Effect of Poliovirus Infection on Histone Synthesis in the HeLa Cell.* (30363)

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The original reports of the course of DNA (deoxyribonucleic acid) synthesis in HeLa cells infected with poliovirus(1-3) were followed by studies with other picornaviruses (4-6) which suggested a general characteristic pattern. Synthesis of DNA continues for several hours after infection; during this time viral development is well under way. DNA synthesis then undergoes a progressive, accumulative inhibition and by the fourth hour may be only 20% of normal. The mechanism of this progressive suppression of DNA synthesis is not known. It has been suggested that the virus acts upon the cell through the normal regulatory control mechanism of the cell(7). A role of nuclear histone in the genetic expression of cells was proposed as a control mechanism by Stedman and Stedman(8). More recently, histones were shown to inhibit RNA synthesis by direct interaction with DNA(9,10,11). This in turn may halt enzyme synthesis which, unreplenished, in the normal course of decay, would progressively limit DNA synthesis. Alternatively histones may act directly on DNA replication(12). This line of reasoning prompted the present investigation of nuclear histone as a controlling factor in viral-induced repression of DNA synthesis. During these studies a report of closely comparable findings with a different picornavirus (ME virus of mice related to Columbia SK) was published(13).

Materials and methods. Cells. HeLa cells were grown at 37°C in monolayers in Roux bottles with Eagle's basal medium(14) and 10% calf serum and passaged every 7 days. Periodically cells and fluid were inoculated into special media to ensure that they were free of mycoplasma and bacteria(15).

Virus. The Mahoney strain of type I poliovirus used in these experiments was grown in HeLa cells, sedimented by centrifugation and resuspended in phosphate buffered saline. Virus was assayed by the plaque technique using HeLa cells in 3-ounce prescription bottles.

C^{14}-L-Arginine. Uniformly labeled C^{14}-l-arginine was obtained from Volk Radiochemical Co., Skokie, Ill. (174 millicuries per millimole).

Radioactivity. For measurements of radioactivity, 0.5 ml of sample and 9.5 ml of liquid scintillation fluid(16) were mixed in scintillator vials, counted for 10 minutes in an Automatic Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., model 314).

Protein. Protein was determined by the method of Lowry(17) using crystalline bovine serum albumin as a standard.

Deoxyribonucleic acid (DNA). The method of Burton was used to determine DNA quantitatively(18).

Isolation of nuclei. Experimental cultures were rinsed twice with cold PBS (phosphate buffered saline), the cells were scraped from the bottle with a rubber policeman into 15 ml of Eagle's basal medium with 1% calf serum and centrifuged at 1500 rpm for 10 minutes at 4°C. The cells were resuspended in the same medium, and recentrifuged. The supernatant was decanted and the packed cells stored overnight at —20°C. The cells were then thawed at 37°C and the nuclei isolated according to the method of Hogeboom *et al*(19). In the light microscope little cytoplasmic contamination was seen in the preparation.

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Extraction of deoxyribonucleoprotein (DNP). Nuclei isolated as described above were resuspended in 1.0 ml of 0.15 M NaCl and brought to a final concentration of 2 M by addition of 5 M NaCl. Additional 2 M NaCl was then added to give a final volume 50 times that of the original packed nuclei. By agitation a homogeneous suspension was obtained which was then centrifuged at 2000 rpm for 15 minutes at 4°C. The supernatant was removed, diluted with distilled water to give a 0.15 M solution. After standing overnight at 4°C, the pellet of DNP was collected by centrifugation at 2000 rpm and washed twice with 0.15 M NaCl. This is based upon a procedure of Mirsky and Pollister(20).

Extraction of nuclear histone and DNA. The DNP pellets were washed once with 80% ethanol, then extracted 3 times with 2 ml volumes of a mixture of (80%) ethanol and (20%) 1.25 N HCl. The extracts were combined. The residue was then extracted 3 times with 2 ml volumes of 0.2 N HCl.

The insoluble residue was resuspended in 2 ml of 5% trichloroacetic acid (TCA) heated in a water bath, 100°C for 15 minutes. The extraction was repeated 3 times and the supernatants containing DNA were combined.

The remaining pellet insoluble in TCA, was extracted twice more with 3 ml volumes of 0.1 N NaOH. The residue was then discarded.

Efficiency of extraction. To establish that in reality the extraction procedure does separate distinct classes of proteins from the deoxyribonuclear complex, the amount of radioactivity removed with each successive extraction was determined. The total radioactivity which was obtained with each extraction is recorded in Table I for a typical experiment. It will be noted that on the average 90% of the radioactivity which could be removed with the ethanol-HCl mixture was obtained on the first extraction (*e.g.*, 4214 counts from the control sample), while the third extraction removed only 1.2% or 51 counts. Further, the first subsequent extraction with 0.2 N HCl removed a total of 2178 counts, indicating protein with different properties was now being extracted. Likewise, the extractability of the remaining radioactivity becomes progressively limited indicating the method is exhaustive. Three extractions are sufficient under these conditions to separate cleanly these DNA-associated proteins which have been shown elsewhere to be characterized by high arginine or lysine contents, respectively(21,22).

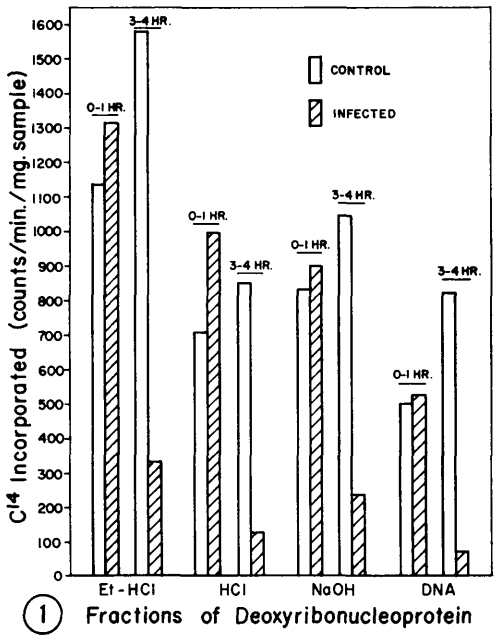
TABLE I. Efficiency of Successive Extractions of C^{14}-Arginine Labeled Histones from DNP of HeLa Cell Nuclei.

Extraction*		C^{14} (cpm) removed by successive extractions: 0-1 hr Control†	0-1 hr Infected†
Ethanol-HCl	1st	4214	9230
	2nd	173	630
	3rd	51	901
HCl	1st	2178	5370
	2nd	530	1300
	3rd	352	684

* Samples of DNP (deoxyribonucleoprotein) were successively extracted 3 times with 80% ethanol plus 20% 1.25 N HCl followed by 3 successive extractions with 0.2 N HCl.

† One sample of DNP was isolated from an uninfected control culture exposed to C^{14}-arginine for 1 hr; a second sample was isolated from a culture exposed to both C^{14}-arginine and poliovirus for 1 hr.

Experimental and results. Experimental design. The influence of poliovirus infection on the incorporation of C^{14}-arginine into the deoxyribonucleoprotein of HeLa cells was determined. In a series of similar experiments, 8-day-old cultures of HeLa cells containing approximately 5×10^7 cells in a monolayer in Roux bottles were used. In some experiments (Fig. 2) the cultures were incubated 2 hours before use with Eagle's medium that was free of arginine and non-radioactive arginine was not used during the experimental period. This did not produce optimal conditions for the cells, nor completely reproducible rates of incorporation of C^{14}-arginine. When the arginine in the medium was increased nearly 3000-fold, the incorporation of isotope was reduced only by 50%. In later experiments (Fig. 1 and 2) unmodified Eagle's medium was used. In the usual experiment the medium was removed from the cultures, 30 ml of fresh medium was added with 1.5 ml of poliovirus containing approxi-



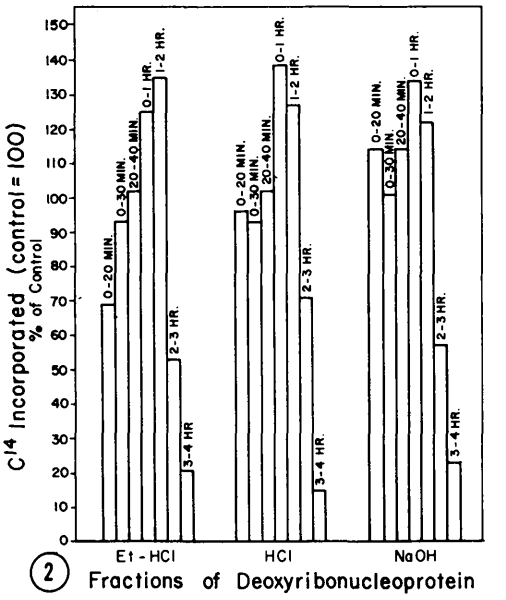


FIG. 1. Incorporation of C^{14}-arginine (counts/min/mg sample) during one-hour intervals, 0 to 1 hr and 3 to 4 hr, into fractions of DNP (Ethanol-HCl and HCl soluble histones, and insoluble protein and not TCA soluble DNA) isolated from control and poliovirus infected HeLa cells. Time is measured from addition of virus. All data from a single experiment using full complement of arginine in the medium.

FIG. 2. Incorporation of C^{14}-arginine during intervals (measured after addition of virus) into fractions of DNP (Ethanol-HCl and HCl soluble histones and acid insoluble nucleoprotein) isolated from control and poliovirus infected HeLa cells. Incorporation of infected cultures is expressed as percentages of corresponding controls. Data for each time interval from separate experiments. With the exception of the 1-2-hr interval, a medium depleted of arginine was used.

mately 10^9 plaque forming units per ml. After one hour (Fig. 1) or in some cases 20 minutes (indicated in Fig. 2) adsorption, the infecting fluid was removed, titered for virus and the input multiplicity determined (in most experiments 40 PFU per cell). The cultures were then overlayed with 60 ml of Eagle's medium plus 1% calf serum and incubated further at 37°C. At selected times after addition of virus, the volume of medium was reduced to 30 ml and C^{14}-arginine (1 microcurie) was added. After an appropriate interval of further incubation, the experiment was terminated and the incorporation of the isotope during this interval determined. Control cultures which were not infected were treated in a similar manner. By comparison with these cultures, the effect of poliovirus upon the cell could be determined throughout the first 4 hours of viral development.

The data of Fig. 1 are from one experiment. The nucleoprotein from infected and control cultures was separated into DNA, 2 acid soluble histone fractions and an acid insoluble protein. The incorporation of radioactive carbon into each is recorded (counts/min/mg sample) for the intervals from 0 to 1 hour and 3 to 4 hours. The data compiled from several experiments are plotted in Fig. 2 and expressed as percentage incorporation based upon specific activities of the corresponding controls.

Distribution of C^{14}-arginine. C^{14}-arginine was found to be readily incorporated into the DNP of both infected and ordinary HeLa cells (Fig. 1). It is incorporated at a significant level into the DNA as well as the associated histones. The specific activity of the DNA is at least one-half that of the individual protein fractions and since by weight the DNA is nearly 50% of the DNP, the pathway of arginine to DNA is a significant one in the HeLa cell. Incorporation into the ethanol-HCl soluble histone is more rapid than into the other fractions, but each incorporates at nearly the same level.

In one set of control experiments the histones were hydrolyzed with 5.5 N HCl and the resulting amino acids chromatographed, 2-dimensionally on paper. The C^{14} activity was found exclusively in the arginine position. This would indicate the incorporation of C^{14}-arginine is a measure of the rate of histone synthesis. The distribution of the radioactivity among the nucleotides of the DNA has not been determined, but this fraction is nearly free of protein as determined by the Lowry method. Contamination by the histone fractions could not account for the large activity in DNA fraction.

Effect of poliovirus on utilization arginine. In all experiments (Fig. 1 and 2) the incorporation of C^{14}-arginine into both histone fractions is enhanced in the interval of 1 to 2 hours after infection. The stimulation varies from one experiment to another in the range of 120 to 140%. This may reflect the normal variation in the biologic time scale and the fact that the enhancement does not occur at once and later, 3-4 hour, is followed by a strongly depressed utilization (15 to 20% of normal). The pathway of arginine into DNA (Fig. 1) likewise is strongly depressed by the third hour of infection but not initially. In the experiments summarized in Fig. 2, the DNA was not separated from the NaOH soluble fraction. However, from these data and those of Fig. 1 it is clear that arginine is not being utilized effectively in the synthesis of this acid insoluble nuclear protein after the third hour of infection.

Arginine is a distal precursor of DNA and its incorporation cannot be regarded as a measure of the rate of DNA synthesis. However, it does correspond to the synthesis of DNA as determined in other experiments using more proximal precursors.

Discussion. The early stimulation of histone synthesis and subsequent inhibition described in cells infected with poliovirus, cannot be *a priori* causally related to the corresponding inhibition of DNA synthesis. The pattern is, however, consistent with a hypothesis wherein histones are assigned a role of repressor to DNA function. In cell-free enzyme systems, histones can block both the replication of DNA(12) and its function in RNA synthesis(9,10,11). Further, *in vivo* experiments have suggested that DNA synthesis is blocked when high histone to DNA ratios are attained in nuclei preparing for mitosis(23). In the experiments reported in the present study, the cells are not in synchronous division and some of the progressive accumulative changes accompanying viral infection may represent the arrest of cell growth at a normal stage which is achieved only when various cells in the population undergo further degrees of development and a high histone to DNA ratio is attained.

If histones act intracellularly as they do *in vitro,* the early increased rate of histone synthesis could lead to the block in DNA synthesis observed subsequently at the third hour of infection(1). Similarly, the later depressed rate of histone synthesis may merely reflect the inhibitory action of the newly formed histone on the function of DNA in RNA synthesis and the subsequent indirect effect on protein synthesis.

The synthesis of DNA, RNA and protein is interrelated; inhibition of one will be reflected eventually in the others. When the synthetic pattern of the 3 is disrupted, the prime cause should be ascribed to the one first observed to be altered. This requirement is met by the histone. However, specific inhibition of synthesis of a few key enzymes or their corresponding DNA and RNA templates can subsequently stop all polymer synthesis and alteration in these key components would not be reflected initially in the total activity of protein, DNA or RNA.

The arginine-rich and lysine-rich histones observed in these experiments are each presumed to contain multiple components. It is not known if all components in either are stimulated and subsequently depressed. Yet when the 2 are compared, they show much the same pattern. The initial depression of the ethanol-HCl fraction, Fig. 2 (0-20 min), is not regarded as meaningful because of technical difficulties in operating in the short interval after infection. Further examination by electrophoretic separation is necessary to answer whether single key components are altered prior to the general population of molecules and whether these are new compo-

nents produced under direction of the viral genome.

Summary. The distribution of incorporated C^{14}-arginine was determined among fractions (acid soluble histones, acid insoluble protein and DNA) of deoxyribonucleoprotein from HeLa cells. During the early stages of infection of cells with poliovirus, the synthesis of the histones was stimulated. This was followed (third hour) by a depressed rate of synthesis of both histones and a markedly reduced incorporation of arginine into DNA. The interrelations of these effects of infection on components of the histone-DNA-RNA synthetic sequence of the host cell are considered.

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Testosterone in the Urine of Castrated Men.* (30364)

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The futility of evaluating the "androgen burden" in women or children or the "effective androgen concentration" in men on the basis of the levels of androgens or 17-ketosteroids in urine and/or blood or on the basis of the individual 17-ketosteroids in these fluids was discussed(1). It was further indicated that titers of testosterone levels in plasma and urine may be a more significant parameter. This has been established now by the finding of a rather large difference between plasma levels of free testosterone for men and women(2-5) and highly significant differences between the urinary titers of testosterone for men and women. The present communication extends these studies

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