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THE EFFECT OF A NOVEL INHIBITOR OF ALDEHYDE DEHYDROGENASE ON VIRAL REPLICATION

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Abstract—The effect of AMPAL (4-amino 4-methyl 2-pentyne 1-al), an inhibitor of aldehyde dehydrogenase, on adenovirus type 5 (Ad5) replication was studied. AMPAL at 2×10^{-4} M clearly reduced the cytopathic effect on HeLa cells but had no effect on cell growth at this concentration. Viral adsorption, penetration and protein synthesis were not affected by adding AMPAL at 2 hr post infection. When viral DNA synthesized in the presence of AMPAL was investigated, no significant inhibition was observed on either synthesis or the physicochemical properties of the neosynthesized DNA. However, there was a 4-fold increase in the amount of condensed DNA. In addition, AMPAL inhibited intracellular viral production (40%) and brought about concomitant inhibition of virus release (70%) into the medium. The absence of a quantitative relationship between the inhibition of viral DNA synthesis on one hand and that of viral production on the other may imply that the antiviral effect of AMPAL is indirectly mediated by the action of the malondialdehyde which has accumulated on some, as not yet identified, membrane constituent.

Increased aldehyde dehydrogenase (ALDH)‖ activity is associated with cell transformation induced by chemical carcinogens [1–3] and by viruses [4]. AMPAL (4-amino 4-methyl 2-pentyne 1-al), a synthetic unsaturated amino aldehyde inhibitor of ALDH has been shown to be a selective inhibitor of the growth of transformed cells [4].

In cell metabolism, ALDH is responsible for the oxidation of exogenous and endogenous aldehydes among which are 3-aminopropionaldehyde and malondialdehyde (MDA) which itself can arise from 3-aminopropionaldehyde by the action of diamine oxidase [5]. Thus, the inhibition of ALDH activity by a product such as AMPAL will induce an accumulation of MDA. This has been proven in NRK cells [4]. MDA which is a product of polyamine degradation [5] as well as of lipid peroxidation [6] can interact with DNA *in vivo* and *in vitro* forming both DNA–DNA [7] and DNA–protein crosslinking [8, 9]. These damages to the DNA can interfere with macromolecular syntheses [10] and thus inhibit cell growth and possibly viral replication.

To investigate the latter, we undertook studies on the effect of AMPAL on HeLa cells infected with adenovirus type 5 (Ad5).

‖ Abbreviations used: Ad5, Adenovirus type 5; ALDH, aldehyde dehydrogenase; AMPAL, 4-amino 4-methyl 2-pentyne 1-al; MDA, malondialdehyde; ID, infectious doses; CPE, cytopathic effect; Tris, Tris (hydroxymethyl) aminomethane; PBSd, Ca^{2+} Mg^{2+} depleted phosphate buffered saline.

MATERIALS AND METHODS

AMPAL was synthesized as described in [4].

Cell culture. HeLa cells from the American Type Culture Collection were growth twice weekly as monolayer cultures in Eagle's MEM (Gibco, Uxbridge, U.K.) supplemented with 10% foetal bovine serum (Flow Laboratories, Irvine, Scotland).

Virus. Human Adenovirus type 5 (Ad5) was propagated in monolayer cultures of HeLa cells with Eagle's MEM containing 2% foetal bovine serum.

Infection, labelling and measurement of viral infectivity. At 24 hr after seeding 3×10^6 cells/Petri dish (10 cm dia.), the cells were inoculated with Ad5 at a multiplicity of infection of 10–20 ID cell. Adsorption was allowed to proceed at 37° for 2 hr and cell–virus complexes were washed twice with cold medium. AMPAL was added to some cultures (control and infected) and incubated at 37°. At 24 hr post infection, DNA was labelled by adding [methyl-^{3}H] thymidine (1–2 μCi/ml, 37–74 kBq/ml, 45 Ci/mmole, 1665 GBq/mmole; CEA) for 3 hr or 72 hr. Proteins were labelled with [^{35}S]methionine (5 μCi/ml, 185 kBq/ml, 800 Ci/mmole, 29,600 GBq/mmole; Amersham, Amersham, U.K.).

The infectious titre of virus in media, washings and cells was assayed on HeLa cells in 96-well plates by the end-point dilution method and titers were expressed in infectious doses (ID).

Total uptake and specific incorporation of isotopes into DNA and proteins were determined by measuring total and acid-precipitable radioactivity.

Studies on adsorption and penetration of virus were carried out with [^{3}H] purified Ad5 labelled with [^{3}H]thymidine.

Viral DNA synthesis. At the end of the labelling period, cells were washed twice with ice-cold 0.14 M NaCl and scraped with a rubber-coated rod. The cells were centrifuged at 600 *g* for 5 min and the pellet was stored at −70°.

The pellet was resuspended in TES [0.03 M Tris (hydroxymethyl) aminomethane pH 8.0, 0.005 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M NaCl]. An equal amount of a solution containing 2 mg/ml of pronase and 1% sarkosyl in TES was added and incubated for 1 hr at 37°. The DNA was partially sheared by repeated pipetting before centrifugation.

Analysis of intracellular DNA by isopycnic sedimentation in neutral CsCl *density gradients.* A volume of 1 ml of the DNA preparation was layered on the top of 9.5 ml of a CsCl solution consisting of 9.9 g of CsCl in 7 ml of TES in ultra-clear tubes. After sedimentation at 106,000 *g* for 60 hr at 18° using a 50 Ti rotor in a Beckman model L8 preparative ultracentrifuge, fractions (0.4 ml) were collected from the top of the tube. After addition of an equal volume of cold 20% trichloracetic acid (TCA), precipitable material was collected by filtration on glass fiber discs (GF/C Whatman) and washed three times with 2% TCA and once with 95% ethanol. Discs were dried, placed in toluene scintillator (Packard) and radioactivity was determined in a Packard tri-Carb scintillation counter model Minaxi *β*.

Analysis of intracellular DNA by zonal sedimentation in alkaline sucrose density gradients. After the labelling period, cells were washed twice with 0.14 M NaCl scraped, counted and resuspended in PBSd at a concentration of 1×10^6 cells/ml. They were then placed on top of an alkaline sucrose density gradient [11]. The SW41 polyallomer tubes contained a 1 ml cushion of alkaline CsCl solution of density = 1.8 g/cm^3 in 0.7 M NaCl, 0.3 M NaOH and 0.005 M EDTA, 10 ml of a 5–20% (w/v) alkaline sucrose density gradient in 0.7 M NaCl, 0.3 M NaOH and 0.005 M EDTA, 0.35 ml of the lysis solution (0.5 M NaOH, 0.005 M EDTA) and 50,000 cells to be lysed. [^{14}C] thymidine labelled Ad5 marker DNA was also added on the gradient. Lysis was allowed to proceed for 17 hr at 4° prior to centrifugation in the SW41 rotor at 150,000 *g* for 150 min at 4°.

Fractions (0.3 ml) were collected from the top of the tube and counted directly in 5 ml picofluor (Packard).

Polyacrylamide-gel electrophoresis. Proteins were denatured and separated on 12% gels according to Laemmli [12].

After electrophoresis, gels were fixed, dried and exposed to Agfa Gevaert Curie X-ray film at −70°.

Analytical procedures. DNA concentration was determined using the Hoescht dye [13] and protein by the method of Lowry *et al.* [14].

MDA was measured as its fluorescent derivative formed with thiobarbituric acid [5] (MDA–TBA adduct) at 532 nm excitation and 553 nm emission in a KONTRON analytical SFM25 spectrofluorimeter.

Effect of adding AMPAL at different times pre or post infection on virus production. To study the time dependent effect of AMPAL on Ad5 multiplication, the compound (2×10^{-4} M) was added to HeLa cells either 1 hr before infection or just after adsorption (2 hr post infection). Cultures were harvested at 28 hr or 72 hr post infection. In other experiments, AMPAL was added at 2 hr, 31 hr and 48 hr post infection respectively or repetitively at 2 hr, 24 hr and 48 hr or at 2 hr and 48 hr.

Reversibility studies. Reversibility of AMPAL action was investigated by adding the drug 2 hr post infection and removing the medium at 24 hr or 48 hr, continuing the incubation with fresh medium devoid of AMPAL until 72 hr. At this time, the cultures were harvested, sonicated and analysed for infectious virus.

RESULTS

Effect of AMPAL on cellular growth. Since AMPAL selectively inhibits the growth of transformed cells (HeLa) but not that of normal cells (MRC_5) we first had to determine the concentration of AMPAL which could be used to investigate Ad5 replication on HeLa cells without affecting cellular growth.

Accordingly, HeLa cells were cultured for 4 days in the presence of AMPAL at concentrations ranging from 0.5×10^{-4} M to 6×10^{-4} M. No differences in viability or cell morphology were detectable by microscopic examination with AMPAL at concentrations ranging from 0.5×10^{-4} M to 2×10^{-4} M. As shown in Table 1, the growth of HeLa cells assessed by the measurement of proteins was inhibited by only 18% with AMPAL at 2×10^{-4} M. This concentration of AMPAL was selected for all future experiments on Ad5 replication.

Effect of AMPAL on Ad5 infection according to the moment of addition and removal. To determine the optimum conditions for an effect on viral replication, the action of sequential addition, repetitive additions and sequential removal of AMPAL was studied as described in Materials and Methods.

When AMPAL (2×10^{-4} M) was added either 1 hr before infection, at the time of infection or at 2 hr after infection and maintained until the end of

Table 1. Effect of AMPAL on growth of normal (MRC5) and transformed (HeLa) cells in culture

Final concentrations of AMPAL	% Inhibition of growth	
	MRC_5*	HeLa
0.5×10^{-4} M	0	5
1×10^{-4} M	0	11
2×10^{-4} M	0	18
4×10^{-4} M	4	54
6×10^{-4} M	11	82

At 4 hr after seeding 10^6 cells in 100 mm dia. plastic Petri dishes for tissue culture with 10 ml of medium, AMPAL was added to give the final concentrations indicated. After 4 days incubation at 37° in a 5% CO_2 humid atmosphere, cellular growth was assessed by cell numeration and protein measurements.

* From Ref. 4.

the experiment, the cytopathic effect (CPE) was greatly inhibited and the amount of infectious extracellular virus was lowered. Pretreatment of cells with AMPAL during 18 hr before infection, followed by infection without AMPAL, resulted in CPE and virus release which were similar to those observed on untreated infected cells. This was also the case when AMPAL was added as late as 24 hr or 31 hr after infection.

The inhibition of CPE and of virus release was greatest when AMPAL was added 2 hr post infection and subsequently added once at 48 hr or twice at 24 hr and 48 hr.

The diminution of CPE and of the amount of infectious virus released into the medium 72 hr post infection was most effective when AMPAL was present throughout infection. When it was removed at 24 hr or at 48 hr post infection the inhibition of CPE was reduced.

In all these experiments, only CPE and release of virus into the medium were modified, whereas the amount of intracellular infectious virus was unchanged regardless of the moment when AMPAL was added.

As the inhibitory effect of AMPAL was greatest when it was maintained throughout the experiment all further studies were made by adding it 2 hr post infection and maintaining it all during experimentation.

To determine whether the diminution of CPE was due to a virucidal effect of AMPAL on the infectivity of the inoculated virus, Ad5 was incubated in 1 ml

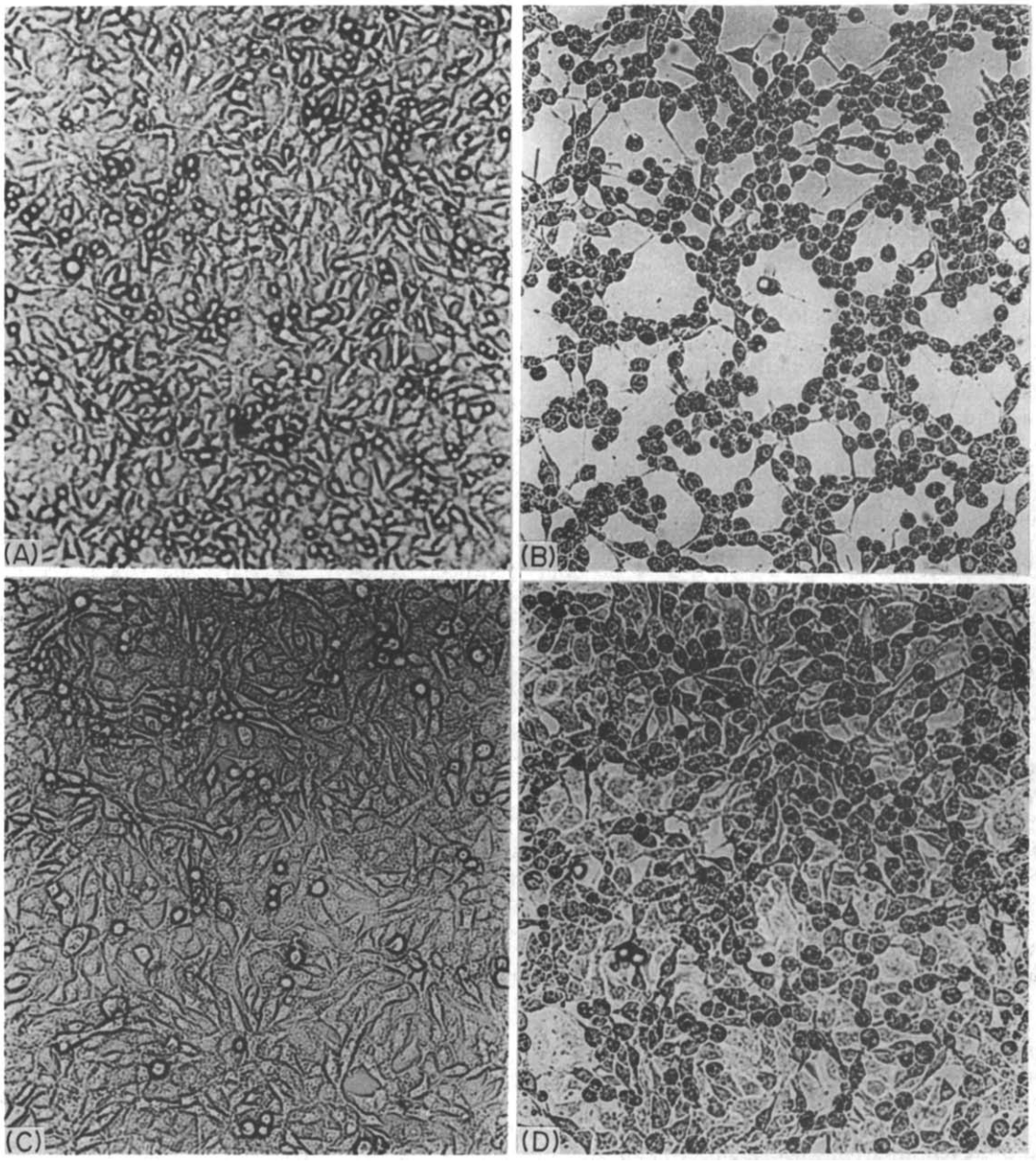

Fig. 1. Effect of AMPAL on CPE of Ad5 in HeLa cells three days after infection. (A) Uninfected control cells; (B) infected cells; (C) uninfected and treated cells; (D) infected and treated cells. Magnification ×100.

Table 2. Effect of AMPAL on Ad5 production and Ad5 release

Sample	Infectious doses		
	Total Ad5 production at 72 hr P.I.*	Ad5 release at 72 hr P.I.†	Ad5 release at 28 hr P.I.†
Infected cells	$38.5 \times 10^6 \pm 3.8$	$11.3 \times 10^5 \pm 0.6$	$13.1 \times 10^4 \pm 1.7$
Infected and AMPAL treated cells (2×10^{-4} M)	$23.1 \times 10^6 \pm 2.8$	$3.4 \times 10^5 \pm 0.3$	$8.5–10^4 \pm 1.0$
Number of experiments	(7)	(7)	(3)
Student *t*-test on the means	$P < 0.001$	$P < 0.001$	$P < 0.001$
% Decrease	40.0	69.9	35.1

The results represent the mean ± SE.
* Intracellular + extracellular infectious virus.
† Extracellular infectious virus.

of maintenance medium with 2×10^{-4} M AMPAL for 24 hr at 37°. AMPAL did not inactivate Ad5 since there was no significant titre difference between treated ($10^{6.50}$ ID/ml) and untreated ($10^{6.75}$ ID/ml) samples.

Effect of AMPAL on Ad5 production. With optimum conditions defined, the effect of AMPAL on Ad5 replication was investigated.

The treatment of HeLa cells infected with 10–20 ID/cell with AMPAL at 2×10^{-4} M beginning 2 hr post infection greatly inhibited the CPE of Ad5 on HeLa cells (Figs 1D vs 1B). The total amount of infectious virus which was produced was lowered by 40% (Table 2). The progressive infection of all cells in a culture depends on the contiguous cell to cell transfer of virus from originally infected cells. It was therefore possible that the decreased CPE and viral production observed here in the presence of AMPAL could have been due to inhibition of secondary infection of cells not infected by the original inoculum.

To investigate this, the effect of AMPAL on viral adsorption, penetration and release on one hand and of macromolecular syntheses on the other was investigated.

Effect of AMPAL on adsorption and penetration of Ad5. To examine this, [^{3}H]thymidine labelled virus was prepared and purified as described in Materials and Methods. Cells were treated with AMPAL 1 hr before the virus was added and AMPAL was maintained throughout the experiment. After 2 hr at 37°, at which time adsorption was complete [15], the amount of radioactivity associated with the cells was determined. In the presence of AMPAL, 24.3% of the radioactivity of the inoculum was found associated with the cells compared to 23.9% in non-treated cells. Therefore, AMPAL had no effect on viral adsorption.

After 2 hr adsorption of labelled virus, cells were also washed for 2 min at 4° with 0.05 M glycine–HCl buffer, pH 3.0 containing 0.1 M NaCl, to remove non attached virions and then reincubated at 37° to study viral penetration. No significant inhibition in the rate of penetration of the input virus into cells was observed after 3 hr incubation in the presence of AMPAL (67% of the adsorbed virus vs 68% in untreated cells).

Effect of AMPAL on cellular aldehyde metabolism. To try to determine the effect of AMPAL on cellular aldehyde metabolism, the identification of aldehydes which accumulate in the presence of AMPAL was first investigated. In this context we were guided by our previous results showing that 3-aminopropanal, which arises from spermine oxidation by the action of polyamine oxidase can, in turn, be oxidatively deaminated by diamine oxidase to MDA [5]. However, 3-aminopropanal can also be oxidized to β alanine by ALDH1 which may be related to one of the isoenzymes of ALDH capable of oxidizing aliphatic aldehydes such as propanal [16]. MDA itself can be easily oxidized by the action of ALDH2 to malonic acid.

Now, MDA which can also arise from lipid peroxidation [6] has been shown to be an inhibitor, albeit non specific, of cellular macromolecular syntheses [17]. It therefore seemed reasonable to first compare MDA levels in AMPAL treated and non treated cells before measuring the incorporation of labelled precursors into cellular and viral DNA and proteins.

The amount of total intracellular MDA per mg of DNA was always higher in AMPAL treated cells than in untreated cells regardless of whether they were infected or not (Table 3). These results provided evidence that AMPAL brings about an increase in cellular MDA levels.

To reduce the intracellular content of MDA arising from lipid peroxidation, vitamin E (α-tocopherol) at the concentration of 10^{-4} M was added to HeLa cells in culture for 24–48 hr. At the end of this period, the determination of the intracellular MDA level showed that it had decreased by 80–86% from 0.40 or 0.44 nmole/mg proteins to 0.08 or 0.06 nmole/mg proteins. At this concentration of vitamin E, there was no effect on cell growth and on viral replication. When AMPAL was added to vitamin E treated cells, cellular growth was inhibited and this was accompanied by a concomitant rise of 15–20% in MDA levels. These results strongly suggested that the accumulation of MDA in the presence of AMPAL was not lipid derived. Although ALDH activity was low in HeLa cells, we have found a 15–18% inhibition of the activity of this enzyme after a 10 min *in vitro* incubation of the cytosolic fraction of HeLa cells with AMPAL (2×10^{-4} M) at 37°. At a

Table 3. Effect of AMPAL on MDA content in uninfected and infected HeLa cells after 72 hr

Assays	Uninfected cells			Infected cells		
	Untreated cells	AMPAL treated cells (2×10^{-4} M)	% increase	Untreated cells	AMPAL treated cells (2×10^{-4} M)	% increase
1	6.19	9.62	55.41	9.35	12.20	30.48
2	18.41	20.85	13.25	23.23	25.55	9.99
3	5.10	7.52	47.45	8.33	9.81	17.76
4	3.15	3.53	12.06	4.10	4.21	2.68
5	3.91	4.53	15.86	3.68	4.30	16.85
6	5.26	6.90	31.17	7.29	7.40	1.51
Significance (Paired *t*-test)		$P < 0.02$			$P < 0.05$	
Mean percentage increase†			29.20 ± 17.08			13.20 ± 9.91

The values are expresses as nmoles MDA/mg DNA.
† Mean ± SE.

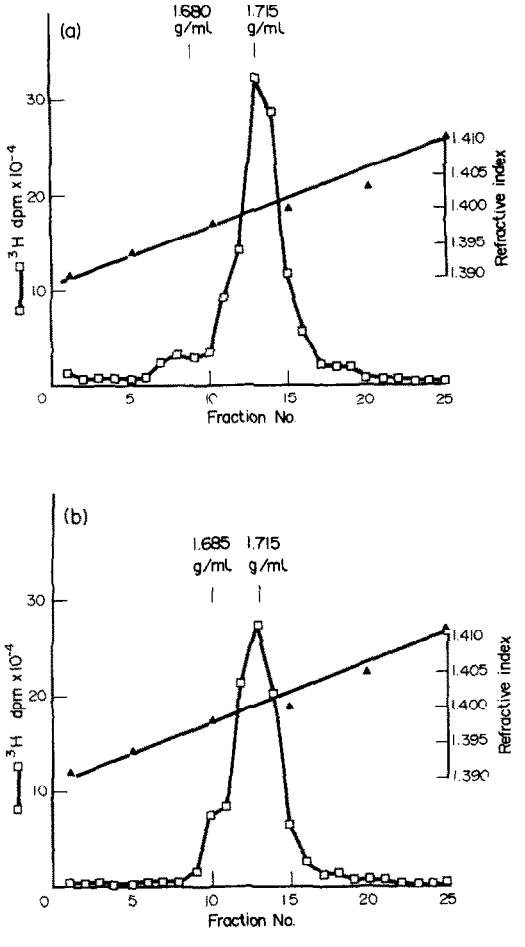

Fig. 2. Effect of AMPAL on DNA synthesis. Monolayers of HeLa cells were infected with Ad5 at a moi of 15 ID/cell. After 2 hr at 37° cultures were washed twice with cold medium. Eagle medium containing 2% dialysed foetal bovine serum was added. AMPAL (2×10^{-4} M) was also added in some cultures. Twenty-four hours after infection [^{3}H]thymidine (1 μCi/ml) was added. The cultures were harvested 72 hr after infection and the DNA of (a) untreated or (b) AMPAL treated cultures was analysed in CsCl gradient. Density increases to the right (cf. refractive index).

higher concentration (10^{-3} M) 80–85% inhibition was observed thus showing that the decrease in ALDH activity is related to the concentration of AMPAL.

Effect of AMPAL on precursor uptake and incorporation into DNA and protein in uninfected cells. Before determining whether AMPAL was affecting viral DNA and protein synthesis, we had to determine if cellular synthesis was modified. Uptake of [^{3}H]thymidine was inhibited by about 30% in the presence of 2×10^{-4} M AMPAL in uninfected cells whereas there was no significant effect on [^{35}S]methionine uptake. Incorporation of [^{3}H]thymidine was inhibited by about 15% into cellular DNA without any significant effect on cellular protein.

Effect of AMPAL on viral DNA and protein synthesis. Replication of Ad5 DNA was monitored by CsCl density gradient analysis of total DNA. This method gives a clear separation of viral DNA from host DNA.

The results presented in Fig. 2(a) showed that in untreated infected cells we could detect both, viral DNA as a very prominent peak at a density of 1.715 g/ml and cellular DNA at a density of 1.680 g/ml. In the presence of AMPAL (Fig. 2b), the cellular DNA was detected as a shoulder at a density of 1.685 g/ml and the viral DNA peak (1.715 g/ml) was reduced by 7.3% (92.7% of the amount of trichloracetic acid-insoluble radioactivity recovered from the peak of viral DNA in untreated cells).

In the experiment performed with labelled [^{35}S]methionine, the analysis of polypeptides by polyacrylamide gel electrophoresis showed that AMPAL had no effect on the number and distribution of viral structural polypeptides since the capsidal polypeptides, i.e. II (hexons), III (pentons base) and IV (fibers) were detected in treated as well as in untreated cells (Fig. 3). The other viral polypeptides were less clearly apparent both in treated and in untreated cells. Further, densitometer tracings of the electropherogram revealed no quantitative difference between treated and untreated cells (data not shown). Thus, protein synthesis was not affected by AMPAL in both non infected and infected cells.

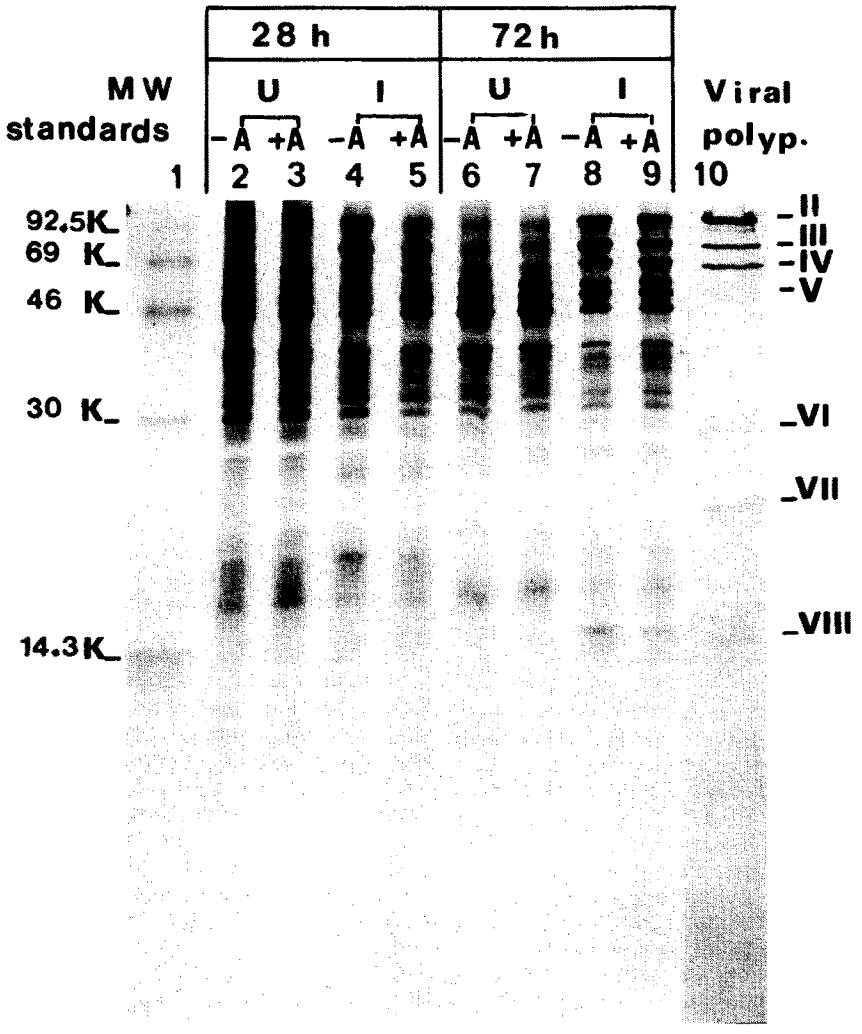

Fig. 3. Synthesis of polypeptides. HeLa cells were infected as in Fig. 2. After adsorption Eagle medium containing 5% of the usual concentration of methionine and 2% dialysed foetal bovine serum was added. Cultures received AMPAL (2×10^{-4} M) (+A) or not (−A). Twenty-four hours after infection [^{35}S]methionine (5 μCi/ml) was added. The cultures were harvested 28 hr and 72 hr after infection. The proteins were disrupted by SDS and mercaptoethanol and the polypeptides were analysed by 12% polyacrylamide gel electrophoresis. A preparation of [^{35}S]methionine labelled purified Ad5 was similarly disrupted and analysed (lane 10). Molecular weight standards (lane 1). Uninfected cells (U) or infected cells (I) were harvested 28 hr (lanes 2 to 5) or 72 hr (lanes 6 to 9) after infection. Photograph of the X-ray film.

With [^{3}H]thymidine, there was a consistent decrease (24%) in the specific activity of viral DNA at 18 hr after infection in AMPAL treated cells whereas the specific activity of cellular DNA was increased by 17%. At 72 hr, however, the situation was different in that the specific activity of cellular and viral DNA increased in AMPAL treated cells (20.5 and 3.5%, respectively).

In view of these observations, we tried to verify whether these small but consistent differences in [^{3}H]thymidine incorporation into DNA were accompanied by any changes in the physical properties of the DNA synthesized in the presence of AMPAL.

Effect of AMPAL on the melting temperature (T_m) and hyperchromicity of cellular and viral DNAs. Melting profiles and T_m values were obtained at low ionic strength (10 mM PO_4, pH 7.0 and 1 mM EDTA). Cellular and viral DNAs showed a biphasic profile (Fig. 4) corresponding to two parts of different stability. A slight decrease both in the melting point and in the hyperchromicity of cellular and viral DNAs only in the region melting at 67–70° was observed in AMPAL treated cells. It is not clear if this slight decrease is significant. If it were in fact significant, then there should be a difference in sedimentation profiles of DNA neosynthesized in the presence of AMPAL.

Effect of AMPAL on the sedimentation coefficient of neosynthesized viral DNA. This was investigated by zonal sedimentation in alkaline sucrose density gradients after direct lysis of Ad5 infected cells in

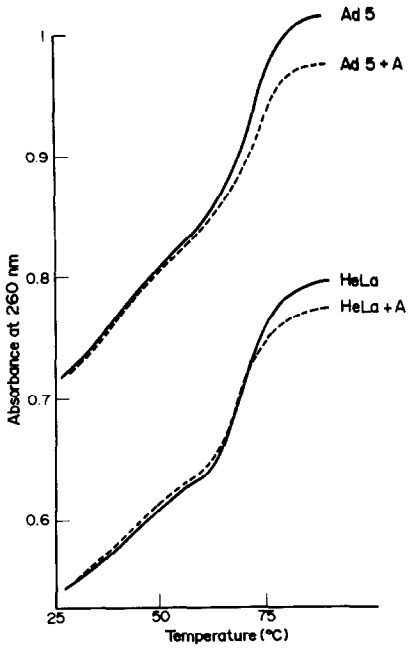

Fig. 4. Effect of AMPAL on the thermal melting profiles of cellular and viral DNA.

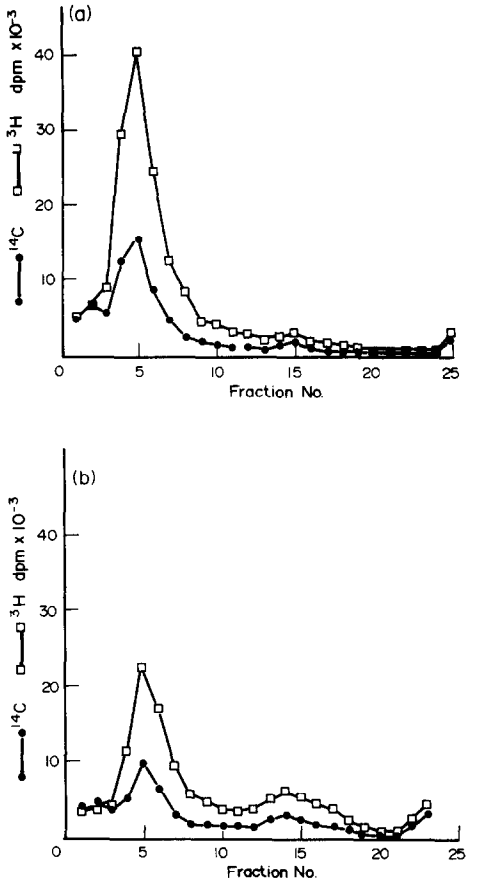

Fig. 5. Analysis of intracellular DNA by zonal sedimentation in 5 to 20% alkaline sucrose density gradients. HeLa cells were infected and labelled as in Fig. 2. Seventy two hours after infection, cells were washed twice with PBSd and were lysed for 17 hr on the top of the gradient, together with [^{14}C]thymidine-labelled Ad5 marker DNA (●). Untreated (a) or AMPAL treated (b) cultures. Sedimentation is from left to right.

0.5 M NaOH on the top of the gradients. Three size classes of DNA were separated in both untreated and AMPAL treated cells (Fig. 5): an important peak of viral DNA which co-sediments at 34S with ^{14}C labelled Ad5 marker DNA extracted from purified virus, DNA sedimenting between 50 and 90S and DNA sedimenting on the bottom of the gradient. Two points clearly appeared: in the sample from AMPAL treated cells, the main peak of viral DNA was reduced (46% of intracellular DNA vs 63% in untreated cells) and the peak sedimenting at 50–90S was increased (19.5% of intracellular DNA vs 5.5% in untreated cells).

These results show quite clearly that the most significant effect of AMPAL on neosynthesized DNA is the 4-fold increase in the highly condensed form of DNA.

To determine whether the difference in DNA condensation was the only change between AMPAL treated and untreated cells which might explain the reduction of CPE or whether differences also appeared in the last event in the viral cycle, i.e. release of progeny virus, this stage was examined.

Effect of AMPAL on virus release. If one assumes that late in the infection, CPE is the reflection of an alteration of host cell membrane allowing the escape of neovirions, the great decrease in the CPE found in AMPAL treated cells should be accompanied by a diminution in virus release. This is precisely what was observed since the amount of infectious extracellular virus recovered after 3 days of infection was lowered by 70% in infected cells treated with AMPAL as compared with that in untreated cells (Table 2). A 35% decrease in virus release was also observed at 28 hr, i.e. the length of a single infectious cycle (Table 2).

DISCUSSION

The most evident effect of AMPAL on Ad5 replication is the important reduction of the cytopathic effect with a 70% inhibition of infectious virus release into the medium at 3 days and a 40% diminution of the total production of infectious virus (intra- plus extracellular virus). As adsorption and penetration of Ad5 were not affected and all viral structural proteins were synthesized (Fig. 3), it is clear that it is not by its action on these events that AMPAL reduces CPE and viral production.

Another point of impact of AMPAL could have been its effect on the contiguous cell to cell transfer of virus from originally infected cells. This, however, would seem to be unlikely since the multiplicity of infection (10–20 ID/cell) was more than sufficient to infect all the cells in the cultures.

Another possible reason for the diminution of CPE and viral production could have been a defect in viral DNA synthesis. We verified this possibility, but the 7% reduction found in viral DNA synthesis

was insufficient to explain the 40% drop in Ad5 production in the presence of AMPAL.

In an attempt to understand the mechanism whereby AMPAL affects the yield of infectious virus, we looked at the activity of ALDH and at the metabolism of intracellular MDA. As far as ALDH activity is concerned, a 15–18% inhibition was brought about by AMPAL at 2×10^{-4} M and at 10^{-3} M this inhibition was about 80%. Since this inhibition of ALDH activity was thought to be involved in increasing cellular MDA levels we first confirmed that in AMPAL-treated cells, cellular MDA did increase by 29% over that of non-AMPAL-treated cells. Further investigations into the origin of MDA, using vitamin E, showed that it was not lipid derived MDA which was responsible for the effect observed on cell growth and viral replication.

The role of MDA in the biological function of DNA has been well established [7–10]. Indeed, Reiss and Tappel [7] have shown that MDA can crosslink DNA with impairment of both its physicochemical characteristics and its biological activity. When this was investigated by T_m measurements on cellular and viral DNA extracted from AMPAL treated cells, it was found that there was no marked effect on either T_m or hyperchromicity.

Further, when the sizes of the viral DNA synthesized in the presence or absence of AMPAL were determined on sucrose alkaline gradients, it appeared quite clearly (Fig. 5) that the viral DNA synthesized in the presence of AMPAL showed a 4-fold increase in the 50–90S region compared to that synthesized in the absence of AMPAL. This result provides good evidence for DNA crosslinking which would explain the increase in sedimentation coefficient.

Why then is there not a greater effect on T_m in the viral DNA derived from AMPAL treated cells? This may be due to the fact that the 50–90S sedimenting DNA corresponds to no more than 20% of the viral DNA synthesized hence the increase in T_m could be masked by the 80% non-crosslinked DNA. At any rate, this fast sedimenting form of viral DNA had previously been observed by Burger and Doefler [11] in cells infected by Ad2. They explained its presence as viral DNA covalently integrated into cellular DNA. In our experiments we provide one possible mechanism for the formation of such crosslinks between cellular and viral DNAs, i.e. via MDA.

Nevertheless it is difficult to attribute to these small changes in the physicochemical properties of DNA the 40% reduction in viral production.

At this stage of experimentation, the only hypothesis which can reasonably be put forward to explain the decrease in CPE was the inhibition of virus release into the medium. When this was evaluated by the titration of extracellular virus, it was found effectively that in AMPAL treated cells there was a 70% inhibition of the amount of extracellular infectious virus as compared to untreated cells.

One mechanism which can be proposed to explain this decrease in the release of progeny virus out of the cells involves the modification of some membrane constituent by the action of MDA.

There is some evidence for this in the literature. Thus, human erythrocytes treated with MDA show changes in membrane constituents such as phospholipids, particularly phosphatidylethanolamine and phosphatidylserine which have been shown to be target molecules for MDA [18]. If the MDA which has accumulated after AMPAL treatment has effectively brought about condensation of cellular membrane phospholipids, there could result the decrease observed in the release of progeny virus.

Regardless of the mechanism(s) (condensation of viral DNA or of membrane phospholipids) responsible for the decrease in the release of infectious virus, compounds which enhance such condensations should be examined for their antiviral properties. Among this category, alkylating agents such as nitrosoureas are well known but are plagued by their toxicity and secondary side effects.

The synthesis of molecules which can mimic the effect of AMPAL in the accumulation of MDA in cells may represent another way for developing a new family of antiviral compounds.

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