NUCLEIC ACID METABOLISM OF VIRUS-INFECTED HELA CELLS*

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The study of the chemistry of virus-infected cells cannot yet claim a molecular interpretation for the genesis of the viral cytopathic effect. However, many striking observations have been made that must be accounted for in any molecular theory of viral cytopathology. From the viewpoint of comparative virology, the most consistent viral-induced aberrations involve the nucleic acids. The findings that we describe here concern the several classes of these important compounds, their relations to the cell architecture, and their dynamics relative to a single cycle of infection. These experiments with HeLa cells and poliovirus are part of a continuing investigation and are contributions made in collaboration with our colleagues. Some results have been published; others have not been made available previously.

Part of the record in the literature (Payne *et al.*, 1958) describes how a single cycle of infection of monolayers of HeLa cells may be effected with high concentrations of poliovirus. It suffices here to say that interpretations at the cellular level of the present observations made on multicellular cultures have been justified.

The first experiments concern the stability of the cell structure after infection, particularly in regard to those elements that will be considered in the metabolic studies.

Transudation Phenomenon

HeLa cells are readily labeled with P^{32} by allowing them to grow in a medium containing radioactive inorganic phosphate. The P^{32} is incorporated into a number of large molecules and structures such as the nucleic acids, microsomes, and mitochondria. When such labeled cells are transferred to unlabeled maintenance medium (Scherer, 1953) and incubated, some of the radioactive phosphorus leaks from the cell. Initially, the process is rapid, but it soon subsides and, by 5 hours, an equilibrium seems to be established between this exchangeable phosphate of the cell and that of the medium. After this time the P^{32} of the medium ceases to rise and remains constant (FIGURE 1).

When poliovirus is added to such a culture of labeled cells, the process of P^{32} exchange closely parallels that of the control until the sixth hour of infection. At this time, the curves for the release of P^{32} diverge (FIGURE 1). Progressively, the transudation of labeled cellular elements is accelerated in the infected culture. A preliminary examination of this transudate shows it to be composed of both acid-soluble and acid-insoluble elements. The soluble fraction is a complex mixture of nucleotides, nucleosides, and inorganic phosphate.

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This transudation phenomenon closely parallels the release of virus and the progressive loss of cytoplasmic area seen in time-lapse cinemaphotography (unpublished results, W. W. Ackermann and H. Kurtz). It is probably related to the mechanism of viral release and due to specific virus action, since the extent of the process in any culture is dependent upon the concentration of the viral

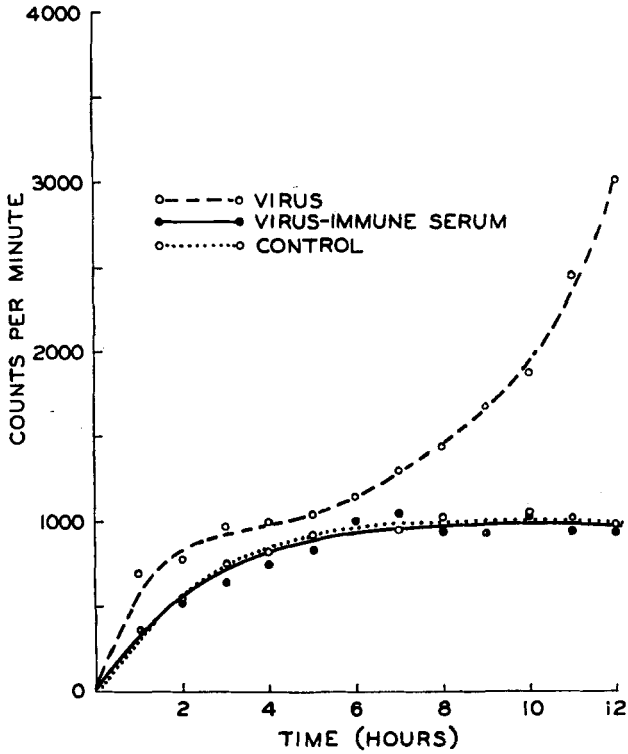

FIGURE 1. The action of virus neutralized with antiviral serum on the release of radioactivity from HeLa cells labeled with radioactive phosphorus. A viral inoculum containing $10^{7.5}$ $TCID_{50}$ was neutralized with an equal amount of undiluted antiviral serum, and the mixture incubated for 1 hour at 37° C. The neutralized mixture was added to a culture of HeLa cells. To a second culture an equal amount of unneutralized poliovirus was introduced. The uninfected control received an equivalent amount of maintenance medium. At hourly intervals samples were taken from each culture and the amount of radioactivity released was determined. The results are expressed as counts per minute per milliliter.

inoculum and can be prevented by prior incubation of the virus with viral-specific immune monkey serum.

These observations tell us little of the biochemical alterations leading to this selective loss of cellular integrity, but they do determine the maximum interval after infection that can be studied most profitably. This period comprises the first seven hours when nearly all the newly formed virus is intracellular and the essential integrity of the cell is intact.

Results that are more easily subject to interpretation are obtained by following the incorporation of P^{32}, which is introduced after infection.

The object of the next experiment is to follow the incorporation into various tissue components of the labeled phosphate that was introduced at various times after infection. The essential part of the observation has already been recorded in the literature (Maassab *et al.*, 1957).

In a typical experiment, a series of cultures was exposed to a large inoculum of poliovirus. After 1 hour of incubation at 37° C., the cultures were washed free of the residual inoculum and incubated further. At various times between 1 and 7 hours after initiation of infection, each culture was exposed to P^{32} in the form of inorganic phosphate. One-half hour after this addition the experiment was terminated. The cells were fractionated into cytoplasm and nuclei. Both morphologic fractions were subjected to chemical analysis. In addition, a sample of the cytoplasmic fraction was taken for virus assay. Thus the accumulated increase of nucleic acids, the rate of incorporation of P^{32} over short-time intervals, and the appearance of the virus were followed.

The Nucleic Acid Composition of Normal HeLa Cells and Their Incorporation of P^{32}

When ordinary HeLa cells were fractionated into cytoplasm and nucleus and analyzed, the total ribonucleic acid (RNA) was found to be 1.5 to 2.5 times greater than the desoxyribonucleic acid (DNA). The amounts of nuclear RNA and cytoplasmic RNA were quite similar. The uptake of P^{32} into various nucleic acid fractions follows the pattern of cells from other species described previously (Marshak, 1948, Smellie *et al.*, 1953). The highest rate of incorporation is seen in the nuclear RNA, followed by the cytoplasmic RNA, and DNA. The amount of P^{32} incorporated into the total RNA is 17.8 times greater than that in DNA. This phenomenon generally has been found for resting cells (Smellie *et al.*, 1953). In the experimental period studied, from 0 to 7 hours, it was found that the cell number, composition with regard to DNA and RNA, and the pattern of incorporation of the isotope did not vary significantly.

Incorporation of P^{32} into the Nucleic Acids During the Infectious Cycle

In contrast to the constant activities of the normal cell, striking changes in uptake of P^{32} occur after infection. The data in FIGURE 2 illustrate the rate of uptake of P^{32} during various intervals of the infectious cycle. Since the pattern of incorporation of the isotope by the uninfected controls did not vary significantly throughout the period studied, the values obtained for the incorporation of P^{32} are recorded as percentage change relative to the control. The enhanced incorporation of P^{32} by the 3 nucleic acid fractions is detectable by the first hour after infection. It is sustained in the DNA for 2 hours, after which it steadily declines, but more abruptly at the fourth hour of infection. In contrast, the cytoplasmic RNA increases in activity until the sixth hour, after which it also decreases. Becker *et al.* (1958) have observed also increased incorporation of P^{32} into human amnion cells following infection. Similarly, Miroff *et al.* (1957) found an increased incorporation of P^{32} into the total nucleic acids of HeLa cells during the first 2 hours of infection with poliovirus.

Changes in Nucleic Acids, Protein, and Virus Following Infection

Cultures of HeLa cells were analyzed for changes in the nucleic acid phosphorus at various times after infection. The nucleic acids of the nuclear fraction show a slight rise for a period of 2 hours after infection and remain constant thereafter. The increase in cytoplasmic RNA-P starts by the first hour and is progressive until the sixth hour, at which time the RNA-P may have increased 250 per cent. Each fraction of the cytoplasm examined during the period of infection studied also shows increases in protein. The amount of increase in protein in 7 hours represents nearly a doubling of the cytoplasmic

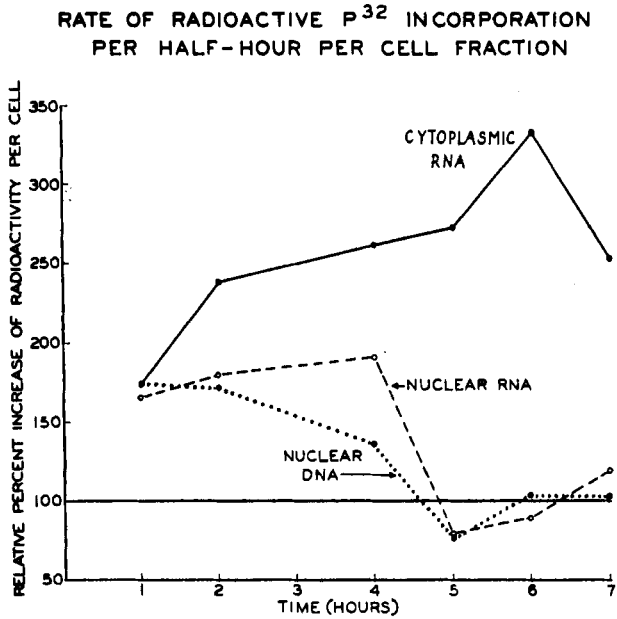

FIGURE 2. Rate of P^{32} incorporated per half hour into the 3 nucleic acid fractions. Cells were exposed to an undiluted inoculum containing a total of 1×10^9 PFU of virus for a period of 1 hour. Cultures were washed 3 times with Hank's balanced salt solution, supplied with fresh medium, and incubated at 37° C. The additions of P^{32} contained a total of 125 μc. per culture. The amount of P^{32} incorporated is expressed as the percentage of change related to the control.

material. The first increase in viral activity in the cell was not detected until the fourth hour; extracellular virus starts to appear at about the sixth hour. It should be noted that at the seventh hour, when 90 per cent of the total viral yield has been formed, only 1 per cent of the new virus is in the extracellular state. These data clearly indicate an intracellular phase in the development of poliovirus, as has been reported by other investigators (Roizman *et al.*, 1958, Darnell, 1958).

The increases in nucleic acids and protein described here are huge in proportion to the size of the cell. The significance of the changes can be deduced from several lines of reasoning.

First, the increase in cytoplasmic RNA appears in excess of what would be expected on the basis of the viral yield of the cell. If the incremental RNA and

protein were all virus, the yield would be 10^6 particles/cell, and the protein would correspond to 10^7/cell. However, the yield of virus seldom exceeds 1000 plaque-forming units (PFU)/HeLa cell. According to Schwerdt and Fogh (1957), the number of characteristic particles in a preparation that are countable with the electron microscope is not less than 30 PFU/cell under the best plating conditions. This would set a lower limit of 30,000 particles/HeLa cell. The increase in RNA and protein in the cytoplasm actually found at the sixth hour of infection is of a different order of magnitude than the increase in viral activity.

Second, the composition of the cytoplasmic RNA is not altered in the direction expected by the accumulation of additional RNA of the virus type. The RNA of the cytoplasm obtained after 6 hours from both infected and normal HeLa cells was hydrolyzed to a mixture of nucleotides by the method of Volkin and Carter (1951) and chromatographically separated as described by Hurlbert *et al.* (1954). The chromatographic fractionation of the hydrolyzate

TABLE 1

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

Nucleotide	μM of nucleotide $\times 10^{-9}$/cell		
	Normal	Infected	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

Samples for analysis were obtained from HeLa cells 6 hours after initiation of infection with poliovirus and from normal HeLa cells treated in the same manner without exposure to virus. Values recorded here are averages of data from 5 experiments.

* The difference between normal and infected cells in the amount of each nucleotide in the cRNA.

of the nucleic acids yielded the usual 4 nucleotides. The number of micromoles per cell of each nucleotide was determined. The results are presented in TABLE 1. There is approximately 150 per cent more of each nucleotide in the cytoplasmic RNA of the infected as compared to the normal cell. The nucleotide composition of the RNA isolated from the cytoplasm of normal HeLa cells is very similar to that reported for RNA isolated from other mammalian tissues in that it is rich in guanylic and cytidylic acids and poor in adenylic and uridylic acids. The RNA of the cytoplasm of infected cells was found to have a nucleotide composition that does not differ significantly from that of normal cells. However, values obtained by Schwerdt (1957) for the nucleotides of RNA from poliovirus, Mahoney strain, are quite different in composition from those of normal cells. The values are lower especially for cytidylic and guanylic acids. Thus, while viral RNA must be synthesized in the infected cell, the major portion of the newly formed RNA induced by virus infection appears not to be of the viral type, but resembles more closely that of the ordinary cell cytoplasm.

Third, the distribution of the incremental materials among the various fractions does not correspond to the distribution of virus. Furthermore, in two of

the fractions the incremental protein and RNA are not present in the same proportions as in the virus.

The cytoplasmic RNA is a composite of several species of RNA (Smellie, 1955) associated with various subcellular components. By differential centrifugation the subcellular elements may be fractionated and thus indicate where the incremental RNA exists in the cytoplasm of infected cells. At the sixth hour of infection, the cytoplasm of normal and infected HeLa cells was fractionated by differential centrifugation, following the method of Hogeboom and Schneider (1955), into 3 fractions: Fraction I, the sediment after 6600 g for 20 min.; Fraction II, the sediment after 41000 g for 1 hour; and Fraction III, the supernatant fluid above Fraction II. Each of these fractions was analyzed for RNA-P, protein and virus activity, and the rate of incorporation of P^{32} into RNA.

TABLE 2

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

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

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

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

The data are presented in TABLE 2. In the normal cell, the distribution of the cytoplasmic RNA among the 3 fractions is 7, 18, and 75 per cent, respectively. P^{32} was incorporated into the RNA of each fraction of the uninfected cell. The specific rates of incorporation of the isotope vary; Fraction III containing 75 per cent of the cytoplasmic RNA is the least active. After infection there is an increase in the rates of incorporation of the isotope in all of the fractions, Fraction II showing the greatest increase (130.4 per cent). The distribution of total RNA-P follows the same pattern seen with the radioactivity data. After infection there is an increase in all fractions but, by the sixth hour, the greatest percentage of accumulation is again in Fraction II (191 per cent), followed by Fraction I (65 per cent), and Fraction III (25 per cent). Likewise, all fractions show an increase in protein content. The largest accumulation is in Fraction III, but the percentages in fractions I and III are the same and are greater than in Fraction II. The virus activity is found predominantly in Fraction II.

There is a characteristic value for the ratio of protein N to RNA-P for each of the components of the normal and infected cell cytoplasm and for the purified virus as computed by Schwerdt (1957). A comparison of these values de-

termines whether the newly formed materials resemble the composition of the normal component with which it is associated or that of the virus, or whether the materials are anomalous. Only in Fraction II does the incremental material resemble that of the virus. The incremental materials of Fraction I more closely resemble the normal components than the virus. Fraction III of the infected cells is seen to contain an increment that is relatively rich in protein as compared to the normal component. From these considerations we conclude that the accumulated protein and RNA do not constitute poliovirus and, at least for RNA, do represent material of the virual type (Ackermann *et al.*, 1958).

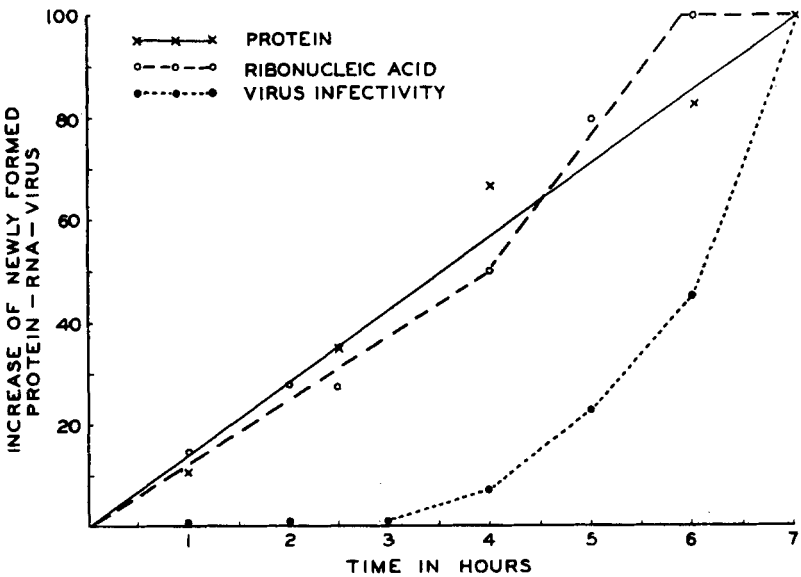

FIGURE 3. The increase of newly formed cytoplasmic protein, RNA, and virus in HeLa cells at various times following infection with poliovirus. The newly formed, or Δ, material represents the difference between normal and infected cells in the amount of each material. Values plotted are expressed as a percentage of the amount of Δ material found at seven hours following infection.

Key: X——X——X protein of Fraction III of cytoplasm; O - - O - - O ribonucleic acid of total cytoplasm; ●- - -●- - -● virus infectivity of total cytoplasm.

Kinetics of Synthesis of RNA, Protein, and Virus

The preceding sections describe the biochemical situation in the cell at the sixth hour of infection. The following experiment is concerned with the sequence of steps in time by which the situation developed. Replicate cultures of cells were analyzed at various times in the interval from the initiation of infection to the seventh hour. In order that the characteristics of development of several materials that differ in absolute amount may be compared, the incremental amount of each at the seventh hour was assigned the value of 100, and the amounts at other times, some proportion of 100. In this form, data concerning the newly formed protein of Fraction III of the cytoplasm, the total cytoplasmic RNA, and virus infectivity are plotted in FIGURE 3. The

synthesis of protein proceeds at a constant linear rate from the first hour until the seventh. The synthesis of RNA also begins close upon the initiation of infection and appears to be linear; the rate in the first 4 hours closely parallels that of the protein synthesis. The rate between the fourth and sixth hours proceeds at a further increased rate, and the process stops at the sixth hour. It appears that 50 per cent of the protein and RNA are formed before the appearance of any virus activity. A further 50 per cent of the virus appears after synthesis of RNA stops.

Currently, a similar study is under way. The amounts of RNA and virus were determined in HeLa cells at various times during a single sequence of infection with hemadsorption Type I virus. Some of the preliminary observations are presented in TABLE 3. From these data the following interpretations are drawn concerning the biosynthetic activities of the infected cell.

TABLE 3
PHOSPHORUS DISTRIBUTION OF HELA CELLS INFECTED WITH HEMADSORPTION VIRUS TYPE I

Hours after infection	mg. $\times 10^{-10}$ per cell*				Virus titer†	
	CRNA-P		Total NA-P of nuclei		$TCID_{50}$/ml.	
	C	I	C	I	Extra-	Intra-
2	21.59	29.9	26.90	30.90	2.5	3.5
6	—	28.33	—	32.10	2.7	3.3
12	26.13	52.45	34.2	41.50	4.3	7.0
18	—	50.22	—	49.9	5.0	7.9
24	25.99	35.2	31.0	33.0	6.8	7.7

* The amount of nucleic acid is expressed as milligrams of phosphorus contained in each fraction. The CRNA-P and total nucleic acid phosphorus (NA-P) fractions correspond respectively to cytoplasmic RNA phosphorus and nucleic acid phosphorus of the nucleus.

† The cultures were infected with a large inoculum of virus ($10^{7.0}$ $TCID_{50}$). After 2 hours of incubation at 37° C., the residual inoculum was removed by washing with Hank's balanced salt solution. Samples of the fluid overlay and of the cytoplasm after fractionation of the cells were taken for virus assay.

The virus induces nucleic acid synthesis in both of the major morphologic fractions of the cell (nucleus and cytoplasm). There is an increase in the cytoplasmic RNA fraction even at the second hour and a marked accumulation of RNA-P between the sixth and twelfth hours. After reaching its maximum value at the twelfth hour of infection, the level of RNA-P of the cytoplasm declines and the release of the virus from the cell is apparent. The nucleus, on the other hand, shows a sustained accumulation of nucleic acid for a period of at least 18 hours, and then declines.

Associated studies of viral activity relate the appearance of new virus and nucleic acid synthesis. Viral increase parallels more closely the induced changes of the nucleic acid of the nuclear fraction. There is also an intracellular phase of viral development, as found previously for poliovirus. Thus the virus seems to induce an increase of the nucleic acid of the nucleus, as well as the cytoplasm of the infected cell, in contrast to poliovirus, where the incremental material is

located primarily in the cytoplasm. A further characterization of this incremental material is in progress.

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