Nucleic Acids. III. Antiviral Activity of Nucleotides and Dinucleoside Phosphates Containing *ara*-Cytidine

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ara-Cytidine (cytarabine, 1-β-D-arabinofuranosylcytosine, CA, *ara*-C) has shown antiviral activity against a broad spectrum of DNA-containing viruses *in vitro*, in certain experimentally induced infections in animals, and also in man. The antiviral activity of 27 phosphorylated derivatives (including 24 dinucleoside phosphates) containing *ara*-C has been studied *in vitro* and *in vivo*. Using a plaque-inhibition test as a measure of *in vitro* antiviral activity, all compounds containing *ara*-C were inhibitory for herpes simplex virus (a DNA-containing virus), whereas no inhibition was noted for five RNA viruses. Quantitative *in vitro* studies revealed that herpes virus multiplication was more readily inhibited by some isomers than by others, *e.g.*, *ara*-C 3′-monophosphate was much more inhibitory than either the 2′- or 5′-monophosphates. Similarly, those dinucleoside phosphates containing *ara*-C linked from the 3′ position to the 5′ position of the second nucleoside were somewhat more active than those with 5′→5′ linkages, while those with 2′→5′ linkages were least active. Reduced activity resulted from those compounds containing *ara*-C linked from the 5′ position to either the 2′ or 3′ position of the second nucleoside. These data, taken with the observation that deoxycytidine reversed the activity, suggest that the nucleotides and the dinucleoside phosphates are cleaved to the free *ara*-C, the active moiety. The differences in activity which are observed probably reflect the stability of the compound to enzymatic hydrolysis. Compounds were tested for activity in mice by giving a single intracerebral treatment 3 hr after intracerebral inoculation of herpes virus. Several dinucleoside phosphates showed activity under these conditions; however, there was no consistent correlation between results *in vitro* and in mice, suggesting that in the brain, the fate of the molecule may be different than *in vitro*. Several compounds showed activity against experimental herpes infection of rabbit eyes. In the various tests, none of the phosphorylated compounds was more active than *ara*-C.

1-β-D-Arabinofuranosylcytosine (cytarabine, *ara*-cytidine, CA, *ara*-C) is a nucleoside with antiviral properties. In cell culture, *ara*-C has been shown to inhibit the intracellular multiplication of all viruses containing DNA, yet has no effect on several RNA-containing viruses.[1-4] The antiviral activity is partially prevented by deoxycytidine. *ara*-Cytidine has been shown to be effective in the treatment of herpes keratitis in experimentally infected rabbits[5] and also in man.[6]

Recently, a large number of phosphorylated derivatives (as nucleotides and dinucleoside monophosphates) of *ara*-C have been prepared.[7] With the goal of uncovering agents with improved activity over that of *ara*-C, these compounds were studied *in vitro*, and in many cases *in vivo*, for their antiviral effects.

Methods

In Vitro **Antiviral Activity by Plaque Inhibition.**—Monolayers of susceptible cells were prepared in 60-mm plastic Petri dishes and infected with an appropriate dilution of each virus. Embryonic chick kidney cells served as the host for influenza A (PR-8), infectious bronchitis virus, and Newcastle disease virus; ML monolayers served as the host for Coe (Coxsackie A-21) virus, and HEp-2 monolayers for parainfluenza 3 (HA-1) virus. Primary rabbit kidney monolayers were used for the growth and plaque assays of herpes (MRS) virus which was originally isolated from an oral herpetic lesion.[2]

The method of Siminoff[8] was slightly modified for the detection of antiviral activity. Monolayers were incubated at 37° for 1 hr after the addition of virus. The infected monolayers received 4 ml of medium 199 containing 5% calf serum and 1% agar. A well cut into the agar medium received 50 μg of the test compound in 0.2 ml. After incubation to permit plaque formation, the monolayers were examined for protection of the cell sheet from virus destruction. The antiviral activity was scored from 0 to 4 depending on the diameters of the zones of protected monolayers.

Compounds.—The compounds, supplied by W. J. Wechter,[7] were used in aqueous solution.

Virus Multiplication Studies.—The growth medium was removed from the monolayers and, after washing the monolayers with 2 ml of Hanks' solution, 0.5 ml of the virus was added to each culture. The virus was allowed to infect the monolayers at 37° for 1 hr. The excess virus was removed and the plates were again washed with Hanks' solution. To each plate was added 4.5 ml of Eagle's medium[9] containing 3% calf serum (BME-3% CS) and 0.5 ml of the drug in Hanks' solution. The cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Virus was harvested 24–36 hr after infection and stored at −70° until the time of assay.

Virus Titrations.—Virus titrations were made using the plaque method. Tenfold dilutions of the virus samples were prepared in Hanks' solution and 0.5 ml of each dilution was added to monolayer cell cultures. After 60 min to allow for virus attachment, an agar medium was added. The agar medium contained 1% agar, medium 199 supplemented with Eagle's vitamins and amino acids, 5% calf serum, and antibiotics. Plaque counts were made 3–4 days after infection and are expressed as PFU/0.5 ml.

Bioautography.—Whatman No. 1 paper was spotted with 24–28 μg of each dinucleoside phosphate. Each paper also contained 30 μg of *ara*-C as a marker. The papergrams were developed in a solvent consisting of EtOH–1 *M* NH_4OAc (5:2 v/v).[10] The developing time was 13 hr. The uv absorbing zones were located, then lines were drawn through the origin and the center of the uv absorbing spot, and 0.5-in. strips were cut with the line at the center. The paper strips were then placed on rabbit kidney monolayers infected with herpes virus. Zones of antiviral activity were detected after incubation at 37° to allow for plaque formation.

Antiviral Activity in Mice.—Herpes virus (MRS) inoculated into mice had been serially passed nine times in rabbit kidney cell cultures. The virus stock was diluted 5000-fold so that 0.03 ml contained approximately 40 LD_{50} (50% lethal doses). This volume was inoculated intracerebrally (ic) into 12-g white mice.

(1) H. E. Renis and H. G. Johnson, *Bactericol. Proc.*, **45**, 140 (1962).
(2) D. A. Buthala, *Proc. Soc. Exptl. Biol. Med.*, **115**, 69 (1964).
(3) S. Salagi, *Cancer Res.*, **25**, 144 (1965).
(4) L. A. Feldman and F. Rapp, *Proc. Soc. Exptl. Biol. Med.*, **122**, 243 (1966).
(5) G. E. Underwood, *ibid.*, **111**, 660 (1962).
(6) H. E. Kaufman and E. D. Maloney, *Arch. Ophthalmol.*, **69**, 626 (1963).
(7) W. J. Wechter, *J. Med. Chem.*, **10**, 762 (1967).
(8) P. Siminoff, *Appl. Microbiol.*, **9**, 66 (1961).
(9) H. Eagle, *Science*, **122**, 501 (1955).
(10) S. S. Cohen, private communication.

Three hours later the test compound as an aqueous solution or suspension was also inoculated intracerebrally in a volume of 0.03 ml.

Deaths occurring within 72 hr after inoculation of compound were attributed to toxicity or trauma. Survivors were checked twice daily from day 3 through day 10 postinoculation and deaths were recorded. At the conclusion of the experiment, the 50% survival time of the mice that died (ST_{50}) and the per cent survivors (% S) were calculated for each test group of 20 mice. Three groups of water-treated controls were run on each experiment and the mean ST_{50} and % S values for these 3 groups were determined. For significant activity ($p = .95$), the ΔST_{50} (treated minus control) must be at least 20 hr or the Δ% S must be at least 20. When toxic on the first experiment, samples were retested at a lower level, usually one-tenth the original concentration. Samples found active on the first experiment were retested in duplicate at the original concentration.

Antiviral Activity in Rabbit Eyes.—An experimental herpes keratitis was produced, treated, and scored as previously described.[6] Treatment with aqueous solutions of the test samples was initiated 24 hr after virus inoculation and was repeated hourly from 8:00 AM to 4:00 PM for 5 consecutive days. There were four rabbits in each group.

Results

Using the modified plaque inhibition test, all of the compounds containing *ara*-C protected the cell monolayer from destruction by herpes virus (Table I). Under the conditions of this test, the compounds all appeared to have approximately the same activity, *i.e.*, the zones of protection were of approximately the same diameter except for CA^pCA,[12] which was somewhat less active than the others. None of the compounds was active against the RNA-containing viruses: Newcastle disease, influenza A (PR-8), parainfluenza 3, Coe, or infectious bronchitis virus. These results indicate that the spectrum of antiviral activity of the *ara*-C-containing nucleotides is the same as that previously reported for *ara*-C.[1-3]

Previous studies have shown that at low concentrations, *ara*-C inhibits plaque formation of DNA viruses when added to the agar medium covering the infected monolayer.[1,2] Since all of the compounds under investigation contained *ara*-C, it was essential to establish that the compounds were not being degraded to free *ara*-C during storage. Bioautograms of some of the compounds are shown in Figure 1. The solvent system used separated the nucleotide derivatives from free *ara*-C. Free *ara*-C could not be detected in any of the preparations either by examining the papergram under ultraviolet light or by biological activity. It is concluded from this experiment that the antiviral activities observed are due to the nucleotide derivative and cannot be attributed to free *ara*-C contamination. Whether the compounds under investigation enter the cell intact or are first broken down to the nucleoside cannot be ascertained by this experiment.

To compare the antiherpes activity of *ara*-C and some of the nucleotide derivatives, rabbit kidney monolayers were infected with high multiplicities of herpes virus (virus:cell ratio about 10) and treated with different levels of each compound. The virus yields were determined after a single growth cycle. The effect of

TABLE I

INHIBITION OF PLAQUE FORMATION BY NUCLEOTIDES AND DINUCLEOSIDE PHOSPHATES CONTAINING *ara*-CYTIDINE

Compd	Abbrev[a]	Antiherpes act.[b]
ara-Cytidine	CA	3
ara-Cytidine 5′-phosphate	pCA	3
ara-Cytidine 2′-phosphate	CA_c	4
ara-Cytidine 3′-phosphate	CA_p	4
ara-Cytidylyl-[2′ → 5′]-thymidine	CA^pT	4
ara-Cytidylyl-[2′ → 5′]-deoxyuridine	CA^pdU	3
ara-Cytidylyl-[2′ → 5′]-deoxyadenosine	CA^pdA	3
ara-Cytidylyl-[2′ → 5′]-uridine	CA^cU	4
Uridylyl-[2′ → 5′]-*ara*-cytidine	U^cCA	4
ara-Cytidylyl-[2′ → 5′]-*ara*-cytidine	CA^pCA	2
ara-Cytidylyl-[2′ → 5′]-adenosine	CA^pA	3
Adenylyl-[2′ → 5′]-*ara*-cytidine	A^cCA	4
Thymidylyl-[3′ → 5′]-*ara*-cytidine	T_pCA	3
ara-Cytidylyl-[3′ → 5′]-thymidine	CA_pT	4
ara-Cytidylyl-[3′ → 5′]-deoxyuridine	CA_pdU	3
Deoxyuridylyl-[3′ → 5′]-*ara*-cytidine	dU_pCA	4
ara-Cytidylyl-[3′ → 5′]-deoxyadenosine	CA_pdA	4
ara-Cytidylyl-[3′ → 5′]-uridine	CA_pU	4
Uridylyl-[3′ → 5′]-*ara*-cytidine	U_pCA	4
ara-Cytidylyl-[3′ → 5′]-*ara*-cytidine	CA_pCA	4
ara-Cytidylyl-[3′ → 5′]-adenosine	CA_pA	4
Adenylyl-[3′ → 5′]-*ara*-cytidine	A_pCA	4
ara-Cytidylyl-[5′ → 3′]-*ara*-cytidine	pCACA	3
ara-Cytidylyl-[5′ → 3′]-*ara*-uridine	pCAUA	3
ara-Cytidylyl-[5′ → 3′]-deoxycytidine	pCAdC	4
ara-Cytidylyl-[5′ → 3′]-cytidine	pCAC	3
ara-Cytidylyl-[5′ → 3′]-deoxyuridine	pCAdU	4
ara-Cytidylyl-[5′ → 3′]-adenosine	pCAA	4

[a] The abbreviations are taken from ref 7. [b] Activity expressed as relative diameters of zones of protected cells from 0 to 4.

different concentrations of *ara*-C and its nucleotides on the yield of herpes virus is shown in Figure 2. These results indicate that CA_p was much more inhibitory than either CA^p or pCA, although *ara*-C was the most active compound.

The virus yields from monolayer cultures infected with herpes virus and treated with different concentrations of pCACA, pCAdC, and pCAC are shown in Figure 3. All three compounds have approximately the same inhibitory effect on herpes virus multiplication at the

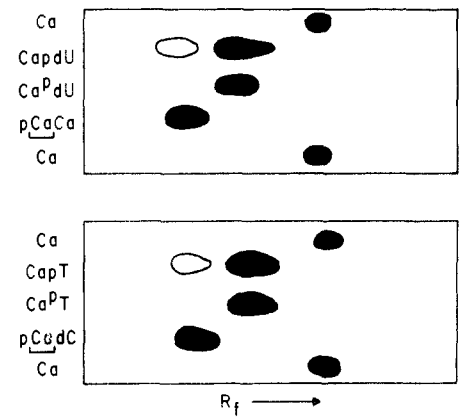

Figure 1.—Bioautography of selected dinucleoside phosphates. Whatman No. 1 paper, spotted with *ca.* 30 μg of each compound, was developed for 13 hr in EtOH–1 *M* NH_4OAc (5:2 v/v). The uv absorbing zones are indicated as clear areas; those zones showing both uv absorption and bioactivity are indicated as the dark areas.

(11) G. E. Underwood, G. A. Elliott, and D. A. Buthala, *Ann. N. Y. Acad. Sci.*, **130**, 151 (1965).

(12) The nomenclature used in this paper is as follows: a p to the left of the symbol indicates a 5′ linkage, a superscript p to the right of the symbol indicates a 2′ linkage, and a subscript p to the right of the symbol indicates a 3′ linkage.

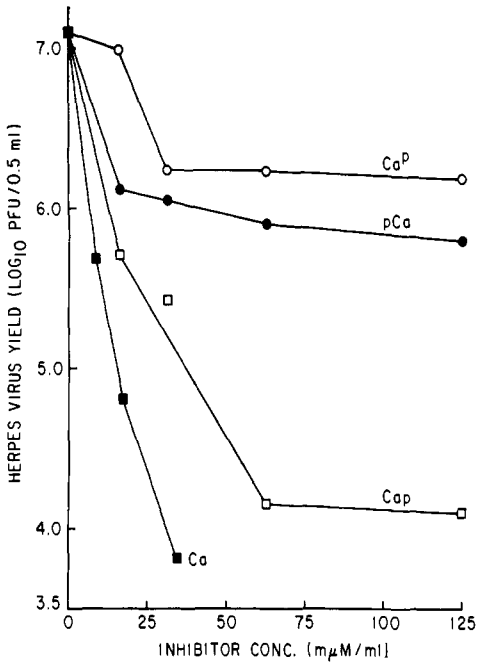

Figure 2.—Herpes virus yields from cultures receiving different concentrations of *ara*-C and nucleotides of *ara*-C.

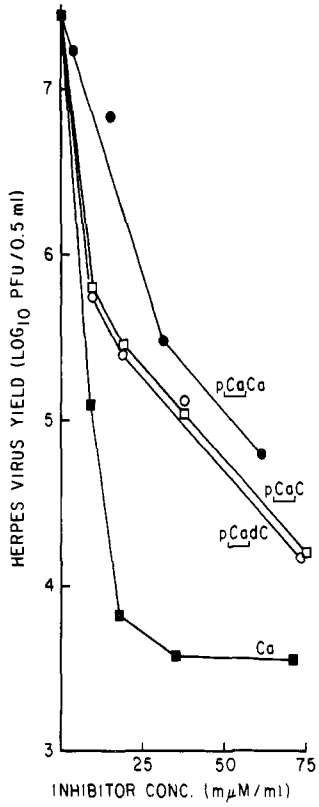

Figure 3.—Effect of different concentrations of dinucleoside phosphates containing *ara*-C and cytidine or deoxycytidine on herpes virus yields.

higher concentrations (60–75 μM). At the lower concentrations (less than 30 mμmoles/ml) pCACA may have been somewhat less active than either of the other derivatives. It is of interest that the activity of pCAdC was not reversed by incorporating deoxycytidine, the antagonist of *ara*-C, into the molecule. The activity of pCAdC was the same as that of the cytidine-

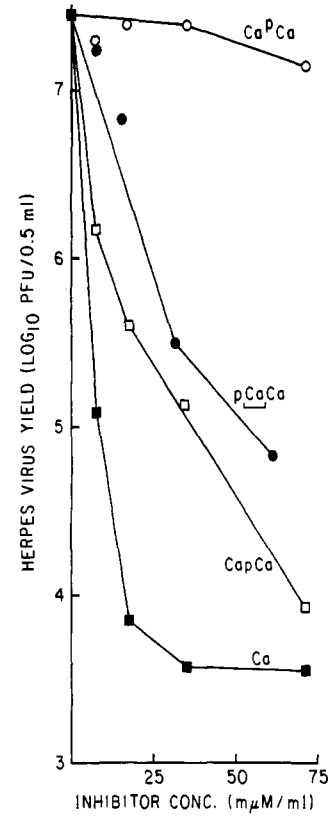

Figure 4.—Effect of different concentrations of dinucleoside phosphates containing *ara*-C on herpes virus yields.

containing dinucleoside phosphate. Cytidine is not effective in reversing the antiviral activity of *ara*-C.[1] Similarly, the dinucleoside phosphate containing two molecules of *ara*-C (pCACA) was no more active than the other compounds with only one molecule of *ara*-C. *ara*-Cytidine was included as a positive control.

A comparison of the inhibitory effect of three isomeric dinucleoside phosphates containing two molecules of *ara*-C esterified from the 5′ position of one molecule to either the 2′, 3′, or 5′ of the other is shown in Figure 4. Of these three compounds, CA_pCA was the most active, followed by pCACA. The CA^pCA was only slightly inhibitory in this test at concentrations where both of the other compounds were quite active. Less than 0.5 log reduction in virus titers resulted with the CA^pCA, whereas greater than 2 and 3 log reductions were noted with CA_pCA and pCACA, respectively. All of these compounds were less active than *ara*-C itself.

The effect on virus yield of different concentrations of a series of dinucleoside phosphates containing deoxyuridine 5′-phosphate esterified to *ara*-C at the 2′, 3′, or 5′ positions is shown in Figure 5. At high levels (75 and 38 μM), the compounds containing *ara*-C esterified at the 3′ and 5′ positions appeared to have about equal activity and were nearly as active as *ara*-C itself. At lower concentrations, it appears that the pCAdU was slightly more active than the CA_pdU. The least inhibitory compound was CA^pdU. Thus, the activity of this series of compounds was similar to that seen with the dinucleoside phosphates containing only *ara*-C as shown in Figure 4.

The data shown in Figure 6 are a comparison of the virus yields from cultures treated with different con-

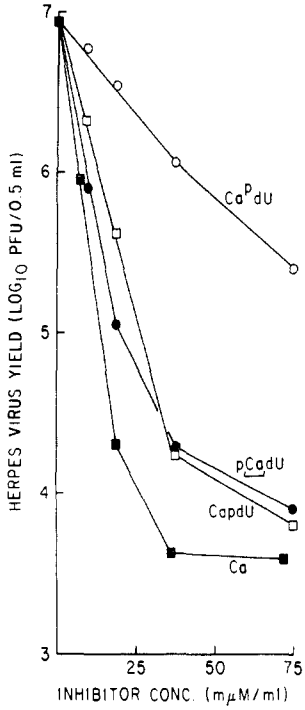

Figure 5.—Effect of different concentrations of dinucleoside phosphates containing *ara*-C and deoxyuridine on herpes virus yields.

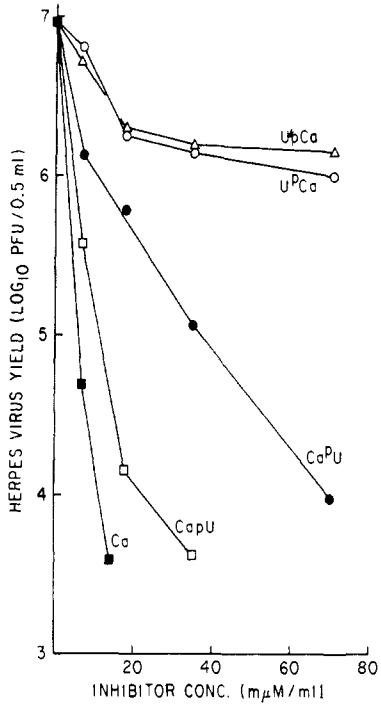

Figure 6.—Effect of different concentrations of dinucleoside phosphates containing *ara*-C and uridine on herpes virus yields.

centrations of four dinucleoside phosphates containing *ara*-C and uridine. The most active *ara*-C derivative was the one containing *ara*-C linked from the 3′ position of *ara*-C to the 5′ position of uridine, followed by the 2′→5′ derivative. Both of these compounds were more active than those in which the uridine was linked from the 5′ position of *ara*-C to either the 2′ or 3′ position of uridine. While none of the compounds was as active as *ara*-C itself, it is clear from these data that the position of the hydroxyl group on the molecule involved

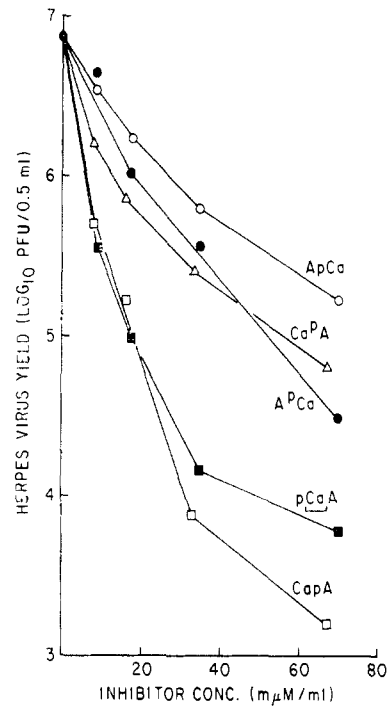

Figure 7.—Effect of different concentrations of dinucleoside phosphates containing *ara*-C and adenosine on herpes virus yields.

in ester formation is important to the antiherpes activity.

The dinucleoside phosphate derivatives containing *ara*-C and adenosine were compared for their antiherpes activity (Figure 7). The most active compound was the one containing the *ara*-C linked from the 3′ position to the 5′ position of adenosine; however, the pCAA was less active only at the higher concentrations. The CA^{p}A was more active than either the A^{p}CA or the A$_p$CA at the lower concentrations (8–16 mμmoles/ml), whereas, at the highest concentration tested (68–70 mμmoles/ml), CA^{p}A was less active than A^{p}CA. At the lower concentrations, A$_p$CA and A^{p}CA were of nearly equal activity.

The reversal of the antiherpes activity of several *ara*-C-containing dinucleoside phosphates by deoxycytidine has been studied (Table II). The presence

TABLE II

REVERSAL OF ANTIHERPES ACTIVITY BY DEOXYCYTIDINE

Compd	Plaque-forming units (PFU)/0.5 ml	
	Agent[a]	Agent + dCdR[b]
Control	1.8×10^7	1.8×10^7
CA	3.05×10^3	4.65×10^5
CA$_p$CA	1.4×10^5	1.45×10^7
pCACA	9.7×10^5	1.2×10^7
pCAC	1.9×10^5	5.0×10^6
pCAdC	2.1×10^5	1.2×10^7
CA^{p}dU	2.8×10^6	1.8×10^7
CA$_p$dU	2.4×10^4	2.6×10^7

[a] The agent was added at a concentration of 20 μg/ml immediately after infecting rabbit kidney monolayers with herpes virus. [b] Titers from cultures receiving deoxycytidine HCl (50 μg/ml).

of deoxycytidine caused an increase in virus yields of 1–2 logs compared to those treated cultures not receiving the reversing agent. *ara*-Cytidine was included as a positive control. The results indicate that in all cases studied, deoxycytidine reversed the antiherpes activity similar to that observed for *ara*-C.

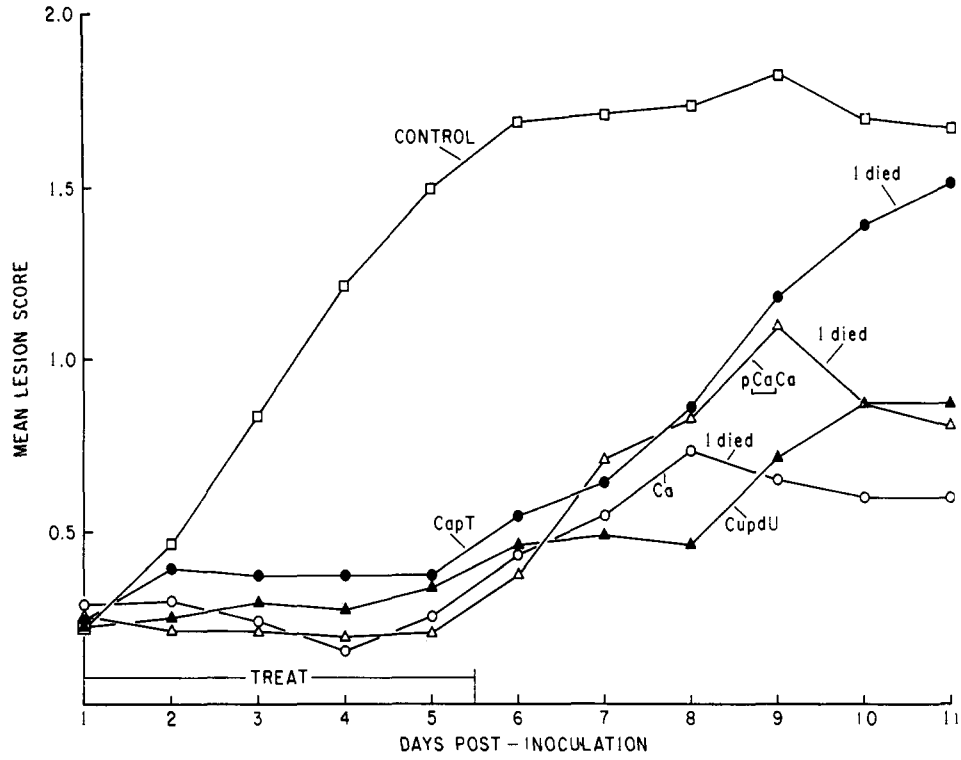

Figure 8.—*ara*-C dinucleoside phosphates *vs.* herpes keratitis in rabbits.

Mice.—Since *ara*-C showed consistent antiherpes activity in the intracerebral mouse test ws well as marked activity against experimental herpes keratitis in rabbits,[5] it was used as a positive control in *in vivo* testing of the dinucleoside phosphates. Results obtained in mice (Table III) show that *ara*-cytidylyl-

TABLE III

PHOSPHORYLATED DERIVATIVES OF CA *vs.* HERPES IN MICE

	Expt 1		Expt 2	
Compd	mg/mouse	Result[a]	mg/mouse	Result[a]
Ca^pU	0.3	+	0.3	+
U^pCA	0.1	+	0.1	++
U_pCA	0.1	+	0.1	+
CA_pU	0.4	−		
CA^pdU	0.4	+	0.4	++
CA_pdU	0.4	Toxic	0.04	−
pCAdU	0.3	Toxic	0.03	−
CA^pA	0.4	+	0.4	+
CA_pA	0.5	+	0.5	+
CA_pdA	0.2	−		
CA_pT	0.4	Toxic	0.1	+
CA_p	1.0	Sl toxic	0.3	+
pCA	1.0	Toxic	0.1	+
pCAC	1.0	Toxic	0.1	−
pCAdC	1.0	Toxic	0.1	−
CA	0.9	++	0.9	++

[a] −, ΔST_{50} and $\Delta\%$ S each <20; +, ΔST_{50} or $\Delta\%$ S 20–35; ++, ΔST_{50} or $\Delta\%$ S 36–50. ST_{50}, calculated as antilog of average log survival time, is an estimate of the 50% point in survival time in hours, assuming the survival time is log normal. S is survival, expressed in per cent.

uridine phosphates were active with CA^pU, U^pCA, or U_pCA linkages, but inactive at 0.4 mg/mouse when the bond was CA_pU. With the *ara*-cytidylyldeoxyuridine phosphates, CA^pdU was active but CA_pdU and pCAdU were inactive at nontoxic levels.

Adenosine 5′-phosphate esterified to either the 2′ or 3′ position of *ara*-C was active, but CA_pdA was inactive at the concentration tested. CA_pT was toxic at 0.4 mg, but active at 0.1 mg/mouse.

Both CA_p and pCA were toxic at 1 mg/mouse, but both showed some activity at lower levels. It is of interest that *ara*-C was considerably less toxic than the phosphate derivatives; thus, *ara*-C was tolerated at least up to a level of 3 mg/mouse.

Two compounds were toxic at 1 mg/mouse and negative at 0.1 mg/mouse: pCAC and pCAdC. None of the compounds tested was more active than *ara*-C itself. Negative controls averaged 4% survivors and an ST_{50} of 118 hr.

Rabbits.—Three *ara*-C-containing dinucleoside phosphates were tested for activity against herpes keratitis in rabbits. These were CA_pT, CA_pdU, and pCACA. Each showed (Figure 8) antiviral activity approximately equivalent to that of *ara*-C, at least during the time of treatment.

Discussion

Based on the spectrum of viruses inhibited and the results of the reversal studies, the antiviral activity of the nucleotides and the dinucleoside phosphates containing *ara*-C is like that of *ara*-C itself. Quantitative studies *in vitro* revealed that these compounds containing *ara*-C were less inhibitory for herpes virus multiplication than was the free nucleoside. This decreased activity probably reflects the necessity of prior enzymatic cleavage of the molecule to the nucleoside, since it has been demonstrated that the cell is much less permeable to the nucleotide than to the nucleoside.[13,14] The greater activity of the 3′-phosphate of *ara*-C compared to the 2′- and 5′-phosphates suggests that the

(13) (a) P. Reichard, *J. Biol. Chem.*, **234**, 1244 (1959); (b) D. G. Parsons and C. Heidelberger, *J. Med. Chem.*, **9**, 159 (1966).

(14) A. Bloch, M. H. Fleysher, R. Thedford, R. J. Maue, and R. H. Hall, *ibid.*, **9**, 886 (1966).

former is more readily hydrolyzed at the cell surface. These results are consistant with those of Mukherjee and Heidelberger,[15] who reported that the thymidine antagonists 5-fluoro-2′-deoxyuridine 3′- and 5′-monophosphates were both less active than the nucleoside in Ehrlich ascites cells; the 3′-phosphate was more active than the 5′-phosphate.

The dinucleoside phosphates containing *ara*-C 5′-phosphorylated to the 5′ position of cytidine or deoxycytidine were found to have nearly equal inhibitory activity toward herpes virus multiplication and were somewhat more active than the pCACA. All three 5′→5′ dinucleoside phosphates appeared to be more active than pCA, but less active than *ara*-C. Thus, the presence of deoxycytidine, the reversing agent for *ara*-C,[1,16] in the molecule did not appreciably reduce its activity, and the presence of two molecules of *ara*-C, as in pCACA, did not enhance the activity. These data are similar to those obtained in studies using dinucleoside phosphates containing FUDR[13b] or FUR;[14] it was concluded in both cases that the dinucleoside phosphates were first cleaved to the nucleoside and nucleotide prior to entering the cell. Montgomery, *et al.*,[17] showed that a dinucleoside phosphate containing two molecules of 6-mercaptopurine riboside phosphorylated through the 5′ position of each molecule was highly active against HEp-2 cells resistant to 6-mercaptopurine. It was concluded that the molecule entered the cell as such and was then cleaved to the corresponding nucleotide. Smith, *et al.*,[18] have shown that the dinucleoside phosphates containing *ara*-C phosphorylated from the 5′ position to the 5′ position of deoxycytidine or cytidine were equal to *ara*-C with respect to the inhibition of KB cells in culture, whereas the pCACA was somewhat less active. It has been shown[7] that 5′→5′ dinucleoside phosphates containing *ara*-C are cleaved by venom diesterase to *ara*-C and the 5′-nucleotide. The antiherpes activity obtained with the 5′→5′ dinucleoside phosphates containing *ara*-C suggests that the compounds do not enter the cell without prior hydrolysis. In view of the resulting activity, it would appear that the hydrolysis is selective, producing *ara*-C and the corresponding nucleotide. If the hydrolysis were random, producing two nucleosides and two nucleotides, the deoxycytidine-containing dinucleoside phosphate would be expected to be reduced in activity compared to the cytidine-containing compound. The lesser antiherpes activity of the pCACA may simply reflect a slower rate of hydrolysis.

The antiviral activity of the *ara*-C-containing dinucleoside phosphates is dependent on the linkage as well as the order of the nucleosides. The greatest activity was observed in those compounds containing *ara*-C linked from the 3′-hydroxyl to the 5′-hydroxyl of the second nucleoside. The 5′→5′-linked compounds were also highly active, whereas the 2′→5′-linked compounds were much less active. The dinucleoside phosphates containing *ara*-C linked from the 5′-hydroxyl to either the 2′ or 3′ of the second nucleoside were less inhibitory for herpes virus multiplication *in vitro* than those compounds in which the 2′ or 3′ positions of *ara*-C were linked to the 5′-hydroxyl of the second nucleoside. These data suggest that in infected rabbit kidney monolayers, the 3′→5′ linkages are most easily cleaved, the products probably being the nucleoside and the 5′-nucleotide. Thus, the products expected from the cleavage of CA_pU would be *ara*-C and uridine 5′-phosphate, whereas U_pCA would yield uridine and *ara*-C 5′-phosphate. The lesser activity of those compounds containing *ara*-C phosphorylated at the 2′ or the 5′ position of *ara*-C probably reflects the greater stability of these linkages to enzymatic hydrolysis. In mice, U_pCA, U^pCA, as well as CA^pU, were quite active, whereas the CA_pU was inactive at the levels tested. It would appear that the fate of these compounds in the mouse brain is quite different from that in cell culture. It is tempting to speculate that if hydrolysis occurs in the brain, it occurs so that the compounds containing *ara*-C esterified at the 5′ position yield *ara*-C, while those containing 2′ or 3′ esters of *ara*-C are not as readily hydrolyzed. Both CA_p and pCA are toxic to mice at high doses and show some antiherpes activity at lower doses. The most active compounds in mice were those containing 2′→5′ linkages. The *ara*-C dinucleoside phosphates containing 3′→5′ linkages were degraded by venom diesterase to nucleosides and 5′-nucleotides, whereas the products of spleen phosphodiesterase action were 3′-nucleotides and nucleosides.[7] Therefore, it appears that the hydrolysis *in vitro* resembles that shown for the venom diesterase, whereas it is quite likely that in the brain the hydrolysis resembles that observed with the spleen phosphodiesterase.

A comparison of the *in vitro* antiherpes activity of the dinucleoside phosphates containing *ara*-C linked to adenosine showed some deviation of the expected inhibitory activity of this series of compounds compared to those containing pyrimidine ribo- or deoxyribonucleosides. The most active contained *ara*-C linked from the 3′-hydroxyl to the 5′-hydroxyl of adenosine; less active was the 5′→5′ derivative. However, A^pCA was more active than A_pCA and about as active as the CA^pA, suggesting that the presence of adenosine causes a change in the fate of the molecule. Using a "kinaseless" mutant of L51784 cells which is resistant to *ara*-C,[19] Smith, *et al.*,[18] have shown that CA_pA and CA^pA are partially cross-resistant, whereas this cell was resistant to 16 other *ara*-C-containing compounds. CA^pA and CA_pA were tested in mice and both were found active. Thus, it appears that the mechanism of action of these compounds in inhibiting herpes virus multiplication *in vitro*, *in vivo*, or as toxic agents for KB cells may vary.

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