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STUDIES *IN VITRO* ON SOME PRECURSORS OF 1-β-D-ARABINOSYLCYTOSINE*

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Abstract—Several modifications of 1-β-D-arabinosylcytosine (ara-C) are described with particular regard to prolonging its presence in the tissues and lowering its toxicity. Precursors containing an isourea-ether or amidine group and a phosphate group were synthesized and studied *in vitro*. The most promising compound satisfying these criteria is O^2:2′-anhydro-1-β-D-arabinosylcytosine 3′-phosphate (anhydro-ara-CMP). We have followed the disappearance of the anhydro-ara-C chromphore spectrophotometrically in phosphate, bicarbonate, citrate, lactate and Tris–HCl buffers of various concentration and pH. We found that the ultraviolet change was due to two concomitant first-order reactions. The main process was a general-base catalyzed hydrolysis leading to the formation of 1-β-D-arabinosylcytosine 3′-phosphate (ara-CMP), but the formation of 2′,3′-cyclic CMP was also observed to some extent. The rate constants and the arabino to ribo ratio depended on the ionic strength and the pH. In the course of studies of ^{14}C-labeled compound in human blood *in vitro*, the presence of ara-C and its favorable rate of formation could be observed. It was concluded that the administration of a single dose of anhydro-ara-CMP will produce an effect similar to continuous infusion of decreasing amounts of ara-C.

1-β-D-ARABINOFURANOSYLCYTOSINE (ara-C)‡ has shown considerable promise in the treatment of human leukemia.[1–3] However, the high toxicity[4,5] and metabolic instability[6–8] of this compound limit its application and have provided the incentive for a search for improved versions. It appeared desirable to obtain some non-toxic derivatives which would eventually be metabolized to ara-C at such a rate that a sustained drug action under the toxic level could be maintained. Along these lines, we had earlier proposed a scheme of derivatization of ara-C which might rectify the above-mentioned shortcomings of this drug.[9,10] On a theoretical basis, we anticipated that anhydro-ara-C derivatives could be suitable storage forms for the slow release of ara-C. Preliminary accounts of our studies, including biological testing data on anhydro-ara-CMP, were presented orally.[9,10]

Our major modification was to protect the amino group which is rapidly lost due to the high deaminase activity present in several tissues.[7,8] We envisioned a type of

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‡ The abbreviations used are: ara-C, 1-β-D-arabinosylcytosine; ara-U, 1-β-D-arabinosyluracil; ribo-C, 1-β-D-ribosylcytosine; ribo-U, 1-β-D-ribosyluracil; anhydro-ara-C, O^2:2′-anhydro-1-β-D-arabinosylcytosine; anhydro-ara-CMP, O^2:2′-anhydro-1-β-D-arabinosylcytosine 3′-phosphate; ara-CMP, 1-β-D-arabinosylcytosine 3′-phosphate; ara-UMP, 1-β-D-arabinosylcytosine 3′-phosphate.

protection that could be removed by general-base catalyzed hydrolysis, for example, by using amidines and isourea-ether groups. We assumed that the concentration of bicarbonate and phosphate in the blood and tissue fluids would be sufficient to ensure a convenient rate of hydrolysis. We also noted that the major route to the synthesis of ara-C[11,12] involves an anhydro-ara-C derivative (Fig. 1; **1**), e.g. a cyclic isourea-ether in which the original C^4-NH_2 is protected in the form of an immonium group. Theoretically, the common synthetic precursor of ara-C appeared to be a suitable precursor under conditions *in vivo*.

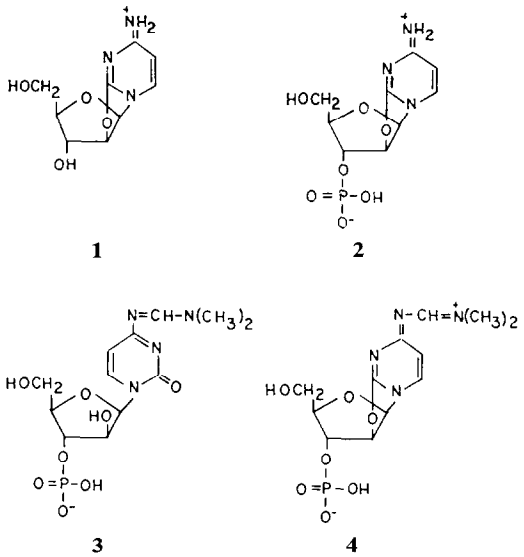

FIG. 1. Anhydro-ara-C, **1**; anhydro-ara-CMP, **2**; N^4-dimethylaminomethylene arabinosylcytosine 3′-phosphate, **3**; N^4-dimethylaminomethylene-O^2:2′-anhydro-arabinosylcytosine 3′-phosphate, **4**.

A second modification which might modulate the rate of biotransformation of the modified drug or precursor was accomplished by introducing a 3′-phosphomonoester group. Even though a phosphate ester is hydrolyzed rather rapidly, it can provide a temporary protection against the deaminase in the blood, liver and spleen. Increased levels of phosphatase activity in some tumor systems[13] were also an argument for the use of phosphate ester, since some selective activation, however small, might be achieved.

When we designed the precursors of ara-C depicted below we had hoped that, in addition to prolonging the presence of ara-C in the body, some favorable effects on tissue distribution, absorption and excretion would be observed.

MATERIALS AND METHODS

O^2:2′-anhydro-1-β-D-arabinosylcytosine 3′-phosphate (anhydro-ara-CMP; Fig. 1; **2**) was prepared on a large scale by our published procedure.[14] For the preparation of smaller quantities (0·01–1 m-mole) of ^{14}C-labeled compound, a slight modification was introduced. Tri-*n*-butylammonium cytidine 2′,3′-cyclic phosphate[15] was dried by repeated evaporation from a mixture of dimethylformamide (DMF) and pyridine.

The final reaction mixture comprised in pyridine as the solvent 2 equiv of tri-*n*-butylamine and more than 10 equiv of trimethylsilyl chloride. It was kept at 80° for 1 hr, and then concentrated *in vacuo*. The resulting gum was dissolved in cold 95% ethanol at a concentration of 0·2 M and, after 15 min of standing at 0°, the nucleotide was precipitated with 5 vol. of ether.

The precipitate was dissolved in ice-cold water and the pH carefully adjusted to 7·1 with ethanolic triethylamine. The solution was immediately poured onto a Dowex-1 column (formate form, ca. 30 ml resin/m-mole of nucleotide) and the column was washed with 1 bed-volume of distilled water at 5°. The title compound was eluted with another bed-volume of 0·1 M acetic acid and freeze-dried (80 per cent yield). Subsequent elution with 0·1 M formic acid produced a mixture of cytidylate and aracytidylate (less than 5 per cent). Fast evaporation of the etheric mother liquor and electrophoresis of the residue in 0·05 M NH_4OOCH_3 at pH 5 can produce additional quantities of anhydro-ara-CMP.

O^2:2′-anhydro-1-β-D-arabinosylcytosine (anhydro-ara-C) was obtained by dephosphorylation of the 3′-phosphate or the 3′,5′-diphosphate[11,12] by an excess of acid phosphatase (Worthington, AP) in 0·05 M CH_3COONH_4, pH 6. The incubation mixture was applied to a Dowex-50 column (pyridinium, 30 ml resin/m-mole of nucleotide) and, after washing with water, anhydro-ara-C was eluted with 0·1 M pyridinium formate. The freeze-dried powder of the formate was dried over P_2O_5. The ultraviolet and nuclear magnetic resonance (NMR) spectra were in accordance with the structure and published data.[11,12,16]

More recently, commercial preparations (Terramarine) were also used. Only the method of Walwick *et al.*[11] was considered practicable at the time this work was initiated. Since then, new syntheses have been published.[17–20]

N^4-dimethylaminomethylene 1-β-D-arabinosylcytosine 3′-phosphate[21] (ara-CMP; Fig. 1; **3**) was prepared by alkaline hydrolysis of anhydro-ara-CMP.[12] The Bu_3NH salt of ara-CMP in anhydrous DMF was treated with dimethylformamide dimethyl acetal according to Zemlicka[22] and the nucleotide precipitated with ether. The u.v. spectrum in H_2O, pH 7, exhibited the characteristic bathochromic shift of λ_{max} to 314 nm. The nucleotide was stored in aqueous solution at −20° for no longer than a week.

N^4-dimethylaminomethylene-O^2:2′-anhydro-1-β-D-arabinosylcytosine 3′-phosphate (Fig. 1; **4**) was obtained by the method of Nagyvary and Tapiero.[23]

Analytical methods

Paper chromatography was carried out on Whatman No. 3 MM paper in the solvent mixtures: *n*-butanol–H_2O (86:14) and ethanol-1 M ammonium acetate (5:2). High voltage electrophoresis was performed in a Savant flat-plate apparatus using 0·05 M ammonium acetate, pH 5·5 and 7·0, for the separation of nucleotides. The arabino- and ribonucleosides were separated in sodium borate buffer, pH 8, according to Gordon *et al.*[24]

Ultraviolet measurements were carried out on a Beckman DU instrument equipped with a Gilford recorder or on a Cary 15 recording spectrophotometer. The ORD spectra were taken on a Cary 60 spectropolarimeter. The radioactivity of nucleosides

and nucleotides was determined in a Beckman LS-250 liquid scintillation counter using 0·4% PPO–0·05% POPOP* in toluene mixture over paper disks.

Determination of the overall rate of anhydro-ara-CMP disappearance in various buffers. Anhydro-ara-CMP was dissolved at convenient spectral concentration (1·5 to 1·8 $\times$ 10^{-4} M) in phosphate, bicarbonate, lactate and citrate buffers, pH 7·1, 7·5 and 8·2, and the decrease of absorption at 262 nm with time was recorded at 37°. A decrease of 30 per cent in the initial absorption of anhydro-ara-CMP (A_0) was taken as the end-point of hydrolysis (A_∞) on the basis of the corresponding extinction coefficients of the pure compounds (anhydro-ara-CMP, ϵ_{262} = 10,400; ara-CMP, ϵ_{262} = 7300). For any given time, the log (A_t – A_∞) value was calculated and plotted against time. The half-time ($T_{\frac{1}{2}}$) of anhydro-ara-CMP disappearance was determined from the graphs, and the observed first-order constant (k_{obs}) was calculated according to the formula $k_{obs} = 0{\cdot}693/T_{\frac{1}{2}}$.

Determination of the percentage of arabino- and ribonucleotide formed from anhydro-ara-CMP. Studies were carried out in phosphate and bicarbonate buffers of various concentration at pH 7·5. Samples of ^{14}C-labeled anhydro-ara-CMP (1·5 $\times$ 10^{-4} M; 2 $\times$ 10^6 counts/min) were kept in sealed vials at 37° for 1 week. Subsequently, the solutions were treated with pancreatic ribonuclease to hydrolyze and 2′,3′-cyclic-CMP formed, diluted and passed through 20 ml Dowex-1 resin (formate form), which was then washed with 200 ml water followed by 200 ml of 0·1 M formic acid. This latter fraction was freeze-dried, the nucleotides were dissolved in 0·1 M Tris–HCl, pH 8·5, and incubated with alkaline phosphatase overnight. The resulting nucleosides were separated by paper electrophoresis in borate buffer, using ribo-C and ara-C standards. The u.v.-absorbing spots were cut out and counted in a Beckmann LS-250 scintillation counter.

The per cent ara-C and ribo-C values thus obtained were used to calculate the rate constants for hydrolysis (k_H) and rearrangement (k_R).

Analysis of products formed during the incubation of [^{14}C]-anhydro-ara-CMP in human blood. Heparanized human blood (0·5 ml) containing 1·0 $\times$ 10^5 dis/min (1·5 $\times$ 10^{-4} M) [^{14}C]-anhydro-ara-CMP was sealed in a vial and incubated at 37° for 3 hr. The content was treated with 1 ml of cold 5% trichloroacetic acid (TCA), the precipitate centrifuged off and washed twice with 1-ml portions of TCA. One mg each of the following carriers was added to the combined TCA washings: ribo-U, ara-U, ribo-C, ara-C, anhydro-ara-CMP, 2′(3′)-UMP, ara-UMP, 2′(3′)-CMP, ara-CMP and anhydro-ara-CMP. This solution was passed through a column of 10 ml Dowex-50 (H^+) resin which was connected to an Isco u.v.-analyzer. Two peaks were obtained on washing the column with 150 ml distilled water. A third fraction was obtained with 100 ml of 2% pyridine, and finally, the column was eluted with ca. 100 ml of 0·1 M pyridinium formate.

The first fraction was concentrated *in vacuo* and chromatographed on paper in butanol–H_2O. In this process, ribo-U and ara-U were separated, while 2′(3′)-UMP and ara-UMP remained at the start. These nucleotides were eluted, dephosphorylated, and the resulting ara-U and ribo-U were separated by borate electrophoresis as described above. The paper spots were directly counted as before.

The second fraction of the Dowex-50 column containing anhydro-ara-CMP

* PPO = 2,5-diphenyloxazole; POPOP = 1,4-*bis*-2-(4-methyl-5-phenyl-oxazolyl) benzene.

2′(3′)-CMP and ara-CMP was subjected to electrophoresis in 0·05 M ammonium acetate, pH 5·5. The zwitterionic anhydro-ara-CMP was separated and its amount determined. The mixture of the nucleotides was dephosphorylated, and the nucleosides were quantitated as described above.

The third peak contained the nucleosides ara-C and ribo-C, which were separated by borate electrophoresis. The fourth fraction contained only anhydro-ara-CMP, which was also counted on paper.

Degradation of anhydro-ara-CMP in rat serum. Two mg anhydro-ara-CMP was dissolved in a mixture of 0·05 ml of 0·1 N $NaHCO_3$ and 0·15 ml of rat serum, and the pH was adjusted to 7·5. A drop of toluene was added as a bacteriostat and the solution was kept at 37° for 24 hr. The serum proteins were precipitated by shaking with $CHCl_3$, and a part of the aqueous phase was chromatographed in butanol–H_2O (86:14). Observation of the chromatogram under u.v. light revealed only one nucleoside spot, which was eluted with water. This nucleoside was identical with ara-C in several chromatographic systems and on borate electrophoresis. The u.v. data (λ_{max}^{pH7} 272, λ_{max}^{pH1} 280) and the characteristic ORD peak ($[M]_{290}^{25°} = 1{\cdot}5 \times 10^4$, H_2O) provide further proof for the formation of ara-C. The technique used here was not suitable to detect small quantities of ribo-C or ara-U.

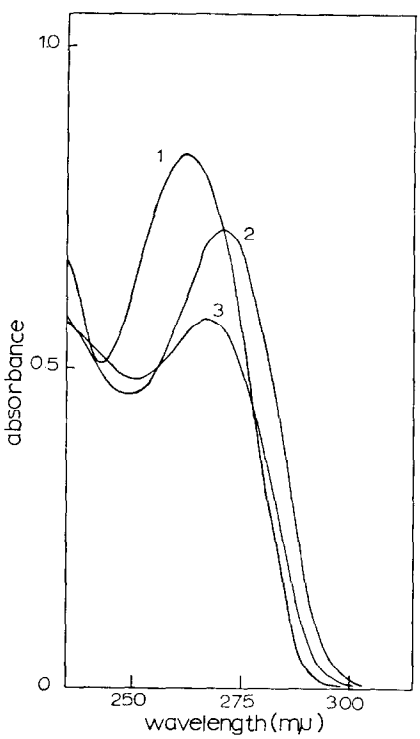

FIG. 2. Ultraviolet spectra of anhydro-ara-CMP (1), ara-CMP (2) and 2′,3′-cyclic CMP (3) in 0·1 M Tris–HCl, pH 7·5. The spectra 1 and 2 were taken at equimolar concentration.

RESULTS

The hydrolysis of the anhydronucleoside and of the amidine group was first studied in simple buffer systems in order to gain basic information on the underlying kinetics and mechanism of this critical step. Only buffers of physiological interest—bicarbonate, phosphate, lactate and citrate in the pH range of 7·1–8·2—were studied.

Studies on anhydro-ara-CMP

A spectral dilution of anhydro-ara-CMP was prepared at several buffer concentrations (0·05, 0·1, 0·2 and 0·5 M) at pH 7·1, 7·5 and 8·2. The hydrolysis was followed spectrophotometrically at the wavelength maximum of the anhydro-ara-C (λ_{max} 262·5, ϵ 10,400) for 3–6 hr. The A_∞ value, which indicates complete hydrolysis to ara-CMP, was obtained by adjusting the pH to 12 (ϵ_{262} 7300). The characteristic u.v. spectra are shown in Fig. 2. First-order kinetics was observed throughout. A characteristic log ($A_t - A_\infty$) vs time plot that served to determine the apparent rate constant k_{obs} is shown in Fig. 3. The rate constants (k_{obs}) observed in various buffers are compiled in Table 1. Most accurate were the data measured in phosphate buffers. Bicarbonate and lactate solutions have little buffering capacity at pH 7·1 and the error becomes > 10 per cent.

Even a cursory evaluation of these data shows the strong influence of pH on hydrolysis of the O^2:2′-anhydro-1-β-D-arabinosylcytosine system. Instant hydrolysis on

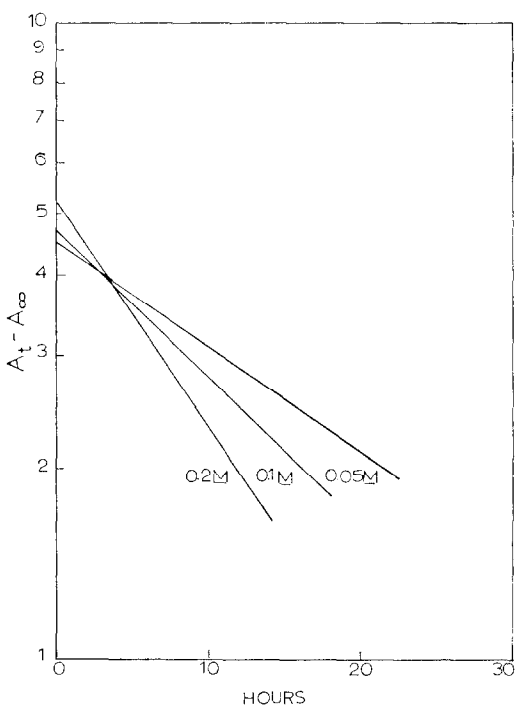

FIG. 3. Log (A_t–A_∞) vs. time plot of 1·5 to 1·7 × 10^{-4} M anhydro-ara-CMP solutions in 0·05 M, 0·1 M and 0·2 M phosphate buffer, pH 7·5, at 37°.

TABLE 1. APPARENT FIRST-ORDER RATE CONSTANT (k_{obs}) OF ANHYDRO-ARA-CMP TRANSFORMATIONS*

Buffer	pH 7·1	pH 7·5	pH 8·2
0·05 M phosphate	0·33	6·52	21·09
0·10 M phosphate	0·93	9·06	21·00
0·20 M phosphate	3·30	12·83	23·10
0·30 M phosphate		13·34	
0·50 M phosphate		11·00	
0·05 M bicarbonate		7·97	25·67
0·10 M bicarbonate		8·56	25·67
0·20 M bicarbonate	5·25	10·11	26·89
0·10 M lactate		2·31	
0·10 M citrate		3·73	

* $k_{obs} \times 10^4$ min^{-1}. The apparent first-order rate constants (k_{obs}) of the transformation of anhydro-ara-CMP in the various buffers at 37° were obtained from a plot of log ($A_t - A_\infty$) vs. time, where A_t is the absorbance at 262 nm observed at time t, and A_∞ is the absorbance taken at the endpoint of hydrolysis. The halftime ($T_{\frac{1}{2}}$) of anhydro-ara-CMP disappearance was determined from the graphs and the k_{obs} values were then calculated according to the formula $k_{obs} = 0{\cdot}693/T_{\frac{1}{2}}$.

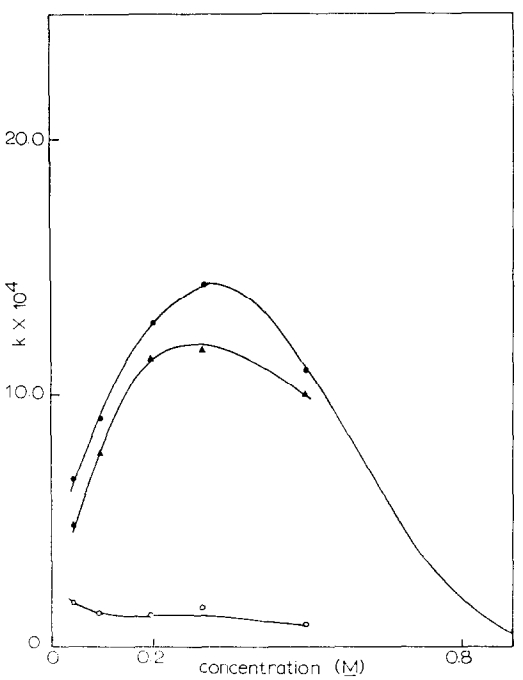

FIG. 4. Plot of k_{obs} (●), the overall apparent rate of transformation of anhydro-ara-CMP, k_H (▲), the apparent rate of hydrolysis of anhydro-ara-CMP, and k_R (○), the apparent rate of rearrangement of anhydro-ara-CMP vs concentration of phosphate buffer, pH 7·5, at 37°.

titration with alkali has been known for some time.[11,12,16] The increase in rate with increasing pH must be due, in part, to the higher concentrations of the conjugate base in the $H_2PO_4^-$–HPO_4^{2-} and H_2CO_3–HCO_3^-–CO_3^{2-} systems. Indeed, raising the buffer concentration from 0·05 to 0·2 M increases the k_{obs}, although in nonlinear fashion. With further increase of buffer concentration, k_{obs} actually decreases (Fig. 4). This finding is compatible with either general-base or nucleophilic catalysis, or both, coupled with a strong salt effect. The addition of NaCl to a 0·05 M phosphate solution, pH 7·5, exhibits the same stabilizing effect of ionic strength on the zwitter-ion. No effort was undertaken to carry out a detailed kinetic analysis at constant ionic strength. The order of activity of the various buffers under identical conditions is as follows: bicarbonate ~ phosphate > citrate > lactate. It appears that the relative basicity and nucleophilicity of the anion is important. Polybasic anions are particularly effective in catalyzing the hydrolysis; therefore, intramolecular catalysis may also be operative as shown below (Fig. 5).

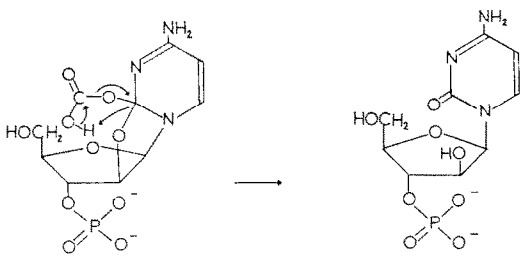

FIG. 5. Probable mechanism of intramolecular catalysis by bicarbonate ion in the hydrolysis of anhydro-ara-CMP.

In the course of identifying the hydrolysis products by paper chromatography and electrophoresis, we found that a varying amount of 2′,3′-cyclic-CMP or 2′(3′)-CMP or of both was present. The prebiotic significance of this finding has been emphasized.[25] The formation of the ribose derivative is negligible in bicarbonate and phosphate buffers above pH 7·5 (less than 1 per cent), but it becomes significant in all dilute buffers at neutrality. The highest amount of ribo-C was obtained after incubating anhydro-ara-CMP in distilled water at pH 7 for 1 week. The ribo/arabino ratio was also determined in various buffers with the help of ^{14}C-labeled nucleotide as described in Methods. According to the data in Table 2, the concentration of buffer has an effect on the ribo/arabino ratio which slightly decreases with increasing ionic strength. It can also be seen that the ratios are similar for bicarbonate and phosphate buffers.

The discovery of the formation of cytidine phosphates by an intramolecular displacement has an important bearing on both kinetic analysis and the expectation of biological activity. Since this competing process is slow and the u.v. spectra of ribo-C and ara-C are almost identical, the error in calculating k_{obs} is negligible. However, k_{obs} merely reflects the rate of disappearance of the O^2:2′-anhydro-1-β-D-arabinosylcytosine chromophore. It can be considered to be the sum of the pseudo first-order hydrolysis constant (k_H) and the intramolecular rearrangement constant (k_R): $k_{obs} = k_H + k_R$. The ratio k_R/k_H should be identical to the before mentioned ribo/arabino ratio, and thus the individual rates can be determined (Fig. 4).

TABLE 2. RIBO/ARABINO RATIO OBSERVED AFTER THE TRANSFORMATION OF ANHYDRO-ARA-CMP*

Buffer	Ribo/ara
0·05 M phosphate	0·34
0·10 M phosphate	0·17
0·20 M phosphate	0·11
0·30 M phosphate	0·13
0·50 M phosphate	0·09
0·05 M bicarbonate	0·17
0·50 M bicarbonate	0·09

* The ribo/arabino ratios resulting from the transformation of anhydro-ara-CMP were obtained from various concentrations of phosphate and bicarbonate buffers at pH 7·5 by incubating ^{14}C-labeled anhydro-ara-CMP ($1{\cdot}5 \times 10^{-4}$ M, 2×10^{6} count/min) in sealed vials at 37° for 1 week. The solutions were then treated with pancreatic ribonuclease to hydrolyze and 2′,3′-cyclic-CMP formed, diluted, and passed through 20 ml Dowex-1 resin (formate form), which was then washed with 200 ml water followed by 200 ml of 0·1 M formic acid. This latter fraction was freeze-dried, the nucleotides were dissolved in 0·1 M Tris–HCl, pH 8·5, and incubated with alkaline phosphatase overnight. The resulting nucleosides were separated according to the method of Gordon *et al.*[24] and counted in a scintillation counter.

Hydrolysis of anhydro-ara-CMP in blood, in vitro

The studies described above have clearly established that the isourea system can be hydrolyzed in dilute buffers at physiological pH at a favorable rate.

Next we have demonstrated that the same process takes place in the blood serum of rat. In this experiment, which is described in Materials and Methods, anhydro-ara-CMP was used at a high concentration to facilitate the isolation of the unlabeled material. Ara-C was unequivocally identified as the major hydrolytic product after 24 hr.

More information could be gained using ^{14}C-labeled anhydro-ara-CMP which was added to heparinized human blood at the concentration of 0·16 μmole/ml. After incubation at 37° for 3 hr, the following hydrolytic products were isolated: anhydro-ara-C, ara-C, ribo-C, ara-U, ribo-U, ara-CMP and 2′(3′)-CMP. The separation of these compounds was achieved by chromatography on a Dowex-50 ion-exchange column, as described in Materials and Methods. The percentage of these metabolites is given in Table 3.

TABLE 3. PERCENTAGE OF METABOLITES FORMED DURING THE INCUBATION OF ^{14}C-LABELED ANHYDRO-ARA-CMP IN HEPARINIZED HUMAN BLOOD*

Metabolite	% Total radioactivity (counts/min)
Anhydro-ara-CMP	61·1
Anhydro-ara-C	14·7
Ara-C	7·6
Ara-CMP	10·8
Ribo-C	0·8
2′(3′)-CMP	0·1
Ara-U	0·8
Ara-UMP	1·1
Ribo-U	1·2

* Heparinized human blood (0·5 ml) containing $1·0 \times 10^5$ dis/min ($1·5 \times 10^{-4}$ M) [^{14}C]-anhydro-ara-CMP was sealed in a vial and incubated at 37° for 3 hr. The content was treated with 1 ml of cold 5% TCA, the precipitate centrifuged off and washed twice with 1-ml portions of 5% TCA. One mg each of the following carriers was added to the combined TCA washings: ribo-U, ara-U, ribo-C, ara-C, anhydro-ara-C, 2′(3′)-UMP, ara-UMP, 2′(3′)-CMP, ara-CMP and anhydro-ara-CMP. The nucleotides and nucleosides were separated and the radioactivity was determined as described in Materials and Methods.

Action of deaminase on anhydro-ara-CMP

Deamination of ara-C is the main reason for its deactivation *in vivo*.[6,7] The immonium group of anhydro-ara-C was not expected to undergo hydrolysis on the basis of earlier studies with *Escherichia coli*.[26] We have studied the action of deaminase present in white blood cells of leukemic patients and in spleen, in collaboration with Dr. D. S. Ho of the M. D. Anderson Hospital and Tumor Institute, Houston, Texas. [^{3}H]-Ara-C was used as a control, and the products were examined by paper chromatography and electrophoresis. We found that the same conditions causing 30 per cent deamination of ara-C left 96 per cent of the anhydro-ara-CMP intact. We conclude that the anhydro-ara-CMP and anhydro-ara-C are stable to deaminase, and deamination may take place only after hydrolysis to ara-C. Anhydro-ara-C was also found to be stable in the presence of mouse kidney cytidine deaminase, as reported by Hoshi *et al.*[27]

Biological results

A typical effect of ara-C, the deformation of the extremities of chick embryos, as described by Karnofsky and Lacon,[28] appeared to us the best suitable preliminary

test for the biotransformation of anhydro-ara-CMP into ara-C. In collaboration with Dr. R. C. Fanguy of our Poultry Science Department, we injected a single dose of anhydro-ara-CMP into each of several dozen embryos on days 3–9. The deformations of claws, wings and beaks were analogous to those observed with ara-C. However, the dose necessary for the same degree of response was two to three times higher.

Preliminary results are available on the effect of anhydro-ara-CMP on leukemia of mice. In work with Dr. R. J. Pienta, we found that the compound was active i.p. on Rauscher virus-induced leukemia in Balb/c mice. Consequently, a comparison of ara-C (NSC 63878) and anhydro-ara-CMP (NSC 128687) was carried out in the L1210 system by A. D. Little, Inc.* Some representative results are compiled in Table 4.

TABLE 4. COMPARISON OF ANHYDRO-ARA-CMP AND ARA-C IN THE L1210 SYSTEM*

Intraperitoneally				Orally			
Anhydro-ara-CMP		Ara-C		Anhydro-ara-CMP		Ara-C	
Dose	T/C (%)	Dose	T/C (%)	Dose	T/C (%)	Dose	T/C (%)
278	86	30·0	71	139·0	273	60·0	216
139	221	15·0	215	69·6	291	30·0	218
69·6	228 (418)	7·50	272	34·8	193	15·0	187
34·8	229 (283)	3·75	147	17·4	163	7·5	164
17·4	217 (225)						
8·7	163 (177)						

* The data were obtained by A. D. Little, Inc., during the period of Oct. 2, 1968 through Feb. 12, 1970. Anhydro-ara-CMP (NSC 128687) and ara-C (NSC 63878) were administered intraperitoneally in saline and orally in water to CDF_1 mice 1 day after inoculating them with L1210 leukemia. The dose schedule involved eight administrations daily at 3-hr intervals on days 1, 5 and 9. The evaluation was done after 30 days in terms of T/C (%): the ratio of mean survival time of the test animals to control animals. Data in parentheses are from a different set of experiments which were evaluated after 60 days.

According to their finding, anhydro-ara-CMP seems to be superior to ara-C when administered orally, while it was found slightly less active via the i.p. route. However the intraperitoneal T/C per cent value of anhydro-ara-CMP had almost doubled in a different set of experiments. Apparent is the wide therapeutic index and low toxicity of anhydro-ara-CMP. Daily injections of 600 mg/kg for 7 days into six mice have not caused any visible toxic effect.

The effect of anhydro-ara-CMP on HeLa cells was studied by Dr. F. Gyorkey at the VA Hospital in Houston. Practically no activity was found.

Studies of other ara-C derivatives

Among the other compounds studied by us, only anhydro-ara-C (formate and chloride) was stable enough to be stored conveniently. The rate of hydrolysis of this

* Screening data summary is available on request from the Drug Research and Development, National Cancer Institute.

compound at pH 7·5, in phosphate and bicarbonate buffers, was virtually indistinguishable from that of its 3′-phosphate. This compound is an immediate precursor of, ara-C and, unlike the nucleotide, it cannot rearrange its carbohydrate moiety. Therefore, it was expected to be somewhat more toxic on a molar basis. Subsequent to the testing of anhydro-ara-CMP, the Drug Research and Development found anhydro-ara-C clearly superior to ara-C in the L1210 system. Hoshi *et al.*[20] and Gish *et al.*[27] have since published short communications confirming these results.

N^4-dimethylaminomethylene 1-β-D-arabinosylcytosine 3′-phosphate can be hydrolyzed by general-base catalysis, a process which was conveniently following by measuring the decrease in the u.v. max at 314 nm. The half-times obtained in $NaHCO_3$ buffers, pH 7·5, at 37° (0·05 M, 3·2 hr; 0·1 M, 4·5 hr; 0·2 M, 5·1 hr) and in phosphate buffer, pH 7·5, at 37° (0·1 M, 4·1 hr) are considerable lower than the corresponding values for the anhydrocytidines. The characteristic u.v. spectra are shown in Fig. 6. In contrast to anhydro-ara-C, this compound is also labile to acid; it can be stored only in anhydrous organic solvents.

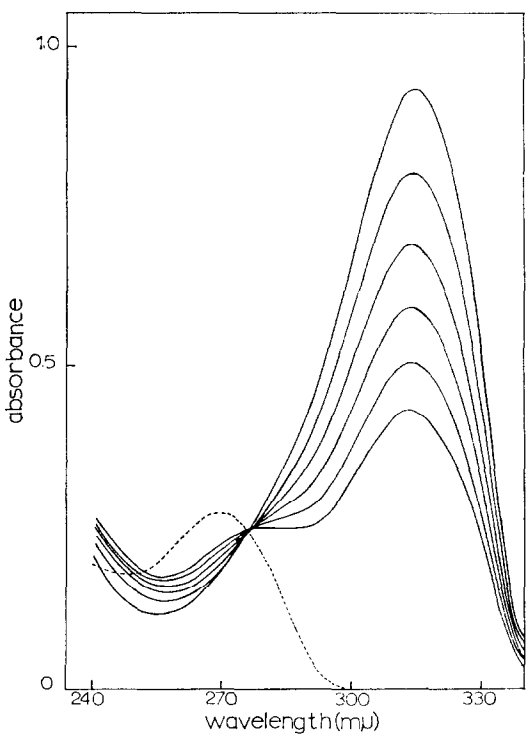

FIG. 6. Ultraviolet spectra of N^4-dimethylaminomethylene arabinosylcytosine 3′-phosphate in 0·1 M phosphate buffer, pH 7·5 at 37° at 0 time, 1, 2, 3, 4 and 5 hr. Ultraviolet spectrum (– – –) of the same sample after it was adjusted to pH 10 and incubated at 37° for 24 hr.

N^4-dimethylaminomethylene-O^2:2′-anhydro-1-β-D-arabinosylcytosine 3′-phosphate is a more complex ara-C derivative, since it requires three hydrolytic steps to form ara-C. The positively charged amidine group can be removed at both acidic or neutral pH without hydrolyzing the anhydrocytidine. In bicarbonate and phosphate buffers,

the hydrolytic processes involving the amidine and anhydrocytidine group occur concomitantly. According to a preliminary spectra study, the rate of formation of ara-CMP is close to that found for anhydro-ara-CMP.

DISCUSSION

Ara-C itself is a precursor of the biologically active forms, ara-CDP and ara-CTP, which are rapidly formed through the action of kinases.[29,30] The utilization of more distant precursors or derivatives can be successful only if the rate of biotransformation is conveniently slow. Recently, the 5′-adamantoyl derivative of ara-C was found to represent a sustained action form of the "parent" compound.[31] Phosphorylation of ara-C, on the other hand, did not provide sufficient stabilization against deamination because the phosphate group was removed too quickly.[32] One of our modifications is sensitive only to chemical, i.e. hydrolytic, removal and therefore it primarily affects the rate at which ara-C is released. The 3′-phosphate group can cause only a minor delay in the appearance of the active agents. At this time, we have no actual proof that the use of the nucleotide form would result in an improvement of therapeutic usefulness over the nucleoside. Recently published results of Hoshi *et al.*[27] and the testing data available through the National Cancer Institute do not seem to support our original assumption in this respect.

The study of chemical hydrolysis *in vitro* gives only preliminary information concerning the biological consequences of a modification, but valuable predictions can be made. All the compounds studied exhibited a half-life of 4–6 hr, which we consider very favorable. It is unfortunate that the low stability of the amidines (Fig. 1; **3** and **4**) during storage virtually precludes their use as therapeutic agents, although their biological activity might be satisfactory.

It is remarkable that, although anhydro-ara-C itself and its 3′,5′-diphosphate had been known for over 13 years, their therapeutic usefulness has only recently been recognized. The reason for this might have been the limitation of testing to a few established cell lines such as HeLa cells. The failure of this important primary screening system in the case of anhydro-ara-CMP is indicative of the pitfalls of relying heavily on cell cultures. According to studies, the hydrolysis of the anhydronucleoside moiety in anhydro-ara-CMP is extremely slow at pH 7, the pH of the HeLa cell medium. At this pH, the rearrangement to 2′,3′-cyclic-CMP could become significant in the metabolism of this drug. The cytidine derivative could then be eventually converted to deoxycytidine, which is known to reverse the action of ara-C.[29] On the basis of our studies on the hydrolysis of anhydro-ara-CMP in buffers, we were able to predict that the compound must be active *in vivo* by slowly releasing ara-C. We have unequivocally identified ara-C in rat serum and in heparinized human blood after 3 hr of incubation, but it appears probable that anhydro-ara-C may have a more prolonged existence in blood. It is predictable that the administration of a single dose of anhydro-ara-C or anhydro-ara-CMP will produce an effect somewhat similar to continuous infusion of decreasing amounts of ara-C.

It can be assumed that anhydro-ara-C will be bound to proteins in blood and tissues by ionic or covalent linkages. This binding could well slow down the excretion and confer some protection against chemical hydrolysis for a much longer time than

expected on the basis of nucleophile content. This effect is too complicated to be calculated and detailed metabolic studies on the ^{14}C-compound were necessary.*

The discovery of the rearrangement[25] as an inherent part of the chemical behavior of anhydro-ara-CMP indicated that this compound was more than a mere precursor of anhydro-ara-C and of ara-C. Very low toxicity and even specific reversal of toxic effects in the more acidic tissues are to be expected. Further studies of various anhydro-ara-C derivatives as a means of improving the ara-C action seem to be warranted. The full potential of these compounds should be apparent in systems of high cytidine deaminase activity.

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