SYNTHESIS OF VIRUS-SPECIFIC POLYMERS IN ADENOVIRUS-INFECTED CELLS: EFFECT OF 5-FLUORODEOXYURIDINE*

By JOHN F. FLANAGAN,‡ M.D., and HAROLD S. GINSBERG, M.D.

(From the Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia)

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A significant increase in cellular deoxyribonucleic acid is associated with adenovirus multiplication in tissue culture cells (1–5). Previous work has shown that a major part of the increased deoxyribonucleoprotein in cells infected with this virus is soluble in 0.15 M sodium chloride in contrast to normal host cell DNA-protein which is relatively insoluble at this salt concentration (2, 3). Radioisotope incorporation studies indicated that this DNA fraction is synthesized *de novo* after infection and does not represent degraded cell DNA (3). Analysis of base content of the nucleic acid of infected cells suggested that major structural differences exist between the newly synthesized DNA and host cell DNA (2). The accumulated evidence indicates that this unique DNA is a product of specific virus infection and raises the possibility that it is precursor material for synthesis of virus particles. The present investigation was designed to define further the relationship between synthesis of the new DNA in infected cells, production of virus antigens, and assembly of infectious particles. For these purposes a potent inhibitor of DNA synthesis, 5-fluoro-2'-deoxyuridine (6, 7) (hereafter referred to as 5-FUDR) was utilized.

5-FUDR acts as an inhibitor of DNA synthesis by its effect on the enzyme, thymidylate synthetase, (6, 7). This enzyme, which converts deoxyuridylic acid to thymidylic acid by methylation, is susceptible to strong competitive inhibition by 5-fluorodeoxyuridine phosphate (7). The capacity of 5-FUDR to inhibit DNA synthesis rapidly depends on the speed with which the intracellular pool of thymine is exhausted after enzymatic synthesis of thymidylic acid is halted by the analogue. The pool of thymidylic acid and thymine within HeLa cells is extremely small (8); it could be expected, therefore, that addition of 5-FUDR to the cells would lead to rapid cessation of DNA synthesis. In studies on multiplication of vaccinia virus, Salzman found this situation to pertain in HeLa cells: Addition of the analogue to cells infected with vaccinia led to immediate and total suppression of DNA synthesis (9).

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‡ United States Public Health Service Postdoctoral Fellow.

The investigations reported in this communication indicate that 5-FUDR rapidly and completely inhibited propagation of adenoviruses; that, within limits, the quantity of infectious virus synthesized was a linear function of the concentration of the analogue; that new DNA synthesis commenced 5 to 6 hours before appearance of new infectious virus; and that the amount of infectious virus produced was directly related to the quantity of DNA synthesized following infection.

Materials and Methods

Tissue Culture.—Experiments were performed with monolayer cultures of HeLa cells (Gey strain) propagated in duraglass prescription bottles of 4 ounce or 32 ounce volume with Eagle's basal medium containing 10 per cent human serum or in screw-capped test tubes with 40 per cent human serum in Hanks' balanced salt solution (BSS) by methods described previously (10).

Virus and Virus Infection.—The virus stocks were prototype strains of type 4 and type 5 adenoviruses. Virus pools for these experiments were prepared by inoculating $10^{6.5}$ TCD_{50} of virus into bottle cultures containing 50 ml of maintenance mixture consisting of 67.5 per cent Scherer's amino acid–vitamin mixture, 25 per cent tryptose phosphate broth (Difco), and 7.5 per cent chicken serum (11). Infected cultures were incubated until cytopathic effects were completed (4 to 6 days). The cells were harvested, centrifuged at 1000 RPM in an International PR-2 refrigerated centrifuge for 30 minutes at 4°C, and suspended in Hanks's balanced salt solution (BSS). The suspensions were frozen and thawed six times to release virus particles from the cells. The cell debris was sedimented by centrifugation of 3000 RPM for 30 minutes and the supernatant fluids, containing infectious virus, were used for the experiments to be described.

A minimal medium consisting of Eagle's basal medium supplemented with 7.5 per cent dialyzed chicken serum was utilized for all experiments. The virus inoculum contained a multiplicity of 1 to 4 infectious particles per cell. To destroy the soluble toxin, virus suspensions were treated with trypsin (0.1 mg/ml) for 1 hour at 37°C (12, 13) and the trypsin neutralized by addition of an equal quantity of soybean trypsin inhibitor before inoculation onto the cells. The experiments, unless otherwise stated, encompassed the initial virus multiplication cycle of 32 hours.

Virus Infectivity, Toxin, and Complement Fixation Titrations.—Infectivity titrations were performed in duplicate tube cultures of HeLa cells using 1:3.2 $(10^{-})^{0.5}$ serial dilutions as described previously (10). Virus titers are expressed as 50 per cent tissue culture doses (TCD_{50}).

Toxin titrations were carried out in tube cultures using 2-fold dilutions of virus suspensions. The endpoint of the titration was considered to be the last dilution producing distinct cytopathic effect on the cells of the monolayer at the end of 6 hours (14).

Complement-fixing activity was measured by a standard method using 1.5 exact units of complement per tube and heat-inactivated (56°C for 30 minutes) type 4 or type 5 specific rabbit antiserum or human convalescent antiserum. The titers are expressed as the greatest dilution of antigen producing complete fixation of complement.

Determinations of DNA, RNA, and Soluble Protein.—Cells were harvested, centrifuged at 1000 RPM for 30 minutes, and washed twice with 0.15 M sodium chloride buffered at pH 7.2 with 0.01 M phosphate (hereafter referred to as buffered saline). The washed cells were resuspended in 0.15 M sodium chloride containing 0.01 M sodium citrate and homogenized for 10 minutes with alundum in a motor-driven teflon grinding apparatus. The homogenates, which by microscopy contained no intact cells, were allowed to stand overnight at 4°C, and

then centrifuged at 3000 RPM for 30 minutes. The sediment was discarded and the supernatant fluid, termed the saline-soluble fraction, was chilled in chipped ice. The saline-soluble fraction was submitted to a modified Schmidt-Thannhauser procedure for separation of DNA, RNA, and protein (2). DNA was determined by the diphenylamine reaction of Burton (15), using thymus DNA as the standard. RNA was measured by the orcinol method, utilizing D-ribose as the standard (16). Protein content was assayed by the Lowry phenol method employing as the standard bovine serum albumin (17).

Analogue Preparation.—A stock solution of 10^{-3} M 5-fluoro-2'-deoxyuridine (5-FUDR) was prepared in distilled water and diluted to the appropriate concentration for each experiment. The stock solution was stored at −20°C.

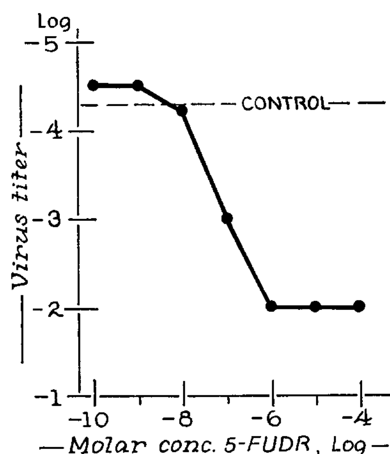

FIG. 1. Concentration of 5-FUDR required to inhibit multiplication of type 4 adenovirus. 5-FUDR was added to tube cultures at the time of infection with 2 TCD_{50} of virus per cell. Incubation period was 30 hours. Each group contained 4 cultures; endpoints represent geometric means of results from 2 experiments.

EXPERIMENTAL

Relationship Between Concentration of 5-Fluorodeoxyuridine (5-FUDR) and Virus Multiplication.—Initial experiments were designed to determine the effect of 5-FUDR concentration on the quantity of infectious virus produced.

Tube monolayer cultures of HeLa cells were overlaid with minimal medium, containing 5-FUDR in concentrations varying from 10^{-4} M to 10^{-10} M, and trypsin-treated type 4 adenovirus to produce a multiplicity of 2 infectious units per cell. 6 hours after infection, the cells were washed with Hanks' solution. 1 ml of minimal medium, containing the same concentrations of 5-FUDR as before, was placed on the cells for the remainder of the experimental period. Four matched tubes were used with each concentration of 5-FUDR. The experiments were terminated 30 hours after infection. The suspensions from the four tubes of each group were pooled and infectivity titrations performed as described previously.

The results of a representative experiment summarized in Fig. 1 indicate that production of virus was reduced by more than 99 per cent during a single

multiplication cycle by 10^{-6} M 5-FUDR. An increase in the concentration of analogue beyond that point failed to lower the titer of virus further. In relation to this finding, it was also noted that even where 5-FUDR was added after the cells were washed free of unadsorbed virus, 6 hours past infection, maximal inhibition of virus was effected. These data suggest that 10^{-6} M 5-FUDR completely inhibited virus multiplication, and that the virus measured in the presence of the inhibitor was residual virus remaining from the original inoculum.

Effect of Thymidine on Inhibition of Adenovirus Multiplication by 5-Fluorodeoxyuridine.—To ascertain the specificity of inhibition of adenovirus multi-

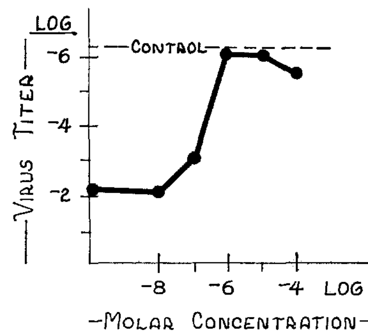

FIG. 2. Concentration of thymidine required to reverse effect of 5-FUDR. Cultures were infected as in Fig. 1. 5-FUDR in 2×10^{-6} M concentration and thymidine in the indicated concentrations were added to cultures for the experimental period of 30 hours.

plication by 5-FUDR, thymidine was utilized to determine whether addition of this nucleoside to inhibited cultures could completely overcome the analogue effect.

For these experiments 1 ml of minimal medium was added to the tube cultures, containing 2×10^{-6} M 5-FUDR, thymidine in concentrations varying from 10^{-4} M to 10^{-8} M, and trypsin-treated type 4 adenovirus to produce a multiplicity of 2 infectious units per cell. 6 hours after infection the cells were washed with Hanks' solution and minimal medium, containing the same concentrations of 5-FUDR and thymidine as before, was added to the cells for the remainder of the experiment. Four matched tubes were used with each concentration of thymidine. The experiments were terminated 30 hours after infection and infectivity titrations were carried out.

The results of several experiments summarized in Fig. 2 indicate that 10^{-6} M thymidine added with the analogue at time of infection permitted synthesis of infectious virus to the level of the untreated control. Greater amounts of thymidine were without added effect. Uridine in a concentration as high as 10^{-4} M in the medium did not reverse the inhibition of virus synthesis by 5-FUDR. These data imply that 5-FUDR effects its inhibition of virus propa-

gation by specific inhibition of DNA synthesis through thymine deprivation and not by non-specific cytotoxic properties.

Relationship Between Time of Addition of 5-Fluorodeoxyuridine and Multiplication of Type 4 Adenovirus.—Previous measurements of the rate of P^{32} incorporation into DNA indicated that synthesis of the new saline-soluble DNA begins in type 4 adenovirus–infected cells at 10 to 11 hours after infection, distinctly preceding the increase in infectious particles (2). The capacity of 5-FUDR to arrest virus production indicated that this analogue might be useful in describing further the sequence of early DNA synthesis and production of infectious particles by serial addition of the analogue to cultures at specific times during the multiplication cycle of type 4 adenovirus. If DNA synthesis were inhibited rapidly, addition of the analogue early in the multiplication cycle should stop production of precursor nucleic acid for virus particles, leaving only that DNA made prior to addition of the inhibitor for incorporation into infectious units. This hypothesis was investigated.

Monolayer tube cultures of HeLa cells were infected with trypsin-treated type 4 adenovirus to produce a multiplicity of 2 infectious units per cell. Unadsorbed virus was washed from the cells 6 hours after infection. The medium was replaced and 5-FUDR, in a final concentration of 10^{-6} M, was added to groups of four matched tubes at 2 hour intervals from 6 to 28 hours after infection. At the time of each addition of 5-FUDR to infected tubes, four infected control tubes were harvested and pooled. All groups of infected cultures containing analogue were harvested at 32 hours after infection and infectivity titrations were performed.

In these experiments, summarized in Fig. 3, it is apparent that when 5-FUDR was added at any time up to 10 hours after infection complete inhibition of virus production occurred. Inhibition decreased rapidly beyond that point, and addition of the analogue to infected cells more than 16 hours after infection failed to reduce the final titer of virus below that in untreated control cultures. It can be inferred from these data that synthesis of precursor DNA for virus synthesis began approximately 10 hours after infection and that sufficient DNA was made by 16 hours after infection for a maximal production of infectious particles. Control virus multiplication cycles showed that (with the minimal maintenance medium used for these experiments) the initial increase in virus titer began about 16 hours after infection.[1] From comparison of these two curves, it is clear that DNA synthesis must precede assembly of infectious particles by at least 5 to 6 hours. Studies of protein synthesis within adenovirus-infected cells indicate that a significant part of this lag between DNA synthesis and virus particle assembly is associated with an event which can be inhibited by *para*-fluorophenylalanine (19). The inhibitable process is considered to be the synthesis of protein subunits for the infectious virus particle.

[1] The eclipse period for type 4 adenovirus is of the order of 14 hours when complete maintenance mixture is employed (18). Utilizing the minimal maintenance medium for these experiments, the eclipse period was prolonged 2 to 3 hours.

The Effect of 5-FUDR on DNA, RNA, and Protein Synthesis in Type 4 Adenovirus-Infected Cells.—Inhibition of virus multiplication by 5-FUDR, and the temporal relationship between addition of the analogue and quantity of virus produced, indicated the necessity to investigate the direct effect of 5-FUDR on synthesis of the unique saline-soluble DNA in adenovirus-infected cells.

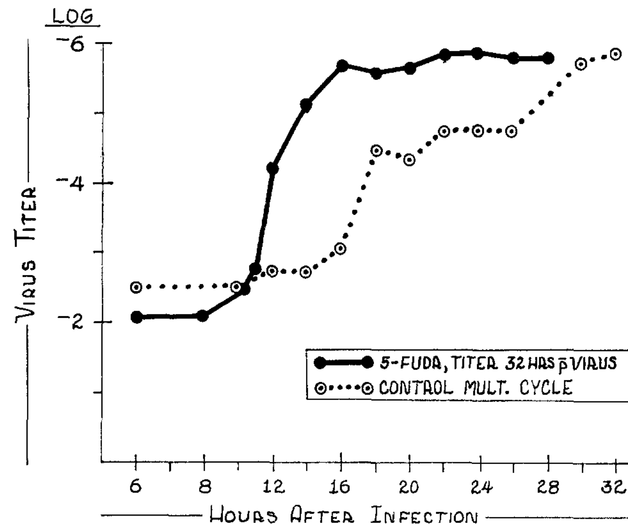

FIG. 3. Inhibition of type 4 adenovirus multiplication by 5-FUDR added at intervals after infection. Tube cultures were infected with 2 TCD_{50} per cell. 5-FUDR in 10^{-6} M final concentration was added at the indicated times following infection. Points along solid line indicate virus titer 32 hours after infection; points on broken line indicate virus titer in untreated infected tube cultures harvested at the indicated times after infection to establish a control multiplication cycle. Each endpoint represents the geometric mean of the results from 4 experiments. Each infectivity titer was obtained from a pool of cells from 4 cultures.

Monolayer cultures in 32 ounce bottles were infected with a multiplicity of 1 to 4 infectious units per cell. Unadsorbed virus was removed 6 hours after infection. The medium was restored and 2×10^{-6} M 5-FUDR was added to one pair of bottles. Other experimental groups included (each consisting of 2 cultures) were: infected cultures withsut 5-FUDR, uninfected cultures without 5-FUDR, and uninfected cultures with 2×10^{-6} M 5-FUDR. All groups were harvested 36 hours after infection. Fractionation and chemical determinations were carried out as described in Materials and Methods.

The saline-soluble DNA under conditions of normal infection showed an 8-fold increase above the level present in uninfected control cells (Table I). No significant increase in saline-soluble DNA was measured in infected cells treated with 2×10^{-6} M 5-FUDR.

The RNA content of 5-FUDR-treated infected cells, 5-FUDR-treated

uninfected cells, and untreated (control) infected cells showed approximately the same level of increase (32 to 38 per cent) over the quantity in untreated, uninfected (control) cells. A similar pattern of increase in protein was noted in infected cells treated with 5-FUDR, uninfected cells treated with 5-FUDR, and untreated (control) infected cells as compared to normal cells.

The experiments demonstrated that 5-FUDR completely suppressed synthesis of saline-soluble DNA concomitant with inhibition of infectious virus formation. The reason for an increase in RNA and protein, which was of similar magnitude in untreated infected cells or in infected and uninfected cells which received 5-FUDR, is not completely clear since synthesis of virus-specific proteins was arrested by the analogue as described later in this paper.

TABLE I

Effect of 5-FUDR on Saline-Soluble Deoxyribonucleic Acid, Ribonucleic Acid, and Protein in Uninfected and Type 4 Adenovirus–Infected HeLa Cells

	DNA	RNA*	Protein
	μg/10⁶ cells	*μg/10⁶ cells*	*μg/10⁶ cells*
Uninfected cells	0.34	1.38	84.2
Infected cells‡	2.71	1.80	110
Infected cells‡ + 5-FUDR§	0.59	1.96	116
Uninfected cells + 5-FUDR§	0.43	1.80	114

* Expressed as ribose.

‡ Infected with 2 TCD_{50} of virus per cell, and incubated for 36 hours after infection.

§ 2×10^{-6} M 5-FUDR.

Investigation of the Earliest Step in DNA Synthesis Inhibited by 5-FUDR.— Experiments were next conducted to define the temporal relationship between the first step inhibitable by 5-FUDR and the onset of DNA synthesis in infected cells. If thymidylic acid synthesis were under the control of genetic factors associated with the virus particle, it is conceivable that the inhibitable step would occur before synthesis of DNA begins. On the other hand, if production of thymidylate were accomplished under controls already in operation in the cell, feed-back regulation would probably be the controlling mechanism for this synthesis. In the latter case, the inhibitable step would begin immediately after synthesis of DNA had begun, at the time that thymidylic acid was removed from the cell pool, and the essential enzyme system was activated for synthesis of new thymidylate.

In experiments to examine this point an inhibitory concentration of 5-FUDR was added to infected cells early in the virus multiplication cycle. After specific time intervals the analogue effect was reversed by addition of excess thymidine to the medium. Under these conditions treatment with 5-FUDR should permit virus infection to proceed only to the point at which thymidylate synthesis

begins. Initiation of virus DNA production should depend entirely on the addition of thymidine. Adjustment of the time span between treatment of infected cells with the analogue and reversal by thymidine, therefore, should permit determination of the earliest DNA synthesis vital for virus production.

In the first experiments of this type, 2×10^{-6} M 5-FUDR was added to monolayer bottle cultures of HeLa cells at the time of virus inoculation. 8 hours after addition of analogue and

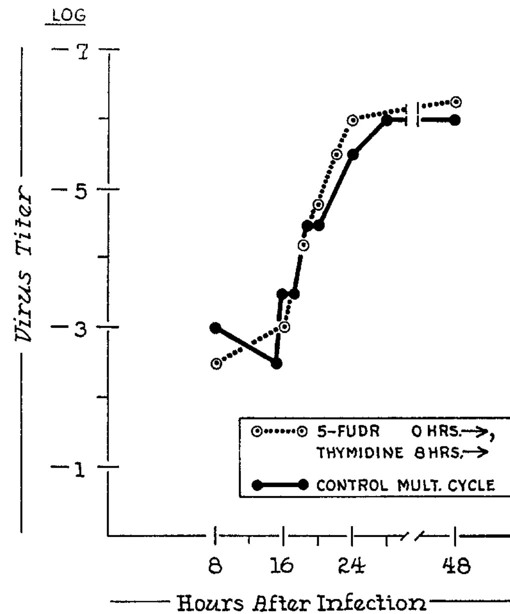

FIG. 4. Reversal of 5-FUDR inhibition by thymidine 8 hours after infection. 5-FUDR in 2×10^{-6} M final concentration was added to cultures at time of infection with 2 TCD_{50} of virus per cell. Cultures were washed 3 times and the fluid was replaced with minimal medium containing 4×10^{-6} M thymidine 8 hours after infection. 5-FUDR–treated and control cultures were harvested at times indicated. Results are expressed as the geometric means of 2 experiments.

virus to the cultures, medium was removed, cells washed, and the fluid replaced with new medium containing 4×10^{-6} M thymidine. Paired infected bottles treated with 5-FUDR and infected control bottles were harvested at intervals up to 48 hours after infection. Infectivity titrations were performed on the suspensions to determine whether an extension of the virus latent period had resulted from the analogue treatment.

The results of this experiment, summarized in Fig. 4, indicated that no lag occurred in the appearance of new virus in infected cells treated with 5-FUDR for 8 hours as compared with control infected cells. In addition, the final titer of virus was the same in both groups. These findings indicate clearly that

no essential thymidylic acid synthesis had occurred during the first 8 hours after infection.

The next experiment consisted of adding the inhibitor 6 hours after virus infection and reversing the effect with 4×10^{-6} M thymidine 16 hours after infection (Fig. 5). A significant increase in virus titer occurred in the inhibited-reversed cultures at 22 hours after infection. This represented a delay of 6 hours beyond the appearance of newly synthesized virus in the control infected

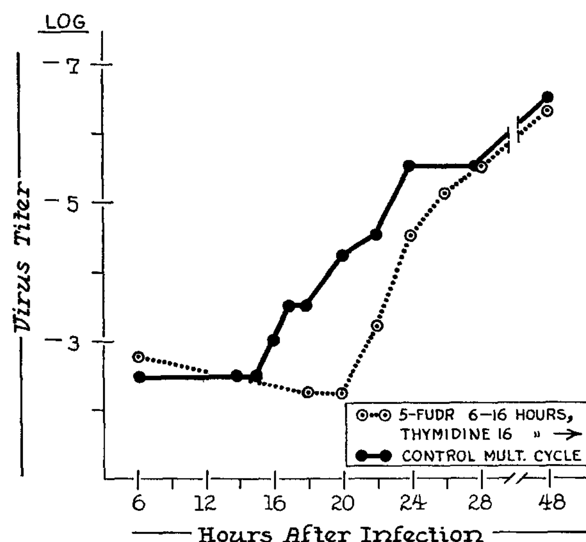

FIG. 5. Reversal of 5-FUDR inhibition by thymidine 16 hours after infection. 5-FUDR in 2×10^{-6} M concentration was added to cultures 6 hours after infection with 2 TCD_{50} of virus per cell. The fluid was replaced 16 hours after infection with medium containing 4×10^{-6} M thymidine. Each group consisted of 2 bottle cultures. Control and 5-FUDR-treated cultures were harvested at indicated times. Each endpoint represents the geometric mean of results from 3 experiments.

cultures. The results imply that an essential step inhibitable by 5-FUDR began 6 hours before inhibition was reversed, *i.e.*, 10 hours after infection. It may be deduced from these data that the onset of thymidylic acid synthesis essential for DNA virus production began at approximately 10 hours after infection. This nucleotide synthesis coincides with the time of the initial production of precursor DNA for virus multiplication (Fig. 3). It seems likely, therefore, that the onset of DNA synthesis removed thymidylic acid from the intracellular pool, and that enzymatic production of new thymidylate occurred as a result of removal of the end-product.

Effect of 5-FUDR on Type 4 Adenovirus Multiplication with Prolonged Incubation.—It was found that the concentration of 5-FUDR which effectively

inhibited virus synthesis during the initial multiplication cycle failed to suppress production of infectious virus when the incubation period with 5-FUDR exceeded 40 hours. Several explanations seemed possible to account for this finding: (*a*) an alternate pathway for synthesis provided small amounts of thymidylic acid for DNA synthesis under normal conditions and gradual increase in activity of this pathway might occur when the major enzyme was inhibited by 5-FUDR; (*b*) the inhibitor, at the concentration used for these experiments, was insufficient for complete suppression of enzyme activity and permitted a constant, small amount of thymidylic acid to be synthesized by

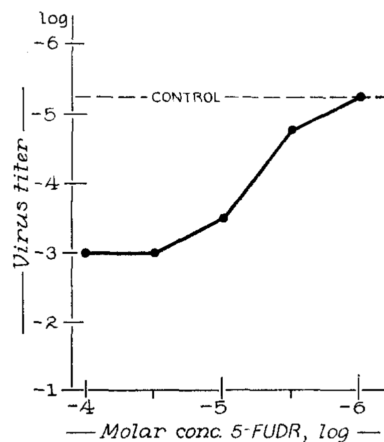

FIG. 6. Inhibition of type 4 adenovirus multiplication by 5-FUDR with prolonged incubation of cultures. 5-FUDR was added in indicated concentrations to cultures at time of infection with 4 TCD_{50} of virus per cell. All cultures were harvested 60 hours after infection. Each group contained 2 bottle cultures. The geometric mean of results from 2 experiments are presented.

the normal route; (*c*) the effective concentration of 5-FUDR gradually decreased following introduction of inhibitor into the culture, either by simple degradation, or by enzymatic conversion to other fluorouracil derivatives.

The initial group of experiments to explore the cause of this "escape" from inhibition consisted of adding 5-FUDR to infected cells at several concentrations and measuring the quantity of virus present at the end of 60 hours. It can readily be seen by the data summarized in Fig. 6 that 10^{-4} M to $10^{-4.5}$ M 5-FUDR in the medium maintained virus titers equal to the background level, while concentrations of 10^{-5} M to 10^{-6} M permitted release from inhibition. It is unlikely, in view of this finding, that an alternate pathway for thymidylate synthesis could account for the escape from 5-FUDR inhibition in prolonged experiments. If an alternate pathway for synthesis existed, in-

creasing the concentration of inhibitor specific for thymidylate synthetase should have been without effect, whereas, the experiments described (Fig. 6) clearly showed that synthesis during prolonged periods could be totally suppressed by a greater concentration of 5-FUDR in the medium. The escape seemed to be related either to insufficient 5-FUDR to produce complete inhibition from the outset or to gradual disappearance of the inhibitor during incubation. Experiments were performed in which medium containing 5-FUDR was maintained on infected cells for 24 hours or in the incubator in a closed bottle without being in contact with cells for the same period of time. These two types of media were then tested for inhibitory capacity on newly infected cell cultures which were allowed to incubate 30 hours after addition of the test

TABLE II

Effect of Preincubation of 5-FUDR on Capacity to Inhibit Type 4 Adenovirus Multiplication

5-FUDR	Conditions of preincubation	Virus titer
None	—	$10^{-5.5}$*
2×10^{-6} M	None	$10^{-3.5}$*
2×10^{-6} M	37°C/24 hrs. with normal cells‡	$10^{-5.3}$*
2×10^{-6} M	37°C/24 hrs., no cells‡	$10^{-5.15}$*

* Determined 30 hours after infection.

‡ After preincubation 5-FUDR placed on cells infected with 2 TCD_{50} virus per cell.

media. It was found that both of these media permitted virus multiplication (Table II). These data indicated clearly that 5-FUDR was changed to an inactive form in media maintained at 37°C whether in contact with cells or not. A concentration of 5-FUDR which was just sufficient to suppress virus production through one cycle of multiplication did not effect suppression for longer periods. Increasing the initial concentration of 5-FUDR obviated this escape from inhibition during prolonged experiments.

For experiments involving prolonged inhibition of DNA synthesis, the concentration of 5-FUDR in the medium was increased to $10^{-4.5}$ M.

HeLa cells grown in monolayer culture in 4 ounce bottles were infected with a multiplicity of 1 to 3 infectious units per cell. The cultures were washed 6 hours after infection and new medium containing $10^{-4.5}$ M 5-FUDR was added. The infected cells were incubated until 24 hours after infection at which time the medium was replaced with fresh medium containing 4×10^{-6} M thymidine. Paired bottles were harvested at intervals up to 48 hours after infection. A control multiplication cycle was performed concomitantly.

When inhibition of DNA synthesis by 5-FUDR was maintained from the 6th to the 24th hour, reversal of inhibition occurred readily upon addition of thymidine (Fig. 7). A significant increase in virus titer was detectable 4 hours

after reversal. This appearance of new virus was approximately 2 hours earlier than might have been expected from the results of previous inhibition-reversal experiments (Fig. 5) and suggests that a minimal amount of DNA synthesis had occurred during the prolonged period of inhibition. The slope of increase in virus titer and final titer at 48 hours in the experimental group paralleled that of the uninhibited control multiplication cycle, indicating that the replicating system for production of infectious particles could remain completely intact and functional in cells for 24 hours despite minimal DNA synthesis.

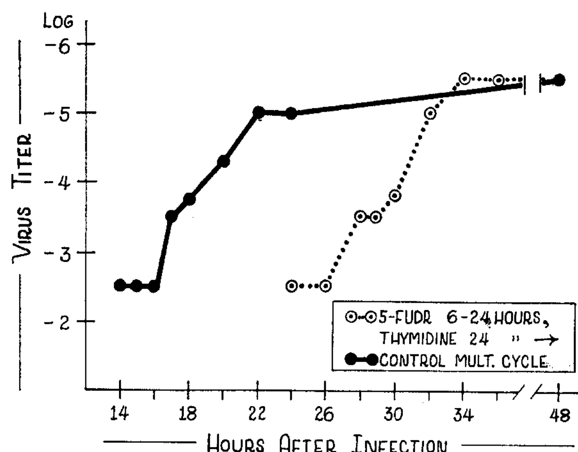

FIG. 7. Reversal of 5-FUDR inhibition by thymidine 24 hours after infection. 5-FUDR in a $10^{-4.5}$ M concentration was added to cultures 6 hours after infection with 4 TCD_{50} of virus per cell. Fluid was replaced 24 hours after infection with medium containing 4×10^{-6} M thymidine. Control and 5-FUDR-treated cultures were harvested at indicated times. Each group consisted of 2 bottle cultures. The endpoints represent the geometric mean of results from 3 experiments.

Relationship between DNA Synthesis and Production of Adenovirus-Specific Antigens.—To explore the relationship between 5-FUDR inhibition of virus synthesis in infected cells and production of virus-specific antigens, experiments were performed in which the amounts of complement-fixing antigen and soluble toxin were measured in sequentially inhibited cultures. It seemed possible that synthesis of DNA and specific proteins might proceed independently once the genetic material had passed from the infecting virus particle into the host cells. If this were true, one might expect that 5-FUDR added early in the multiplication cycle would suppress precursor-DNA synthesis but would still permit maximal synthesis of antigens in the infected cell. On the other hand, if the elaboration of these antigens were dependent on DNA synthesis, the synthesis of antigens within infected cells would be susceptible to suppression by 5-FUDR to the same degree as infectious particles.

Experiments with type 4 adenovirus showed that production of group-specific

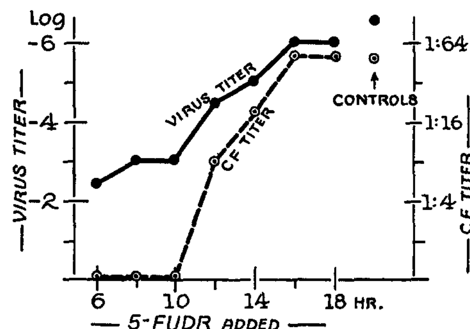

Fig. 8. Multiplication of infectious virus and synthesis of complement-fixing antigen in type 4 adenovirus-infected cells treated at intervals with 5-FUDR. 5-FUDR in 2×10^{-6} M concentration was added to cultures at intervals from 6 to 18 hours after infection with 4 TCD_{50} of virus per cell. All cultures, including untreated, infected controls, were harvested 32 hours after infection. Each endpoint represents results from pool of cells from 2 bottle cultures.

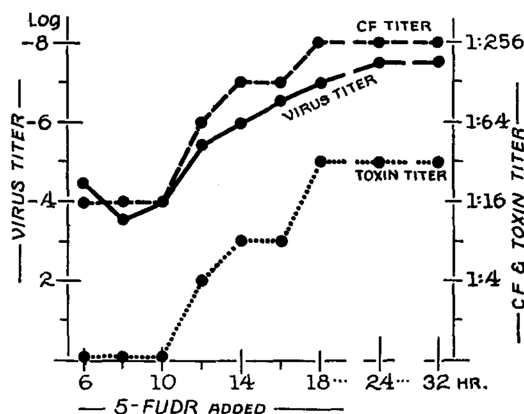

Fig. 9. Multiplication of type 5 adenovirus and production of complement-fixing antigen and toxin in infected cells treated at intervals with 5-FUDR. Cultures were infected as in Fig. 8. 5-FUDR in 2×10^{-6} M concentration was added to cultures and cultures were harvested as in Fig. 8. Each group consisted of 2 bottle cultures. The endpoints represent the geometric mean of results from 2 experiments.

complement-fixing antigen in infected cells was directly related to the time of addition of 5-FUDR (Fig. 8). The data indicate that synthesis of complement-fixing antigens, like infectious virus, was completely inhibited by 5-FUDR when added up to 10 hours after infection. However, when synthesis of DNA was not inhibited until 12 hours after infection, some complement-fixing antigens as well as infectious virus could be produced. Addition of 5-FUDR serially to cultures thereafter showed a definite and apparently fixed relationship between the amount of complement-fixing antigen produced and the quantity of infectious virus formed.

Studies with type 5 adenovirus showed a similar pattern to that of type 4 in respect to the complement-fixing antigen (Fig. 9). In addition these experiments demonstrated that the amount of soluble toxin produced in type 5–infected cells was correlated with the amount of infectious virus found. These findings lead to the conclusion that synthesis of DNA is essential for production of the virus-specific antigens.

DISCUSSION

It is clear that deoxyribonucleic acid (DNA) synthesis is increased in adenovirus-infected cells (2–5, 20). The evidence is strong that the DNA which accumulates in virus-infected cells is of a unique variety, the so called saline-soluble DNA, and is different from the normal host cell DNA in base composition and physical structure (2, 3, 20). Experiments described in this communication, using 5-fluorodeoxyuridine, an inhibitor of thymidylic acid synthesis, and, therefore, of DNA production, demonstrate that DNA synthesis is essential for adenovirus multiplication. The data obtained from this study support the hypothesis, based on preliminary chemical studies of purified virus, that DNA is the nucleic acid constituent of adenoviruses.

Studies demonstrating that virus nucleic acids may be infectious (21–25) indicate that the subunit which carries the genetic information must indeed be the nucleic acid moiety of the virus particle. It may be deduced from the experiments described in this paper that the genetic information of adenovirus can persist for a relatively long time before replication occurs. After virus infection, DNA synthesis and virus multiplication were inhibited by 5-FUDR for periods as long as 24 hours after infection. When inhibition was reversed by thymidine, DNA synthesis was rapidly resumed and virus multiplication followed predictably.

Synthesis of the unique, saline-soluble DNA begins in adenovirus-infected cells approximately 4 hours before the appearance of new infectious virus, a finding which suggests that the saline-soluble DNA might be a precursor of virus DNA (3). Inhibition of DNA synthesis by 5-FUDR at varying times after infection indicates that the production of DNA essential for fabrication of infectious virus commences about 10 hours after infection, at approximately the same time as the first detectable synthesis of saline-soluble DNA (3). These data strengthen the postulate that the saline-soluble DNA is a precursor of virus DNA.

Not only is there an accumulation of newly synthesized DNA, but also there is a distinct increase in protein content in adenovirus-infected cells (2, 14). An appreciable part of this protein consists of soluble, virus-specific antigens (14). Detailed studies on separation and purification of these adenovirus antigens indicate that there are at least three immunologically discrete proteins: (*a*) a group-specific complement-fixing antigen, (*b*) a toxin, which is

group-specific, and *c*) a type-specific complement-fixing antigen (14, 26). In the experiments described in this paper, it was shown that synthesis of the virus-specific antigens required DNA synthesis. Under the conditions of the experiments reported, a net increase in cell protein and RNA content occurred in both infected and uninfected cells when they were treated with 5-FUDR. It seems possible that addition of 5-FUDR produced immediate arrest of mitosis without a simultaneous inhibition of cell RNA and protein synthesis. This state of unbalanced growth could lead then to a general increase in cell mass, RNA, and protein content. Despite the fact that an increase in cell protein occurred in infected cells treated with 5-FUDR, these experiments clearly indicated that production of the virus antigens was arrested when DNA synthesis was inhibited by the analogue.

Investigation of the synthesis of adenovirus-specific antigens utilizing the amino acid analogue *para*-fluorophenylalanine indicates that synthesis of the antigens followed synthesis of DNA by approximately 2 to 3 hours and preceded the appearance of infectious virus by approximately 2 hours (19). This series of events is similar to findings described for T-even bacteriophages (27, 28).

From these studies one may postulate the sequence of events occurring in adenovirus-infected cells which leads to fabrication of infectious virus. Inherent in this hypothesis is the implication derived from this and previous investigations (2, 3, 14), that the saline-soluble DNA and adenovirus-specific antigens are precursors and possibly subunits of the completed virus particle. Following adsorption to and penetration of the host cell, the virus particle is disrupted to permit the nucleic acid to transmit its information to the nucleus of the infected cell, the site for synthesis of virus antigens and infectious particles (29–32). A relatively long interval ensues after infection during which no synthesis of DNA or protein necessary for virus propagation can be detected. Synthesis of viral precursor DNA begins approximately 10 hours after infection and production of the virus-specific antigens follows DNA synthesis by 2 to 3 hours. Finally, new infectious virus begins to appear 4 to 6 hours after the onset of DNA synthesis and 2 to 3 hours after antigen production. Thus, the sequential synthesis of DNA, protein, and finally, the infectious particles, is compressed into the last 25 per cent of the eclipse period. Each step in this pattern of synthesis appears to be closely dependent on the preceding one.

SUMMARY

Biochemical synthesis in adenovirus-infected HeLa cells was studied utilizing 5-fluorodeoxyuridine (5-FUDR), a potent inhibitor of deoxyribonucleic acid production. Synthesis of saline-soluble DNA and infectious virus was completely suppressed by addition of the analogue to cells as late as 10 hours after infection. The inhibitory effect of this compound was totally reversed

by addition of 10^{-6} M thymidine to the culture medium. Synthesis of DNA essential for virus production began 10 hours after infection and was completed by 16 hours after infection. These data support the hypothesis that the saline-soluble DNA is a precursor of infectious virus particles. Studies of antigen production indicated that formation of virus-specific proteins was directly dependent upon synthesis of DNA.

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