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STUDIES OF THE EFFECTS OF 5-IODO-2′-DEOXYURIDINE ON THE FORMATION OF ADENOVIRUS TYPE 2 VIRIONS AND THE SYNTHESIS OF VIRUS-INDUCED POLYPEPTIDES*

JUNE KAN-MITCHELL and WILLIAM H. PRUSOFF

Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

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Abstract—The antiviral activity of 5-iodo-2′-deoxyuridine (IdUrd) on human adenovirus type 2 (Ad2) was examined. The loss of infectivity, resulting from the presence of IdUrd in the medium, was greater than either the decrease in the number of viral particles produced or the increase in yield of incomplete particles. A "new" species of viral particle with a lower buoyant density than other known incomplete particles was identified at the higher concentrations of IdUrd tested. In the presence of 10 μg/ml of IdUrd, the specific infectivity of the complete particles was 0.04 per cent that of control. No difference in mobilities or molar ratios of the structural proteins derived from these completed virions was detected by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The buoyant density of the viral DNA derived from virions grown in the presence of IdUrd was higher in CsCl than that of control viral DNA, although both samples of DNA sedimented at the same rate in neutral and alkaline sucrose density gradients. The loss of infectivity after replication in media supplemented with IdUrd is associated not only with a reduced yield in total particles formed and an enhanced formation of incomplete particles, but also with the production of noninfectious substituted complete virions. The biosynthesis of virus-induced polypeptides in the presence or absence of IdUrd was analyzed. No apparent inhibition of early virus-induced proteins was detected even at very high doses of the analog. On the other hand, the synthesis of late viral proteins was inhibited markedly; the degree of inhibition was related directly to the concentration of IdUrd in the medium. Our studies suggest that IdUrd exerts its antiviral effect after incorporation into viral progeny DNA. The loss in the biological integrity of such substituted DNA molecules is not accompanied by fragmentation. The molecular basis for the inhibitory effect of IdUrd on the replication of Ad2 remains to be elucidated.

5-Iodo-2′-deoxyuridine (IdUrd), a metabolic analog of thymidine, inhibits the replication of over twenty DNA viruses in cell culture [1], and is of clinical utility in the therapy of herpes keratitis in man. IdUrd [2], as well as 5-bromo-2′-deoxyuridine [3, 4] inhibits the replication of adenovirus in cell culture, and IdUrd also suppresses the development of adenovirus 12 induced tumors in new born hamsters [5]. However, the mechanism of action of IdUrd is yet to be fully elucidated. This analog and its phosphorylated derivatives exert no substantial inhibition on the various enzymes concerned with DNA biosynthesis in infected and noninfected cells which could otherwise account for the magnitude of the antiviral activity observed, although a deleterious effect on gene expression after incorporation of this halogenated analog into viral DNA has been invoked [6].

The effect of IdUrd on the replication of herpes virus [7, 8], pseudorabies virus [9] and SV40 [10, 11] has been studied extensively. Smith and Dukes [8] found that IdUrd did not affect the production of herpes virus components, but rather their assembly. Replacement of 90 per cent of dThd in pseudorabies virus DNA by IdUrd did not impair significantly the production of viral DNA or viral coat proteins; however, viral particles were not formed, presumably because viral DNA containing IdUrd caused the synthesis of nonfunctional proteins required for viral assembly [9].

In contrast, studies with SV40 virus revealed a relationship between the reduction in the formation of viral coat protein and the amount of DNA–dThd replaced by IdUrd [11]. The decreased production of infectious SV40 virus by IdUrd was attributed to both suppression of viral DNA synthesis and reduced infectivity of substituted progeny DNA molecules.

Although IdUrd is incorporated into progeny viral DNA, different modes of inhibition have been proposed for the antiviral effect, namely, an inhibition of virus assembly of herpes and pseudorabies viruses, and a decreased synthesis of less infectious SV40 progeny DNA. Wigand and Klein [12] demonstrated that tritium-labeled IdUrd is incorporated into adenovirus virions, and analysis of the capsid proteins of these virions showed no difference in several physiological and immunological characteristics relative to control virions. Adenovirus type 5, grown on a medium supplemented with 5-bromo-2′-deoxyuridine, has a decreased infectivity of more than 4,000-fold, is sensitized to ulraviolet radiation by 6-fold, and based on electron microscopy has a central core of nucleic acid in the virion [4].

The present report examines the effect of IdUrd on the replication of adenovirus type 2 (Ad2), a DNA virus which is intermediate in size and complexity relative to herpes and SV40 viruses, to determine

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whether the differences in the mode of inhibition of these two viruses by IdUrd reflect inherent biological variations in the replication cycle of these viruses. Specifically, we studied the properties of the Ad2 preparations grown in the presence of IdUrd and the effect of this analog on the synthesis of early and late viral polypeptides.

MATERIALS AND METHODS

Cells and virus. KB cells were the generous gift of Dr. H. S. Ginsberg of Columbia University. The cells were maintained in suspension with Eagle's medium without calcium chloride, supplemented with 7% horse serum, or as monolayer cultures in Eagle's medium containing 10% fetal calf serum. Cells were routinely screened for mycoplasma contamination.

Ad2, free of identifiable small adeno-associated virus, was obtained from Dr. L. D. Hodge of Yale University. The infection of cells and preparation of virus have been described previously [13]. The virus stocks were diluted to 1×10^{12} particles/ml in a solution of 0.1 M Tris [(hydroxymethyl)-aminomethane-hydrochloride] (pH 8.1)–0.1% bovine serum albumin–0.85% sodium chloride and were used within 1 month of preparation.

Studies on the inhibition of infectious virus in the presence of IdUrd. Identical monolayers of KB cells in 25 cm^2 Falcon tissue culture flasks were infected simultaneously with 0.5 ml of virus suspension diluted in Eagle's medium for 30 min at 37° at an infecting dose of 200 particles/cell. After the inoculum fluid was removed by aspiration, the cells were washed three times with pre-warmed Eagle's medium, and then 5 ml of complete medium containing the designated concentration of IdUrd was added to each flask. At appropriate times the flasks were frozen at −20°. To release virus for the plaque assay, the cells and medium were thawed, transferred with a sterile Pasteur pipet to a 15-ml glass culture tube and then extracted with an equal volume of sterile-filtered Freon-113 (trichlorotrifluoroethane, Miller–Stephenson Chemical Co. Inc., Danbury CT). The sterile aqueous layer was assayed for plaque-forming units by a modified plaque assay described by Ensinger and Ginsberg [14].

Quantitation of viral particles after growth in the presence of IdUrd. Exponentially growing KB cells in suspension were concentrated by centrifugation to 2×10^7 cells/ml and infected with a dose of 200 particles/cell for 20 min at ambient temperature. Aliquots of these synchronously infected cells were introduced into spinner flasks containing various concentrations of IdUrd. At 15 hr post-infection, an equal amount of a mixture of twenty tritiated amino acids (Schwartz Mann, Orangeburg NY) (1 μCi/ml) was added to each flask. When maximal growth was attained (36–40 hr after infection), the virus was harvested and purified. Table 1 summarizes the method of viral purification used for these studies, which is based on the procedure of Green and Pina [15].

The degree of contamination by host cellular proteins was determined by an isotope-dilution experiment. The acid-insoluble fraction (1.68×10^6 cpm) derived from non-infected KB cells, grown in the presence of the same mixture of twenty tritiated amino acids, was added to unlabeled virus-infected cells before purification. From the amount of radioactivity recovered in the purified virus preparation, it was estimated that less than 0.001 per cent (less than 100 cpm) of the host cellular protein was present in the purified virus sample used for quantitation. The reproducibility of duplicate samples was determined by the recovery of purified radioactive virions added to virus-infected cells at Step 2 (Table 1).

Since identification of the bands of virions from the discontinuous cesium chloride (CsCl) gradients was done visually, purified non-radioactive Ad2 was added as carrier to the first Freon-extracts of Ad2 derived from growth in the presence of 10 μg/ml of IdUrd or more. Virus from the second CsCl gradient was collected directly into nitrocellulose tubes for equilibrium centrifugation. Four-drop fractions (about 40 μl) were taken from the bottom of the punctured tubes directly into scinitillation vials and counted in Aquasol (New England Nuclear Corp., Boston, MA).

Extraction of viral DNA from purified virions. The virus band derived from the CsCl gradient was dialyzed extensively against 0.01 M Tris–HCl (pH 8.1) and 0.01 M ethylenediaminetetra-acetate (EDTA), and then the virions were dissociated with 1% sodium dodecyl sulfate (SDS) at 37° for 1 hr. The preparation was then adjusted to 0.25 M mercaptoethanol, and for each ml, 500 μg of self-digested pronase (CalBiochem, San Diego, CA) was added (pre-incubated at 5 mg/ml for 1 hr at 80°, pH 5.5; neutralized to pH 7 and incubated for 2 hr at 37°). After 30 min at 37°, a similar quantity of the self-digested enzyme was introduced and the incubation was allowed to proceed for another

Table 1. Procedure for the purification and quantitation of Ad2 particles

Step	Procedure
1	Infected cells were harvested and washed three times in cold PBS. The cell pellet (5 to 7.5×10^7 cells) was resuspended and lysed in 2.5 ml of 0.01 M Tris–HCl (pH 8.1).
2	Non-radioactive Ad2 (approximately 10^{13} particles) was added as carrier to virus preparations derived from cell grown in media containing more than 10 μg IdUrd/ml. The cell lysate was extracted with an equal volume of Freon-113. After contrifugation at 1000 *g* for 10 min, the aqueous layer containing the virus was saved.
3	Step 2 was repeated.
4	The aqueous layer (approximately 2 ml) was layered onto a discontinuous CsCl gradient containing equal volumes of CsCl solution at densities of 1.2 and 1.4 g/ml. The sample was centrifuged at 30,000 rev/min (75,000 *g*) for 1 hr at 4° in a SW 50.1 rotor. The virus bands, which were very close together, were collected.
5	After dilution with an equal volume of 0.01 M Tris–HCl (pH 8.1)–0.01 M EDTA, Step 4 was repeated. Virus was collected, as described above, directly into the nitrocellulose tube used for equilibrium density gradient analysis.

30 min. The digested solution was extracted three times with redistilled phenol saturated with a solution of 0.5 M Tris–HCl (pH 8.1), 0.1 M sodium chloride and 0.01 M EDTA. Viral DNA was precipitated by the addition of 2 vol. of cold absolute ethanol and stored at −20° overnight. The precipitate was collected by centrifugation at 10,000 *g* for 20 min, dissolved in a solution of 0.05 M Tris–HCl (pH 8.1), 0.1 M sodium chloride and 0.001 M EDTA, and then stored frozen at −20°. The A_{260}/A_{280} ratio was found generally to range between 1.98 and 2.00.

Analysis of viral DNA in density gradients. The size of viral DNA was analyzed by centrifugation in 13-ml linear neutral and alkaline 5–20% (w/v) sucrose density gradients [16]. The gradients were centrifuged in a Spinco SW 40 Ti rotor at 24,000 rev/min for 16 hr at 4°. After centrifugation, fractions were collected dropwise from the bottom of the tubes directly into scintillation vials. When fractions were collected from an alkaline sucrose density gradient, they were neutralized with an equivalent amount of HCl before Aquasol was added for the determination of the amount of radioactivity in a Packard liquid scintillation counter.

The buoyant density of viral DNA was determined by equilibrium density sedimentation. CsCl (4.75 g) was added to 3.6 ml of a solution of viral DNA (5–50 μg) to obtain a final concentration of 1.7 g CsCl/ml. The gradients were centrifuged at 30,000 rev/min at 20° for 60 hr in a Spinco SW 65 Ti rotor, and fractions were collected as described above.

Preparation of [^{35}S]-methionine-labeled cell lysates. KB cells grown to confluency in 60-mm^2 petri dishes were infected as described above and labeled with [^{35}S]methionine (20 μCi/ml) for 1 hr at different times after infection, according to the procedure of Anderson *et al.* [17]. After the labeling period, the cells were scraped off the petri dish with a rubber policeman, and washed five times by suspension in ice-cold phosphate buffered saline followed by centrifugation. The cell pellet was finally solubilized in SDS buffer (0.05 M Tris–HCl, pH 6.8, 1% SDS, 1% mercaptoethanol and 10% glycerol).

SDS polyacrylamide gel electrophoresis. The method of Laemmli [18] was used with modifications. A detailed description of the SDS-disc and SDS-slab techniques of polyacrylamide gel electrophoresis has

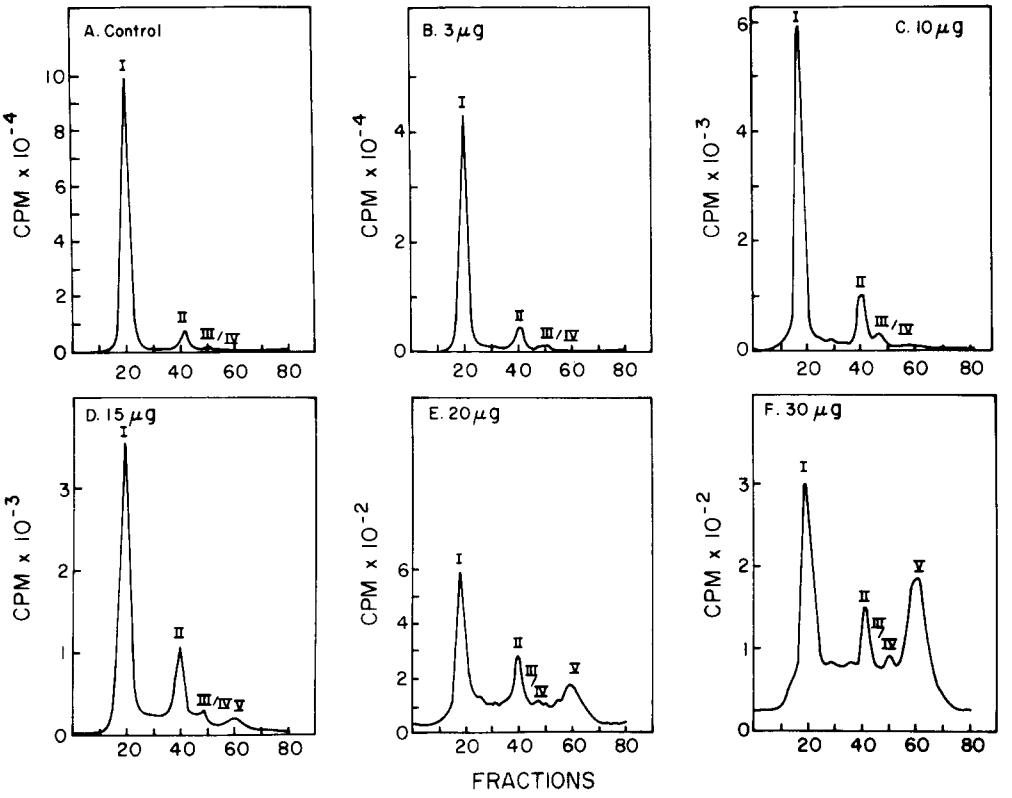

Fig. 1. Quantitation and analysis of radioactive Ad2 particles produced by an equal number of infected KB cells grown in the absence and presence of different concentrations of IdUrd in the culture media. Ad2 was labeled by the addition of a mixture of twenty [^{3}H]amino acids (1 μCi/ml) at 15 hr post-infection and harvested after maximal growth at 36 hr post-infection. Each preparation, purified according to the method outlined in Table 1, was further resolved into subpopulations of complete (peak I) and incomplete (peaks II, III/IV and V) particles by equilibrium CsCl buoyant density gradient centrifugation. Panel A represents the radioactivity profile of the control Ad2 preparation; panels B, C, D, E and F represent that of virus preparations grown in media supplemented with 3, 10, 15, 20 and 30 μg/ml of IdUrd, respectively. The amount of radioactivity associated with each peak is assumed to be proportional to the number of particles present.

been described [19]. After electrophoresis, tube gels were fractionated into 1-mm slices with a Gilson gel fractionator, dissolved in Aquasol, and the amount of radioactivity in each fraction was determined by scintillation counting. When samples containing ^{3}H and either ^{35}S or ^{14}C were analyzed, the spillover of these higher energy isotopes into the ^{3}H-channel (8–10 per cent) was calculated using appropriate standards, and the apparent ^{3}H-radioactivity was corrected accordingly.

Slab gels after electrophoresis were dried without heating under vacuum, and autoradiography was performed by exposing Kodak RP Royal X-Omat X-ray films to the dried gels for 2–7 days.

RESULTS

Inhibition of virus production. After infection of KB cells for 48 hr, the yield of virus (plaque-forming units) was maximal both in the absence and presence of IdUrd. Furthermore, IdUrd did not affect the kinetics of virus production. A dose–response relationship showing the percentage reduction of infectious virus at various concentrations of IdUrd is shown in Fig. 3 (curve C). Maximal inhibition was achieved with an IdUrd concentration of 10 μg/ml or greater. These results show that IdUrd is not only a very effective antiviral agent against Ad2, but also that the low titers resulted from a lack of production of infectious Ad2 virus without attenuation of the viral replication cycle.

The effect of IdUrd on the number of viral particles produced was determined with viruses labeled in their structural proteins after growth in the presence or absence of the analog and a mixture of twenty radioactive amino acids. Noninfectious incomplete viral particles were resolved from the infectious complete viral particles by CsCl equilibrium density gradient centrifugation [20]. Assuming that IdUrd did not significantly alter the uptake, metabolism and distribution of the labeled amino acids by the infected cells, the amount of radioactivity under each peak is proportional to the number of particles present. Figure 1 illustrates a typical set of radioactivity profiles of virus preparations obtained in a single experiment in which Ad2 was grown in the presence of different concentrations of IdUrd. Based on their buoyant densities, peak I was identified as complete viral particles and peaks II, III and IV as incomplete viral particles.

These sedimentation profiles provide two types of information. First, because the various preparations were infected simultaneously and an equal number of cells were used in each preparation, the reduction of any particular virus peak, as a result of the presence of IdUrd, may be expressed as a percentage of the control. This normalization to the control permits the comparison of data obtained on different days despite biological variations in viral yields. It is obvious that increasing IdUrd concentrations resulted in progressively fewer radioactive virions.

Second, the effect of IdUrd on the distribution of the subpopulations of the virus can be examined. Figure 2 summarizes the effects of IdUrd on the percentage distribution of these particles. Because peaks III and IV were not sufficiently resolved from each other, they were grouped together as one species. With increasing concentrations of the drug, progressively smaller percentages of peak I and higher percentages of peaks II and III/IV were found. Of particular interest is a "new" species of virions, represented by peak V, which appeared only at the higher drug concentrations.

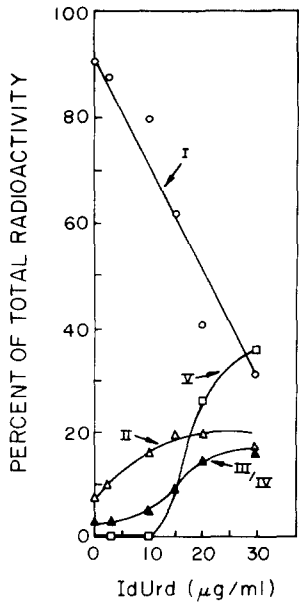

Fig. 2. Percentage distribution of complete and incomplete particles in preparations of Ad2 grown in the absence and presence of various concentrations of IdUrd. This figure summarizes data obtained from experiments similar to those described in Fig. 1.

The antiviral effect can now be expressed by two parameters, namely a decrease in the production of infectious virus and a decrease in the yield of particles. Figure 3 compares these changes at increasing concentrations of IdUrd. First, the percentage reduction of total yield of particles (curve A) is less than that of the infectious virus (curve C). This indicates a proportional increase of assembled but noninfectious particles with increasing concentrations of IdUrd. Second, the percentage increase of incomplete particles (which is given by the difference between curves A and B) cannot account for the decrease of infectious virus; otherwise curves B and C would be superimposed. Lastly, since the percentage loss of infectious virus (curve C) is much more pronounced than that of complete particles (curve B), a substantial fraction of the complete particles grown in media supplemented with IdUrd is not infectious. For example, at 10 μg/ml of IdUrd, the infectious virus yield was reduced by 4.5 logs, while complete viral particle assembly was only reduced by 1 log. In other words, the specific infectivity (that is, the ratio of the infectious virus to the number of particles) of IdUrd-treated complete particles was always a small fraction of that of the control (0.037 per cent at 10 μg/ml).

With many animal viruses, increased percentages of incomplete or defective virions have been reported at high multiplicities of infection [21]. However, the production of incomplete particles after infection by Ad2 has been shown to be independent of the infecting dose of virus [22]. We have compared the yield of complete and incomplete particles when Ad2 was grown in

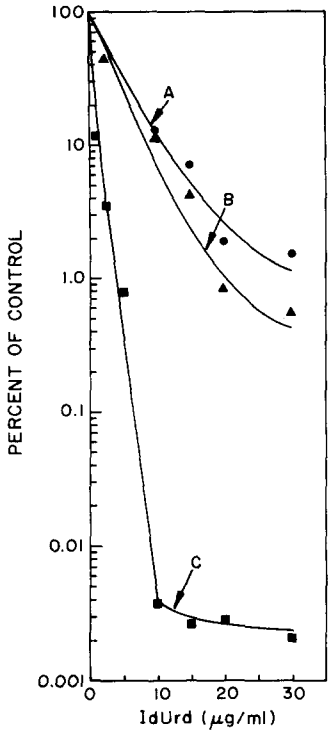

Fig. 3. Comparison of the effects of increasing concentrations of IdUrd in the growth media on the yields of total viral particles (complete plus incomplete), complete particles and infectious viral particles from Ad2-infected KB cells. The yield of virus from infected cells cultured in the presence of IdUrd was expressed as a percentage of that from the same number of infected cells grown in the absence of the drug. Data on the reduced yields of viral particles (●) and complete particles (▲) were obtained from experiments described in Fig. 1. The dose–response curve (■) shows the percentage reduction of plaque-forming units of Ad2 derived from infected KB cell grown in the presence of different concentrations of IdUrd.

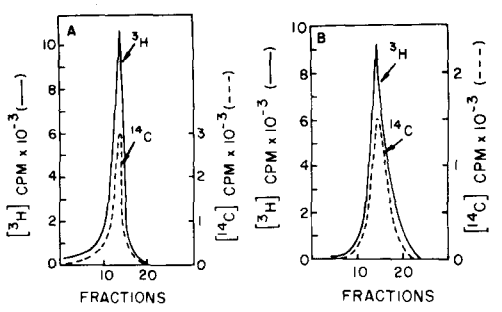

Fig. 4. Sucrose density centrifugation analysis of Ad2 DNA derived from complete particles grown in the presence and absence of IdUrd in neutral and alkaline sucrose linear density gradients. Particles grown in the presence of 10 μg IdUrd/ml were labeled with [^{3}H]deoxyadenosine (1 uCi/ml; 150–250 mCi/m-mole) 4 hr post-infection, and control particles at this time were labeled with [^{14}C]deoxyadenosine (0.2 μCi/ml; 30–50 mCi/m-mole). Extraction of viral DNA from the purified complete particles is described in Materials and Methods. Panel A: the neutral sucrose gradient (5–20%) contained 1 M NaCl, 0.01 M Tris–HCl (pH 7.2) and 0.01 M EDTA, and was centrifuged in a Spinco SW 40 Ti rotor at 24,000 rev/min for 46 hr at 4°. Panel B: the alkaline sucrose gradient (4–20%) contained 0.2 N NaOH, 0.1 M NaCl and 0.01 M EDTA, and was centrifuged under the same conditions. After centrifugation, fractions were collected dropwise from the bottom of the tubes for radioactivity determination.

IdUrd supplemented media (10 μg/ml) at several infecting doses of virus (2,000–20,000 particles/cell) and found that IdUrd does not affect the production of incomplete particles under these conditions.

Properties of complete particles grown in medium supplemented with IdUrd. Both the genome and the structural proteins of purified complete virions from cells treated with 10 μg/ml of IdUrd were analyzed in an attempt to explain the drastic loss of specific infectivity (99.94 per cent).

[^{3}H]Deoxyadenosine-labeled viral DNA from the IdUrd-treated preparation exhibited a higher buoyant density in CsCl than [^{14}C]deoxyadenosine-labeled control DNA (1.735 g/ml vs 1.716 g/ml). This increase in density indicates that 7–10 per cent of the dThd residues in the IdUrd-treated complete virions were replaced by IdUrd.

The size of the viral DNA molecules extracted from complete particles grown in IdUrd-supplemented medium was also compared to those from the control virions in linear 5–20% sucrose density velocity gradients. Both the IdUrd-containing and control samples sedimented as single peaks and at the same rate under both neutral (pH 7.4) and alkaline denaturing (pH 12) conditions (Fig. 4A and B, respectively). Therefore, the viral genome in which 7–10 per cent of the dThd residues have been substituted by IdUrd was not fragmented. Both DNAs migrated at the same rate as discrete bands when subjected to 0.3% agarose gel electrophoresis, indicating that their molecular weights could not differ by more than 10 per cent [23].

Finally, a mixture of complete particles was lysed directly onto an alkaline sucrose gradient; a profile similar to that depicted in Fig. 4B was obtained. Thus, no small fragments had been formed or lost during the extraction of DNA from the virions.

The polypeptides of IdUrd-substituted and control complete particles were analyzed on 13% SDS polyacrylamide gels. At 12 hr after infection, the control cell culture was labeled by the addition of [^{14}C]leucine (2.5 μCi/μmole), while the culture growing in the presence of 10 μg/ml of IdUrd was labeled simultaneously with [^{3}H]leucine at the same specific radioactivity. Twenty-four hr later, the two cultures were pooled, and the labeled complete particles were purified as one preparation. The combined virions were solubilized and then subjected to coelectrophoresis in the same cylindrical gel. Figure 5 illustrates the distribution of radioactivity in each gel fraction as well as the ratios of ^{3}H to ^{14}C dis./min. Both profiles contained the same number of polypeptides (denoted in the figure as II, III, III$_a$, IV, VII, VIII and IX, according to the convention of Anderson *et al.* [17]. No differences in the molar ratios and electrophoretic mobilities of these viral structural proteins could be detected in the two viral preparations. The same result was obtained when the particles were labeled with [^{35}S] and [^{3}H]methionine in a similar experiment. Our data show that all the detectable molecular species of the viral protein coat of IdUrd-containing complete particles are present in the correct propor-

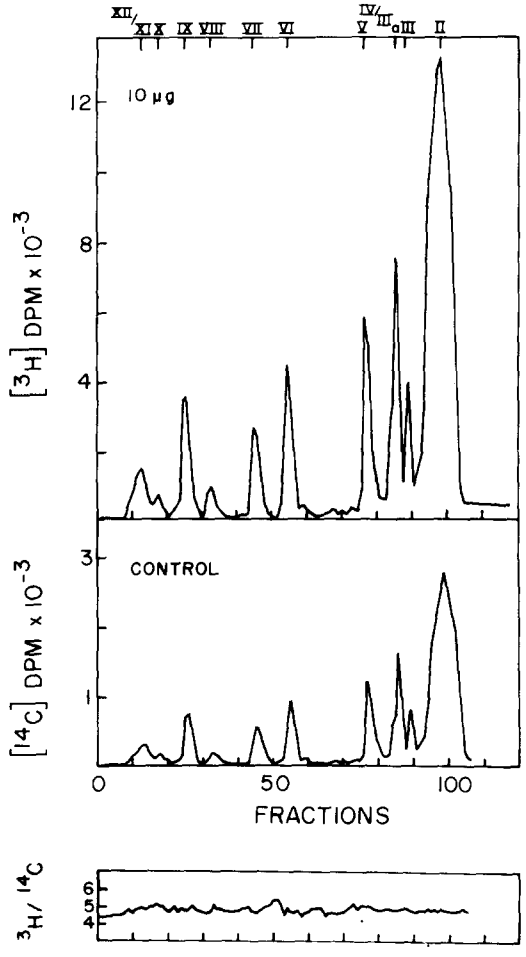

Fig. 5. Polypeptides of IdUrd-substituted and control complete particles. A mixture of SDS-dissociated complete Ad2 particles purified from control and IdUrd-supplemented (10 μg/ml) cultures were coelectrophoresced. Complete particles from infected cells grown in the absence and presence of 10 μg/ml of IdUrd were labeled at the same specific radioactivity (2.5 μCi/μmole) at 12 hr post-infection with [^{14}C]leucine and [^{3}H]leucine respectively. The viral proteins were electrophoretically separated on a 13% polyacrylamide tube gel. The amount of ^{3}H- and ^{14}C-radioactivity in each fraction and the ratio of ^{3}H- to ^{14}C-radioactivity were determined by liquid scintillation counting. The gel was 0.8 × 11 cm; each fraction was 1-mm thick. The anodal direction is to the left.

tions relative to the control virions, and are apparently not different in molecular weight from the expected values.

Properties of incomplete particles grown in medium supplemented with IdUrd. Attempts to isolate and analyze the individual subpopulations of Ad2 after replication in the presence of IdUrd were thwarted by the low yield of viral particles. Nonetheless, studies with [^{3}H]deoxyadenosine and labeled amino acids support the presence of DNA in these virions. Figure 6A depicts the distribution of ^{14}C-amino acid labeled virions after the first CsCl equilibrium density gradient (see Table 1). Fractions 30–75 (marked by the horizontal bar) contain all the incomplete Ad2 particles and were pooled for analysis on a second CsCl density gradient. The distributions of ^{3}H- and ^{14}C-radioactivity derived from [^{3}H]deoxyadenosine and ^{14}C-labeled amino acids in the fractions of this gradient are depicted in panel B.

The subpopulations of Ad2 are represented by peaks of ^{14}C-radioactivity. Each band was identified by its buoyant density and its relative position to the other viral components. The present of ^{3}H-radioactivity in each peak is assumed to denote DNA and apparently all the incomplete particles (peaks II, III, IV and V) contained some DNA. Particularly interesting is the presence of a new well-defined band of ^{3}H-radioactivity—Peak V. Essentially the same results were obtained with the preparation of Ad2 grown in the presence of 20 μg/ml of IdUrd.

The composition of the protein capsid of the incomplete particles was also analyzed by SDS polyacrylamide gel electrophoresis. The incomplete viral particles were prepared by the procedure described in the legend of Fig. 7. The radioactivity profiles of a mixture of lysates derived from [^{3}H]methionine-labled incomplete particles grown in the presence of 10 μg/ml of IdUrd and [^{35}S]methionine-labeled incomplete particles grown in the absence of the analog are shown in Fig. 7. The radioactivity profile of the polypeptides derived from the drug-treated populations is identical to the control with respect to the number of peaks resolved, their molar ratios and electrophoretic mobilities.

Effect of IdUrd on proteins induced by Ad2 infection of KB cells. Ad2 infected KB cells, grown in the presence of different concentrations of IdUrd, were pulse-labeled with [^{35}S]methionine, collected at various times after infection, solubilized and then subjected to SDS polyacrylamide electrophoresis. The amounts of radioactivity taken up into the various bands of proteins after each 1 hr pulse were assumed to be representative of their rates of synthesis. The assumption is also made that the presence of IdUrd did not affect the uptake or pool size of methionine in the infected cells.

At early times after infection (2–6 hr), the appearance and rates of synthesis are not apparently inhibited by IdUrd. Figure 8 shows the autoradiogram of a linear 7.5 to 22% gradient SDS polyacrylamide gel containing control cell lysate (sample a) and lysates of cells grown in the presence of 10 μg/ml (sample b) and 20 μg/ml (sample c) of IdUrd at 6 hr after infection. Several polypeptides which were absent or less conspicuous in the mock-infected cells were identified. Three of these polypeptides appeared to migrate in the positions corresponding to polypeptide 71K [17], 67K [24] and 45K [25]. In addition, four other bands were detected, which were designated as EP in Fig. 8. Even the presence of 20 μg/ml of IdUrd did not affect the rate of synthesis of these early virus-induced products, although the yield of viral particles in these cultures was reduced greatly.

In contrast, IdUrd markedly reduced the rate of synthesis of late viral polypeptides. Figure 9 depicts the synthesis of late viral polypeptides in the absence (samples a, c, e, g, i and k) and presence of 10 μg/ml of IdUrd (samples b, d, f, h, j and l). By comparing the autoradiograms of the cell lysates at each time after infection, it is evident that viral polypeptides are made at much reduced rates when Ad2 are grown in the presence of IdUrd.

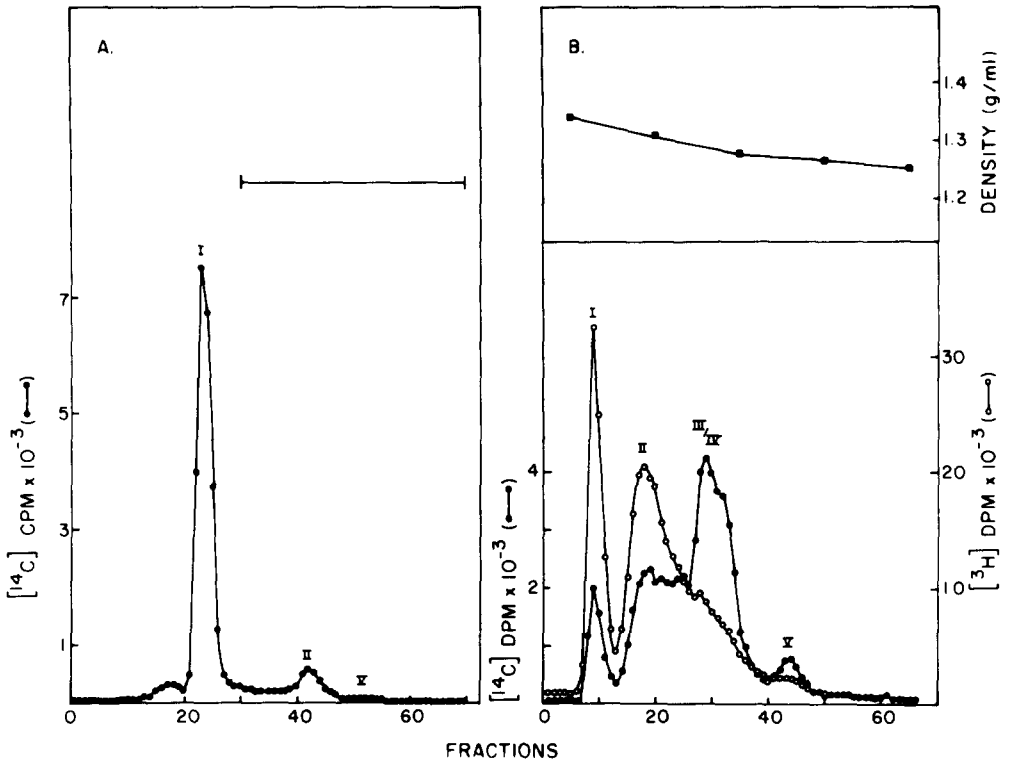

Fig. 6. Detection of DNA in the subpopulations of Ad2 purified from infected cell grown in the presence of 20 μg/ml of IdUrd. KB cells (3×10^6) were infected with Ad2, and 7 hr post-infection [^{3}H]deoxyadenosine (1 μCi/ml; 250 Ci/mole) was introduced into the culture to label viral DNA. Five hr later, a mixture of ^{14}C-amino acids (0.1 μCi/ml) was added to label the viral proteins. The labeled virus was purified 36 hr post-infection and centrifuged to equilbrium in a CsCl buoyant density gradient. Panel A: the distribution of the subpopulation of the ^{14}C-labeled virus after CsCl density gradient centrifugation. The fractions under the horizontal bar were collected and pooled for a second equilbrium CsCl density gradient. Panel B: radioactivity profile of the second equilibrium density gradient in which viral DNA was identified by the presence of [^{3}H]deoxyadenosine under each peak of ^{14}C-labeled virus.

This inhibition by IdUrd of the expression of late viral polypeptides was examined further by labeling for different lengths of time. We found that all of the viral particles (II, III, IIIa, IV, IV_{a2}, VI and IX) or their precursors (P-VI, P-VIII and P-VII) as well as non-virion proteins such as 71K, 100K and 50K were all inhibited by the presence of IdUrd (data not shown). However, at the end of the viral replication cycle (24 hr after infection), all of the late viral polypeptides could be detected in the IdUrd-treated cultures, even when the concentration of IdUrd was 20 μg/ml. No one viral polypeptide identified under these conditions appeared to be completely or preferentially inhibited.

The inhibitory effects of varying concentrations of IdUrd (5, 10 and 20 μg/ml) on the synthesis of late viral polypeptides at representative times late in the productive replication of Ad2 are shown in Fig. 10. The decrease in the rate of synthesis of a particular virus-induced product is greater at higher concentrations of IdUrd.

DISCUSSION

The subpopulations of Ad2 derived from growth in media supplemented with IdUrd were quantitated. Our results show that a decreased production of viral particles in general, an enhanced percentage yield of incomplete particles, and the production of complete particles of very low specific infectivity together contribute to the observed loss of infectious virus after growth in the presence of IdUrd. Furthermore, these aberrations are more exaggerated with increasing concentrations of IdUrd in the culture media.

Although the proportion of incomplete particles was increased in the IdUrd-treated Ad2 preparations, the number of these virions was actually smaller than that of the control. The distribution of these subpopulations of incomplete particles was also a function of the concentration of IdUrd. At 30 μg/ml, incomplete virions represented 70 per cent of the total yield of viral particles. For example, peak II, which was present as 8 per cent in the control viral preparation, represented 18 per cent of the population. Similarly, peak III/IV increased from 2.5 to 16 per cent. Of particular interest was the appearance of peak V, which constituted 40 per cent of the preparation. While peak V could not be isolated readily for characterization, isotope-labeling revealed the presence of protein and DNA. Its buoyant density in CsCl was lower than the other incomplete virions; hence, the size of the DNA is probably less than 15 per cent of the viral genome, based on the method of analysis of Daniell [26]. The origin of this peak is not known; the question here is whether peak V was created by IdUrd or was present in control Ad2 preparations in amounts too low to be detected.

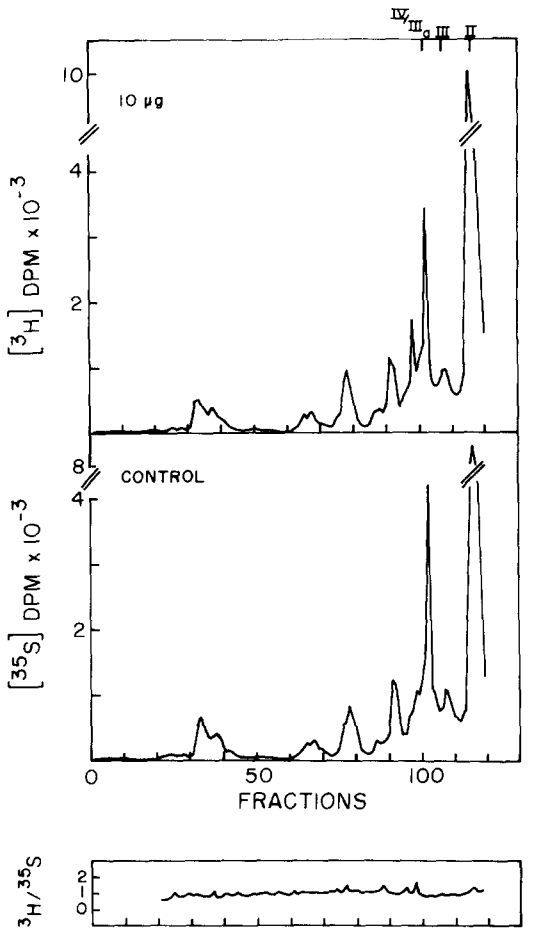

Fig. 7. Polypeptides of IdUrd-substituted and control incomplete particles. A mixture of SDS-dissociated incomplete Ad2 particles purified from control and IdUrd-supplemented (10 μg/ml) cultures were coelectrophoresed. The infected cells grown in the absence and presence of 10 μg/ml of IdUrd were labeled 12 hr post-infection with [^{35}S]methionine (2.5 μCi/μmole) and [^{3}H]methionine (2.5 μCi/μmole) respectively. After purification, the incomplete particles were separated from complete particles by an equilibrium CsCl buoyant density gradient. The incomplete particles were concentrated by precipitation with trichloroacetic acid, and subsequently solubilized with SDS buffer for analysis by polyacrylamide gel (13%) tube electrophoresis. The amount of ^{35}S- and ^{3}H-radioactivity and their ratios were determined by liquid scinitillation counting.

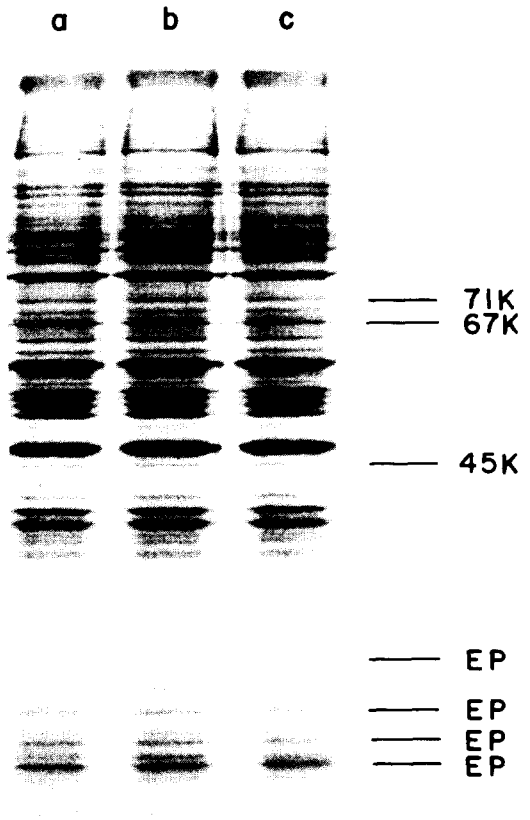

Fig. 8. Effect of IdUrd on the expression of early Ad2-induced proteins separated in a linear (7.5 to 22.5%) polyacrylamide gel. Panels a–c are SDS-polyacrylamide gel autoradiograms of Ad2-infected KB cells labeled at 6 hr post-infection after growth in the absence and presence of IdUrd. The infected KB cell monolayers were labeled for 1 hr in medium containing [^{35}S]methionine (20 μCi/ml) and carrier methionine (3 μg/ml). Samples were processed as described in the text, and approximately 2 × 10^5 cpm were applied to each sample well. The sample order is: (a) infected cells grown in the absence of IdUrd; (b) infected cells grown in the presence of 10 μg/ml of IdUrd; and (c) infected cells grown in the presence of 20 μg/ml of IdUrd.

The specific infectivity of complete particles formed over the range of IdUrd concentrations studied (3–30 μg/ml) was always a fraction of that of the control. At 10 μg/ml, the yield of infectious virus was decreased by 4.5 logs and that of complete particles by 1 log; hence, the specific infectivity, defined as the ratio of infectious virus to complete particles relative to that of the control, is 0.04 per cent. At 30 μg/ml, the specific infectivity was 0.4 per cent, which represented a 10-fold increase. This apparent rise in specific infectivity with increasing concentrations of IdUrd above 10 μg/ml was the consequence of progressive reduction of the formation of virions but without a concomitant progressive inhibition of infectious virus. We conclude that, with increasing concentrations of IdUrd after maximal inhibition of infectivity was attained, there was a progressive decrease of the assembly of noninfectious complete Ad2 particles.

The reason for the absence of further reduction of infectivity at IdUrd concentrations greater than 10 μg/ml is unknown. Even at a concentration of 80 μg IdUrd/ml, approximately 2 × 10^4 plaque-forming units were present in each ml of cell lysate. Therefore, this residual infectivity could not be attributed to a limiting sensitivity of the plaque assay. Similar dose–response curves were obtained in our laboratory when other antiviral compounds were tested against herpes simplex type 1. The possibility exists that these residual infectious viruses represented part of the original infecting inoculum, despite extensive washings of the cells after the adsorption period and incubation of the infected cultures for 48 hr. However, upon reinfection of KB

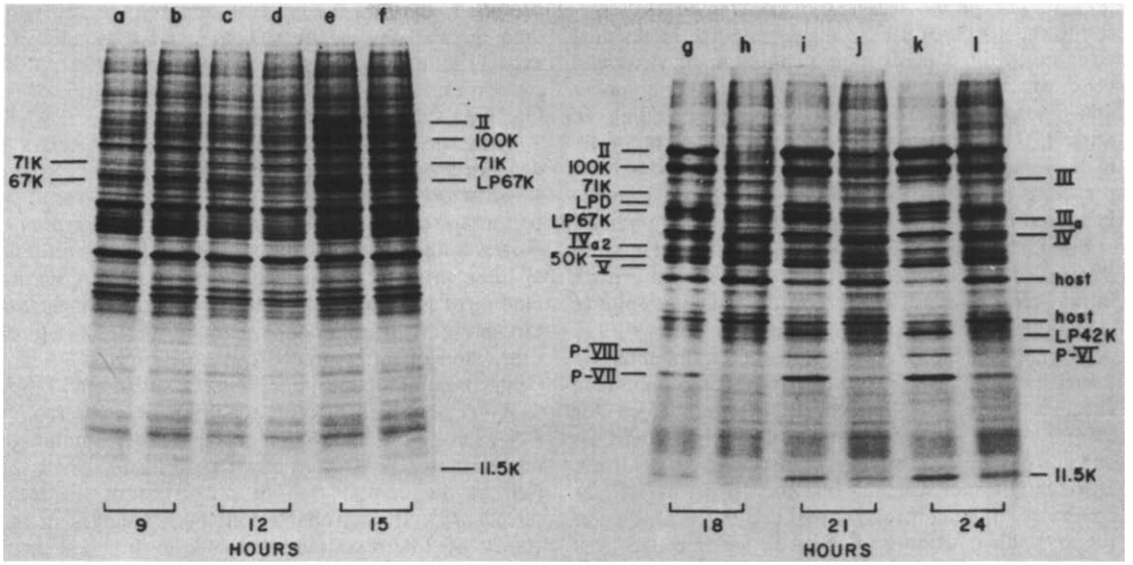

Fig. 9. Effect of IdUrd on the expression of early Ad2-induced proteins. SDS polyacrylamide gel autoradiogram of polypeptides derived from Ad2-infected cells pulse-labeled with [^{35}S]methionine late (9–24 hr) after infection: a, c, e, g, i and k, infected cells grown in the absence of IdUrd and pulse-labeled for 1 hr at 9, 12, 15, 18, 21 and 24 hr post-infection respectively; and b, d, f, h, j and l, infected cells grown in the presence of 10 μg IdUrd/ml and pulse-labeled for 1 hr at 9, 12, 15, 18, 21 and 24 hr after infection respectively.

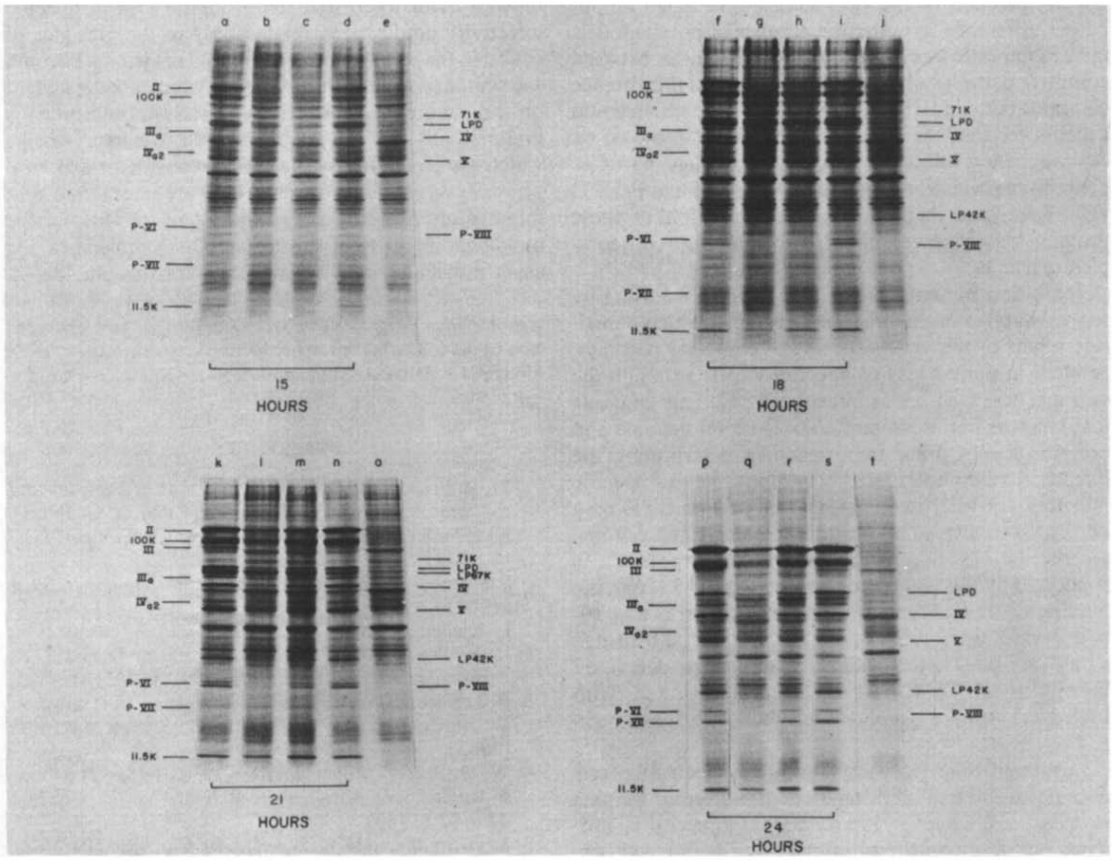

Fig. 10. Effect of IdUrd on the expression of late Ad2-induced proteins. SDS polyacrylamide gel autoradiogram of infected cells grown in media supplemented with different concentrations of IdUrd and pulse-labeled at representative times late after infection to illustrate the dose-related inhibition of the synthesis of virus-induced proteins: a, f, k and p, cells grown in normal media and pulse-labeled at 15, 18, 21 and 24 hr after infection respectively; b, g, l and q, cells grown in the presence of 20 μg/ml and pulse-labeled at 15, 18, 21 and 24 hr after infection respectively; c, h, m and r, cells grown in the presence of 10 μg/ml and pulse-labeled at 15, 18, 21 and 24 hr after infection respectively; d, i, n and s, cells grown in the presence of 5 μg/ml and pulse-labeled at 15, 18, 21 and 24 hr after infection respectively; and e, j, o and t, mock-infected cells.

cells, these residual viruses were found to be less sensitive to the action of IdUrd, suggesting that the residual virus constituted a resistant population of virus and hence was biologically distinct from the original inoculum. These results are comparable with the findings of Smith [27] that resistance to IdUrd is present naturally in the herpes simplex population to the extent of 0.5 to 4 per cent, and that the resistant population may be increased to more than 40 per cent by serial passage.

Some characteristics of the DNA and protein components of the complete particles derived from growth in the presence of IdUrd were studied in an attempt to explain the observed loss in specific infectivity (99.996 per cent). The DNA genome was not fragmented, in contrast to the findings with IdUrd substituted DNA in vaccinia virus [28] but consistent with those for SV40 [11]. In addition, its molecular weight could not differ from the control by more than 10 per cent. However, the increase in its buoyant density in CsCl, as compared to the control, showed that 7–10 per cent of the thymidine residues of the Ad2 genome had been replaced by IdUrd. Because reduced infectivity of IdUrd-containing SV40 DNA has been proven [11], the loss of specific infectivity of Ad2 complete particles may be related to such substitution of the viral genome thymidine with IdUrd.

SDS polyacrylamide gel electrophoretic anaylsis of the composition of the viral structure revealed no apparent difference in either the number of polypeptides, their molar ratios, or electrophoretic mobilities between complete particles obtained from growth in the absence and presence of IdUrd (10 and 20 μg/ml), which could account for the loss of specific infectivity in the latter. Although non-infectious "young" complete particles containing precursor proteins (P-VI, P-VII and P-VIII) have been described [29], no accumulation of these proteins was observed in IdUrd-containing Ad2 complete particles.

Inhibition by temperature shift of the processing by tsAd2 mutants of the precursor proteins, which constitute a part of the viral structure of complete particles, resulted in a total loss of infectivity [30] although the viral genome by itself is infectious [31]. This suggests that changes in the interaction of the viral genome and proteins are of great importance in determining the infectivity of complete particles. Thus, the low specific infectivity of IdUrd-containing complete particles may be related to altered affinity of the substituted genome for the viral structural proteins. This hypothesis is supported by the findings of Lin and Riggs [32] that the binding of the *lac* repressor to the *lac* operon was 10-fold greater after the bacterial DNA was substituted with 5-bromo-2′deoxyuridine. However, the decreased infectivity of SV40 DNA when substituted with IdUrd [11] supports a potential effect on the genome in addition.

Although SDS polyacrylamide gel electrophoresis does not resolve small differences in molecular weights or measure changes in the functional integrity of proteins, our study on the synthesis of virus-induced proteins over the course of Ad2 replication demonstrates clearly that late viral products are inhibited preferentially by replication in the presence of IdUrd. Early viral proteins are coded for by the parental genome and their expression depends on the host cellular synthetic apparatus. Since IdUrd does not appear to have any profound effect on the enzymes involved with thymidine metabolism in the cell [6], it is reasonable to expect that all viral proteins made prior to viral DNA replication are unaffected by the presence of this drug. Our data substantiate this proposition.

Since the synthesis of late Ad2 messenger RNAs is dependent on the formation of progeny DNA [33], the substitution of IdUrd into viral DNA can result in perturbation of the synthesis of late viral proteins. However, the molecular basis for the reduced synthesis of these proteins remains to be elucidated. A tighter binding of substituted DNA to chromosomal or regulatory proteins in cells could result in the inhibition of expression of differentiated cell functions [34], and a tighter binding of specific regions of substituted DNA to RNA polymerase could affect not only the rate of RNA formation [35, 36] but also the relative amount of the various RNAs normally transcribed [27–40] as well as the composition or properties of the transcripts [41]. It is probable that both changes in the ability of IdUrd-containing DNA to function as a template during transcription and the reduced yields of such templates contribute to the overall observed loss of viral proteins synthesized late in infection.

In conclusion, our studies suggest that IdUrd exerts its antiviral effect only after its incorporation into viral progeny DNA. This hypothesis is supported by two lines of evidence. First, the dramatic loss of specific infectivity could be correlated only to the presence of IdUrd in the viral genomes of complete virions. Second, the synthesis of late viral proteins, which are dependent on the synthesis of progeny viral DNA molecules, is preferentially inhibited as compared to early proteins which are coded for by the parental unsubstituted viral genome. Although we were able to demonstrate that fragmentation is not a prerequisite to this loss in the biological integrity of substituted DNA molecules, the exact molecular mechanism remains to be elucidated.

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