 10 per cent equine serum and 90 per cent Scherer's maintenance solution (5).

*Phosphorus Isotope.*—Sterile solutions containing $P^{32}$ (specific activity 52 to $100 \times 10^3$ mc./gm.) were obtained from Oak Ridge National Laboratories. Determinations of radioactivity were obtained by plating samples of material on an aluminum dish with a depression $3/4$ inch in

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diameter and ⅛ inch in depth. The samples were dried under a heat lamp and the dishes were placed on a shelf which fitted exactly under a Geiger tube.

*Separation of Cytoplasm and nucleus.*—The nucleus and cytoplasm were separated by treating the HeLa cells with a neutral solution of 0.1 molar sodium chloride containing 0.05 molar citrate as described by Crampton *et al.* (6). The number of cells in each preparation was determined by counting a suspension of the nuclei in a hemocytometer.

*Extraction of Nucleic Acids.*—The isolation of cytoplasmic RNA (ribonucleic acid) and the RNA and DNA (deoxyribonucleic acid) of the nucleus was accomplished using a combination of several published methods. The method of Schneider (7) employing cold and hot 5 per cent trichloroacetic acid was used to separate the RNA from the cytoplasmic proteins. The nuclei were extracted with a boiling alcohol-ether mixture (3:1) to remove lipide. The protein and nucleic acid residues were dissolved in alkali at room temperature and the DNA was separated from the hydrolyzed RNA as described by Schmidt and Thannhauser (8).

*Phosphorus Determination.*—The phosphorus content of the nucleic acid fractions was determined according to the method of Fiske and Subbarow (9).

*Virus Assays.*—The amounts of intracellular virus were determined by assay of the citrate extracts using the plaque technique of Dulbecco (10) modified to use HeLa cells (11). All titers are expressed as PFU[1] (plaque-forming units) of virus.

## EXPERIMENTAL

### All data presented were obtained from experiments of one design.

Cultures composed of monolayers of cells were exposed to high concentrations of virus for an interval of time during which nearly all cells in the culture became infected. The completeness of infection was verified by maximal yields of virus obtained at 7 hours and also by supporting experiments in which the spread of infection was restricted by the use of immune serum (12). The study of replicate cultures allowed the cumulative changes in nucleic acid to be followed and correlated with a single cycle of viral development. The addition of radioactive phosphorus to cultures at various times followed shortly by disruption and extraction of cells allowed the incorporation of $P^{32}$ to be determined during brief intervals of the growth cycle.

In the typical experiment a series of cultures each containing 8 to 10 $\times$ 10⁶ cells were overlaid with maintenance solution and incubated 2 hours at 37°C. before infection. This was found necessary in order to prevent changes from occurring in the control cultures during the experimental period. The cultures were then infected by the addition of 1 $\times$ 10⁹ PFU of virus. The final volume of the overlay was 6 ml. and the approximate depth 0.5 ml. After 1 further hour of incubation at 37°C., the original medium was discarded and each culture was washed 3 times with 10 ml. of Hanks's balanced salt solution to remove the residual inoculum.

At various times between 1 and 7 hours later, individual cultures received 0.4 ml. of $P^{32}$, about 200 μc. of activity. One-half hour after this addition, the experiment was terminated. Each culture was washed three times with 10 ml. of buffered saline to reduce the contaminating $P^{32}$. The cells were then removed with a rubber policeman into a cold saline-citrate mixture.

The cytoplasm was thus separated from the nucleus and the nucleic acids were separated and extracted from the two morphologic components. In addition samples of the cytoplasm were assayed for virus. In this manner the cumulative change in

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[1] PFU, plaque-forming units.

nucleic acid and virus could be determined, as well as the incorporation of $P^{32}$ during various one-half hour intervals of the growth cycle.

## RESULTS

*Nucleic Acids of and $P^{32}$ Incorporation by HeLa Cells.*—When ordinary HeLa cells were fractionated into cytoplasm and nucleus and analyzed, the nuclear RNA was found to be 1.5 times greater than the cytoplasmic RNA. The amount of nuclear DNA was similar to the cytoplasmic RNA. In the 2nd column of Table I, the actual amounts of the nucleic acids per cell fraction are given.

TABLE I

*The Phosphorus and $P^{32}$ Distribution in a HeLa Cell*

| Fraction* | Control | | |
|---|---|---|---|
| | Phosphorus | Counts‡ × $10^{-10}$/cell | Relative specific activity |
| | $mg. \times 10^{-10}$ /cell | | $\dfrac{counts/cell}{mg. \ P/cell}$ |
| cRNA-P | 21.0 | 2.02 | 96.19 |
| nRNA-P | 31.3 | 19.4 | 619.81 |
| nDNA-P | 20.0 | 1.2 | 60.0 |

* The amount of nucleic acid is expressed as milligrams of phosphorus contained in each fraction. The cRNA-P, nRNA-P, and nDNA-P fractions correspond respectively to cytoplasmic RNA-phosphorus, nuclear RNA-phosphorus, and nuclear DNA-phosphorus.

‡ The rate of uptake of $P^{32}$ was determined over a half-hour period. The $P^{32}$ added contained a total of 143 μc. of activity.

They are expressed as milligrams of nucleic acid phosphorus. The uptake of $P^{32}$ into the various nucleic acid fractions is tabulated in the 3rd column of Table I. The uptake is expressed in counts per 3 minutes per fraction of a single cell. It will be noted that the highest rate of uptake is into the nuclear RNA This is in agreement with the data of other investigators using other cell systems. The incorporation of $P^{32}$ into the nuclear DNA is similar to the uptake by the cytoplasmic RNA. In column 4 is given the specific activity of each fraction calculated by dividing the number of counts per cell fraction by the milligrams of nucleic acid phosphorus in that fraction. The relative incorporation is seen to be greatest in the nuclear RNA and least in the nuclear DNA.

*Changes in Nucleic Acids Following Infection.*—When cultures of ordinary HeLa cells were analyzed for nucleic acids after various periods of incubation they were not found to differ significantly during a 7 hour period. However, infected cultures showed striking progressive changes. The resulting data are recorded in Fig. 1, in which the per cent changes in the infected cells are plotted relative to the uninfected control. By the 1st hour after infection there appeared a small increase in nuclear RNA and DNA. From then until the 4th hour, the

amounts of DNA and nuclear RNA remained fixed. After the 4th hour, there was a small percentage decline in the nuclear nucleic acids of the infected cell. Usually by the 1st hour after infection, an increase in cytoplasmic RNA was detectable. The actual increase varied from one experiment to another. It
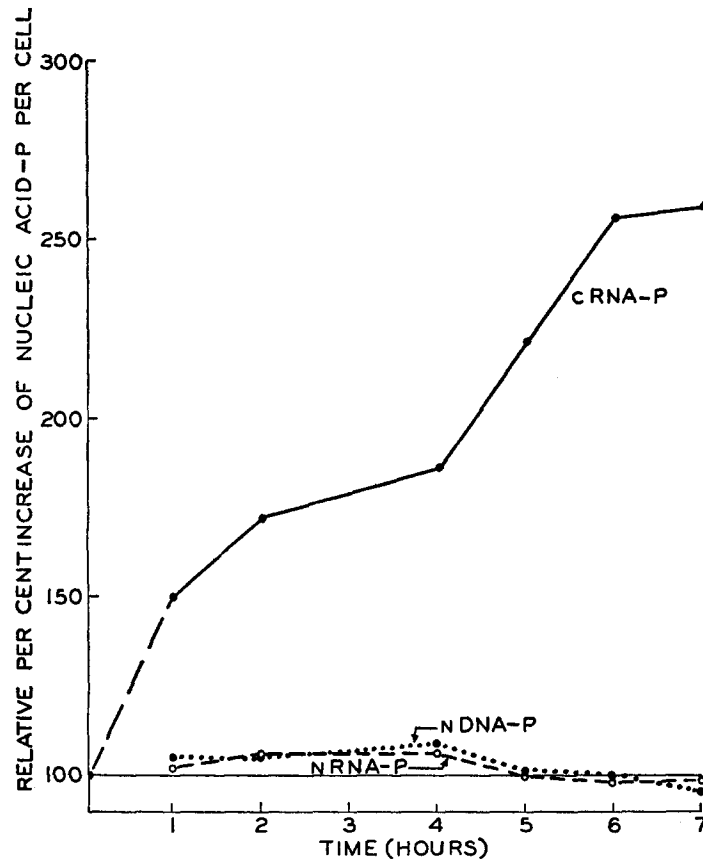


FIG. 1. Changes in the nucleic acid–phosphorus of cell fractions consequent upon infection. The cells were exposed to an undiluted inoculum containing a total of $1 \times 10^9$ PFU of virus for a period of 1 hour. Then the culture was washed three times with balanced salt solution, replaced with fresh medium and incubated, at 37°C. Nucleic acid was determined as nucleic acid–phosphorus, and expressed as the per cent change relative to the control.

might be from 110 to 150 per cent of the control. The accumulation of RNA continued in the cytoplasm until the 6th hour at which time the RNA might have increased by 250 per cent. Between the 6th and 7th hour no further increase occured.

*Incorporation of $P^{32}$ into Nucleic Acids of Infected Cells.*—The data of Fig. 2

show the amount of radioactive phosphorus incorporated into the various nucleic acid fractions of the infected cell. These data represent the uptake of $P^{32}$ over a short period of time at various intervals of the infectious cycle and hence they are an expression of rate. Once again the experimental values are recorded as per cent changes relative to an uninfected control.
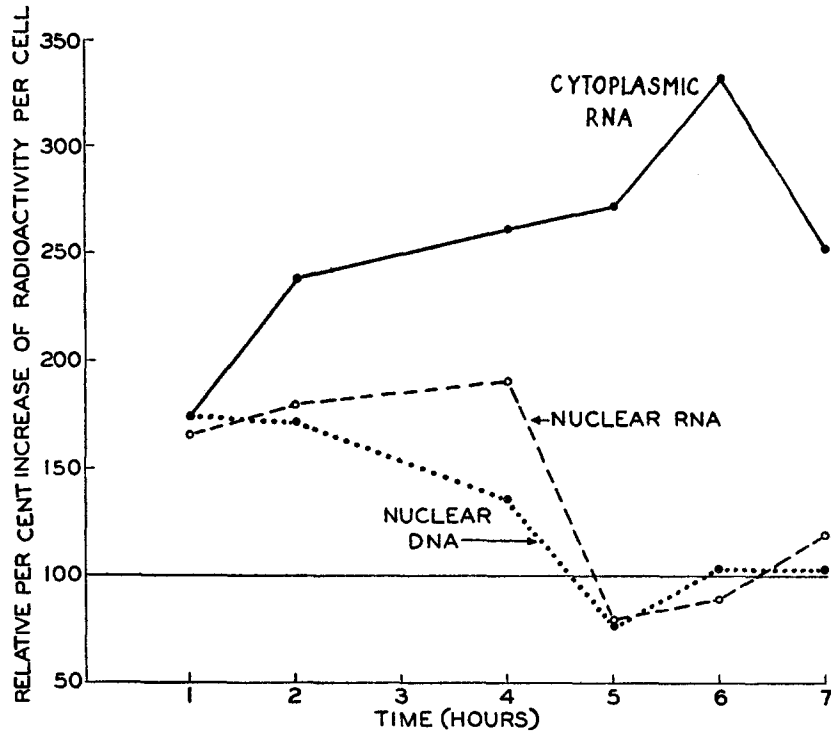


FIG. 2. The rate of $P^{32}$ incorporated into the three nucleic acid fractions was measured over one-half hour periods. The cells were exposed to an undiluted inoculum containing a total of $1 \times 10^9$ PFU of virus for a period of 1 hour. Then the culture was washed 3 times with balanced salt solution, replaced with fresh medium, and incubated at 37°C. The additions of $P^{32}$ contained a total of 143 $\mu c$. of activity. The amount of $P^{32}$ incorporated is expressed as the per cent change related to the control.

By the 1st hour after infection, the rate of incorporation of $P^{32}$ into the two nuclear fractions was from 150 to 160 per cent of the control. This enhanced rate continued for the next 3 hours, after which there was an abrupt decline in this nuclear activity. The uptake of $P^{32}$ into the cytoplasmic RNA was also enhanced by the 1st hour, and the rate increased until it was maximal at the 6th hour. This maximal rate might be 250 to 300 per cent greater than that of the uninfeeted cell. After the 6th hour the activity of the cytoplasmic fraction declined sharply.

*Intracellular Appearance of Viral Activity.*—An attempt to correlate the synthesis of cytoplasmic RNA and the appearance of mature virus is illustrated in Fig. 3. Again the data for RNA in the cytoplasm are presented as per cent increase relative to the control. The results of the viral assay are presented as
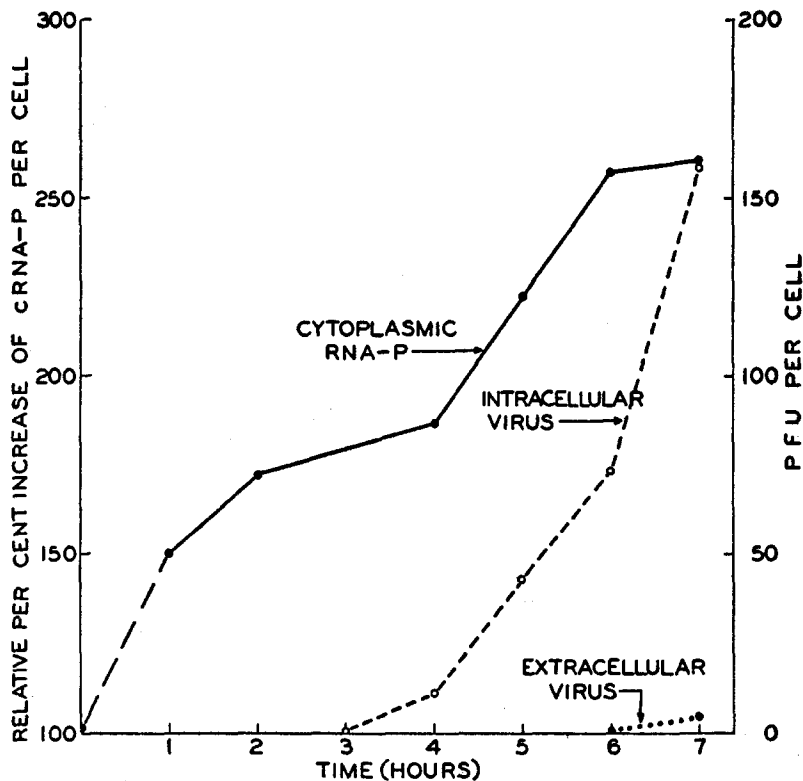


FIG. 3. Increase in the cytoplasmic RNA-phosphorus in relation to the appearance of demonstrated virus. The cells were exposed to an undiluted inoculum containing a total of 1 × 10$^9$ PFU of virus for a period of 1 hour. Then the culture was washed three times with balanced salt solution, replaced with fresh medium, and incubated at 37°C. Both intracellular and extracellular virus samples were taken at hourly intervals, and titered according to the plaque technique. Virus titers are expressed as PFU per cell. The amount of cytoplasmic RNA-phosphorus is expressed as the per cent change relative to the control.

the number of PFU of virus per cell. Upon extraction with the citrate-saline mixture, the preponderance of virus was found in the cytoplasmic fraction. An increase in viral infectivity was not detectable until the 3rd hour after infection. The virus continued to increase and accumulated in the cytoplasm until the 7th hour. It will be noted that at the 7th hour, when 90 per cent of the total viral yield had been formed, only 1 per cent of the new virus was in the extra-

cellular state. These data clearly indicate an intracellular phase in the development of poliovirus as has been reported by other investigators (13, 14). Mature virus appears in the cytoplasm after the synthesis of RNA has begun. The virus increases concomitantly with the RNA synthesis and continues for some time (between the 6th and 7th hour) after the maximal synthesis of RNA has been reached.

## DISCUSSION

The study of the incorporation of $P^{32}$ into the nucleic acids takes on increased significance when viewed together with the quantitative changes which occured in these acids. The sustained high uptake of $P^{32}$ by the nuclear fractions during a period (1 to 4 hours) in which the quantities of RNA and DNA remained fixed indicated an enhanced metabolic activity, rather than net synthesis of nucleic acids. There was either an exchange of $P^{32}$ with the phosphorus of the molecule or synthesis and degradation were proceeding at similar rates. This nuclear activity might be associated with protein synthesis and indeed the data of Ruska et al., obtained with the electron microscope, are suggestive in this connection (15). Pertinent also are the findings of Buckley. She has reported that viral antigen is strongly detectable in or on the nucleus by the use of the fluorescent antibody technique (16). If this interpretation is correct, it is of interest that the synthetic activity subsided between the 4th and 5th hour after infection when the major portion of the viral yield was yet to appear.

The incorporation of radioactivity into the cytoplasmic RNA was undoubted ly a reflection of the actual net synthesis of nucleic acid. Viral material was most probably being synthesized in the cytoplasm. As yet, it has not been possible to demonstrate infectious activity with this RNA and the amount is large relative to the viral activity which develops. Qualitatively the RNA which accumulates appeared different from the usual cellular RNA. It was more susceptible to cold alkaline hydrolysis than is the ordinary cellular nucleic acid. A detailed description of this finding will be presented elsewhere.

This is the first time a progressive synthetic activity induced by a viral infection has been followed in animal cells, uncomplicated by inflammation and the presence of diverse cell types. Clearly a sustained investigation of this system can be most instructive of the viral developmental process.

## SUMMARY

The RNA and DNA contents of the nucleus and cytoplasm of the HeLa cell were determined. The rates of incorporation of $P^{32}$ into the various nucleic acid fractions were established for the ordinary HeLa cell maintained under a set of standard conditions.

The changes in the rates of incorporation of $P^{32}$ and in the amounts of RNA and DNA which occurred subsequent to infection with poliovirus were followed

throughout the infectious cycle. These changes were correlated with the intracellular appearance of the newly formed virus. A net synthesis of RNA occurred in the cytoplasmic component of the cell. The increase was detectable 2 hours before the first appearance of demonstrable virus and reached a maximum (2.5 times normal) at 6 hours. Viral increase was not maximal before the 7th hour after infection.

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