Evidence for a Cellular Ribonucleic Acid Synthesis Inhibitor from Poliovirus-Infected HeLa Cells*

Peter P. K. Ho† and Anna L. Washington

ABSTRACT: An inhibitor of RNA synthesis in HeLa cells was isolated from poliovirus-infected cells. The inhibitor appeared shortly after virus adsorption. The synthesis of DNA and protein was not affected by this inhibitor, although viability of cells declined rapidly after an initial lag period of 5 hr. Properties of this inhibitor are described. It was insensitive to ribonuclease or proteolytic enzymes, but the inhibitory activity was destroyed by phospholipases and heating. The active component can be extracted into an organic solvent and has been characterized as a lipid component. These results suggest that virus infection may induce the release or synthesis of lipid components which operate to shut off cellular RNA synthesis. The inhibitor shortens the latent phase of virus multiplication in treated cells, but has no effect on DNA-dependent RNA polymerases.

Ribonucleic acid synthesis is rapidly inhibited after infection of L cells or HeLa cells with picornavirus (Baltimore, 1969). The inhibition of nRNA synthesis corresponds to a loss of enzyme system responsible for the synthesis of RNA in the uninfected cell (Baltimore and Franklin, 1962). An inhibitor of nDNA-dependent RNA polymerase was reported to appear in the cytoplasm of mengovirus-infected L cells (Balandin and Franklin, 1964). It is trypsin sensitive and has been suggested to be of histone nature (Balandin and Kastrikina, 1967). Unfortunately, this report has never been followed up and is contradicted by the inability of Holland (1962) to find such an effect with lysates of poliovirus-infected HeLa cells.

Contrary to the mengovirus-infected L cells system where RNA synthesis is drastically inhibited within 2 hr after infection, in poliovirus-infected HeLa cells the inhibition is less marked and is not observed until 2 hr after infection accompanied by a slow decline in overall synthesis as infection proceeds (Zimmerman *et al.*, 1963, McCormick and Penman, 1967). The reason for the different results found with different systems seems to reside in the cells and could be accounted for in the above discrepancy.

During our investigation of the inhibitor of cellular RNA synthesis present in the sonicate of poliovirus-infected cells, we observed that synthesis of RNA in intact HeLa cells as measured by tritiated uridine incorporation was rapidly inhibited. As a result of this rather unexpected observation, we have attempted to purify this inhibitor and have partially characterized it as a lipid-containing substance. It appears to differ from the previously reported inhibitor in that it does not inhibit either nDNA-dependent RNA polymerase or partially purified RNA polymerase from *Escherichia coli in vitro*.

Materials and Methods

Cells and Viruses. Large quantities of HeLa cells were propagated in S-MEM[1] with 10% fetal calf serum. The poliovirus used was type I, and was grown according to the methods of Levintow and Darnell (1960). The titer of virus in the lysate was measured by the method of Cooper (1961).

Preparation of Poliovirus-Infected Cell Extract. HeLa cells were concentrated to a density of 1×10^7 cells/ml and were infected with poliovirus type I at a multiplicity of 20 pfu/cell. Following virus adsorption at 37° for 30 min, the infected cells were washed three times with S-MEM and were incubated at 37° for 4 hr in S-MEM containing 10% fetal calf serum at 2×10^5 cells/ml. The infected cells were then harvested by centrifugation, washed three times with cold S-MEM, and kept frozen at −20°. About 1 ml of the frozen infected cells was suspended in 10 ml of S-MEM, and the suspension was sonicated in a Branson sonifier (Heat Systems Co., Melville, N. Y.) at 4° for 5 min. The lysate was centrifuged for 18 hr at 105,000*g*, and the clear supernatant was kept at −20°.

Extraction of Lipids. The clear infected cell extract (10 ml) was injected into 500 ml of ether–ethanol mixture (3:1, v/v) at −20° and kept at −20° for 2 hr with occasional shaking. After centrifugation to remove precipitate, the clear solution was evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml of chloroform ($CHCl_3$) for further separation and assay.

Lipids were separated by chromatography on a column of 10-g silicic acid (Mallinckrodt 100 mesh) and 5-g Hyflo Super Cel (Johns Manville) by elution with 200 ml of $CHCl_3$. The eluate was evaporated to dryness in a rotary evaporator under vacuum. The flask was rinsed with 2 ml of $CHCl_3$ and stored at −20° immediately.

Determination of RNA, DNA, and Protein Synthesis in HeLa Cells. Normal HeLa cells in logarithmic growth were suspended in S-MEM containing 10% fetal calf serum. Inhibitors in infected cell extracts as prepared by the above procedure were added to the suspension at appropriate dilutions. For lipid fractions, a small aliquot (0.05–0.5 ml) of the $CHCl_3$ solution was evaporated to dryness under a stream of nitrogen and the cell suspension was added directly to the vessel. A total of 2.0×10^5 cells/ml was used for the assay and a control cell suspension was performed in every experiment under similar conditions with chloroform.

RNA synthesis was studied using [^{3}H]uridine (2.0 μCi/ml). At various times, 0.5 ml of the suspension was transferred into

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[1] Abbreviations used: S-MEM, Eagle's minimum essential medium for suspension culture; pfu, plaque-formation unit.

2 ml of cold saline. After centrifugation, the cells were suspended in 0.5 ml of water and RNA was precipitated by the addition of 2.5 ml of 10% trichloroacetic acid. The trichloroacetic acid insoluble material was then collected by filtration through Millipore filters. The filters were washed with three 5-ml portions of 5% trichloroacetic acid, dried, and counted in scintillation fluid.

DNA synthesis was studied under similar conditions by using [^{3}H]thymidine (2.0 μCi/ml). When amino acid incorporation ([^{14}C]phenylalanine) was measured, the trichloroacetic acid insoluble material was boiled for 15 min before filtration through Millipore filters.

Enzyme Assay. The assay conditions for DNA-dependent RNA polymerase were measured according to the procedure of Balandin and Franklin (1964) and Nakamoto *et al.* (1964).

Protein and Total Lipid Determination. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Total lipids in the cellular extracts were determined by the method of Entenman (1957). The aqueous solution (1 ml) was lyophilized to dryness, and the residue was extracted twice with 1 ml of hot $CHCl_3$–methanol mixture (3:1, v/v). The combined lipid extract was washed with distilled water, and the dry weight of the organic phase was determined.

Radioisotopes and Enzymes. [^{3}H]Uridine and [^{14}C]L-phenylalanine were purchased from the New England Nuclear Corp. Crystalline ribonuclease, phospholipase C, crystalline trypsin (three-times crystallized), and crystalline chymotrypsin were obtained from Worthington. Wheat-germ lipase and phospholipase D were purchased from Sigma. *Escherichia coli* RNA polymerase was obtained from Miles Laboratories, Inc. HeLa cell nRNA polymerase was prepared according to previously described procedures (Ho *et al.*, 1967).

Results

Inhibition of RNA Synthesis by Poliovirus-Infected Cell Extract. In the initial experiment, RNA synthesis in normal HeLa cells was studied with an extract of infected cells which were concentrated ten times in cell number to that in the assay. The results are shown in Figure 1. RNA synthesis was rapidly inhibited and such inhibition was not reversed by the pretreatment of infected cell extract with ribonuclease. Although the bulk of poliovirus in the infected cell extract has been removed by overnight centrifugation, the virus titers were still 10^3–10^4 pfu/ml, a low but significant amount. The virus/cell ratio present in the assay for inhibitory activity of the extract was 0.01–0.10 pfu/cell. In order to show that this low virus concentration had no effect on cellular RNA synthesis during the first 4- to 5-hr incubation, HeLa cells were incubated with a virus preparation obtained from regular lysate procedures at a titer of 0.5 pfu/cell. No inhibition of RNA synthesis was observed for a period of 4–5 hr (Figure 2). The lack of inhibition at this low multiplicity is within reasonable expectations of poliovirus-infected HeLa cells system, since even at higher multiplicity (100 pfu/cell) no inhibition is observed until 2-hr postinfection (McCormick and Penman, 1967). The poliovirus present in the extract was, therefore, not the factor responsible for the inhibitory activity observed for the duration of the assay (4 hr). An extract of uninfected HeLa cells which had undergone mock-virus infection also did not inhibit RNA synthesis (Figure 1). The amounts of total proteins and lipids in the normal and infected cell extract are not significantly different (2.5 mg/ml of protein and 1.01–1.11 mg/ml of total lipid). The assay media contains approximately

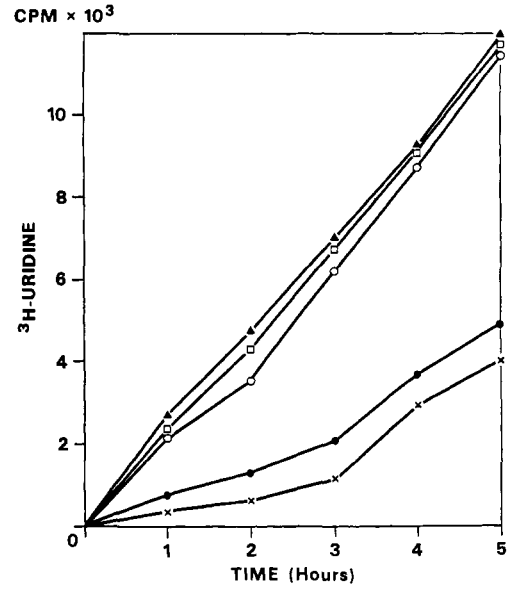

FIGURE 1: Inhibition of RNA synthesis by poliovirus-infected cell extract. Poliovirus-infected cell extract was prepared as described in Materials and Methods. RNA synthesis was measured by [^{3}H]-uridine incorporation into trichloroacetic acid insoluble material at selected intervals after addition of isotope: (○) no addition; (●) = infected cell extract (10-fold dilution); (×) RNase-treated and dialyzed infected cell extract; (▲) heated infected cell extract; (□) normal cell extract.

250 μg/ml of protein and 101–111 μg/ml of lipid. A dilution of the infected-cell extract to 20-fold decreased the inhibitory activity by approximately 50%, while inhibitory activity could not be demonstrated even with an undiluted normal HeLa cell extract.

Effect of Poliovirus-Infected Cell Extract on DNA and Protein Synthesis and Cell Viability. Treatment of cells with the extract under similar conditions for RNA synthesis resulted in slight inhibition of DNA and protein synthesis (Figure 3)

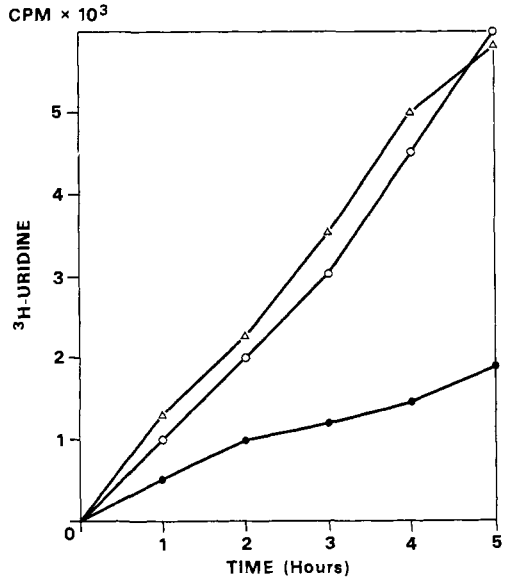

FIGURE 2: Effect of poliovirus on RNA synthesis at low virus/cell ratio (0.5 pfu/cell). RNA synthesis was measured as in Figure 1: (○) no addition; (△) 0.5 pfu/cell; (●) infected cell extract.

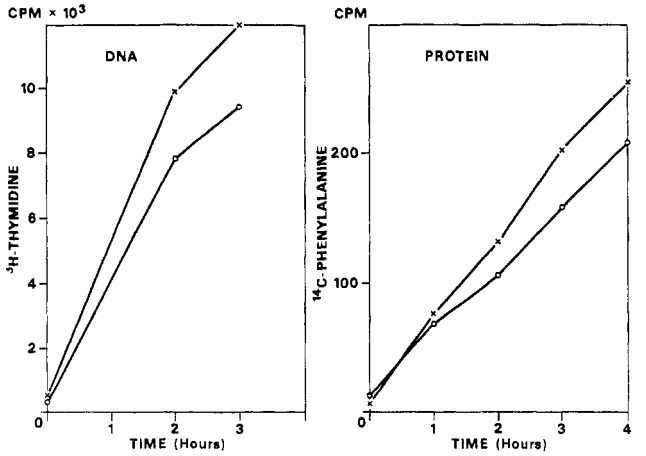

FIGURE 3: Effect of infected cells extract on DNA and protein synthesis: (A) DNA synthesis as measured by incorporation of [^{3}H]-thymidine into trichloroacetic acid insoluble material; (B) protein synthesis, as measured by incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid insoluble material; (×) no addition; (○) with extract (10-fold dilution).

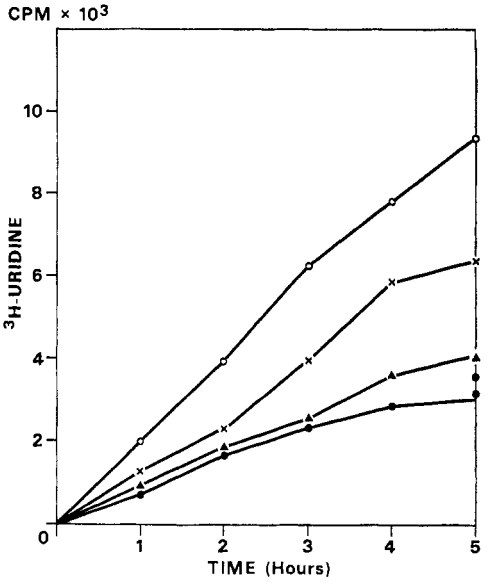

FIGURE 5: Inhibition of RNA synthesis by extracts of infected cells prepared at various times after virus infection. Samples of poliovirus-infected cells were taken at selected intervals after virus adsorption and the extracts of these cells were assayed for their inhibitory activity on normal HeLa cell RNA synthesis at a 10-fold dilution. (○) No addition; (×) 1-hr postinfection extract; (▲) 2-hr postinfection extract; (●) 4-hr postinfection extract.

for a duration of 4 hr. When the cells were incubated in the presence of the extracted inhibitor (see section on Extraction), the number of viable cells remained unchanged for 4–5 hr (Figure 4), but decreased rapidly subsequent to the initial lag period. The remaining poliovirus in the infected-cell extract has been completely removed or inactivated during lipid extraction, and the observed cell death was due possibly to the rapid inhibition of RNA synthesis, although we have not examined DNA and protein synthesis in detail.

Time Dependence on the Appearance of Inhibitor. Extracts were prepared from cells infected for 1, 2, and 4 hr, according to the procedure described in the Materials and Methods section. The inhibitory activity was observed at 1 hr and increased rapidly to maximum at 2–4 hr (Figure 5). Under our assay conditions the amounts of inhibitor present in the extract is difficult to be determined quantitatively. The experimental results, however, do indicate a twofold increase in activity between the extracts from 1- and 2-hr infection. Thus, the inhibitor would probably represent an early component synthesized during virus multiplication.

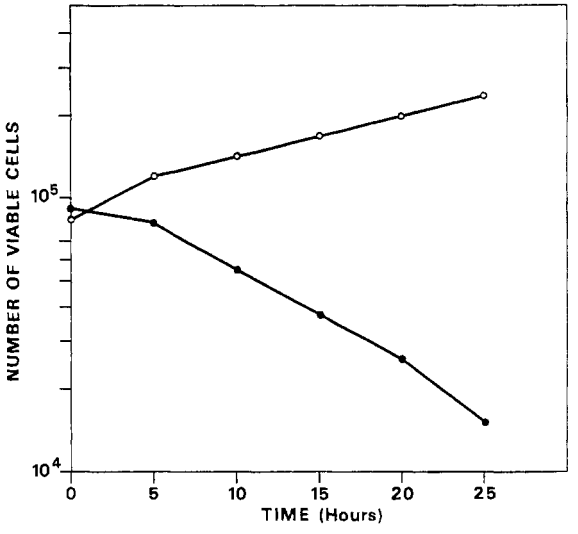

FIGURE 4: Cell death curve in the presence of inhibitor. HeLa cells at 8–9 × 10^4 cells/ml were grown in S-MEM containing 10% fetal calf serum. Viable cells were counted in tryptan blue. (○) No addition; (●) extracted lipid inhibitor, 30 μg/ml.

Effects of Enzymes and Temperature on the Inhibitor. Some information was sought concerning the nature of the inhibitor in the infected cells. In light of the previously reported inhibitor being a protein, the infected cell extract was dialyzed overnight against S-MEM. No loss of inhibitory activity was observed (Figure 1). Although the inhibitor is very stable at 4°, its activity is destroyed by heating at 98° for 15 min. The inhibitor could be precipitated by adjusting the extract to 80% saturation with $(NH_4)_2SO_4$. Since these results tend to imply that the inhibitor is protein in nature, attempts were therefore made to inactivate the inhibitor by proteolytic enzymes. The results shown in Figure 6 reveal that the inhibitor is not destroyed by trypsin or chymotrypsin even after incubation at 37° for 1 hr. Control cells were assayed in the presence of trypsin or chymotrypsin, and their RNA synthesis patterns were similar to those assayed under normal procedures. The experimental results imply that the inhibitor is probably not a protein, but would suggest that it is either a lipoprotein or lipid. Macromolecules such as DNA and RNA have been excluded previously by the treatment of extract with nucleases (see Figure 1).

This hypothesis is substantiated by the observation that incubation of infected cell extract with phospholipase C or phospholipase D completely destroys the inhibitory activity (Figure 6). From these results, one can infer that the inhibitor is a lipid-containing substance.

Extraction of Lipid from Infected Cell Extract. As a working hypothesis, we postulated that the inhibitor could be a lipoprotein. The infected cell extract was, therefore, extracted in an ether–alcohol mixture (3:1, v/v) according to the method of Scanu *et al.* (1958), for the extraction of lipid component from lipoproteins.

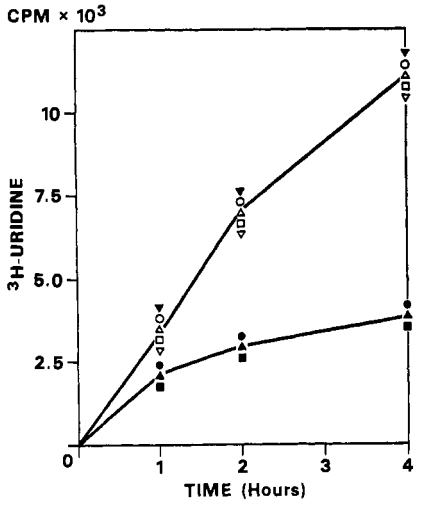

FIGURE 6: Effects of enzymes on the inhibitor. The infected cell extract in S-MEM was incubated at 37° with 100 μg/ml of enzyme for 2 hr. Fetal calf serum was then added to 10% in concentration, and the mixture was assayed for its inhibitory activity on RNA synthesis as described in Figure 1. A control of each enzyme was carried out with S-MEM. (○) No addition; (△) in the presence of trypsin; (□) in the presence of chymotrypsin; (▽) in the presence of phospholipase C or D; (●) with extract; (▲) extract treated with trypsin; (■) extract treated with chymotrypsin; (▼) extract treated with phospholipase C or D.

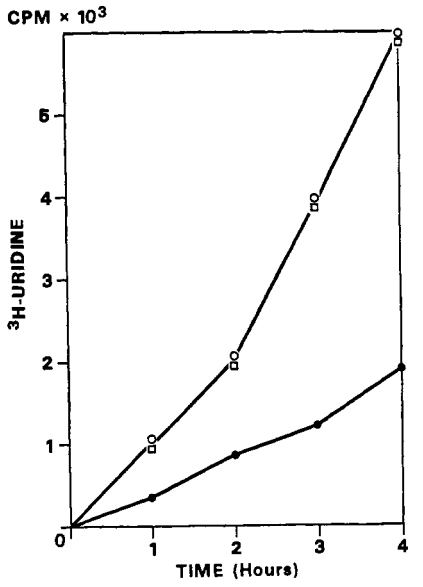

FIGURE 7: Inhibitory activity of fractions obtained by organic solvent extraction. The lipid and protein fractions were prepared as described in Materials and Methods. The insoluble protein fraction was suspended in S-MEM containing 10% fetal calf serum and diluted 10-fold before the addition of HeLa cells (2×10^5 cells/ml). The lipid fraction in $CHCl_3$ was evaporated to dryness and diluted 10-fold with serum containing S-MEM. (○) No addition; (●) lipid fraction, 30 μg/ml; (□) protein fraction.

The data in Figure 7 reveal that the inhibitory activity was recovered in the organic phase which contains 50–75% activity of the original extract, and 300 μg/ml of dry weight. The protein fraction was totally inactive. The extracted inhibitor was also sensitive to phospholipase C treatment. Furthermore, no poliovirus was detected in the organic phase.

Residue recovered from the organic solvent was further purified by column chromatography on silicic acid. Initially, after the application of lipid fraction in $CHCl_3$ onto the silica gel column (1 × 30 cm), the column was eluted with 200 ml of $CHCl_3$. The eluate was shown to contain the inhibitory activity when assayed in the HeLa cells system. No further inhibitory activity was observed in fractions eluted with the following solvents: chloroform–methyl alcohol 4:1, 200 ml; 2:1, 200 ml; and 2:3, 200 ml; and pure methyl alcohol, 200 ml. These results suggested that the inhibitor is not a known phospholipid, since phospholipids are not ordinarily eluted by chloroform (Chang and Sweeley, 1963). Interestingly, phospholipases C and D do inactivate this inhibitor. But since the purity of both enzyme preparations has not been established, other types of lipases may account for the inactivation of this inhibitor.

Examination of the products in the eluate by thin-layer chromatography in chloroform reveals two components either by phosphomolybdate reagent or sulfuric acid spray. Further purification and total characterization of this inhibitor have been hindered by availability of material.

Effect of the Inhibitor on DNA-Dependent RNA Polymerase. The experiments presented thus far had established the inhibition of RNA synthesis in intact HeLa cells, and the mechanism of such inhibition was considered. Previous investigators have detected an inhibitor in infected cell extract by assay with nuclear RNA polymerase (Balandin and Franklin, 1964). When the extract isolated under our procedures was assayed with HeLa nuclear or *E. coli* RNA polymerase, no significant inhibition was observed (Table I). These data suggest that the virus-induced inhibitor reported here is different from that studied by Balandin and Kastrikina (1967). The question of its function in the virus multiplication cycle was therefore considered.

Effect of the Inhibitor on the Latent Period in Virus Production. For 2 hr after infection, no new virus was detectable. During the latent period, not only is no virus produced, but there is very little viral RNA or protein synthesis. Release of viral genome from the particles and synthesis of viral RNA polymerase are generally presumed to account for such a lag period. However, since one of the earliest events during picornavirus infection is the rapid inhibition of cellular RNA and protein synthesis, an alternate determinating factor for the

TABLE I: Effect of Infected Cell Extract on DNA-Dependent RNA Polymerases.[a]

System	[^{14}C]GMP (cpm Incorp)	
	A	B
Complete	1240	6670
Omit CTP	160	0
Add 0.1-ml extract	1100	5920

[a] The assay conditions for column A are those of HeLa nDNA-dependent RNA polymerase (Balandin and Franklin, 1964). To each reaction mixture, 0.2 ml of nuclear preparation (3 mg of protein/ml) was added. The assay conditions for column B are those of *E. coli* RNA polymerase (Nakamoto *et al.*, 1964). Purified enzyme (3 μg) was added to each reaction mixture. The extract was prepared according to the method described in the procedures.

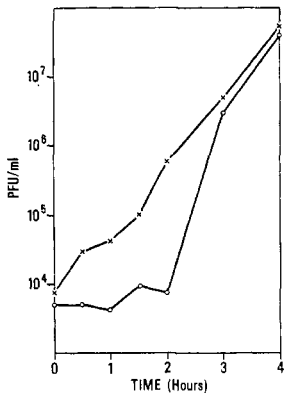

FIGURE 8: Effect of inhibitor on latent phase in virus multiplication. (×) Cultures of 20 ml (2×10^5 cells/ml) were suspended with 2 ml of S-MEM containing 300 μg/ml of the lipid fraction at 37°. After 2 hr, the cultures were centrifuged, washed twice with S-MEM, and resuspended in type I poliovirus at input multiplicity of 1.0. After 30 min at 37°, the cultures were centrifuged and resuspended in 20 ml of virus-free media containing 10% fetal calf serum. At various times 1.0-ml samples were removed, frozen, and thawed twice and the supernatants were assayed for plaque formation units as described previously (Cooper, 1961). (○) The control cultures (20 ml) were treated with S-MEM under the same conditions for infected cell extracts. After 2 hr, the cultures were infected and resuspended and samples were taken as described above for virus titration.

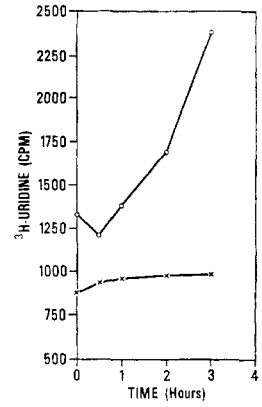

FIGURE 9: RNA synthesis from intracellular uridine pool. Cultures (7 ml; 2×10^5 cells/ml) were incubated at 37° in the presence of 2 μCi/ml of [^{3}H]uridine. After 1 hr, the cultures were centrifuged, washed twice with S-MEM and resuspended in 2.0 ml of S-MEM containing 10% fetal calf serum. (○) The labeled cultures (1 ml) was suspended with 2.5 ml of S-MEM containing 10% fetal calf serum. At various times, duplicate samples of 0.25 ml were removed and RNA synthesis was measured as described in Materials and Methods. (×) The labeled culture (1 ml) was suspended in the serum media but containing 30 μg/ml of lipid fraction.

length of the latent period could be the extent of inhibition of cellular RNA and protein synthesis. In fact, Baltimore (1969) has suggested that picornavirus eclipse should be viewed as a priming period, a time when host functions are being eliminated and viral functions are accelerating. The validity of this theory could now be tested by studying virus growth cycle in the presence of the isolated inhibitor. The results in Figure 8 show that virus is produced 30- to 60-min postinfection in the treated cells, although there is no increase in total virus production at the end of the growth cycle. By adsorbing the virus at 37° for 30 min, the eclipse period due to the uncoating of virus particles has probably taken place before the infected cells are resuspended in fresh media. Thus, under our assay conditions the removal of latent period by the inhibitor suggests that the inhibitor may participate in some of the early events of virus multiplication, such as inhibition of cellular RNA synthesis. However, such inhibition cannot be accounted for as the only contributing factor for the latent period, since inhibition of cellular RNA synthesis by actinomycin D does not either abolish or shorten the latent period. We believe that our inhibitor has yet another unknown function and that its effect on cellular RNA synthesis is part of the mechanism in accelerating virus synthesis.

Inhibition of RNA Synthesis from Intracellular Uridine Pool by the Lipid Component. In the experiments presented here, the incorporation of [^{3}H]uridine into cellular RNA is used to measure RNA synthesis and the effect of such inhibition. The observed inhibition could therefore be either due to an alteration on uridine uptake or RNA synthesis. However, the fact that uptake of thymidine and amino acids are not affected in the presence of inhibitor would suggest that cell membrane permeability to metabolites is not drastically altered. Furthermore, we doubt that inhibition of uridine uptake by this inhibitor would affect the latent period of virus multiplication.

An experiment to study the direct effect of the inhibitor on the rate of cellular RNA synthesis from the intracellular uridine pool was therefore performed. After exposure of HeLa cells to [^{3}H]uridine for 1 hr, 25–50% of the labeled precursors taken up by the cells were present as acid-precipitable material (P. P. K. Ho and A. L. Washington, unpublished data). The isotope was removed from the media and the cells were grown for 3 hr in the presence and absence of inhibitor. The radioactivity in the RNA was followed in the two samples. The removal of labeled precursor did not stop further uridine incorporation into the RNA of untreated cells (Figure 9) In contrast, immediate cessation of RNA synthesis occurred in the presence of inhibitor.

Discussion

Poliovirus infection of HeLa cells in suspension culture results in a rapid decline in the rate of synthesis of both RNA and protein. One of the viral specific proteins made early in infection has been suggested to be the inhibitor of RNA synthesis in host cells (Penman and Summer, 1965). This hypothesis is supported by the evidence that both puromycin and *p*-flurophenylalanine can suppress the inhibition of host cell RNA synthesis (Baltimore *et al.*, 1963). Thus the inhibition is manifested only after a period of protein synthesis following virus adsorption. The exact nature of this inhibitor has never been clearly studied, although the cytoplasm of poliovirus-infected cells has been shown to contain a trypsin-sensitive factor which will inhibit DNA-dependent RNA synthesis in nuclei from uninfected cells (Balandin and Franklin, 1964).

In the interpretation of the experiments presented in this report, the inhibitory agent produced by poliovirus is suggested to impart a direct effect on cellular RNA synthesis. The exact site of this action, however, is not certain. Experiments with DNA-dependent RNA polymerases show that the enzymes are not inhibited *in vitro*. A plausible explanation of the finding reported here, then, is that the inhibitor, being lipid in nature, triggers a shut-off mechanism in RNA synthesis *via* its interaction with nuclear membrane or as a component in the membrane structure of the replicative complex. However, direct evidence is lacking to support this hypothesis.

The possibility that this inhibitor may have an effect on uridine utilization, mediated through its uptake or conversion into other uridine-containing metabolites, cannot be excluded. Our present studies are directed toward further characterization of this inhibitory factor and to a further study of its mode of action.

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Reaction of Pseudouridine and Inosine with *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide*

Nancy W. Y. Ho and P. T. Gilham†

ABSTRACT: Inosine or its 5'-phosphate reacts with the *p*-toluenesulfonate salt of *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)-ethylcarbodiimide in aqueous solution at pH 8–8.5. The product of the reaction is a 1:1 adduct in which the nucleoside or its phosphate is attached to the substituted carbodiimide cation (presumably at the N_1 position of the nucleoside base). The reaction of the reagent with pseudouridine is more complicated in that two monosubstituted compounds (N_1 and N_3) are formed initially and these react further to give the N_1,N_3-disubstituted derivative. This product is unstable at the pH of the reaction mixture and partly hydrolyzes to give the stable N_3-substituted compound. The N_3 derivative is stable under the hydrolytic conditions which will remove carbodiimide groups from uridine, guanosine, inosine, and 5-methyluridine, and this property thus constitutes a method for the specific chemical modification of pseudouridine positions in RNA molecules. In addition, the N_3 substitution of pseudouridine in a polynucleotide confers resistance to the action of pancreatic ribonuclease at the phosphodiester linkage attached to the 3' position of the pyrimidine nucleoside. Pseudouridylyl-(3'–5')-adenosine derivatized in this way is not attacked by this ribonuclease whereas derivatized cytidylyl-(3'–5')-pseudouridine is converted by action of the enzyme into cytidine 3'-phosphate and the carbodiimide derivative of pseudouridine.

Those ribonucleosides and deoxyribonucleosides whose bases have p*K* values in the vicinity of 9 have been shown to react with the Cmc[1] cation *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide (I) (see Chart I) (Gilham, 1962; Lee *et al.*, 1965; Naylor *et al.*, 1965; Ho and Gilham, 1967; Ho *et al.*, 1969). The reactions proceed rapidly in water at pH 8–9 and under these conditions nucleosides such as adenosine and cytidine and their deoxyribo counterparts do not react. The products are thought to be stable guanidine derivatives in which the carbodiimide group of the Cmc cation is substituted at the N_3 positions in pyrimidines (uridine and thymidine) and at the N_1 positions in the purines (guanosine and deoxyguanosine) (Gilham, 1962; Ho and Gilham, 1967). The phosphate derivatives of these nucleosides undergo a similar reaction as do the guanine, uracil, and thymine moieties in polynucleotides and nucleic acids. In the case of polynucleotides and nucleic acids, the conditions under which these chemical modifications are made and the conditions necessary for the subsequent removal of the Cmc groups are of a sufficiently mild nature that the structural integrity of the poly-

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† To whom to address correspondence.

[1] Abbreviation used is: Cmc, *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide.