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Adenosine Dialdehyde: A Potent Inhibitor of Vaccinia Virus Multiplication in Mouse L929 Cells

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SUMMARY

Adenosine dialdehyde (2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-(*R*)-glyceraldehyde), formed by periodate oxidation of adenosine, is a potent inhibitor of *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1.) in mouse L929 cells. Consequently, the dialdehyde produces an increase in intracellular levels of *S*-adenosylhomocysteine and subsequent inhibition of *S*-adenosylmethionine-dependent macromolecular methylations. In the present study we show that adenosine dialdehyde is also a potent inhibitor of vaccinia virus plaque formation in monolayer cultures of L cells. When added to the culture medium immediately following attachment of the virus, concentrations of the dialdehyde as low as 0.5 μM produce greater than 90% inhibition of plaque formation after 72 hr. The efficacy of the compound is greatest when added within 8 hr of virus attachment and gradually decreases in a time-dependent manner when added after this point. Treatment of L cells with 5 μM adenosine dialdehyde for 60 min prior to virus infection causes a transient, but virtually complete loss of *S*-adenosylhomocysteine hydrolase activity and subsequent 3-fold increase in the intracellular *S*-adenosylhomocysteine/*S*-adenosylmethionine ratio. Continuous exposure of infected cells to the dialdehyde results in prolonged inhibition of *S*-adenosylhomocysteine hydrolase accompanied by a 10-fold increase in the *S*-adenosylhomocysteine/*S*-adenosylmethionine ratio. Associated with these changes in the dialdehyde-treated, infected cells are an inhibition of early virus-specific protein synthesis and a 13% decrease in methylation of the cytoplasmic poly A^+-mRNA. The antiviral action of this compound thus appears to be related to a decrease in viral mRNA methylation (e.g., the 5′-terminal cap structure) which results in suppressed translation of viral proteins essential for virus replication.

AdoMet-dependent methylation of the 5′-terminal cap structure of many eukaryotic viral mRNAs is necessary for efficient ribosome binding and subsequent translation into viral proteins as a prerequisite for viral replication (1). Consequently, substances which impair these specific mRNA methylation reactions have pharmacological potential as antiviral agents. It has previously been established that the virus-specific methyltransferases which catalyze both the sugar and base methylations at the 5′-terminus of viral mRNAs, like other AdoMet-dependent methyltransferases, are inhibited by the demethylated product, AdoHcy (2). In addition, a variety of structural analogues of AdoHcy have been shown to inhibit these viral mRNA methyltransferases *in vitro,* some of which have also exhibited inhibition of virus multiplication *in vivo* (3–5). A major limitation of many of these analogues, however, is their relatively low permeability into cells, thereby requiring high extracellular levels to obtain an effective intracellular concentration of the potential antiviral agent.

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A more recent and effective approach to the design of methylation inhibitors as antiviral agents against certain classes of DNA and RNA viruses (i.e., those requiring a methylated 5′-cap on their mRNAs) has focused on AdoHcy hydrolase as a pharmacological target (6–9). This cellular enzyme catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and homocysteine (Hcy) which are rapidly removed by further metabolism. Consequently, inhibiting AdoHcy hydrolase results in an intracellular accumulation of AdoHcy, a significant increase in the intracellular AdoHcy/AdoMet ratio, and subsequent inhibition of AdoMet-dependent methylation reactions such as those essential for viral mRNA maturation. The efficacy of this approach in the development of antiviral drugs has been demonstrated with several adenosine analogues which are readily permeable to cells and which exhibit inhibition of AdoHcy hydrolase. The acyclic analogue, (*S*)-DHPA, was observed by De Clercq *et al.* (10) to be active against several DNA and RNA viruses. c^3Ado was similarly shown to have broad antiviral activity against both DNA and RNA viruses, although this compound also exhibited significant toxicity toward host cells (11–13). In contrast, the carbocyclic analogue of c^3Ado, C-

ABBREVIATIONS: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; c^3Ado, 3-deazaadenosine; C-c^3Ado, 3-deazaaristeromycin; EDTA, ethylenediaminetetraacetic acid, disodium salt; HPLC, high pressure liquid chromatography; (*S*)-DHPA, (*S*)-9-(2,3-dihydroxypropyl)adenine; SDS, sodium dodecyl sulfate; EtOH, ethanol.

c^{3}Ado, was found to be more potent and more selective as an antiviral agent, while being less toxic to host cells at higher concentrations than c^{3}Ado (14, 15). In particular, the antiviral activity of C-c^{3}Ado appeared to be directed towards DNA pox viruses (i.e., vaccinia), double-stranded (±)-RNA viruses (i.e., reovirus), and (−)-RNA viruses (i.e., vesicular stomatitis) as opposed to herpesviruses (i.e., simplex, type I) and (+)-RNA viruses (i.e., polio), possibly implicating an impairment in the production and/or maturation of viral mRNA (e.g., methylation of the 5′-cap structure). Our laboratory has reported that another carbocyclic adenosine analogue, neplanocin A, is also a potent inhibitor of AdoHcy hydrolase and of vaccinia virus replication in murine L929 cells (16). Recently, a striking similarity of the antiviral spectrum of neplanocin A to that of (*S*)-DHPA and C-c^{3}Ado has been demonstrated by De Clercq (17). Furthermore, his laboratory has established that a close correlation exists between the antiviral potency of several of these adenosine analogues and their relative inhibitory effects on AdoHcy hydrolase (9).

Based on structural similarities to the various 3′-keto intermediates formed during the enzymatic reaction, Hoffman (18, 19) first demonstrated that periodate-oxidized adenosine, i.e., adenosine dialdehyde, is another potent inhibitor of AdoHcy hydrolase both *in vitro* and *in vivo*. These findings have previously been confirmed by our laboratory using both purified bovine liver AdoHcy hydrolase and cultured mouse L929 cells (20, 21). The K_i of this inhibitor for the purified bovine liver AdoHcy hydrolase was determined to be 2.39 nM in comparison to 8.39 nM for neplanocin A under the same conditions (16, 20). We now report that adenosine dialdehyde is also a potent inhibitor of vaccinia virus multiplication in mouse L929 cells. In addition, we provide evidence that the antiviral action of this compound is due to an inhibition of early viral protein synthesis, presumably resulting from a decrease in methylation of viral mRNA.

Experimental Procedures

Materials. Radiochemicals were obtained from the following sources: [^{3}H]thymidine (50 Ci/mmol), Moravek Biochem., Inc. (Brea, CA); [2,8-^{3}H]adenosine (30 Ci/mmol) and [*methyl*-^{3}H]methionine (15 Ci/mmol), ICN Radiochemicals (Irvine, CA); [U-^{14}C]uridine (453 mCi/mmol), Amersham Corp. (Arlington Heights, IL); and [^{35}S]methionine (1103 Ci/mmol), New England Nuclear. The remaining chemicals and supplies were purchased from commercial suppliers as follows: Waymouth's 752/1 low calcium spinner medium, KC Biologicals, Inc. (Lenexa, KS); bovine calf serum, Hazelton Dutchland, Inc. (Denver, PA); NCS tissue solubilizer (Amersham Corp.); Zorbax C-8 reverse phase HPLC column DuPont Instruments, (Wilmington, DE); and calf intestinal adenosine deaminase, gentamycin, 1-heptanesulfonic acid, poly U-Sepharose, and SP-Sephadex C-25, Sigma Chemical Co.

[2,8-^{3}H]AdoHcy (10–15 mCi/mmol) was prepared by conversion of [2,8-^{3}H]adenosine to [2,8-^{3}H]5′-chloro-5′-deoxyadenosine followed by its condensation with L-homocysteine in sodium and liquid ammonia (22). Adenosine dialdehyde was prepared by paraperiodic acid oxidation of adenosine (22).

Cell culture. Stock cultures of clone 929 mouse cells, strain L (Earle) were grown in suspension at 37° in Waymouth's modified 752/1 spinner medium supplemented with 5% bovine calf serum and 70 μg/ml gentamycin. Experimental cultures were plated in 35-mm (plaque assay), 60-mm (AdoHcy/AdoMet determination), or 100-mm (AdoHcy hydrolase assay) polystyrene tissue culture dishes at the indicated density and allowed to attach for 4 hr. The medium was then replaced by a chemically defined modified version of Waymouth's MD 705/1 medium, i.e., KU-1 (23).

Vaccinia plaque assay. Plaque assays were performed in 35-mm culture dishes containing 1.5×10^6 cells/dish. The medium was removed by aspiration and fresh KU-1 medium containing vaccinia virus (WR) was added. The plates were incubated for 60 min, the inoculum was removed, and 3 ml of fresh medium containing the indicated concentrations of adenosine dialdehyde were added. The infected cultures were then incubated for 72 hr (37°), after which the medium was discarded, the cells were stained with crystal violet, and the plaques were counted over a light box. All samples were carried out in quadruplicate.

Quantitation of the inhibition of virus production by adenosine dialdehyde. Monolayers of L cells (1.5×10^6 cells/35-mm dish) were infected with 100 plaque-forming units of vaccinia virus for 60 min and the virus inoculum was removed by aspiration. The cultures were then refed with 3 ml of fresh KU-1 medium containing either 0, 0.5, 1.0, or 5.0 μM adenosine dialdehyde and returned to 37°. At the indicated times thereafter, duplicate cultures from each group were removed from the incubator and placed directly into the −70° freezer. After all of the cultures were collected, the amount of virus production in each dish was determined using an appropriate dilution of the culture medium as inoculum for a routine plaque assay as described above.

L cell toxicity. [^{3}H]Thymidine incorporation was measured using 60-mm culture dishes containing 3.0×10^6 cells/dish. To start the experiment, the medium was removed by aspiration and replaced with 5 ml of fresh KU-1 medium containing the indicated concentrations of adenosine dialdehyde. At 36 and 72 hr, the medium was removed from duplicate cultures of each sample and 1.5 ml of fresh medium containing 0.33 μCi/ml [^{3}H]thymidine were added. The cultures were incubated for 120 min (37°), then placed immediately on an ice bath. The radioactive medium was removed and the cells were washed with ice-cold phosphate-buffered saline and collected by centrifugation following trypsinization. The cell pellets were suspended in cold 10% trichloroacetic acid and, after several hours, the acid precipitates were collected on Whatman GF/C filters, washed with 95% ethanol, and air dried. The filters were digested with 0.5 ml of NCS tissue solubilizer and counted for radioactivity in 10 ml of Bray's scintillation cocktail (24).

Cell toxicity as determined by growth curves was performed by experiments similar to those described above except that no radioactive medium was used. At the indicated times after exposure to adenosine dialdehyde, cells were removed from duplicate cultures of each sample by trypsinization, diluted in phosphate-buffered saline, and counted on a hemocytometer.

AdoHcy hydrolase assay. Cells (1×10^7) were removed from 100-mm culture dishes by trypsinization and lysed in 400 μl of cold hypotonic buffer (10 mM sodium phosphate, 10 mM sodium chloride, 1.5 mM magnesium acetate, pH 7.6) by rapid freezing on dry ice. The cell debris was removed by centrifugation in an Eppendorf Microfuge (8000 × *g*, 2 min) and the AdoHcy hydrolase activity in the supernatant was determined using a modification of the procedure of Chiang *et al.* (25). In a total volume of 500 μl, the incubation mixture contained 150 mM sodium phosphate (pH 7.6), 1.0 mM EDTA, 40 μM [2,8-^{3}H]AdoHcy, and 4 units of intestinal adenosine deaminase. The reaction was started by the addition of 320 μl of the cytosolic supernatant and incubated for 60 min at 37°. The reaction was stopped by the addition of 100 μl of 5 N formic acid. The reaction mixture and a 500-μl wash of 1 N formic acid were layered onto a 1.2 × 4.0 cm column of SP-Sephadex C-25 equilibrated in 0.1 N formic acid. The column was eluted with 8.0 ml of 0.1 N formic acid and the eluant containing [2,8-^{3}H]inosine (the product of the hydrolysis of [2,8-^{3}H]AdoHcy and subsequent deamination of [2,8-^{3}H]adenosine) was collected. A 2-ml aliquot of the eluant was added to 10 ml of 3a70 scintillation cocktail and the amount of radioactivity was determined by liquid scintillation counting.

Determination of intracellular AdoHcy/AdoMet ratios. Cells (3×10^6) were removed from 60-mm culture dishes by trypsinization

and lysed in 100 μl of 0.4 N perchloric acid by rapid freezing on dry ice. The samples were stored at −70° prior to analysis. In preparation for HPLC analysis, the samples were slowly thawed and the cell debris removed by centrifugation in an Eppendorf Microfuge. The supernatant (100 μl) was injected into a Perkin-Elmer series 3 HPLC equipped with a 25 cm × 4.6 mm Zorbax C-8 reverse phase column. The AdoHcy and AdoMet were separated by a two-step gradient program at a flow rate of 1.0 ml/min—solvent A (acetonitrile), solvent B (50 mM sodium phosphate, pH 3.2, 10 mM heptane sulfonic acid) (program: 5–20% solvent A, 15 min; 20–40% solvent A, 10 min) and quantitated by absorption at 254 nm using a Perkin-Elmer Sigma Series 10-B Console Data Station.

Analysis of [^{35}S]methionine-labeled proteins from vaccinia virus-infected L929 cells. Monolayer cultures of mouse L929 cells (1×10^6 cells/35-mm dish) were pretreated for 12 hr with or without 5 μM adenosine dialdehyde in KU-1 medium and then infected for 1 hr with vaccinia virus (15 plaque-forming units/cell). Following removal of the virus inoculum the cells were again refed with medium containing or lacking the dialdehyde and incubated at 37°. At various times thereafter, the medium was aspirated from representative cultures of each group and the cells were pulse labeled for 15 min with 25 μCi/dish of [^{35}S]methionine. Subsequently, the cells were washed with ice-cold phosphate-buffered saline and solubilized in sample buffer, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (26) using a 10% acrylamide gel.

Analysis of cytoplasmic poly A$^+$-RNA methylation in vaccinia virus-infected L929 cells. Monolayer cultures of mouse L929 cells (1×10^7 cells/100-mm dish) were pretreated for 12 hr with KU-1 medium containing or lacking 5 μM adenosine dialdehyde. The dishes were then infected with vaccinia virus (10 plaque-forming units/cell) for an additional 5 hr. Subsequently, the medium was removed from the dishes by aspiration and the cells were radiolabeled for 2.5 hr in 3 ml of modified KU-1 medium (i.e., 25% of the normal methionine concentration and the addition of 20 mM sodium formate, 20 μM adenosine, and 20 μM guanosine) containing 15 μCi/ml [*methyl*-^{3}H] methionine and 0.4 μCi/ml [^{14}C]uridine. The cells were then removed from the dishes by trypsinization, lysed in hypotonic buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$), and the cytoplasmic RNA was extracted with phenol/chloroform and precipitated in 70% EtOH overnight at −20° (23).

Poly A$^+$-RNA was isolated from the cytoplasmic RNA by poly U-Sepharose chromatography according to the following procedure. EtOH-precipitated RNA was collected by centrifugation and dried under vacuum. The pellet was rehydrated for 10 min at room temperature in 40 μl of sterile water and dissolved in an additional 180 μl of dimethyl sulfoxide. Following the addition of 20 μl of a sterile solution containing 1M LiCl, 50 mM EDTA, 2% (w/v) SDS, and 10 mM Tris-HCl, pH 6.5, the solution was heated to 55° for 5 min and diluted with 2.2 ml of 0.8× TEN buffer (400 mM NaCl, 0.8 mM EDTA, 4 mM Tris-HCl, pH 7.4) containing 0.5% SDS. The RNA solution was loaded onto a 0.8 × 2 cm column of poly U-Sepharose equilibrated in 0.8× TEN + 0.5% SDS and preempted with 100 μg of *Escherichia coli* tRNA in TEN buffer. The column was eluted with 1-ml fractions of 0.8× TEN + 0.5% SDS until the radioactivity in 25 μl of the eluate reached background, then similarly washed with 0.4× TEN + 0.25% SDS. The bound poly A$^+$-RNA was eluted with 1-ml fractions of 75% formamide in 1 mM EDTA, 0.1% SDS, and 10 mM Tris-HCl, pH 7.5. The fractions containing the eluted poly A$^+$-RNA were pooled, 0.5 A_{260} units/ml of unlabeled carrier rRNA was added, the solution was brought to a final concentration of 0.2 M NaCl, and the RNA precipitated overnight in 70% EtOH at −20°. A second passage of this material was performed using the same procedure.

Results

Inhibition of vaccinia virus plaque formation by adenosine dialdehyde. The antiviral activity of adenosine dialdehyde against vaccinia virus was initially assessed by quantitating virus plaque formation in monolayer cultures of mouse L cells. Following a 60-min inoculation with virus, the cells were incubated for 72 hr in growth medium containing the indicated concentrations of the drug after which the cells were stained and the number of plaques on each dish was counted (Table 1). Although 0.25 μM adenosine dialdehyde produced only moderate inhibition of plaque formation (29.7%), a substantial reduction in the appearance of plaques was observed with 0.5 μM (93.4%). At a concentration of 0.75 μM and higher, the dialdehyde appeared to completely prevent plaque formation, as judged by visual observation. Microscopic examination (×100), however, revealed the presence of some small foci (indicative of the onset of plaques) in those cultures treated with 0.75 and 1.0 μM dialdehyde. These foci were detectably smaller and less prevalent in the latter case, and were completely absent in the cultures treated with a 5 μM concentration of the drug.

A more quantitative analysis of the antiviral activity of adenosine dialdehyde is presented in Fig. 1. These data indicate that although vaccinia replication is not completely inhibited by the drug, the yield of virus in the presence of 5 μM dialdehyde is only 0.5% of the control by 40 hr and 1.2% by 75 hr. Even the low concentration of the drug (0.5 μM) substantially suppressed virus production, yielding 8.2 and 9.9% of the control at 40 and 75 hr, respectively.

The effect of adenosine dialdehyde on host L cell growth. For an antiviral agent to have potential clinical significance, it is essential that a drug have minimal effects on host cell growth and metabolism. The toxicity of adenosine dialdehyde on uninfected L cells was examined by measuring the incorporation of [^{3}H]thymidine into cellular DNA after 36- and 72-hr exposures of the cells to various concentrations of the drug. As shown in Table 1, less than 10% inhibition of [^{3}H] thymidine incorporation is observed after 36 hr with 0.25, 0.5, and 0.75 μM dialdehyde, of which the latter two have significant antiviral effects. Even the higher concentrations tested, 1 and 5 μM, exhibit relatively low inhibition of DNA synthesis, 13.4 and 26.1%, respectively. More interesting, however, are the effects seen after 72 hr. While the lower concentrations of the dialdehyde showed little or no alteration of DNA synthesis, a significant stimulation was seen with the two higher concentrations of the compound. These results reflect a transient growth-inhibitory effect of the drug during the first 36–48 hr, after which the cells are able to recover from the dialdehyde-

TABLE 1

Concentration effect of adenosine dialdehyde on vaccinia virus plaque formation and L cell DNA synthesis

Mouse L929 cells were treated with the indicated concentration of adenosine (Ado) dialdehyde and either assayed for vaccinia virus plaque formation (72 hr) or pulse-labeled with [^{3}H]thymidine for 2 hr to measure DNA synthesis (36 and 72 hr). Results for both sets of experiments are expressed as percentage of the untreated control cultures.

Concentration of Ado dialdehyde	Percentage inhibition of plaque formation (72 hr)	Percentage of control incorporation of [^{3}H] thymidine	
		36 Hr	72 Hr
μM			
0.25	29.7	96.6	106.8
0.50	93.4	93.3	103.3
0.75	95.7	92.2	120.6
1.0	100	86.6	195.3
5.0	100	73.9	208.6

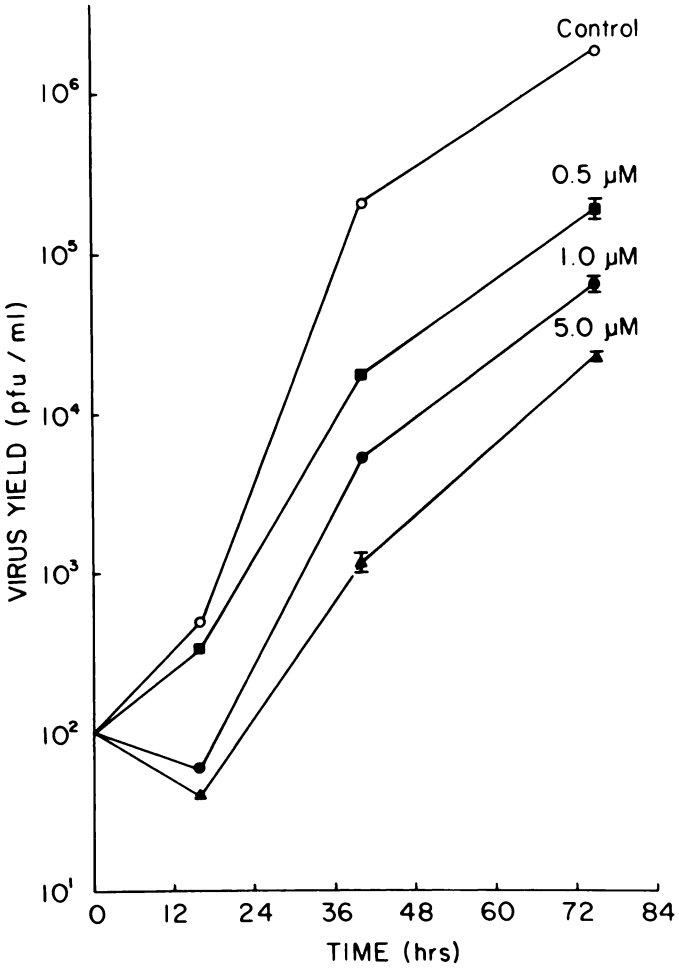

Fig. 1. Vaccinia virus production as a function of adenosine dialdehyde concentration. Monolayer cultures of mouse L cells (1.5×10^6 cells/35-mm dish) were infected with vaccinia virus for 60 min (37°) and the virus inoculum was removed. KU-1 medium containing either 0 (○), 0.5 μM (■), 1.0 μM (●), or 5.0 μM (▲) adenosine dialdehyde was then added and the cultures returned to 37°. After 16, 40, and 75 hr, duplicate cultures from each group were removed and immediately frozen at −70°. Subsequently, these cultures were thawed to 4°, an appropriate dilution was made, and 0.5 ml of the diluted sample was used as inoculum for a routine plaque assay (72 hr) as described under Experimental Procedures.

induced growth suppression. When compared to the untreated cultures (i.e., percentage of control) which have substantially depleted the nutrients in their culture medium by 72 hr, and thus, are growing at a slow rate, the amount of [^{3}H]thymidine incorporation in the treated cells is observed to be approximately 2-fold higher.

To provide further evidence that adenosine dialdehyde is not toxic to L cells, a growth curve of uninfected L cells treated with and without 5 μM dialdehyde is presented in Fig. 2. This concentration represents the effective antiviral dose that was used in most of the following experiments reported here. It can be seen that the onset of growth suppression occurs between 12 and 24 hr after initial exposure to dialdehyde and continues to increase throughout 48 hr. Between 48 and 70 hr, however, the rates of increase in cell number (i.e., the slopes of the lines) for the dialdehyde-treated and untreated cultures become parallel, indicating a similar growth rate for both sets of cultures. Thus, rather than being toxic to L cells, 5 μM adenosine

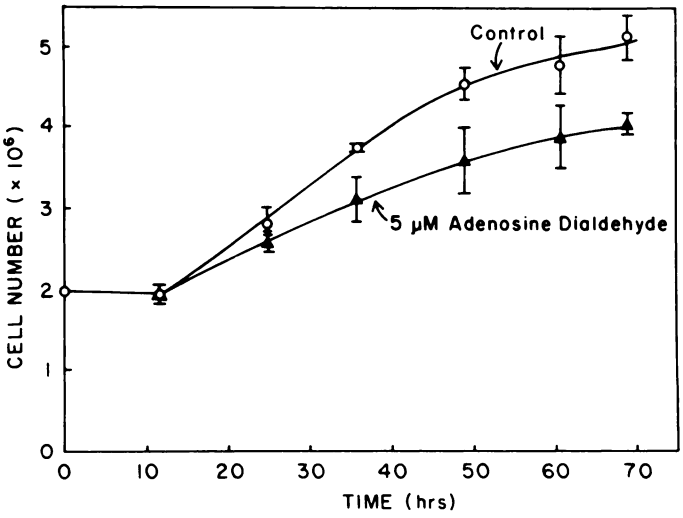

Fig. 2. Growth curves of mouse L929 cells. Monolayer cultures of mouse L cells (2.0×10^6/60-mm dish) were refed at time 0 with fresh KU-1 medium containing (▲) or lacking (○) 5 μM adenosine dialdehyde and returned to 37°. At the indicated times, duplicate cultures of each sample were removed from the incubator and the cells were collected by trypsinization and counted with a hemocytometer. Slopes of the lines were calculated for 10–48 hr and 48–68 hr using linear regression analysis: control 10–48 hr = 0.073, adenosine dialdehyde 10–48 hr = 0.045, control 48–68 hr = 0.028, adenosine dialdehyde 48–68 hr = 0.021.

dialdehyde appears to act as a cytostatic agent causing temporary growth suppression.

The effect of time of exposure to adenosine dialdehyde on inhibition of plaque formation. To determine what point(s) in the course of virus infection is most sensitive to inhibition by adenosine dialdehyde, the following experiment was performed. Monolayer cultures of L cells were pretreated for 60 min with or without 5 μM dialdehyde, then infected for another 60 min with virus inoculum lacking the drug. At selected times after infection cultures not preexposed to the drug were given fresh medium containing 5 μM dialdehyde and those cultures pretreated with the drug were similarly administered a second treatment (5 μM). All cultures were incubated for a total of 72 hr following infection and the amount of plaque formation was determined for each. Fig. 3 indicates that the compound appears to have its most potent antiviral activity when initially administered within 8 hr of virus attachment. Subsequently, the effectiveness of the drug gradually diminishes with time. It is also observed that a 60-min pretreatment with the dialdehyde causes little inhibition of plaque formation by itself and, in combination with a second administration of the drug, does not significantly extend the effective time course of plaque inhibition.

It is noteworthy that a single 60-min pretreatment with adenosine dialdehyde, an interval sufficient to cause maximal inhibition of AdoHcy hydrolase (below), does not appear to impair attachment of the virus to the cells since a significant number of plaques was observed on these cultures. Moreover, it is evident from these studies that early events in the production of new virus components seem to be most sensitive to inhibition by the drug. Considering the effects of this compound on macromolecular methylation reactions (21), transcription and/or maturation of early viral gene products seem to be involved.

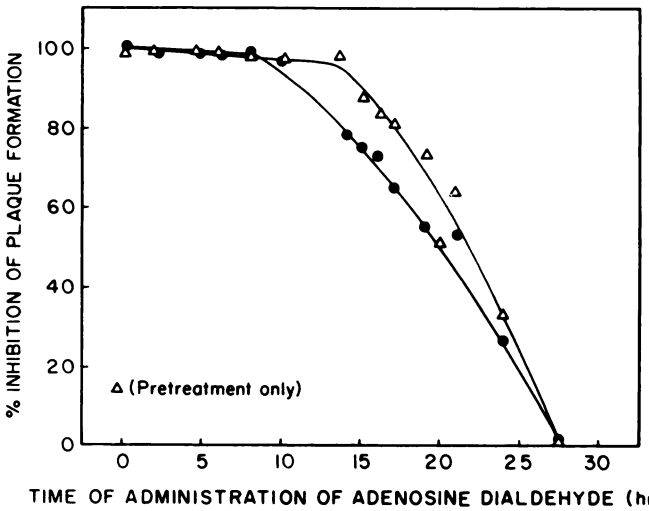

Fig. 3. The effect of the time of adenosine dialdehyde administration on inhibition of vaccinia virus plaque formation. One set of monolayer cultures of mouse L cells (1.5×10^6/35-mm dish) was exposed to 5 μM adenosine dialdehyde in KU-1 medium for 60 min (37°). Following this pretreatment, all of the cultures were infected with vaccinia virus for 60 min and the virus inoculum was removed. KU-1 medium containing or lacking 5 μM adenosine dialdehyde was then added as indicated by the time on the *abscissa* and the cultures were incubated for 72 hr. The cells were stained with crystal violet and the viral plaques were counted. The untreated control cultures were assayed in quadruplicate and had an average of 220 plaques per dish. All other samples were assayed in triplicate and the average value was used to determine the percentage of inhibition of plaque formation. Δ, pretreated cultures; ●, non-pretreated cultures.

The effect of adenosine dialdehyde on AdoHcy hydrolase activity and the intracellular AdoHcy/AdoMet ratio. Adenosine dialdehyde has been shown to inhibit AdoHcy hydrolase (20) and, consequently, to cause intracellular accumulation of AdoHcy in L cells leading to an inhibition of AdoMet-dependent macromolecular methylations (21). To assess the potential effects of this inhibitor of methylation reactions in virus-infected cells, we measured the AdoHcy hydrolase activity and the intracellular AdoHcy/AdoMet ratio of these cells following a short pretreatment with a 5 μM concentration of the drug.

Fig. 4 illustrates that, within 60 min of exposure to the dialdehyde, virtually all L cell AdoHcy hydrolase activity is inhibited. Following another 60 min incubation in the absence of the drug (i.e., during virus infection) the hydrolase activity is still negligible, after which it slowly recovers during the next 16 hr to the level seen in untreated, infected cells. In contrast to the dialdehyde-treated cells, the untreated counterparts show a steady increase in AdoHcy hydrolase activity during the initial 8 hr. This response likely reflects the administration of fresh nutrients which promote the synthesis of new enzyme and cofactor, NAD^+. A high level of hydrolase activity is maintained in these cells throughout the 24-hr period.

Inhibition of AdoHcy hydrolase in these cultures following pretreatment with dialdehyde is accompanied by a cellular accumulation of AdoHcy which is reflected in the intracellular AdoHcy/AdoMet ratio (Fig. 5). As expected from the temporary loss of enzyme activity, the AdoHcy/AdoMet ratio begins to increase within the initial 60 min of treatment, then reaches a plateau during the next hr (following removal of the drug) at a value 3-fold higher than that of the untreated controls. With

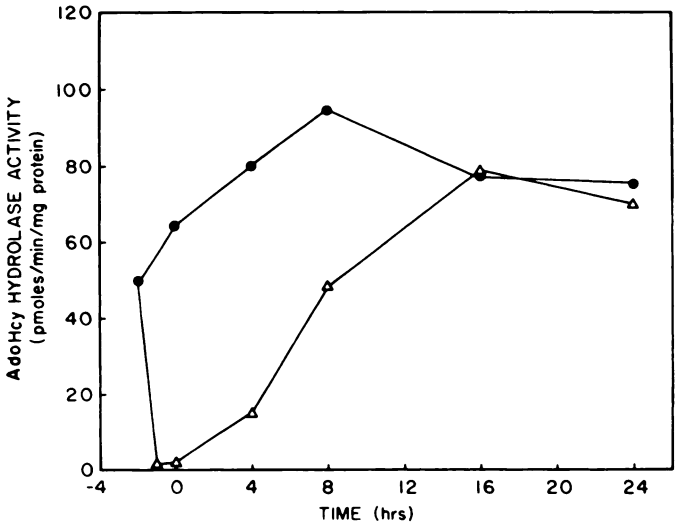

Fig. 4. AdoHcy hydrolase activity of virus-infected cells following pretreatment with adenosine dialdehyde. Monolayer cultures of mouse L cells (1.0×10^7 cells/100-mm dish) were exposed for 60 min to KU-1 medium containing (Δ) or lacking (●) 5 μM adenosine dialdehyde. Subsequently, the medium was removed and the cells were infected with vaccinia virus for an additional 60 min. Following removal of the virus inoculum, the cells were refed with fresh KU-1 medium which did not contain dialdehyde and returned to the 37° incubator. At the indicated times, duplicate cultures were harvested by trypsinization and cell extracts were prepared for determination of AdoHcy hydrolase activity as described under Experimental Procedures.

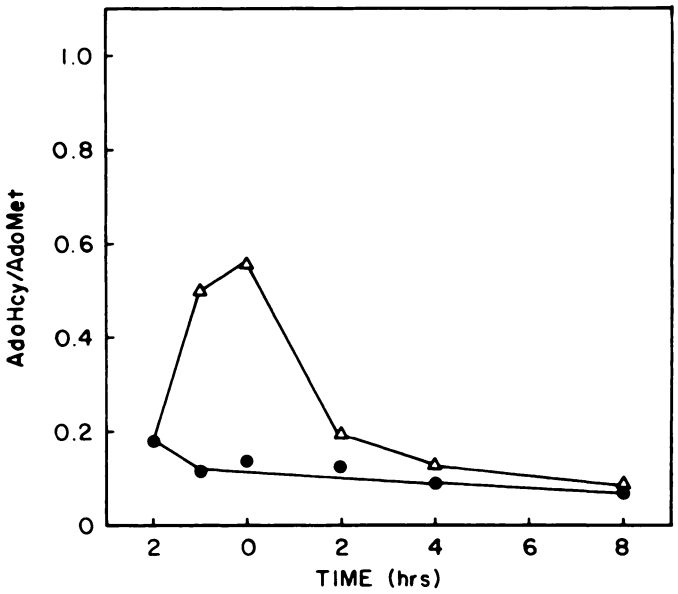

Fig. 5. AdoHcy/AdoMet ratios in virus-infected L cells following pretreatment with adenosine dialdehyde. Monolayer cultures of mouse L cells (3.0×10^6 cells/60-mm dish) were treated with (Δ) or without (●) 5 μM adenosine dialdehyde as described in the legend to Fig. 4. At the indicated times, duplicate cultures were harvested by trypsinization, and the cells were lysed in 125 μl of 0.4 N $HClO_4$ and frozen on dry ice. AdoHcy and AdoMet in the supernatant were separated by reverse phase HPLC as described under Experimental Procedures.

the onset of recovery of hydrolase activity (Fig. 4), the AdoHcy/AdoMet ratio (Fig. 5) rapidly returns to a value similar to that in the controls.

The transient nature of these changes in AdoHcy hydrolase activity and the AdoHcy/AdoMet ratio following adenosine

dialdehyde pretreatment is probably related to the inability of the drug to substantially reduce virus plaque formation, since the inhibition of macromolecular methylation observed under these conditions would be incomplete and for a limited interval. These findings are consistent with the hypothesis that there are methylation events early in the viral replication cycle which are essential for virus production.

In contrast to the effect of a short dialdehyde pretreatment on cellular methylation reactions, continuous exposure of infected cells to the drug elicits more dramatic results illustrated by the potent antiviral action (Table 1, Fig. 3). As shown in Fig. 6, L cell AdoHcy hydrolase activity is again virtually completely inhibited after a 60-min exposure to 5 μM dialdehyde. Under conditions of continuous exposure, however, the enzyme activity remains negligible for at least 48 hr and, by 98 hr, has only recovered to approximately 20% of the activity observed in the control cultures.

Corresponding to the prolonged inhibition of AdoHcy hydrolase activity is a dramatic increase in the intracellular AdoHcy/AdoMet ratio of the cells continuously exposed to 5 μM adenosine dialdehyde (Fig. 7). Within 24 hr of administration of the drug, this ratio increases to a maximum value 10-fold higher than that seen in untreated cells. A high AdoHcy/AdoMet ratio is maintained under these conditions through about 70 hr, after which it gradually decreases to the control value (120 hr). It is not surprising that the decline of this ratio coincides closely with the gradual recovery of AdoHcy hydrolase activity demonstrated in Fig. 6. Moreover, it is noteworthy that a significant reduction in the AdoHcy/AdoMet ratio is attained by 98 hr even though there is only a small (10–20%) recovery of hydrolase activity.

Inhibition of viral protein synthesis and poly A⁺-RNA methylation in adenosine dialdehyde-treated L cells. If the antiviral activity of adenosine dialdehyde is related to an inhibition of viral mRNA cap methylation, this ought to be expressed as subsequent inhibition of translation of these mRNA into proteins. Therefore, as an initial approach to identifying the mechanism of action of this drug, we examined its effect on cellular and viral protein synthesis at several early

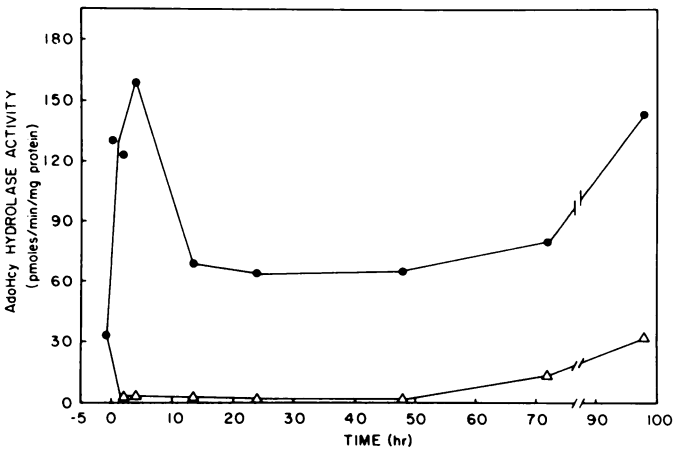

Fig. 6. AdoHcy hydrolase activity in virus-infected L cells continuously exposed to adenosine dialdehyde. Monolayer cultures of mouse L cells (1.0×10^7 cells/100-mm dish) were infected with vaccinia virus for 60 min. Following removal of the virus inoculum, the cells were refed with fresh KU-1 medium containing (△) or lacking (●) 5 μM adenosine dialdehyde. At the indicated times, duplicate cultures were harvested and cell extracts were prepared for determination of AdoHcy hydrolase activity.

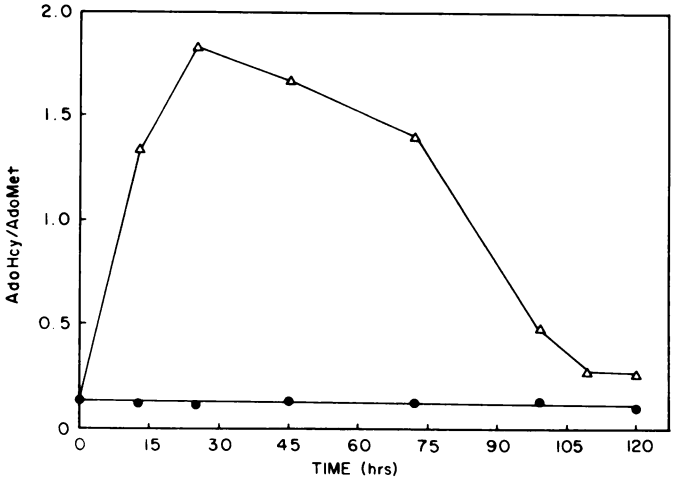

Fig. 7. AdoHcy/AdoMet ratios in virus-infected L cells continuously exposed to adenosine dialdehyde. Monolayer cultures of mouse L929 cells (3.0×10^6 cells/60-mm dish) were infected for 60 min and then exposed to culture medium with (△) or without (●) 5 μM adenosine dialdehyde. At the indicated times, duplicate cultures were harvested by trypsinization, and the cells were lysed in 125 μl of 0.4 N $HClO_4$ and frozen on dry ice. AdoHcy and AdoMet in the supernatant were separated and quantitated as described under Experimental Procedures.

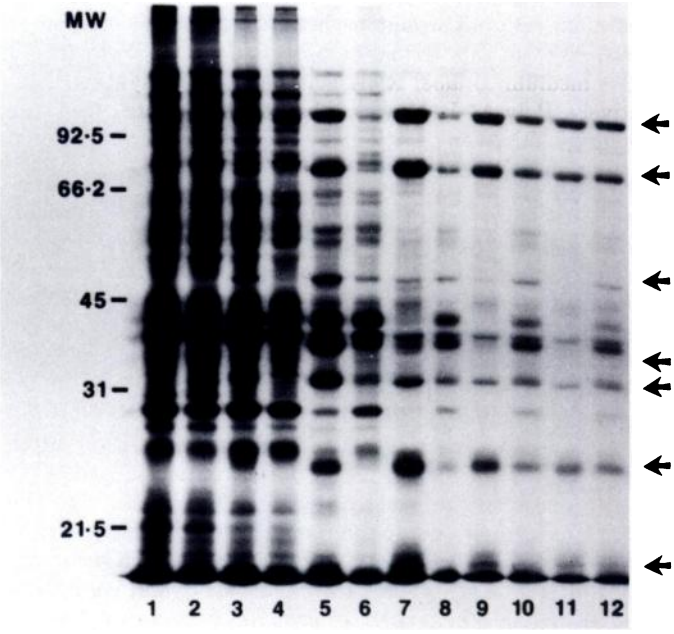

Fig. 8. Autoradiogram of ^{35}S-labeled proteins from vaccinia virus-infected L cells. Monolayer cultures of mouse L929 cells (1×10^6 cells/35-mm dish) were pretreated for 12 hr with medium containing or lacking 5 μM adenosine dialdehyde and then infected with vaccinia virus for 45 min at 30 plaque-forming units/cell. At indicated times thereafter, cultures from each group were pulse-labeled for 15 min with medium containing 25 μCi of [^{35}S]methionine and the cellular proteins subsequently resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. Time after infection: *lanes 1* and *2*, 0 hr; *lanes 3* and *4*, 1 hr; *lanes 5* and *6*, 2 hr; *lanes 7* and *8*, 4 hr; *lanes 9* and *10*, 6 hr; *lanes 11* and *12*, 8 hr. *Lanes 1, 3, 5, 7, 9,* and *11*, untreated controls; *lanes 2, 4, 6, 8, 10,* and *12*, treated with 5 μM adenosine dialdehyde. ←, virus proteins.

times after infection. The incorporation of [^{35}S]methionine into newly synthesized proteins was analyzed at 1, 2, 4, 6, and 8 hr post-infection by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 8). While an inhibitory effect

of the dialdehyde on the initial appearance of at least five distinct viral protein bands ranging in molecular weight from 28,000 to 120,000 (designated by the *arrows* in Fig. 8) is detectable after the first hr of infection (Fig. 8, *lanes 3* and **4**), the effect is much more striking by 2 hr (Fig. 8, *lanes 5* and *6*) and 4 hr (Fig. 8, *lanes 7* and *8*) post-infection. Also apparent during this time frame is a characteristic shutdown in the host L929 cell protein synthesis in response to the vaccinia infection (27). In view of the low level of synthesis of these viral proteins detected in the treated cultures by 4 hr, it appears that the inhibitor does not render the cells completely resistant to the virus at the high multiplicity of infection used in this study.

Another point that is clear from the results of this experiment is that the 12-hr pretreatment with the methylation inhibitor has little, if any, effect on the level of endogenous cellular protein synthesis prior to the onset of infection. This is apparent from the virtually identical labeling pattern of protein bands in *lane 1* of Fig. 8 (untreated) as compared to *lane 2* (12-hr dialdehyde-treated). These findings support the lack of a toxic effect of the inhibitor on the host cell translational system.

To test whether the inhibition of viral protein synthesis in dialdehyde-treated cells is related to a decrease in mRNA methylation (such as that known to occur in the 5′-cap structure of vaccinia virus mRNA), cytoplasmic RNA was isolated from dialdehyde-treated and untreated L cells 7.5 hr after the onset of infection. During the last 2.5 hr, the cultures were pulsed with [*methyl*-^{3}H]methionine and [^{14}C]uridine in the culture medium to label RNA methylation and synthesis, respectively. Poly A$^+$-RNA was subsequently selected from the cytoplasmic RNA preparations by poly U-Sepharose chromatography and the amount of [^{3}H]methyl and [^{14}C]uridine incorporation were determined for each sample. As presented in Table 2, the ratio of ^{3}H/^{14}C incorporation was 34% lower in the total cytoplasmic RNA isolated from the adenosine dialdehyde-treated cells than in the untreated cells. When the poly A$^+$-RNA was selected from these samples, the ratio of ^{3}H/^{14}C was observed to be 13% lower in the treated cultures, indicating that there is a detectable inhibition of methylation in response to the inhibitor. However, whether this inhibition is restricted to the 5′-cap structure or to internal methylation sites, or occurs in both, remains to be determined.

Discussion

Early studies utilizing analogues of AdoHcy showed that inhibition of AdoMet-dependent methylation had significant potential for the development of antiviral agents (3–5, 28–31). As initially demonstrated by Cantoni and his colleagues (11–14), AdoHcy hydrolase has now become the primary target of this approach, particularly in light of the extent of information presently available on its biochemical properties, mechanism of catalysis, and essential role in mammalian AdoHcy metabolism (32, 33). The most convincing evidence in support of this approach, however, has been the recent demonstration by De Clercq and Cools (9) that there is a direct correlation between the antiviral activity and the inhibition of AdoHcy hydrolase by four different adenosine analogues. The results which we present here for adenosine dialdehyde are in agreement with these findings and also provide some evidence for the mechanism of action of these inhibitors.

TABLE 2

Effect of adenosine dialdehyde on RNA methylation in vaccinia virus-infected mouse L929 cells

Monolayer cultures of L929 cells were treated with 5 μM adenosine (Ado) dialdehyde for 12 hr (37°), after which vaccinia virus was added (10 plaque-forming units/cell) and the incubation continued for another 5 hr. The infected cells were then labeled for 2.5 hr with medium containing 15 μCi/ml [^{3}H]methionine and 0.4 μCi/ml [^{14}C] uridine. Total cytoplasmic RNA (cRNA) was extracted from the cells and the poly A$^+$-RNA isolated by poly U-Sepharose chromatography.

5 μM Ado dialdehyde	Sample	dpm [^{3}H] Methionine	dpm [^{14}C]Uridine	^{3}H/^{14}C
−	total cRNA	6.54×10^5	1.96×10^5	3.34
+	total cRNA	4.01×10^5	1.81×10^5	2.22
−	poly A$^+$-RNA	2.04×10^4	1.28×10^4	1.59
+	poly A$^+$-RNA	1.71×10^4	1.24×10^4	1.38

Adenosine dialdehyde, which we have previously reported to be a potent inhibitor of purified bovine liver AdoHcy hydrolase *in vitro* (20) and of mouse L929 cell AdoHcy hydrolase *in vivo* (21), has been shown in the present study to be a potent inhibitor of vaccinia virus replication in this same cell line. When added to the culture medium 1 hr post-infection, the compound produced concentration-dependent inhibition of both total virus production (Fig. 1) and viral plaque formation while exhibiting little cytotoxicty to the host cells (Table 1). Using the latter analysis, the dialdehyde was determined to have an IC_{50} value (concentration which produces 50% inhibition) of 0.175 μM (data not shown) as compared to 0.095 μM for neplanocin A, another antiviral nucleoside analogue and one of the most potent inhibitors of AdoHcy hydrolase reported to date (9, 16, 17), further illustrating its potent antiviral activity. The data in Fig. 2 indicate that, rather than exhibiting cytotoxicity, the compound appears to produce some temporary cytostatic effects. The mechanism of this response is most likely related to a blockage in the production of homocysteine resulting from the inhibition of AdoHcy hydrolase. In vertebrates, this is the only source of homocysteine which is essential for the regeneration of methyltetrahydrofolate in order to maintain purine and pyrimidine biosynthesis. This mechanism has been clearly documented by Kim *et al.* (34) for 3-deazaaristeromycin, another potent inhibitor of the hydrolase enzyme (14).

As seen from the results in Figs. 4–7, treatment of infected cells with 5 μM dialdehyde results in a rapid inhibition of cellular AdoHcy hydrolase activity and subsequent elevation of intracellular AdoHcy. It is evident, however, that the onset of these effects can be readily reversed if the drug is removed from the culture medium after 1 hr, resulting in a marked suppression of the antiviral activity. These findings, in comparison to the antiviral activity observed following prolonged exposure, suggest that the large and sustained increase in the ratio of AdoHcy/AdoMet is important for obtaining the maximal antiviral effect. In addition, the data in Fig. 3 provide initial evidence to indicate that the events which are most susceptible to the increase in this ratio (i.e., some critical methylation reaction) occurr within the first 6–8 hr after infection.

The suppression of virus production by adenosine dialdehyde presumably involves the inhibition of methylation of the 5′-terminal cap structure of early viral mRNA due to the accumulation of cellular AdoHcy. This mechanism of action has been confirmed by de Ferra and Baglioni (35, 36) for the antiviral effects of interferon on vesicular stomatitis virus-infected HeLa cells. In addition, Montgomery and co-workers (14, 15) have shown that other inhibitors of AdoHcy hydrolase which are likewise potent inhibitors of vaccinia virus multiplication fail to inhibit the replication of poliovirus which has a

polypeptide cap on the 5′ end of its mRNA and is not methylated. In support of this mechanism in our system, we have demonstrated that the synthesis of early virus-specific proteins is strongly inhibited by pretreating the cells for 12 hr with the dialdehyde. Moreover, it is clear from the 0-hr control in Fig. 8, *lane 2* (i.e., 12 hr pretreatment), that this is not the result of a general suppression of host cell protein synthesis following exposure to the drug. Also in agreement with these findings are the results in Table 2 indicating that there is a 34% inhibition of methylation in total cytoplasmic RNA and a 13% inhibition of methylation in the poly A^+-mRNA isolated from dialdehyde-treated, infected cells. It is worth noting that the extent of this inhibition of poly A^+-RNA is virtually identical (14%) to that observed when the same experiment is performed with 1 μM neplanocin A in place of 5 μM adenosine dialdehyde. At present, we do not know whether this inhibition is confined to the 5′-terminal cap structure or whether there are internal methylation sites which are also affected. Although it was reported in an earlier study that the dialdehyde was also capable of inhibiting transcription *in vivo* (37), such an effect was not apparent in our cell culture system since we did not observe any inhibition of [^{35}S]methionine incorporation into cellular proteins (Fig. 8) following a 12-hr treatment with the drug. Currently, we are working to isolate and characterize the vaccinia virus-specific, poly A^+-mRNA from dialdehyde-treated and untreated mouse L929 cells in order to define more clearly the specific site(s) of inhibition.

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