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# Effect of 2′-deoxycoformycin on the biologic half-life of 9-β-D-arabinofuranosyladenine 5′-triphosphate in CHO cells

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Ara-A* is a purine nucleoside analogue that has potent antiviral activity [1, 2] and is also a clinically promising antitumor agent [3]. It is phosphorylated intracellularly to the 5′-triphosphate, ara-ATP, and exerts its major action as a competitive inhibitor of DNA polymerase [4, 5]. In addition, ara-ATP is incorporated into DNA [6–8] and inhibits ribonucleotide reductase [9].

The rapid deamination of ara-A by ADA to the relatively inactive ara-Hx severely limits the effectiveness of this drug [10–12]. Inhibitors of ADA that potentiate the action of ara-A have been developed recently. EHNA [13] increased the lethality of ara-A to mouse fibroblasts by 20-fold [14], whereas dCF, a more potent inhibitor of ADA [15], significantly enhanced the toxicity of ara-A to the mouse leukemia L1210, both *in vitro* [16] and *in vivo* [17–19]. dCF is also an inhibitor of adenylic deaminase from erythrocytes [20].

We have studied the biochemical basis for the increased activity of ara-A in the presence of deaminase inhibitors [21, 22]. Higher ara-ATP concentrations were achieved in cells treated with ara-A in the presence of a deaminase inhibitor than were achieved in those cells treated with ara-A alone, both *in vitro* [23, 24] and *in vivo* [22, 25]. The experiments described here extend these observations by investigating the effects of the inhibition of ADA and possibly adenylic deaminase on the biologic half-life of ara-ATP in CHO cells (the time necessary for the decay of one-half the initial concentration of ara-ATP). It seemed likely that, after utilization of ara-ATP as a phosphate donor by a wide variety of phosphate transferring enzymes or cellular phosphatases, the adenine moiety of the analogue might become susceptible to deamination at either the monophosphate [14] or the nucleoside [10] level. Such deamination might lessen the lethality of the incubation and would effectively eliminate the possibility of phosphorylative salvage to ara-ATP. Inclusion of dCF would be expected to block these major routes of deaminative detoxification [20, 26] and should provide an opportunity to evaluate the importance of these catabolic pathways in the maintenance of cellular ara-ATP levels.

All chemicals used were reagent grade. Ara-A was a product of Pfanstiehl Laboratories (Waukegan, IL) and obtained through the NCI. [2-$^3$H]ara-A (18.7 Ci/m-mole) was purchased from New England Nuclear Corp. (Boston, MA). It was 98.7 per cent pure [$^3$H]ara-A, as determined by thin-layer chromatography. dCF was produced by Parke, Davis & Co. (Detroit, MI) and obtained through the NCI.

CHO cells were maintained in monolayer cultures in McCoy's modified 5a medium and supplemented with 20% horse serum (Grand Island Biological Co., Grand Island,

* Abbreviations: ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); ara-A, 9-β-D-arabinofuranosyladenine; ara-ATP, 9-β-D-arabinofuranosyladenine 5′-triphosphate; ara-Hx, 9-β-D-arabinofuranosylhypoxanthine; CHO cells, Chinese hamster ovary cells; dCF, (R)-3-(2-deoxy-β-D-*erythro*-pentafuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-d][1,3]diazepin-8-ol, deoxycoformycin, covidarabine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-adenine; h.p.l.c., high pressure liquid chromatography; and PCA, perchloric acid.

NY). All experiments used exponentially growing monolayer cultures that were detached with 0.005% trypsin and placed in suspension culture.

The cell viability after a 3-hr ara-A incubation, with or without dCF, was determined by cloning. Cells were harvested from the drug-containing medium and resuspended in fresh medium. After appropriate dilution, 1 ml of cell suspension was added to 4 ml of warm medium in 60 mm petri dishes in triplicate and incubated for 5 days, at which time the macroscopic colonies formed were counted.

To determine the rate of ara-A deamination, CHO cells were incubated with 100 $\mu$M [$^3$H]ara-A (sp. act. $1.27 \times 10^7$ dis./min/$\mu$mole) alone or in the presence of 10 $\mu$M dCF. All experimental cultures containing dCF were preincubated with the deaminase inhibitor for 20 min before the addition of ara-A. At various intervals, cell samples were centrifuged and the nucleosides in the medium were extracted with 0.4 N PCA [7]. A portion of the extract was placed on Avicel F thin-layer chromatography plates (Analtech, Inc., Newark, DE) and chromatographed in $NH_4OH$–water-saturated *n*-butanol (5.5:94.5) [27]. The areas containing ara-A or ara-Hx were detected under u.v. light (254 nm) and scraped into scintillation vials; the nucleosides were eluted with 0.01 N HCl for 1 hr. Eleven ml of PCS scintillation fluid (Amersham Corp., Arlington Heights, IL) was added to each vial before counting in a Packard Tri-Carb scintillation spectrometer, model 2650. Quenching was automatically corrected with an external standard; counting efficiency for $^3$H was 35 per cent.

To determine the biologic half-life of ara-ATP in CHO cells, suspension cultures of CHO cells were incubated with 500 $\mu$M [$^3$H]ara-A (sp. act. $8.94 \times 10^6$ dis./min/$\mu$mole) alone or in the presence of 10 $\mu$M dCF for 3 hr. The cells were resuspended in fresh medium and samples were collected at hourly intervals, centrifuged, and the intracellular nucleotides extracted with 0.4 N PCA for analysis by h.p.l.c. as described below.

Arabinosyl nucleotides were analyzed by h.p.l.c. A Waters Associates (Milford, MA) ALC-204 high-pressure liquid chromatograph equipped with two model 6000A pumps, a model 660 gradient programmer, and a column (250 × 4 mm) containing Partisil 10-SAX anion exchange resin (Whatman, Inc., Clifton, NJ) was employed to fractionate PCA-soluble extracts. Samples ($0.5–1.5 \times 10^6$ cell equivalents) were injected by means of the U6K-LC injection system. The extract was loaded onto the column and the nucleoside mono- and diphosphates were eluted isocratically for 10 min with 60% 0.005 M $NH_4H_2PO_4$, pH 2.8, and 40% 0.75 M $NH_4H_2PO_4$, pH 3.7, at a flow rate of 2 ml/min. After 10 min, linear gradient elution from 40 to 100 per cent was begun over 24 min to separate the nucleoside triphosphates. Typical elution profiles of the separation of ara-ATP from normal cellular ribonucleoside 5′-triphosphates in cell extracts are shown in Fig. 1. Eluted compounds were detected at 254 nm by the model 440 detector and quantitated with a CDS-111 electronic integrator (Varian Associates, Palo Alto, CA). In addition, the ara-ATP eluate was fractionated at 0.5-min intervals into scintillation vials containing 0.3 ml $H_2O$ and 11 ml Aquasol (New England Nuclear Corp.), and radioactivity was determined by liquid scintillation counting. The disintegrations/min were used to calculate the nmoles of [$^3$H]ara-ATP and [$^{32}$P]ara-ATP in each sample. The nmoles of ara-ATP were also determined from a standard curve relating the area under each peak to nmoles. Calculations of ara-ATP nmoles, based on peak areas, varied at concentrations of less than 0.1 nmole/sample due to the internal integration program for quantitation of small peak areas. Quantitation of less than 0.1 nmole ara-ATP/sample, as determined by radioactivity, was more reliable. However, both determinations of ara-ATP concentrations yielded the same statistical results concerning the biologic half-life of ara-ATP.

The effect of dCF on ara-A deamination by CHO cells is shown in Fig. 2. In the absence of a deaminase inhibitor, ara-A was deaminated at an initial rate of 8 nmoles/ml/hr at a cell density of $2 \times 10^5$ CHO cells/ml. After 12 hr nearly 80 per cent of the ara-A had been deaminated. Cells incubated continuously with dCF or cells preincubated with dCF for 3 hr followed by harvesting and resuspension in fresh medium before the addition of [$^3$H]ara-A showed insignificant deamination for at least 30 hr.

After incubating the CHO cells for 3 hr with ara-A alone or in the presence of dCF, the dCF-treated cells accumulated 50 per cent more ara-ATP than those cells treated solely with ara-A. Other experiments have produced similar results.

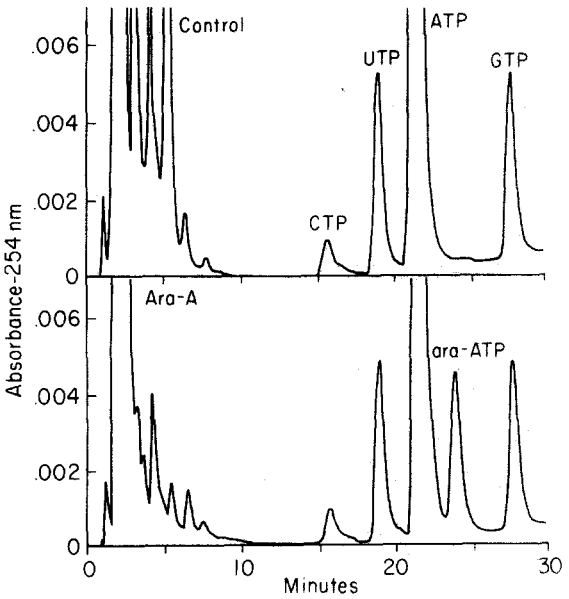


Fig. 1. High-pressure liquid chromatographic analysis of PCA-soluble material from $5 \times 10^5$ CHO cells after a 3-hr incubation in the absence or presence of 500 $\mu$M ara-A. The quantity of ara-ATP present in the ara-A-treated cells was 0.5 nmole.

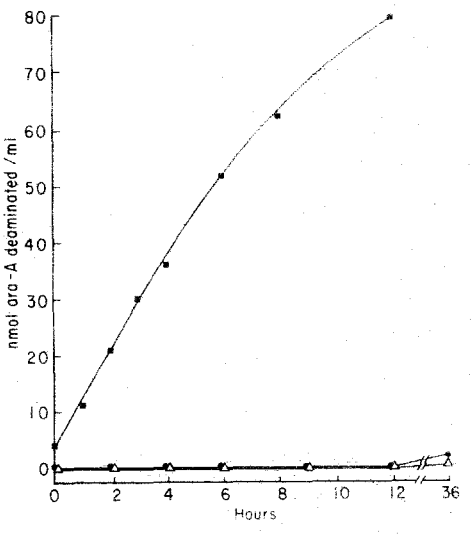


Fig. 2. Deamination of ara-A by CHO cells. CHO cells ($2 \times 10^5$/ml) were incubated with 100 $\mu$M [$^3$H]ara-A (sp. act. $1.27 \times 10^7$ dis./min/$\mu$mole) and no dCF (■); 3-hr preincubation with 10 $\mu$M dCF (●); and 10 $\mu$M dCF continuously (△). The medium was analyzed for ara-A and ara-Hx at the specified intervals.

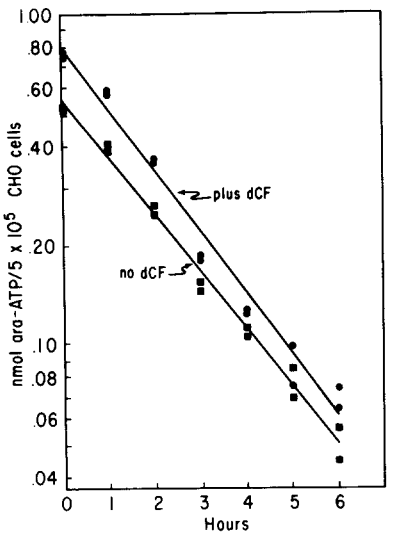


Fig. 3. Effect of dCF on the disappearance of ara-ATP from CHO cells. CHO cells were incubated with 500 $\mu$M [$^3$H]ara-A (sp. act. 8.94 × $10^6$ dis./min $\mu$mole) for 3 hr in the absence (■) or presence (●) of 10 $\mu$M dCF. Cells were harvested and resuspended in drug-free medium. At the indicated times, portions of each culture were prepared for high-pressure liquid chromatographic analysis as described in the text. The standard error of the estimate is 0.09 and 0.13 nmole for the cells treated with ara-A alone or with dCF respectively.

However, the toxicity of ara-A alone or with dCF was comparable, the number of viable cells being 25 and 18 per cent of control cells, respectively, after the 3-hr drug incubation period. We suggest that, although the cells accumulated markedly different amounts of ara-ATP, these high levels were maintained for a relatively short time after the cells were resuspended into ara-A-free medium. Thus, without an exogenous supply of ara-A, the high cellular ara-ATP concentrations quickly decreased to nontoxic levels in both drug-treated cell cultures.

The biologic half-life of ara-ATP in CHO cells was determined after a 3-hr incubation with ara-A in the absence or presence of dCF (Fig. 3), conditions which were toxic to these cells. The disappearance of ara-ATP in both cell cultures was determined to be exponential. The ara-ATP half-life in CHO cells treated with ara-A alone was 1.7 hr while the half-life in cells preincubated with dCF was 1.6 hr. According to Student's $t$-test for comparing two slopes, there is no difference in the rate of the ara-ATP decline in either drug-treated cell culture ($0.10 < P < 0.20$). Thus, there is no significant difference in the ara-ATP biologic half-life, whether ADA is or is not inhibited.

To directly assess the importance of dCF in allowing increased rephosphorylation of catabolized ara-ATP, resynthesis of the triphosphate was quantitated by incorporation of $^{32}P_i$. CHO cells were incubated for 3 hr with 500 $\mu$M [$^3$H]ara-A (sp. act. 1.6 × $10^7$ dis./min/$\mu$mole) alone or in the presence of 10 $\mu$M dCF, then resuspended in fresh medium containing $^{32}P_i$ (sp. act. 8 × $10^7$ dis./min/$\mu$mole, ICN Chemical and Radioisotope Division, Irvine, CA). Cell samples were collected every 2 hr, and the nucleotides were extracted with 0.4 N PCA for h.p.l.c. analysis (Table 1). The levels of [$^{32}$P]ara-ATP and their rates of decay were similar in cells preincubated in the absence or presence of dCF. The incorporation of $^{32}P_i$ into ara-ATP demonstrates that a substantial portion of the cellular ara-ATP pool arises from rephosphorylation of catabolites. However, the similarity of the [$^{32}$P]ara-ATP: [$^3$H]ara-ATP values throughout the incubation suggests that inhibition of the cellular deaminative capacity has little effect on the resynthesis of ara- ATP from its catabolites.

These experiments were undertaken to investigate the possible effects that the inhibition of ADA might have on the biologic half-life of ara-ATP. Although levels of ara-ATP that accumulated in cells incubated with ara-A plus dCF were higher than in those cells treated with ara-A alone, the preincubation with dCF did not affect either the rate of rephosphorylation of catabolites (Table 1) or the biologic half-life of the triphosphate (Fig. 3). This result was observed repeatedly, whether cells were resuspended in dCF-free medium to minimize the possibility of nonspecific effects [20, 28], or whether dCF was present throughout the sampling period. In addition, the results of two separate experiments not shown here in which cells were incubated continuously with $1 \times 10^{-5}$ M EHNA indicated that, similarly, the presence of this deaminase inhibitor did not affect the retention of cellular ara-ATP.

Direct measurements of cellular ara-ATP levels indicate that the presence of a deaminase inhibitor did not affect the flux of ara-ATP. These results show that the phosphorylative salvage of arabinosyl compounds arising from the catabolism of ara-ATP is not supplemented by inhibition of the major routes of deaminative detoxification, in accord with previous findings from this laboratory [21, 22]. Thus, the functional role of deaminase inhibitors in increasing the cytotoxicity of ara-A is to maintain high levels of ara-A as a substrate for subsequent cellular penetration and phosphorylation to ara-ATP.

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Table 1. Incorporation of $^{32}P_i$ into ara-ATP after a 3-hr incubation with [$^3$H]ara-A alone or in the presence of 10 $\mu$M dCF

| Inhibitor | Time after $^{32}P_i$ addition (hr) | [$^{32}$P]ara-ATP (nmoles) | [$^3$H]ara-ATP (nmoles) | [$^{32}$P]ara-ATP: [$^3$H]ara-ATP |
|---|---|---|---|---|
| None | 0 | | 0.536 | |
| | 2 | 0.128 | 0.258 | 0.496 |
| | 4 | 0.103 | 0.124 | 0.831 |
| | 6 | 0.046 | 0.067 | 0.689 |
| dCF(10 $\mu$M) | 0 | | 0.695 | |
| | 2 | 0.154 | 0.328 | 0.470 |
| | 4 | 0.101 | 0.122 | 0.828 |
| | 6 | 0.041 | 0.065 | 0.631 |

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# Initial inhibition by cycloheximide of translational activity of rat liver polysomes *in vivo*

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It has been variously proposed that the primary effect of cycloheximide at the translational level is on the initiation, or elongation, or termination and release of the polypeptide chains. Analysis of numerous literature data on the mechanism of cycloheximide inhibition of protein synthesis shows inconsistent and sometimes contradictory findings depending on the system used, such as yeast [1, 2], L-cells [3], hamster cells [4, 5], chick embryo cells [6], rabbit reticulocytes [7–10], and intact animals such as mouse [11], rabbit [12] and rat [13–16]. It is also difficult to compare, and, therefore, to draw conclusions from systems employing growing versus non-growing cells, cells in tissue culture versus intact animals, and mice versus rats.

In order to define further the mode of action of cycloheximide on protein synthesis in the intact rat during the initial inhibition of liver protein synthesis [17–19], approaches used in the investigation of the stimulated translational activity of rat liver polysomes in our previous paper [20] were employed. The translational state of rat liver polysomes, the proportion of various size classes of polysomes, and their protein-synthesizing activity were examined 2 hr after a single injection of a non-lethal (2 mg/kg body wt) or a lethal (20 mg/kg body wt) dose of cycloheximide.

All studies were performed on 190 ± 10 g male Wistar rats. The maintenance, treatment with cycloheximide, [$^3$H]leucine incorporation (100 $\mu$Ci/100 g body wt), and removal of livers were described previously [19]. Cytoplasmic ribonucleoprotein complexes, polysomal size classes, and puromycin-released polypeptides were isolated and determined as described by Ch'ih *et al.* [20]. Protein was determined by the method of Lowry *et al.* [21]. Trichloroacetic acid-precipitable radioactivity was determined as described [22]. Data were presented as the means ± S.D. of a number of experiments (N). Each experiment consisted of at least two to three rats. The probability that an effect of the experimental condition was due to chance was measured by Students *t*-test on the mean difference between paired observations in a series of different experiments; P values less than 0.05 were considered significant.

Animals were treated with cycloheximide 1 hr prior to the administration of [$^3$H]leucine; after a 60-min labeling period, the livers were removed for processing. Since in our previous study [18] we demonstrated no changes from controls of the specific radioactivity of leucine (0.26 $\mu$Ci/$\mu$mole and 0.24 $\mu$Ci/$\mu$mole for the control and treated), and since leucine is known to be incorporated into the internal sequence of a polypeptide chain, [$^3$H]leucine incorporation was used to measure the rate of protein synthesis in the present study. In