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Effect of Poxvirus Infection on Host Cell Deoxyribonucleic Acid Synthesis

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Deoxyribonucleic acid (DNA) synthesis was studied in poxvirus-infected cells by measuring ^{14}C-thymidine incorporation into viral and host cell DNA. A complete separation of the two species of DNA was achieved by combining the previously used "Dounce method" with a separation method based on different reannealing properties of viral and vertebrate DNA. Shortly after infection of HeLa cells with poxviruses, a burst of viral DNA synthesis occurred in the cytoplasm, but a rapid inhibition of host-cell DNA synthesis in the nucleus was observed. This inhibition of cellular DNA synthesis was also found if an accumulation of viral DNA was prevented. At high multiplicities, ultraviolet-irradiated virus inhibited host-cell DNA synthesis to the same extent as fully infectious poxvirus. Under the same conditions, heating at 60 C for 15 min caused a decrease in the ability of cowpox virus to inhibit host-cell DNA synthesis, but did not produce the same effect on vaccinia virus strain WR.

The effect of infection of a vertebrate cell with a deoxyribonucleic acid (DNA)-containing animal virus on the synthesis of host-cell DNA is of considerable interest, since it may ultimately determine the fate of the infected cell. This problem has therefore been studied in detail with cytocidal viruses, as well as with tumor viruses, and varying phenomena have been observed (5). A prerequisite for an approach to this problem is an experimental method which allows investigation of the incorporation of labeled DNA precursors, such as thymidine, separately into viral and host-cell DNA under various conditions. Because of the similar base composition of poxvirus DNA and DNA from vertebrate cells, separation by physical means, such as isopycnic centrifugation, is difficult (8, 23). Measurements of thymidine incorporation by autoradiographic techniques have established that poxvirus DNA is synthesized in the cytoplasm and that at least in some cell strains replication of host-cell DNA is inhibited shortly after infection; this ability is retained by poxvirus even after inactivation by ultraviolet (UV) irradiation (10, 14, 17, 20).

A more direct approach to this problem became possible with the development of a physicochemical method for separating poxvirus and vertebrate DNA. This method, in combination with the "Dounce method," gave a more satisfactory separation of the two species of DNA than was previously achieved (12). Quantitative experiments performed by this method on DNA synthesis of HeLa cells after infection with poxvirus are described in this report.

MATERIALS AND METHODS

Virus and cell cultures. HeLa S_3 cells were grown on glass in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum. Two days before the experiments, the cells were transferred to spinner flasks and grown in suspension in Eagle's spinner medium with 5% calf serum, at a concentration of 6×10^5 to 8×10^5 cells/ml (4).

For infection, vaccinia virus (strain WR) and cowpox (strain Brighton) were used throughout. The virus was propagated in 10- to 12-day-old chick embryos and was used after purification by the method of Joklik. (7) Infectivity titrations were carried out on primary chick embryo fibroblast cultures either by the method of Lindemann and Gifford (18) or by using the agar-overlay technique described by McClain (21). The two methods gave comparable results. The amount of virus in the purified stocks was also determined by measurement of the optical density (OD) at 260 mμ. From this, the number of virus particles was calculated by assuming that one optical density unit is equivalent to 1.2×10^{10} virus particles (7). The plaque-forming unit (PFU) to particle ratio for the vaccinia virus preparation was determined as 1:150; for the cowpox virus preparation, as 1:450.

Inactivation of virus. UV-inactivated virus was prepared by exposing 4-ml samples of purified stock virus in sucrose to a UV lamp (MR 4, Gates & Co., New York, N.Y.) for 40 and 80 sec at a distance of

31 cm. After irradiation for 5 and 15 sec, infectivity was reduced by 2 and approximately 6 log units. Virus was heat-inactivated by incubation in sealed ampoules at 60 C for 15 min (9). Absence of infectivity was checked by inoculation of 0.05-ml undiluted samples on the chorioallantois membrane.

Adsorption. HeLa cells were infected with different virus multiplicities according to procedure A described by Joklik and Becker (10). Uninfected cells were treated exactly the same as infected cells, but instead of the virus, a similar volume of sucrose was added ("mock infection"). The efficiency of adsorption was assayed with purified virus labeled with ^{14}C-thymidine. Approximately 50% of the infectious or UV-irradiated virus was adsorbed to the HeLa cells. After inactivation by heat, only 30 to 40% of the virus particles were adsorbed.

Pulse labeling and breaking of cells. Thymidine-*2-^{14}C* (1 to 4 μc; specific activity, 55.8 mc/mmole) was added to 25-ml portions of the suspension culture (10^6 cells per ml) for periods of 15 min. The incorporation was stopped by adding frozen phosphate-buffered saline (PBS). Cells were then collected by centrifugation, washed twice with PBS, and suspended in 1 mM phosphate (*p*H 7.4) at a cell concentration of 10^7 per ml. Cells were allowed to swell in the hypotonic buffer and were then broken in a Dounce homogenizer (in 2-ml portions), precalibrated to disrupt over 90% of the cells. Nuclei were separated from cytoplasm by centrifugation at 200 $\times$ *g* for 10 min. After removal of the cytoplasmic fraction, the nuclei were again centrifuged for 10 min at 200 $\times$ *g*, as much supernatant fluid as possible was removed with a Pasteur pipette, and the nuclei were frozen at -20 C until used.

In a portion (equivalent to 10^6 cells) of the cytoplasm, acid-insoluble material was precipitated with trichloroacetic acid (5% final concentration) and filtered (Millipore Corp., Bedford, Mass.); the radioactivity was then determined in an automatic Packard scintillation spectrometer.

Isolation of ^{14}C-HeLa DNA from the cell nuclei. Poxvirus DNA and vertebrate DNA are so similar in their base composition that a satisfactory separation of the two native DNA species cannot be achieved (10). By taking advantage of the fact that cellular DNA retains the density of single-stranded molecules under conditions of renaturation, whereas viral DNA can be reannealed, the original slight density difference between the two types of DNA was magnified. Previously, it was demonstrated that a partial separation of cowpox virus and HeLa cell DNA can be obtained by equilibrium density gradient centrifugation in CsCl after subjecting a mixture of the two types of DNA to denaturation and reannealing. The applicability of the method was demonstrated with mixtures of DNA extracted from purified virions and HeLa cells, and this method was used to purify cellular DNA prepared from nuclei of infected HeLa cells (12).

After thawing, the nuclei from 2.5 $\times$ 10^7 cells were suspended in 1.5 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 7.4), and sodium dodecyl sulfate (SDS) was added to a final concentration of 2%. After 1 hr at room temperature, each sample was extracted twice with an equal volume of phenol and was then extracted with the same amount of chloroform. The DNA was precipitated with ethyl alcohol and redissolved in 3 ml of 0.05 M Tris-chloride buffer (*p*H 7.4) at a concentration of 40 μg/ml. The DNA preparations were then heated for 5 min at 100 C and chilled. CsCl solution was added to a final concentration of 0.5 M, and the samples were further incubated at 60 C for 12 hr. All centrifugations in CsCl gradients were performed for 48 hr at 40,000 rev/min in the R 50 angle rotor of a Beckmann preparative ultracentrifuge. The gradients were collected from the bottom and analyzed for optical density and radioactivity (12).

RESULTS

Rate of viral and cellular DNA synthesis in the virus-infected cell. The rate of DNA synthesis at various intervals after infection was determined by measuring the amount of ^{14}C-thymidine incorporated into acid-insoluble material during short pulses. Because vaccinia virus multiplies in the cytoplasm of cells, it is possible, by pulse labeling and preparation of cytoplasmic extracts, to examine the rate of synthesis of viral DNA. A small amount of the cytoplasmic DNA is, however, centrifuged off together with the nuclei. Because of the large amount of radioactivity incorporated into viral DNA in the first 6 hr after infection, even a small contamination affects the accurate determination of ^{14}C-thymidine incorporation into cellular DNA.

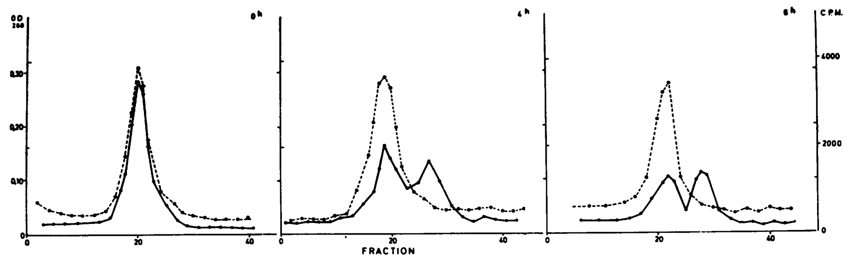

Fig. 1. Distribution of thymidine-2 ^{14}C incorporated into DNA extractable from HeLa cell nuclei at different times after infection. Broken line, optical density. Solid line, radioactivity

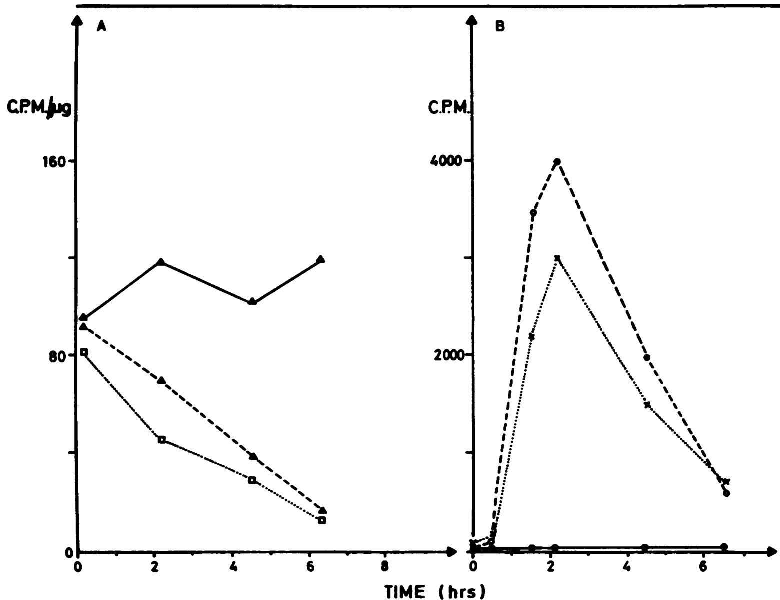

FIG. 2. *Rate of ^{14}C-thymidine incorporation into viral and cellular DNA in uninfected and infected HeLa cells. Cells were infected as described in Materials and Methods. At different times, indicated samples of the cells were exposed to radioactive thymidine for 15 min, and the radioactivity incorporated into each species of DNA was determined. Abscissa, time after infection; ordinate, specific activity of DNA; (A) counts per minute per microgram of HeLa DNA; (B) counts per minute incorporated into cytoplasmic DNA per 10^6 cells. (A) Incorporation into HeLa cell DNA: (▲) uninfected; (△) infected, vaccinia (WR); (□) infected, cowpox. (B) Incorporation into cytoplasmic fraction: (●) uninfected; (○) infected, vaccinia (WR); (X) infected, cowpox.*

Analysis of the DNA in the nuclear fraction by equilibrium density gradient centrifugation revealed the following picture (Fig. 1). In the sample taken from the infected cultures at zero-time, only one peak of radioactivity, corresponding to the UV-absorbing material, was found. This material had the buoyant density of cell DNA (ς = 1.718 g/cm^3). Similar profiles were observed with mock-infected cultures, labeled with ^{14}C-thymidine at different times, as well as with cultures infected with inactivated virus. When an infected culture was labeled with ^{14}C-thymidine between 1 and 6 hr postinfection, radioactivity was not only associated with cellular DNA, but a second peak of radioactivity, with a smaller buoyant density (ς = 1.696 g/cm^3), was also detected. This material formed a band in the CsCl gradient at the same density as reannealed DNA isolated from purified poxvirions or viral DNA isolated from cytoplasm of infected cells (12). A peak of radioactivity was only found in this position when the nuclear DNA had been extracted from infected HeLa cells actively synthesizing viral DNA in the cytoplasm. We therefore concluded that this material represents a small amount of viral DNA contaminating the nuclei. The amount of viral DNA associated with the nuclei depended on the time at which the cultures were pulsed with ^{14}C-thymidine.

The amount of label incorporated into the HeLa cell DNA at various times after infection with vaccinia or cowpox virus is shown in Fig. 2A. Specific activities were calculated by dividing the total amount of radioactivity in cellular DNA by the total amount of cellular DNA recovered from the gradients. When cells were pulse-labeled between 3 and 4 hr after infection, separation from viral DNA was sometimes incomplete, and the specific activity had to be determined from a few fractions of the peak of cellular DNA. Over the experimental period (usually 6 hr), the amount of ^{14}C-thymidine which was incorporated into cell DNA in mock-infected cultures usually increased somewhat. If the HeLa cells were grown before infection at a cell concentration of 6×10^5 to 8×10^5/ml, this increase was usually small and in most experiments was not observed at all. On the other hand, in cell cultures which had been held at a cell concentration around 2×10^5/ml, the increasing rates of DNA synthesis after mock infection were practically always observed. This stimulation of cellular DNA synthesis is most likely caused by the "shock" to which the cells were exposed during the manipulations necessary

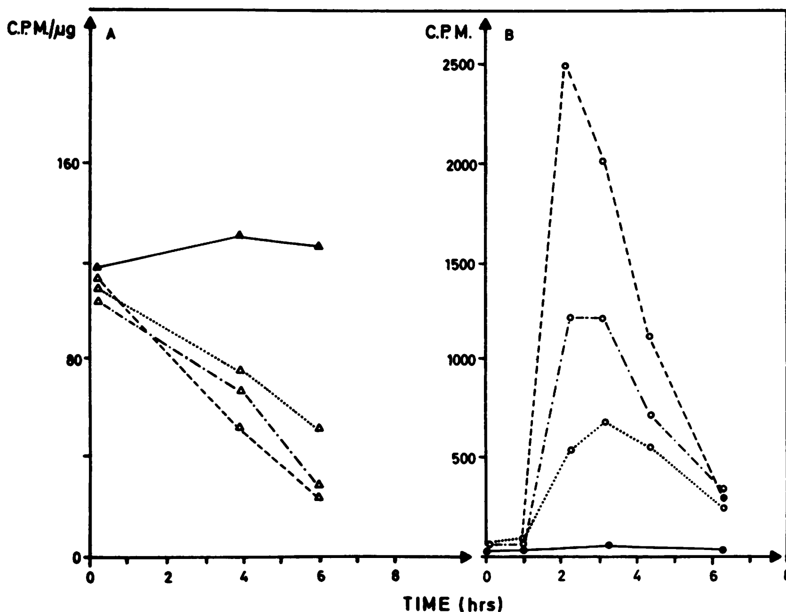

FIG. 3. *Effect of multiplicity of infection on the rate of viral and cellular DNA synthesis. HeLa cell cultures were infected with different multiplicities of vaccinia virus (WR) as described under Materials and Methods. (A) Incorporation of* ^{14}*C-thymidine into HeLa cell DNA.* (▲) *Mock-infected;* (△) *infected with 400 virus particles (dashed line), 80 virus particles (dot-dash line), or 40 virus particles (dotted line). (B) Incorporation of* ^{14}*C-thymidine into cytoplasmic fraction.* (●) *Mock-infected;* (○) *infected with 400 virus particles (dashed line), 80 virus particles (dot-dash line), or 40 virus particles (dotted line).*

for virus adsorption. Similar observations were reported by Kit and co-workers (17). In contrast, after infection with either of the two poxvirus strains (400 virus particles/cell), specific activity of cell DNA decreased. Usually, 6 hr after infection the specific activity of the DNA in infected cells amcunted to only 25% of that in the controls. In some experiments, the time course of inhibition was delayed. Mock-infected control cultures in the same experiment showed a rapid increase in ^{14}C-thymidine incorporation, indicating a degree of "shock" higher than that usually found. This result confirms earlier reports on inhibition of cell DNA synthesis in HeLa cells and various other host cells after infection with different strains of poxvirus (6, 10, 14, 15, 20). In confirmation of earlier observations, a large burst of viral DNA synthesis was found in the cytoplasm of infected cells, beginning at 1 hr and reaching a maximum between 2 and 3 hr after infection (10, 22). After this time, the rate of thymidine incorporation decreased rapidly (Fig. 2B).

Influence of multiplicity of infection on host-cell DNA synthesis. For the eventual elucidation of the mechanism of inhibitory action of poxvirus on host-cell DNA synthesis, it is of importance to know how the multiplicity of infection influences the kinetics of host-cell DNA synthesis.

HeLa cells were infected with 400, 80, and 40 virus particles per cell, and at various intervals after infection the amount of ^{14}C-thymidine incorporated into viral and cell DNA was determined (Fig. 3).

The rate at which host cell DNA synthesis is inhibited after infection with vaccinia virus (WR) was only insignificantly changed at low multiplicities (Fig. 3A). It seems that, under the conditions used, only a few virus particles are needed to trigger the inhibitory effect on host-cell DNA synthesis. Contrary to this, in the range of multiplicities used, the rate of viral DNA synthesis in the cytoplasm clearly depended on the virus input (Fig. 3B). The time course of inhibition of cytoplasmic DNA synthesis, on the other hand, did not vary significantly with the different miltiplicities. Regardless of the amount of virus used for infection, viral DNA synthesis became detectable before 1 hr after infection, and it was complete 5 hr later. This result is in accordance with autoradiographic observations by Cairns and others, which have established that the number of vaccinia factories varies with the multiplicity of infection (2, 14).

Host-cell DNA synthesis in the infected cell in the absence of viral DNA replication. The gradual inhibition of ^{14}C-thymidine incorporation into

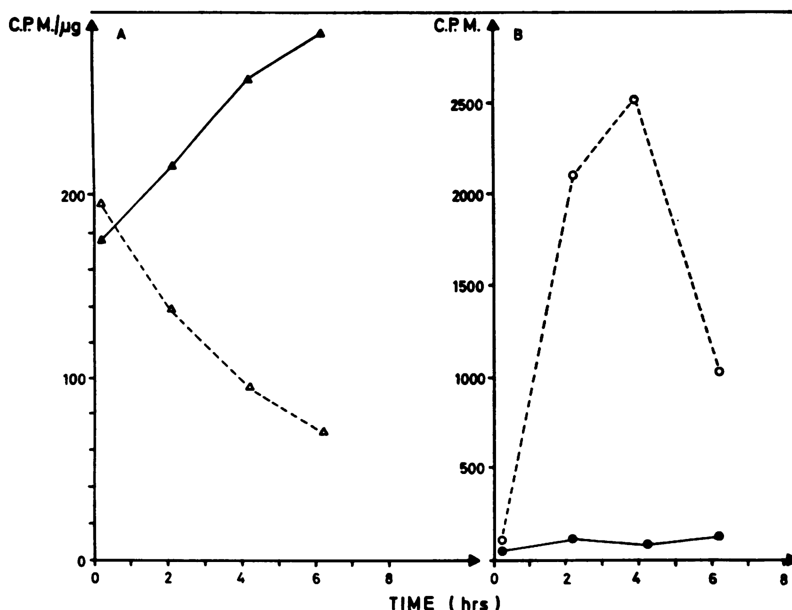

FIG. 4. *Rate of host-cell DNA synthesis in cells treated with FUDR at the time of infection. At the time points shown, inhibition of DNA synthesis was reversed by the addition of* ^{14}*C-thymidine, and the incorporation of the labeled precursor into cell DNA was determined as described under Materials and Methods. Varying the* ^{14}*C-thymidine concentration from* 2×10^{-6} *to* 4×10^{-6} *M had no influence on the rate of thymidine incorporation. (A) Incorporation of* ^{14}*C-thymidine into HeLa cell DNA: ▲ mock-infected; △, infected. (B) Incorporation of* ^{14}*C-thymidine into cytoplasmic fraction: ●, mock-infected; ○, infected with vaccinia (WR).*

host-cell DNA after infection might be caused by a competition of viral DNA with cellular DNA for DNA precursors or templates for enzymes with polymerizing activity (1). To investigate this possiblity, host-cell DNA synthesis was studied under conditions which did not allow accumulation of viral DNA during the infectious cycle.

Addition of the drug fluorodeoxyuridine (FUDR) at the time of infection rapidly inhibited the formation of viral DNA in the poxvirus-infected cell, without influencing the initial steps of virus replication such as adsorption and penetration. FUDR was therefore used to differentiate between "early" and "late" viral functions (13, 24). The effect of FUDR on DNA synthesis can readily be reversed by the addition of thymidine to the medium (22).

Cells were infected with 400 elementary bodies per cell. After adsorption of the virus, cells were diluted 10-fold with Eagle's medium containing 5% dialyzed serum and FUDR at a concentration of 10^{-5} M. At various intervals, the effect of the drug was reversed by the addition of ^{14}C-thymidine, and the amount of radioactivity incorporated into cytoplasmic and nuclear DNA was determined. Figure 4A shows that the rate of ^{14}C-thymidine incorporation into host-cell DNA again decreased with time after infection. The rate of incorporation was approximately the same as when viral DNA accumulated in the cell population. Incorporation into cytoplasmic DNA increased in the first 3 hr after infection at a rate comparable to that in infected cultures not treated with FUDR. Thereafter, the incorporation of labeled thymidine declined in FUDR-treated cultures at a slower rate (Fig. 4B).

The gradual inhibition of ^{14}C-thymidine incorporation into cellular DNA under these conditions cannot be explained by a competition of viral progeny DNA as templates for replication. The possibility that input viral DNA competes as a template cannot be excluded. In addition, this experiment makes it seem very unlikely that the inhibitory effect is caused by progeny DNA itself or by a product coded by it. On the basis of similar experiments, Kaplan and Ben Porat came to the same conclusion for the effect of pseudorabies virus infection on DNA synthesis of rabbit kidney cells (1).

Effect of inactivated pox virus on host-cell DNA synthesis. Insight into the mechanism of the inhibitory activity of the pox-virus particle on cell DNA synthesis was sought by studying the effect of different inactivation methods on the ability of the virus to inhibit host-cell DNA synthesis.

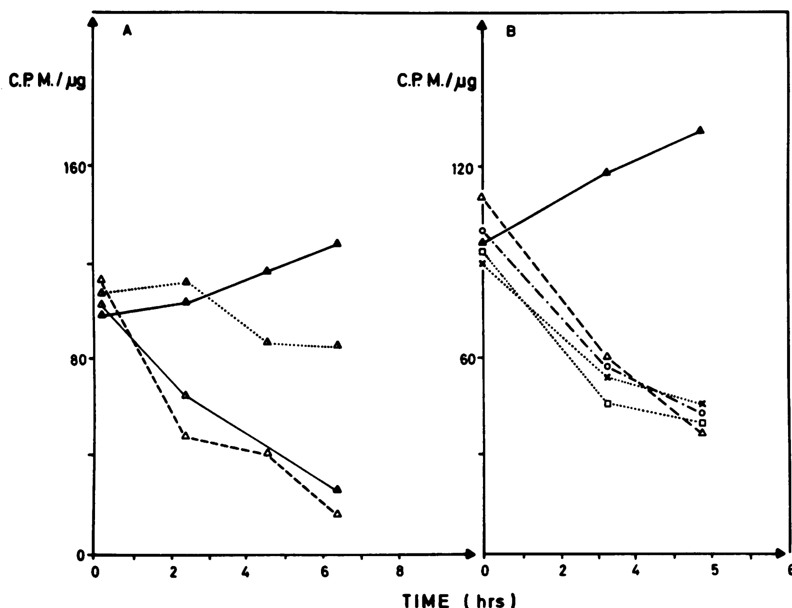

FIG. 5. *Rate of ^{14}C-thymidine incorporation into DNA of HeLa cells infected with inactivated poxvirus. In all experiments, the multiplicity of infection was 400 E.B./cell.(A) Inactivated by heating for 15 min at 60 C: (▲) uninfected; (△) infectious cowpox or vaccinia WR; (▲) heated cowpox (dotted line) or heated vaccinia WR (solid line). (B) Inactivated by UV irradiation: (▲) uninfected; (△) infectious cow pox or vaccinia WR; (X) irradiated vaccinia, 40 sec; (□) irradiated vaccinia, 80 sec; (○) irradiated cowpox, 80 sec.*

Two methods of inactivation were chosen. Heat presumably destroys the infectivity of the virus particle by denaturing a component (most likely a protein) of the complex virus capsid with less effect on the viral genome. Thus, a small proportion of a heat-inactivated vaccinia virus population can be reactivated by infectious pox virus (11). In contrast, UV irradiation exerts its inactivation effect primarily on the DNA of the virus. All experiments with inactivated virus were done at a multiplicity of 400 E.B./cell.

After infection of the HeLa cells with heat-inactivated cowpox virus, the ability to turn off cell DNA synthesis in the nucleus was found to be impaired during the first 3 hr after infection. Thereafter, host-cell DNA synthesis was gradually inhibited, even in cells infected with heated virus (Fig. 5A). The same kinetics of inhibition was also observed when the multiplicity was increased 10-fold. Vaccinia virus strain WR, completely inactivated by heating at 60 C for 15 min, was still able to inhibit cellular DNA synthesis to the same extent as fully infectious virus. Even heating the virus for 30 min at the same temperature did not impair significantly the inhibitory property of vaccinia virus. Since prolonged heating affected the adsorption of the virus particles to the cells, no attempts were made to find more drastic conditions of inactivation with this virus strain. As shown in Fig. 5B, inactivation of both virus strains by UV irradiation had no effect on the ability to interfere with DNA synthesis of the cell, at an irradiation dose which completely inhibits replication of viral DNA. Similar results were reported previously (6, 10, 17). Our experiments with poxviruses inactivated by UV irradiation are compatible with the notion that a component of the virus capsid, rather than an early product of viral DNA, is involved in the effect of the virus on host-cell DNA synthesis.

Host-cell DNA synthesis in infected cells treated with inhibitors of protein synthesis. Experiments have been presented which indicate that protein synthesis is a prerequisite for the inhibitory effect of pseudorabies virus and poxvirus infection on DNA synthesis of the host cell (1, 19). To our knowledge, these reports contain the only experimental evidence that a new protein is necessary to turn off the synthesis of cell DNA in an animal cell infected with a DNA-containing cytocidal virus. We tried to investigate this important question in the virus-cell system under study. Addition of puromycin (50 µg/ml), cycloheximide (30 µg/ml), and streptovitacin (22 µg/ml) at the time of infection or, in the case of the latter two compounds, 1 hr before infection caused a rapid inhibition of cell DNA synthesis. The rate of ^{14}C-thymidine incorporation into the nuclear DNA in

normal cells declined much faster than in untreated, poxvirus-infected cells, and therefore made it impossible to study any virus-specific effect in the presence of these inhibitors. Attempts to study host-cell DNA synthesis in infected cells after reversing the inhibitors have so far been without success.

DISCUSSION

Since poxviruses are a group of DNA-containing animal viruses which replicate in the cell's cytoplasm, it is of particular interest to study the effect of infection on host-cell DNA synthesis (which occurs almost exclusively in the nucleus) and to compare the results with those obtained with DNA viruses multiplying in the cell nucleus.

The decreased incorporation of ^{14}C-thymidine into HeLa cell DNA observed after infection confirms previous results obtained by autoradiographic and biochemical techniques. Preliminary experiments in our laboratory have shown that complete separation of virus and cell DNA can be achieved also in a variety of other cells by isolating nuclei first and then purifying the cell DNA by isopycnic centrifugation. It will be of special interest to see how host-cell DNA synthesis is influenced in stationary cell cultures.

In interpreting the reduced thymidine incorporation into cell nuclei as a virus-specific inhibition of host-cell DNA synthesis, we have to assume that the intracellular pool of thymidine nucleotides remains constant over the experimental period. Because of the low concentration of thymidine nucleotides, attempts to determine the specific activity of the acid-soluble pool in infected cells have so far not been successful. It is, nevertheless, unlikely that eventual variations of the intracellular pool of thymidine influence the incorporation of radioactive thymidine into DNA. The same rate of inhibition is observed even when biosynthesis of thymidine monophosphate is inhibited by FUDR. Degradation of DNA as a source of thymidine nucleotides is also unlikely, because no degradation of viral and cell DNA has been found (16; *unpublished data*).

Several explanations for the observations obtained with inactivated virus are possible. One would be that a component of the virus capsid is responsible for the inhibition of cell DNA synthesis. In keeping with this notion would be the high resistance of the inhibitory ability of the virus particle to UV inactivation. A similar interpretation of the experimental observations was also proposed by Ginsberg for the effect of adenovirus on host-cell DNA synthesis (*personal communications*). It cannot be excluded with certainty, though, that some cistrons on the viral genome have an unusual high resistance to UV inactivation.

Experiments with heat-inactivated virus have have shown that whatever component(s) of the virus causes the effect on host-cell DNA synthesis is not completely inactivated by heat under the conditions used. Why the kinetics of inhibition are altered in the case of infection with heated cowpox but not with vaccinia (WR) virus is not clear.

Only an eventual isolation and chemical characterization of a capsid component responsible for the effect on host-cell DNA synthesis would allow a decision between the two possibilities. Our experiments are compatible with the findings of other workers, who have found a gradual decrease of radioactive thymidine incorporation after infection of L cells with heat-inactivated poxvirus (6, 15). In addition, it can be shown that the effect of heat-inactivated pox virus on host-cell DNA synthesis may vary with the virus strain used in the experiment.

At present, it is impossible to draw any conclusions as to the mechanism of the inhibition of cellular DNA synthesis. Probably the gradual decrease of host cell DNA synthesis is the result of a combination of virus-specific alterations induced in the infected cell. The observation that inhibition of protein synthesis by antibiotics rapidly blocks DNA synthesis makes it perhaps reasonable to investigate the question of cell DNA synthesis inhibition more closely in connection with the inhibition of cell protein synthesis by poxviruses (25).

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