[CANCER RESEARCH 28, 1686-1691, September 1968]

Effects of Hydroxyurea on Polyoma Virus Replication[1]

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SUMMARY

Hydroxyurea inhibits DNA synthesis and growth of mouse embryonic and kidney cells. It is also an effective inhibitor of polyoma virus synthesis in these cells. Uninfected embryo cells are more sensitive to hydroxyurea than infected ones. Several different parameters of polyoma infection, i.e., DNA synthesis, infectivity, and capsid protein formation are affected similarly by hydroxyurea.

INTRODUCTION

Hydroxyurea has been shown to inhibit the growth of rapidly proliferating cells *in vivo* and *in vitro* (12, 21). It was reported in 1963 to inhibit the growth of certain transplanted tumors in mice (22) and subsequently to be active against chronic granulocytic leukemia and inconsistently against other malignant neoplasms in man (8–10, 23). Although it does not appear to be uniquely active against any human tumor, it is of continuing interest because it is an active chemotherapeutic agent of a new class. It has been demonstrated that hydroxyurea inhibits DNA synthesis but not RNA or protein synthesis in cultured mammalian cells (26), bacteria (3, 16), and regenerating rat liver (18). Studies in crude systems have shown hydroxyurea to inhibit the conversion of cytidylic acid to deoxycytidylic acid (2, 24). Further studies (7) using a highly purified enzyme system from *E. coli B.* have shown that hydroxyurea irreversibly inhibits enzyme protein B2 of the ribonucleotide reductase system.

Polyoma virus has been shown to have marked effects on the DNA synthesis of infected cells. In well-contact-inhibited monolayers of mouse kidney cells, infection is followed by an increase in cellular and viral DNA synthesis as well as increased activities of several enzymes concerned with DNA synthesis (1, 5, 6). It has also been shown that infection of mouse embryo cells at high multiplicities can cause a decrease in DNA synthesis (20).

The present studies were undertaken to evaluate the influence of hydroxyurea on growth and DNA synthesis in cultures of mouse kidney and embryo cells and the alterations therein produced by infection with polyoma virus.

MATERIALS AND METHODS

Cell Cultures

Mouse embryo cells were prepared by trypsinizing minced whole mouse embryos (about 14th day of gestation) for 1 hour at 37°C. The cells were suspended in Parker 199 medium with 10% calf serum at a concentration of 3×10^5 cells/ml and grown in Roux flasks, 100 ml/flask. Secondary cultures were made in 60-mm Falcon plastic Petri dishes in Dulbecco's modification of Eagle's medium with 10% calf serum. Mouse kidney cells were prepared from kidneys of two-week-old mice. The kidneys were freed from perirenal fat and capsule, minced, and trypsinized for 2–3 hours at 37°C with intermittent vigorous pipetting. The number of kidney cell clumps plus tubules was adjusted to 10^5/ml in Parker's medium 199 containing 10% fetal calf serum. Cultures were kept in 60-mm Falcon plastic Petri dishes, and the medium was replaced daily for three days. They were employed for experiments on the fourth to seventh day after seeding and were then kept in Dulbecco's modification of Eagle's medium. All Petri dishes were kept in 5% CO_2 in air.

Virus

A single-plaque isolate of polyoma virus obtained in these laboratories by Dr. I. Hellström was used. The original virus source was obtained from Dr. B. E. Eddy and designated 1956-11C. The history of this virus was described elsewhere (4). One single pool of virus titrating 1.8×10^8 plaque-forming units/ml was used in all experiments. This pool was prepared according to Winocour (25).

Infection. Primary kidney cultures and secondary embryo cultures in Petri dishes were incubated for 1 hour at 37°C with 1 ml of virus. Unadsorbed virus was then rinsed away, and the cultures were kept in Dulbecco's medium containing 10% fetal calf serum and varying concentrations of hydroxyurea.

DNA Synthesis

DNA synthesis was evaluated by measuring incorporation of thymidine-^{3}H (16.6 c/mmole, New England Nuclear Co.) into acid-insoluble material using a method modified from Regan and Chu (15). The thymidine was added to Dulbecco's medium in a concentration of 2 μc/ml. This radioactive medium containing fresh hydroxyurea was supplied 24 hours after introduction of virus and hydroxyurea. Thirty-six to forty hours after infection (i.e., 12–16 hours after beginning exposure to

[1] This work was supported by grants from the Swedish Cancer Society, Grant CA-04747 from the National Cancer Institute, USPHS, the Damon Runyon Memorial Fund, and Knut and Alice Wallenbergs Foundation.

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Received February 7, 1968; accepted May 14, 1968.

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thymidine-^{3}H), the medium was poured off and replaced with the same amount (5 ml) of 0.9% NaCl. The Petri dishes were chilled in ice and the cells were disrupted by sonication for 1 minute in a MSE ultrasonic power unit. The sonicates were examined microscopically to determine that the cell disruption was complete.

Aliquots of the sonicate were frozen for subsequent virus assay. One hundred μl of each sample were applied to a 23-mm disc of Whatman No. 3 MM filter paper, which was immediately dropped into cold 5% trichloroacetic acid. The discs were then washed three times for 10 minutes in cold 10% trichloroacetic acid and then three times for 10 minutes in absolute ethanol. After drying, the radioactivity of the discs was measured using dioxane in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Virus Titration

Plaque-assays of virus infectivity were performed in 60-mm Falcon plastic Petri dishes according to Sheinin (19). Hemagglutinating activity was tested as previously described (4). All preparations to be tested for hemagglutination were sonicated in the presence of fluorocarbon just before the test in order to reduce activities of serum inhibitors.

Fluorescent Antibody Tests

The fluorescent antibody technic was used in order to estimate the fraction of cells producing capsid antigen. In such experiments virus and hydroxyurea were introduced as described above into Petri dishes containing mouse kidney or embryo cells growing in monolayers on 20 x 30 mm glass coverslips. At the time of harvest 40 hours after infection the coverslips were rinsed twice in phosphate-buffered saline and once in distilled water, air-dried, and fixed in acetone for 5 minutes. They were then stored frozen prior to test.

In tests with the direct fluorescent antibody technic the coverslips were exposed for 30 minutes to fluorescein-isothiocynate conjugated mouse antipolyoma serum, washed with saline, and mounted on 10% glycerin. The fraction of infected cells was evaluated microscopically using ultraviolet illumination.

Mouse antipolyoma serum was produced by injecting newborn A. SW mice with dilute (5×10^3 in 0.05 ml) polyoma virus. The mice were bled at 4–5 weeks of age. For the indirect technic the method of Minowada (11) was used with serum kindly provided by Dr. Minowada.

Growth Curves

In the experiments to study the effect of hydroxyurea on cell growth, cells were grown in Petri dishes as described above. Medium and hydroxyurea were changed daily. Each day, one dish at each concentration of hydroxyurea was removed, stained with crystal violet (1%), rinsed, and air-dried. The cells in 12 segments of each Petri dish were counted, using a standard ocular grid.

RESULTS

Inhibition of Cell Growth by Hydroxyurea

Mouse Embryo Cells. Secondary embryo cells were seeded into Petri dishes in a dilute concentration (6×10^4/ml). Hydroxyurea was a potent inhibitor of cell proliferation in such cultures (Chart 1*A*). At 10^{-4} M or 10^{-3} M hydroxyurea, there was no detectable proliferation of cells. In 10^{-5} M hydroxyurea the doubling time was about twice as long as in the absence of drug. When fresh medium was supplied to cells kept in 10^{-4} M or 10^{-3} M hydroxyurea after the 4-day exposure, they still failed to grow, indicating an irreversible effect of hydroxyurea after this prolonged exposure.

Mouse Kidney Cells. Clumps or tubules of kidney cells were seeded at a concentration of 10^5/ml. Kidney cells grow in a more dense pattern than embryo cells, giving higher final cell counts. They were more resistant to hydroxyurea than embryo

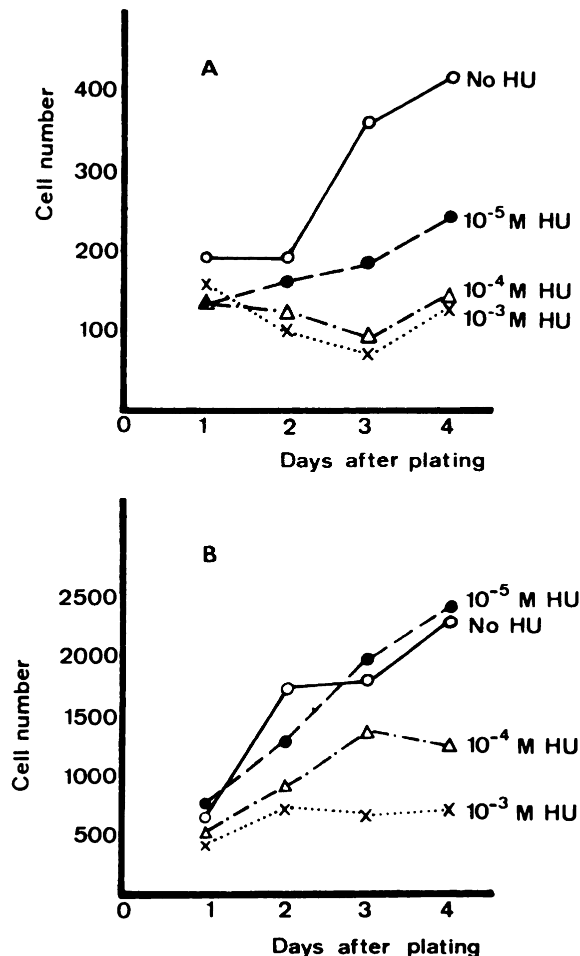

Chart 1*A*. Growth-curves of secondary mouse embryo cells exposed continuously to different concentrations of hydroxyurea (HU). 3×10^5 cells were plated per dish in Dulbecco's medium containing 10% calf serum. Medium containing HU was changed daily. Cells in twelve representative squares of each Petri dish were counted, using a standard ocular grid.

Chart 1*B*. Growth-curves of primary mouse kidney cells exposed continuously to different concentrations of HU. 5×10^5 kidney cell clumps were plated per dish in Dulbecco's medium containing 10% calf serum. Medium containing HU was changed daily. Cells in twelve representative squares were counted using a standard ocular grid.

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cells. In contrast to embryo cells, they were not affected by 10^{-5} M hydroxyurea. 10^{-4} M hydroxyurea inhibited considerably, and at 10^{-3} M there was no significant proliferation (Chart 1*B*). Removal of the drug after incubation for four days in 10^{-3} M hydroxyurea did not result in recovery of the inhibited cells.

Influence of Hydroxyurea on DNA Synthesis

Infection of mouse embryo cells with high multiplicities of polyoma virus is known to interefere with cellular DNA synthesis (20). The relatively low rate of thymidine incorporation, typical for the late phase of polyoma replication in embryo cells, was very resistant to hydroxyurea (Chart 2). Even at 10^{-4} hydroxyurea, at which concentration cell proliferation is completely inhibited, there was no significant effect on DNA synthesis in infected cultures. The uninfected cells, however, were more sensitive to hydroxyurea, which inhibited DNA synthesis by 90% at the 10^{-4} M concentration. This is in perfect agreement with the effect of hydroxyurea on cell growth.

Infection with polyoma virus augmented DNA synthesis in kidney cultures infected at confluence. These cells were similarly

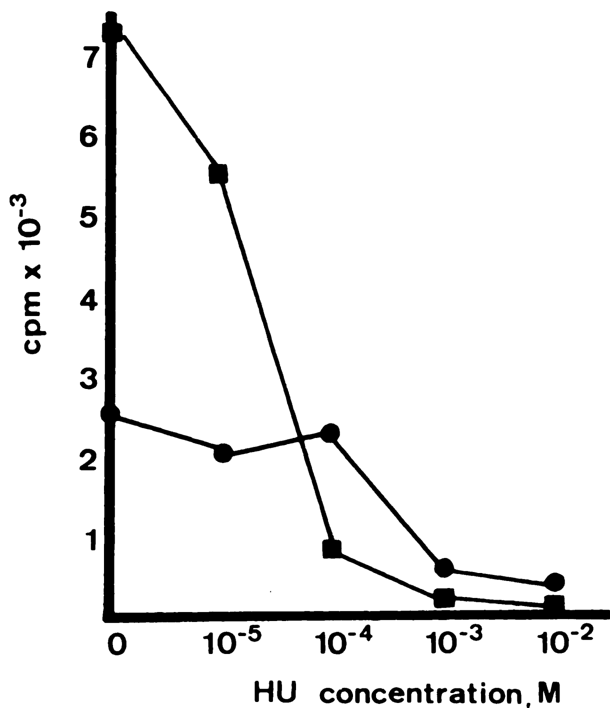

Chart 2. Thymidine-^{3}H incorporation into acid precipitable materials in secondary mouse embryo cell cultures. 1.5×10^6 cells were plated per Petri dish and allowed to settle over night. Control cultures (■) and cultures exposed to 100 pfu of polyoma virus (●) were kept in Dulbecco's medium with different concentrations of HU. Radioactive medium containing 10 μc of thymidine-^{3}H and fresh HU was supplied 24 hours after infection, and the cultures were incubated for 12 additional hours before harvest. Acid-precipitable radioactivity in 0.1-ml aliquots of 5-ml sonicate obtained per dish indicated on the ordinate. pfu, plaque-forming units; HU, hydroxyurea.

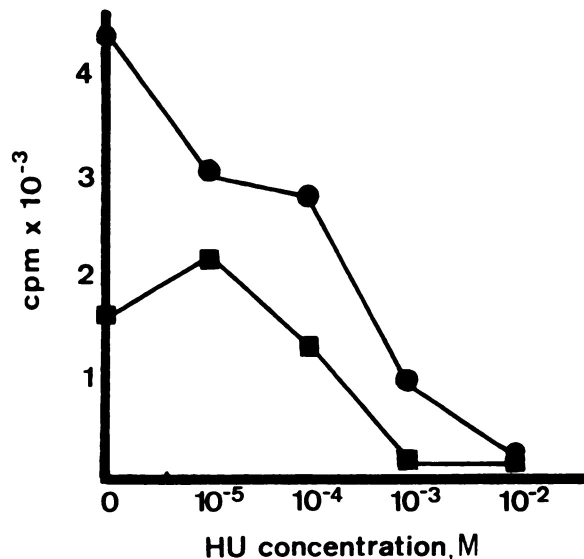

Chart 3. Thymidine-^{3}H incorporation in uninfected (■) and infected (●) confluent mouse kidney cultures. See Chart 2 for experimental procedure. HU, hydroxyurea.

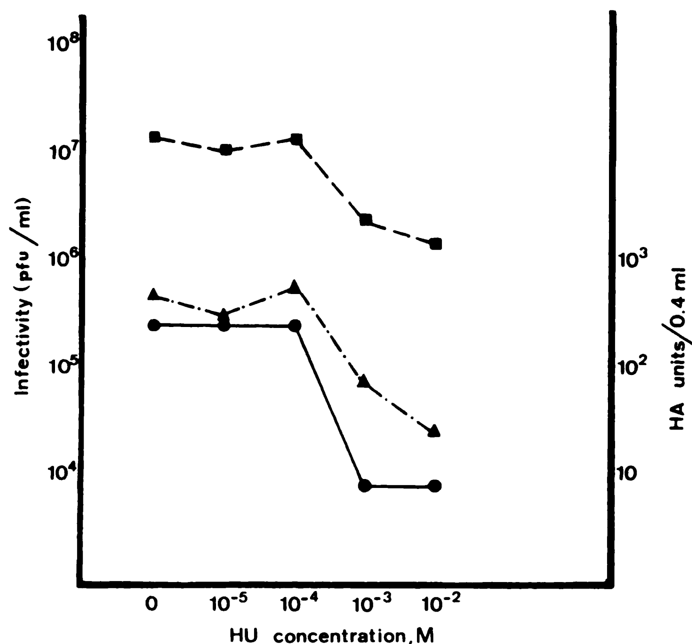

Chart 4. Polyoma virus activity recovered from infected secondary mouse embryo cultures kept in Dulbecco's medium with different concentrations of HU. Just confluent cell cultures were exposed to 100 pfu/cell. Unadsorbed virus was carefully rinsed away and fresh medium containing HU was added. Medium and HU were replaced 24 hours postinfection and the cultures were harvested 40 hours after infection. (■) Infective virus from medium. (▲) Cell-associated infective virus. (●) Hemagglutinating (HA) units per 0.4 ml of medium. HU, hydroxyurea; pfu, plaque-forming units.

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sensitive to hydroxyurea in both infected and uninfected cultures (Chart 3). However, even in this system the data indicate that high (10^{-3} M) hydroxyurea concentrations might inhibit DNA synthesis more completely in uninfected cultures. With kidney cells, too, there is good agreement between the effect of hydroxyurea on DNA synthesis and on cell replication.

Inhibition of Polyoma Virus Production by Hydroxyurea

Hydroxyurea inhibited polyoma virus production in embryonic cells, but the inhibition was incomplete. In the presence of 10^{-2} M hydroxyurea the amount of infective virus released in the medium was only about one log unit less than in untreated cultures (Chart 4). Most of the infectious virus at the time of harvest (40 hours after infection) was found in the medium. The cell-associated virus may represent released virus readsorbed to cells.

It can also be seen from Chart 6 that there is a parallel between hemagglutinin and infective virus production.

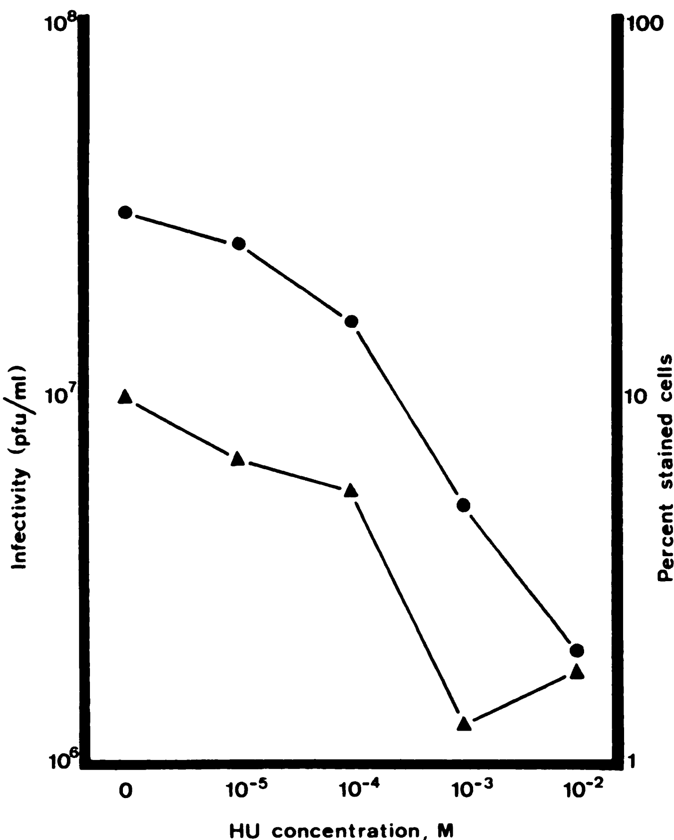

Chart 5. Percent polyoma virus-infected cells in just confluent secondary mouse embryo cell cultures grown in different concentrations of HU. Cultures on glass coverslips in 60-mm Petri dishes were infected with 100 pfu/cell. Forty hours after infection coverslips were removed and subsequently stained with direct fluorescent antibody technic using mouse antipolyoma serum. The fraction of infected cells as judged by the ability to make polyoma antigen (●) and the virus infectivity (▲) recovered from the culture medium were estimated. HU, hydroxyurea; pfu, plaque-forming units.

When the fraction of infected embryo cells was estimated by fluorescent antibody staining, there was a marked decrease of the number of infected cells in hydroxyurea-treated cultures (Chart 5).

In experiments with mouse kidney cells, the inhibition of virus release by hydroxyurea was marked (Chart 6).

Also in these cells hydroxyurea affected hemagglutinin and infective virus production to a similar degree.

In an experiment with kidney cells in which the percentage of infected cells was estimated with the indirect fluorescent antibody technic, the decrease in virus production as a function of hydroxyurea concentration was accompanied by a decrease in the fraction of infected cells (Chart 7).

DISCUSSION

The present experiments with cultured mouse embryo and kidney cells confirm earlier data on the effect of hydroxyurea on DNA synthesis obtained in several different cell systems. They also show that polyoma virus replication and DNA synthesis in polyoma-infected cells can be inhibited by hydroxyurea. Hydroxyurea is known to inhibit ribonucleotide reduction by inactivation of enzyme protein B2 of the ribonucleoside diphosphate reductase system from *E. coli B* (7). The hydroxyurea sensitivity of polyoma virus production makes it highly probable that a ribonucleotide reductase system is active during polyoma infection.

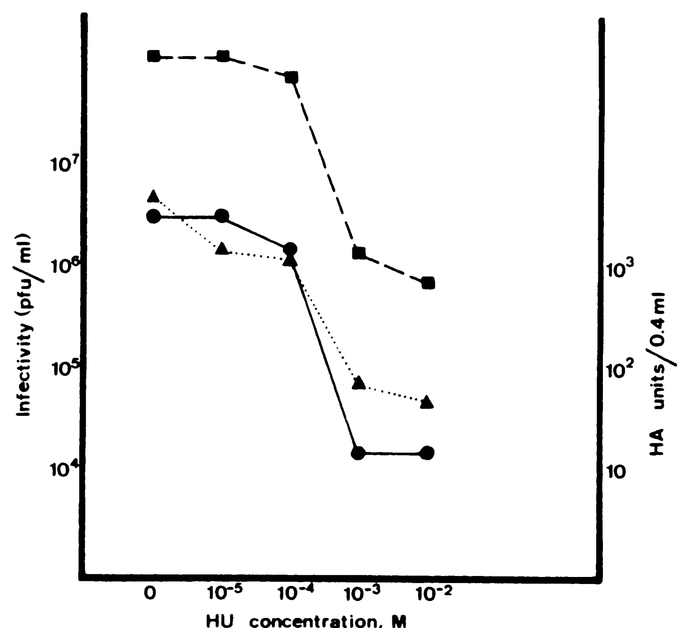

Chart 6. Polyoma virus recovered from confluent primary mouse kidney cultures kept in different concentrations of HU. See Chart 5 for experimental procedure. (■) Infective virus from medium. (▲) Infective virus from cell sonicates. (●) Hemagglutinating (HA) units per 0.4 ml of medium. HU, hydroxyurea; pfu, plaque-forming units.

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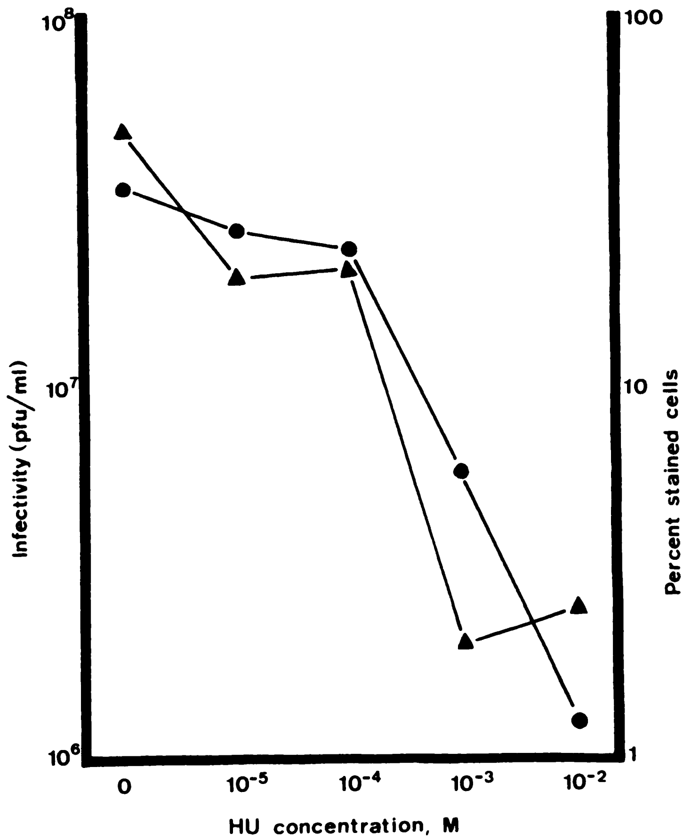

Chart 7. Percent polyoma virus-infected cells in confluent primary mouse kidney cell cultures kept in different concentrations of HU as judged by indirect fluorescent antibody staining of polyoma viral antigen (●). Virus infectivity recovered from medium (▲). See Chart 5 for experimental procedure. HU, hydroxyurea; pfu, plaque-forming units.

Interestingly, the DNA synthesis and cellular replication of uninfected embryo cells are much more sensitive to hydroxyurea than DNA synthesis and virus replication in infected embryo cells. This may be due to a difference in the predominant type of DNA, i.e., cellular or viral, and hydroxyurea in these cells may exert a specific effect on cellular DNA. In kidney cells, no major differences in the sensitivity of DNA synthesis in infected and uninfected cells occurred in response to hydroxyurea.

It is known that excess empty protein shells with hemagglutinating activity are present in normal preparations of polyoma virus (25). It was possible that hydroxyurea, as an inhibitor of DNA synthesis, might preferentially inhibit virus DNA synthesis as estimated by infectivity tests and would, to a lesser degree, interfere with the formation of viral capsid protein, resulting in an excess of empty shell formation. Such results have been described in cells infected with vaccinia and Herpes simplex viruses (13, 17). The observed results argue strongly against this possibility and indicate that in the polyoma system viral DNA and capsid synthesis are closely linked.

The fluorescent antibody tests with both embryo and kidney cells indicate that the decreased virus output in hydroxyurea-treated cultures is not a result of decreased virus formation per cell but primarily of a decrease in the fraction of cells synthesizing virus.

ACKNOWLEDGMENTS

The skillful technical assistance of Miss Gertrud Vejlens is gratefully acknowledged.

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