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Early Inhibition of Cellular DNA Synthesis by High Multiplicities of Infectious and UV-Inactivated Reovirus

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Inhibition of cellular DNA synthesis began 6 to 8 h after reovirus infection at a multiplicity of infection of 10 PFU per cell. However, as the multiplicity of infection was increased to a maximum of 10^3 PFU/cell, inhibition of DNA synthesis began earlier after infection (2-4 h postinfection), and the initial rate of inhibition increased. The enhanced inhibition of DNA replication at high virus multiplicities appeared to be selective since RNA synthesis was not detectably altered as late as 9 h postinfection and inhibition of protein synthesis did not begin until 7 to 9 h after infection. Early inhibition of DNA synthesis did not appear to be related to changes in thymidine pool characteristics, thymidine kinase activity, or detectable degradation of cellular DNA. Even though the particle-to-PFU ratio was increased by ultraviolet light inactivation of virus, the ability to induce early inhibition of DNA synthesis was not diminished.

Inhibition of cellular DNA synthesis begins 8 to 10 h after reovirus infection (9). This inhibition is unique in that similar modifications of the synthesis of cellular RNA and protein apparently do not occur (7, 9, 14). There is no detectable degradation of cellular DNA or alteration of the activity of certain enzymes involved in DNA synthesis (8) and no apparent modification of the rate of DNA chain growth during the early stages of this inhibition process (8, 11, 12). It has been proposed that this selective alteration of the replicative function of cellular DNA during reovirus infection may be due to an inhibition of the initiation of the synthesis of new DNA chains (8, 11, 12).

The selective nature of this inhibition process offers a unique opportunity to attempt to establish a relationship between certain events in the replication cycle of a virus and a specific change in cellular metabolism, and to use this phenomenon to further define the mechanisms which regulate cellular DNA functions. In this study we describe the effects of high multiplicities of infectious and UV-inactivated reovirus on the synthesis of cellular DNA.

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MATERIALS AND METHODS

Cells. Suspension cultures of mouse fibroblasts, strain L-929, were cultivated in spinner-modified minimal essential medium (MEM) supplemented with 7% fetal calf serum (FCS). Suspension cultures were maintained in exponential growth at 37 C. For experimental purposes, cells from stock cultures were resuspended in fresh medium to a cell concentration of 2×10^5 cells/ml.

Monolayer cultures of L cells were prepared by adding 2×10^5 cells from suspension culture to 30-ml plastic culture flasks (Falcon Plastics). After cell attachment (37 C for 1 h) the spinner medium was removed and replaced with 5 ml of prewarmed basal Eagle medium (BME) with 7% FCS. These conditions permitted asynchronous growth of cells out of suspension.

Virus. Reovirus type 3, Dearing strain, kindly provided by P. J. Gomatos, was propagated in L cell suspension cultures. Approximately 10 PFU of virus per cell were added to a suspension culture concentrated to 5×10^6 cells/ml in MEM-Spinner medium containing no serum. After adsorption for 2 h at 37 C, the infected cell suspension was diluted to 5×10^5 cells/ml with fresh, prewarmed spinner medium containing 3% FCS. Sixteen hours after infection, the cells were chilled in an ice bath and centrifuged for 10 min at $1,200 \times g$, and the pellet was frozen at −60 C until virus purification.

Virus purification. All experiments were conducted with virus which was purified from infected cells by the method of Bellamy et al. (2).

Virus titration. Virus was titered on monolayers of L cells. The procedure was essentially that described by Gomatos et al. (10). Approximately 3×10^6 cells from suspension cultures were added to 30-ml plastic culture flasks. After cell attachment, the spinner medium was replaced with 5 ml of BME supplemented with 7% FCS. The cultures were used for titration no later than 24 h after confluency was reached. BME monolayer medium with no serum was used to wash the cultures twice before use and for virus dilution. A fraction (0.25 ml) of each virus dilution was added to a culture and allowed to adsorb for 2 h at room temperature. After adsorption, 5 ml of BME containing 0.9% Ionagar supplemented with 3% FCS was added. Three days after the first overlay, a second 5-ml overlay was added. Three days later the third and final 5-ml overlay was added which included 0.005% neutral red. Plaques were counted 24 h after the third overlay.

UV irradiation of virus. Purified reovirus in calcium-magnesium-free, phosphate-buffered saline was irradiated by using a General Electric UV lamp emitting 95% of its wavelength at 253.7 nm. Virus at a concentration of approximately 10^9 PFU/ml was irradiated at 4 C in the wells of a spot plate (0.4 ml/well) for selected periods of time up to 60 min. The virus preparations were exposed to approximately 11 ergs per s per mm^2.

Thymidine kinase assay. Thymidine kinase activity was determined as described by Ledinko (15). Cellular protein was prepared from 2×10^6 cells. Reaction mixtures of 1 ml were made 3.3 μCi/ml with ^{3}H-thymidine. The reaction was terminated after 30 min, and 5-μliter samples of the reaction mixture were spotted on Whatman no. 1 paper. Thymidine phosphates were separated from thymidine by descending chromatography using an aqueous mixture of isopropanol and ammonia as the solvent (4). Areas with R_f values corresponding to that of thymidine-5'-monophosphoric acid were cut from the chromatograms, and the radioactivity was determined. Protein quantitation was performed as described by Lowry et al. (18).

Measurement of DNA, RNA, and protein synthesis. DNA, RNA, and protein synthesis were measured at selected time intervals after infection by pulse-labeling cells with either ^{3}H-thymidine (0.5 μCi/ml) for 30 min, ^{3}H-uridine (1 μCi/ml) for 1 h, or a mixture of ^{14}C-labeled amino acids (0.045 μCi/ml) for 30 min. At the end of the labeling period, the pulse-labeled samples were mixed with an equal volume of cold 10% trichloroacetic acid, and the radioactivity in the trichloroacetic acid-insoluble material was determined.

Thymidine pool assay. Monolayer cultures in exponential growth were used. Infected (300 PFU/cell) and control cultures were pulse-labeled with ^{3}H-thymidine (0.5 μCi/ml) at selected times after infection. At the end of the labeling period, the medium was removed and the cultures were washed twice with cold BME without serum. After the washing, 2 ml of cold 5% trichloroacetic acid was added to each culture, and the flasks were stored at 4 C for 30 min. The trichloroacetic acid was removed (trichloroacetic acid-soluble fraction); the trichloroacetic acid-insoluble material which remained fixed to the flask surface was washed twice with 2 ml of cold 5% trichloroacetic acid, and the washings were discarded. After the washing, 2 ml of cold 5% trichloroacetic acid was added, and the cells were removed by briefly touching each flask to the probe of a sonic oscillator. The step was repeated, and the samples were pooled (trichloroacetic acid-insoluble fraction). To facilitate radioactivity determination, the trichloroacetic acid-insoluble fractions were hydrolyzed at 95 C for 15 min. The insoluble material remaining after hydrolysis was discarded, and the supernatant fluid was saved. Fractions were removed from trichloroacetic acid-soluble and -insoluble samples, and the radioactivity in each was determined.

Gradient analysis of DNA. The method of Colter et al. (5) was used to extract DNA from control and infected (300 PFU/cell) cells 8 h after infection. Prior to infection, asynchronous suspension cultures were labeled with 0.5 μCi of ^{3}H-thymidine per ml for 48 h. DNA, isolated from control and infected cells, was dissolved in 0.2 M potassium phosphate buffer, pH 7.0. A 0.1-ml amount of each sample (70,000 counts/min) was sedimented for 4 h at 4 C through a 5-ml 5 to 20% linear sucrose gradient (pH 8 or 11.5) at 39,000 rpm by using a Spinco SW-39 rotor. After centrifugation, 7-drop fractions of each gradient were collected directly into scintillation vials, and the radioactivity was determined.

Radioactivity determinations. All radioactivity was measured by scintillation counting in a Beckman DPM-100 liquid scintillation counter using Beckman cocktail D scintillator (5 g of diphenyloxazole, 100 g of naphthalene, and dioxane to 1 liter).

Isotopes. ^{3}H-Thymidine (17.9 Ci/mmol) and ^{3}H-uridine (26.6 Ci/mmol) were purchased from New England Nuclear Corp. L-Arginine (198 Ci/mmol), L-leucine (240 Ci/mmol), and L-glutamic acid (218 Ci/mmol) were purchased from International Chemical and Nuclear Corp.

RESULTS

Effect of high multiplicities of infection on DNA synthesis. To test the effects of high multiplicities of reovirus infection on DNA synthesis in L cells, three cultures were infected with 10, 10^2, or 10^3 PFU/cell, and DNA synthesis was measured at selected time intervals after infection. It was apparent that the multiplicity of infection not only affected the time of inhibition of cellular DNA synthesis, but also the rate at which the inhibition progressed (Fig. 1). At low multiplicities of infection (10 PFU/cell), inhibition of DNA synthesis did not begin until 6 to 8 h after infection which is in agreement with previous reports (14). At higher multiplicities (10^2 or 10^3 PFU/cell), inhibition began as

early as 2 to 4 h postinfection, and the initial rate of inhibition increased with increasing input multiplicities. Cells infected at high virus multiplicities were productively infected, yielding approximately one-half the amount of infectious virus as cells infected with 10 PFU/cell. Infectious progeny virus was detected after 5 h in cells infected with 250 PFU/cell, with maximal virus production occurring at 13 h (unpublished results). Thus at high multiplicities of infection, inhibition of DNA synthesis preceded the detection of infectious progeny virus.

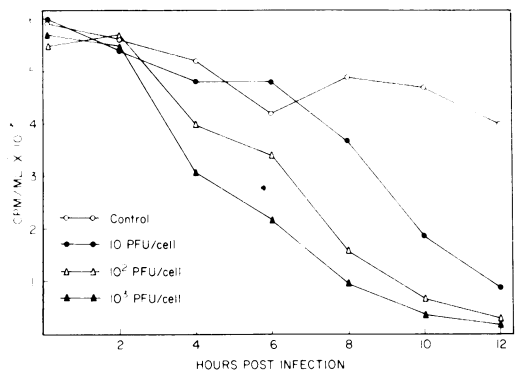

FIG. 1. *DNA synthesis in cells infected with 10, 10^2, and 10^3 PFU/cell of reovirus. At the times indicated, 1.5×10^6 cells from infected and control cultures were pulse-labeled with ^{3}H-thymidine. Trichloroacetic acid-insoluble counts per minute per milliliter of culture are presented.*

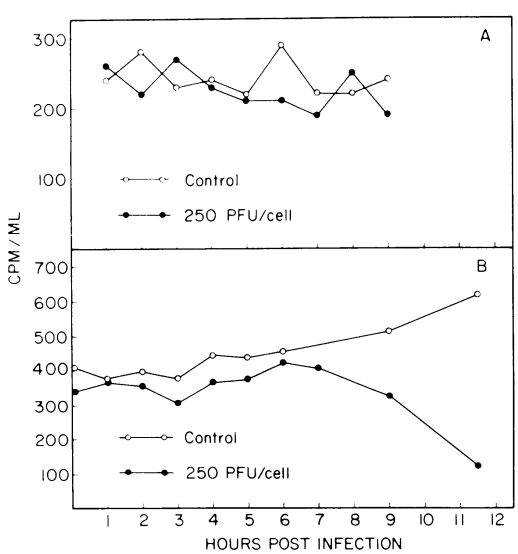

FIG. 2. *RNA and protein synthesis in L cells infected with 250 PFU/cell of reovirus. At the times indicated, 3×10^6 or 1.5×10^6 cells were pulse-labeled with ^{3}H-uridine (A) or ^{14}C-amino acids (B), respectively. Trichloroacetic acid-insoluble counts per minute per milliliter of culture are presented.*

Effect of high multiplicities of infection on RNA and protein synthesis. It was of interest to examine the effect of high multiplicities of reovirus infection on RNA and protein synthesis for two reasons. First, since the alteration of macromolecular synthesis in cells appears to be selective for DNA replication at low multiplicities of infection (7, 9, 14), it was necessary to determine whether this selectivity was maintained at higher multiplicities of infection during accelerated inhibition of DNA synthesis. Second, it was necessary to examine the possibility that early inhibition of DNA synthesis at high input multiplicities might be related to similar modifications of RNA and protein synthesis. There was no significant alteration of total RNA synthesis as late as 9 h after infection in cultures infected with 250 PFU/cell (Fig. 2A). A progressive inhibition of protein synthesis began between 7 and 9 h postinfection at 250 PFU/cell (Fig. 2B). However, inhibition of protein synthesis occurred several hours after the beginning of the inhibition of DNA synthesis. Thus, it appeared that the inhibition of DNA synthesis was selective and was not reflected in immediate changes in RNA and protein synthesis. Since total RNA and protein synthesis was measured in infected cells, obscuration of minor changes in cellular macromolecular synthesis by concomitant synthesis of viral nucleic acids and protein cannot be excluded, particularly in the later stages of the virus replication cycle.

Precursor pool characteristics in cells infected at high input multiplicities. The incorporation of nucleic acid precursors into mammalian cells as a measure of DNA synthesis may yield misleading information, particularly if precursor pool changes occur in cells (22). Modified uptake or leakage of nucleic acid precursors was of particular concern in experiments measuring DNA synthesis in cells exposed to high multiplicities of virus. For this reason, the effect of high multiplicities of reovirus infection on the ability of cells to maintain a constant pool of tritium, supplied exogenously as ^{3}H-thymidine, was examined. The radioactivity in trichloroacetic acid-soluble and -insoluble fractions from infected (300 PFU/cell) and control cultures at selected times after infection was determined. The results (Fig. 3) indicated that inhibition of DNA synthesis (trichloroacetic acid-insoluble counts/min) began 4 to 6 h after infection, whereas the radioactivity in trichloroacetic acid-soluble material remained

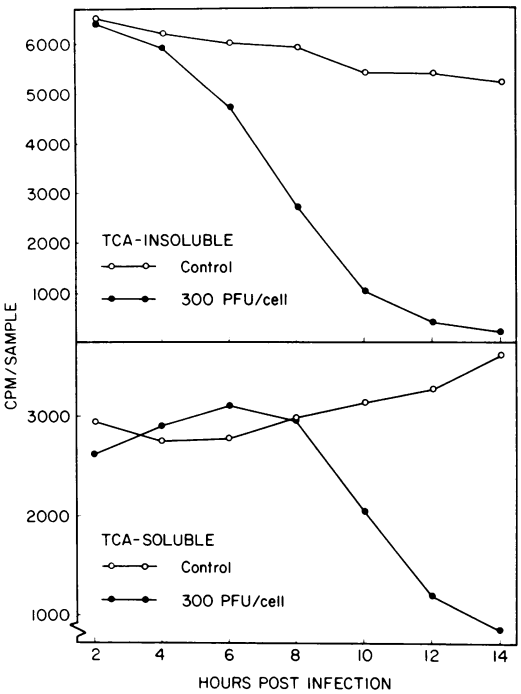

FIG. 3. *Comparison of the incorporation of ^{3}H-thymidine into the trichloroacetic acid soluble and insoluble fractions of control and infected cultures. Monolayer cultures in exponential growth were infected with 300 PFU/cell. At the times indicated, 2 × 10^5 cells from infected and control cultures were pulse-labeled with ^{3}H-thymidine, and the trichloroacetic acid-soluble and -insoluble counts per minute were determined.*

constant as late as 8 h after infection. After 8 h, however, there was a marked and progressive decrease in the radioactivity of the trichloroacetic acid-soluble material of infected cells. These results strongly suggest that the early inhibition of DNA synthesis in infected cells was not due to leakage of nucleic acid precursors.

Thymidine kinase activity of infected cells. The activities of certain enzymes directly involved in the synthesis of DNA are not modified after infection with low input multiplicities of reovirus (8). However, early inhibition of DNA synthesis after infection at high multiplicities could be a reflection of the altered synthesis or activity of such enzymes. For this reason, the specific activity of thymidine kinase, an enzyme which is synthesized immediately before the initiation of DNA synthesis in L cells (16), was examined in cells infected with high multiplicities of reovirus. The results (Table 1) show that the specific activity of thymidine kinase was essentially the same in control cells and cells infected with 250 PFU/cell.

Sedimentation profiles of DNA from infected and uninfected cells. The initial reaction of cells to infection by high input multiplicities of virus could be manifested in partial or complete degradation of cellular DNA, which could account for the observed early inhibition of cellular DNA synthesis following reovirus infection. To investigate this possibility, sedimentation profiles of DNA extracted 8 h after infection were analyzed on sucrose gradients at pH 8 or pH 11.5. Cellular DNA was labeled with ^{3}H-thymidine for 48 h before infection with approximately 300 PFU of reovirus per cell. No significant difference was detected in the sedimentation characteristics of DNA isolated from control and infected cultures (Fig. 4), suggesting that extensive degradation of DNA did not occur after infection. These results are in agreement with the work of Ensminger and Tamm (8) in which no detectable degradation of cellular DNA was seen in cells infected with low input multiplicities of reovirus.

Effect of high multiplicities of UV-inactivated reovirus on DNA synthesis. Since the time of inhibition of DNA synthesis appeared to be related to the number of infectious reovirus particles infecting a cell, it was of interest to determine whether the physical particle alone (noninfectious virus) was capable of altering the synthesis of DNA. To obtain high particle to PFU ratios, a purified suspension of reovirus was irradiated with UV light. The reduction in infectious titer (PFU/ml) of a sample of reovirus after exposure to UV light for time periods up to

TABLE 1. *Specific activity of thymidine kinase in infected (250 PFU/cell) and uninfected spinner cells*[a]

Hours post-infection	Sp act			
	Control[b] (× 10^{-3})	Infected[b] (× 10^{-3})	Control[c]	Infected[c]
1	2.4	3.1	26.7	29.5
2	2.2	2.6	26.3	26.6
3	2.2	2.8	25.3	28.7
4	2.2	3.0	26.9	28.1
5	2.3	2.8	26.7	29.8
6	1.9	2.8	28.1	28.9

[a] Thymidine kinase activity was determined by the procedure of Ledinko (15), using ^{3}H-thymidine. Cell protein from 2 × 10^6 cells was used to determine each figure above.

[b] Picomoles of thymidine (TdR) converted to TdR phosphates per 20 min per μg of cell protein.

[c] Counts per minute of TdR converted to TdR phosphates per 30 min per cell.

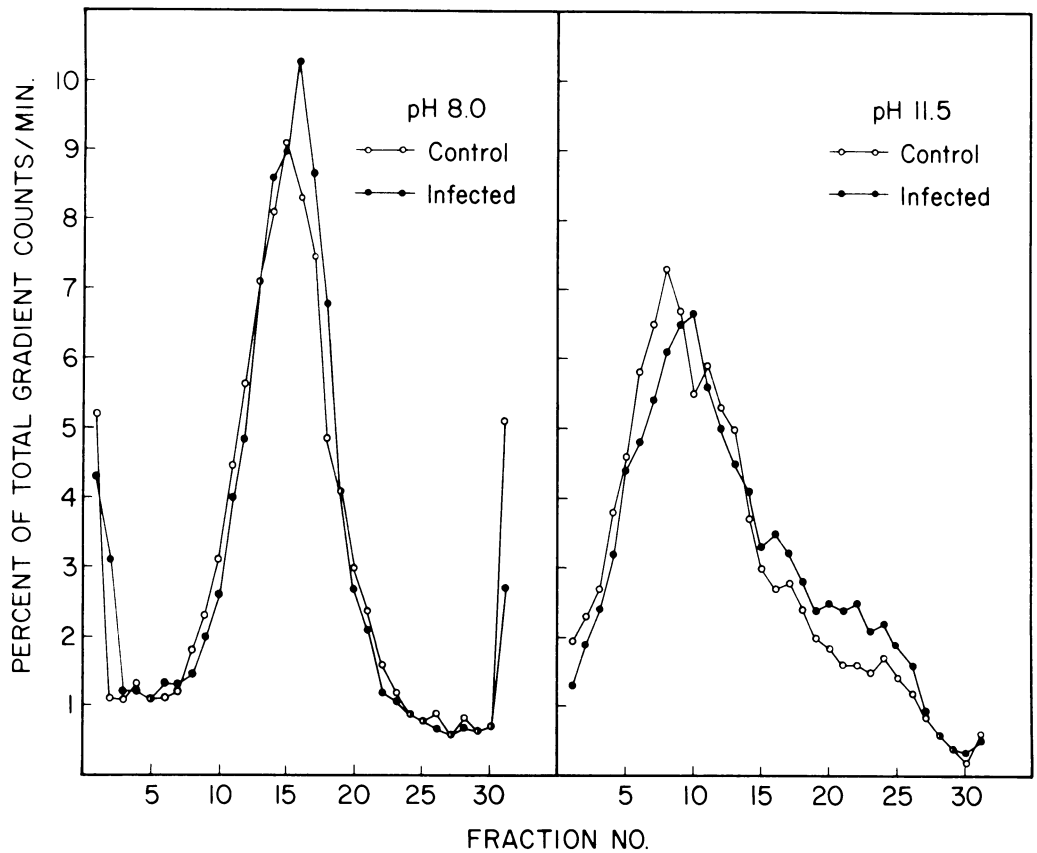

FIG. 4. *Sedimentation profiles of DNA isolated from control and infected (300 PFU/cell) cultures 8 h after infection. Fractions of DNA (70,000 counts/min) isolated from control and infected cultures were sedimented in 5 to 20% linear sucrose gradients at pH 8 or 11.5. Direction of sedimentation is from right to left.*

15 min is shown in Fig. 5. The initial titer was reduced 10^2 to 10^3-fold after 15 min of exposure. The rate of inactivation was greatest during the first 6 min. The significance of this type of inactivation has been discussed by McClain and Spendlove (19).

DNA synthesis was then measured in cultures which had been exposed to 250 UV-inactivated infectious units per cell. The virus was irradiated for 60 min to give a reduction in infectious virus at least equivalent to that measured after 15 mins of irradiation and to insure an input multiplicity of less than 1 PFU/cell. As in cells infected with high multiplicities of infectious virus, inhibition of DNA synthesis was detected early during infection, between 1 and 3 h after exposure to high multiplicities of infectious virus (Fig. 6). These results show that high particle-to-cell ratios are as effective as high PFU-to-cell ratios in inhibiting DNA synthesis early after exposure to virus.

DISCUSSION

The time interval between the initiation of reovirus infection and the beginning of the inhibition of DNA synthesis was reduced approximately 4 h by increasing the multiplicity of infection from 10 to 10^2 PFU/cell. Even though the time at which this inhibition began did not appear to be significantly reduced as the input multiplicity was increased from 10^2 to 10^3 PFU/cell, a marked increase in the initial rate of inhibition was detected.

In addition, the selectivity of this inhibition for the replicative function of cellular DNA was maintained at high virus multiplicities since there appeared to be no alteration of the capacity of DNA to serve as a template for the synthesis of RNA. The progressive inhibition of protein synthesis seen 7 to 9 h after infection confirms an earlier report (7) which established a relationship between reovirus-induced inhibi-

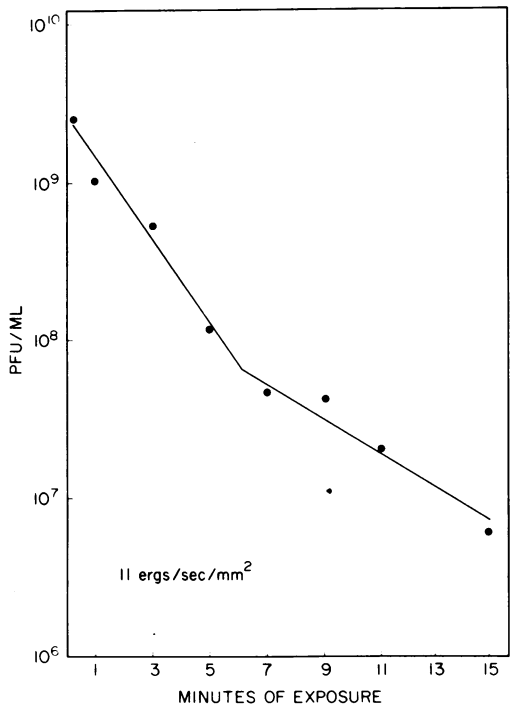

FIG. 5. *UV inactivation of reovirus. A purified suspension of reovirus in phosphate-buffered saline was exposed to UV light as described in Materials and Methods. At the times indicated, fractions were removed, and residual infectious virus was determined by plaque assay. Titers are expressed as PFU per milliliter.*

tion of protein synthesis and cell loss in suspension but not monolayer cultures. It is important to note that inhibition of protein synthesis was observed several hours after the beginning of the inhibition of DNA synthesis, suggesting that the inhibition of DNA synthesis was not a function of early modifications of cellular protein-synthesizing capability. As mentioned previously, it is not clear at the present time whether minor alterations in cellular RNA and protein synthesis were occurring which were obscured by the concomitant synthesis of virus nucleic acid and protein. This problem is presently under investigation.

The maintenance of RNA and protein synthesis capability, the ability to produce infectious virus, the lack of modification of precursor pools, thymidine kinase activity, and the sedimentation characteristics of cellular DNA suggest that early inhibition of DNA synthesis is not due to extensive cytopathology resulting from exposure of cells to high concentrations of reovirus particles. These observations point not only to the selective maintenance of cellular metabolic integrity after infection with high multiplicities of reovirus, but also to the conclusion that the mechanism of reovirus-induced inhibition of cellular DNA synthesis may be related to a specific event which occurs soon after infection and is possibly maintained throughout the virus replication cycle.

The beginning of the inhibition of DNA synthesis does not coincide with the time of accumulation of detectable virus progeny 5 h postinfection when cells are infected at high multiplicities (unpublished results). Rather, it appears that the inhibition may be related to the release of virus components during the uncoating process and/or to the products of early virus-directed transcription and translation. One such component could be reovirus adenine-rich RNA (1, 13, 21). The addition of this viral nucleic acid species to cells results in a selective, but transient, inhibition of DNA synthesis (Monahan and Cox, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 200, 1972; manuscript submitted for publication). However, until the inhibitory capacity of adenine-rich RNA is more rigorously investigated, we cannot exclude the possibility that viral inhibition of DNA synthesis may be the result of the release and subsequent production of virus-specific protein (6).

The maintenance of the inhibitory capacity of high multiplicities of reovirus after UV irradiation suggests that the ability to produce infectious reovirus is not a fundamental requirement for virus-mediated inhibition of DNA synthesis.

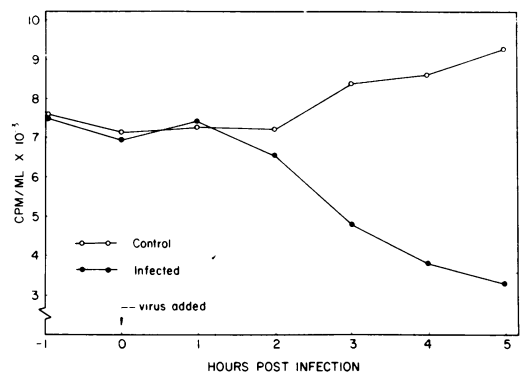

FIG. 6. *DNA synthesis in cells exposed to UV-inactivated reovirus. Virus was irradiated for 60 min, as described in Materials and Methods, and added to a suspension culture at a multiplicity of 250 UV-inactivated PFU per cell. At the times indicated, 2.7 × 10⁶ cells were removed from control and test cultures and pulse-labeled with ³H-thymidine. Trichloroacetic acid-insoluble counts per minute per milliliter of culture are presented.*

Since reovirions contain a transcriptase (3, 20), it is possible that UV-inactivated reovirus may still be capable of limited transcription even though some double-stranded RNA templates are damaged by UV light. However, the results using UV-inactivated virus must be interpreted with caution since UV-treated virus has been reported to produce cytotoxic effects in cells (17).

Nevertheless, these data show that a temporal relationship exists between the multiplicity of infection with reovirus and the time of inhibition of cellular DNA synthesis. High multiplicities of reovirus inhibited DNA synthesis early during infection, while at low virus multiplicities inhibition was delayed by several hours. Since there was no loss of the capacity of UV-inactivated reovirus to inhibit DNA synthesis early after infection at high multiplicities, we suggest that a virus-specific component(s) may be responsible for the inhibition. This would suggest that the virus-specific component(s) would (necessarily) have to be synthesized before inhibition could be detected at low multiplicities. These data, however, cannot rule out that a virus-specific component(s), newly synthesized after infection at high multiplicities, results in early inhibition of DNA syntheses.

ACKNOWLEDGMENTS

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ADDENDUM

While this manuscript was being completed, Hand and Tamm (J. Virol. **48**:223-232, 1973) reported findings which are in agreement with portions of the results presented here.

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