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BIOCHEMICAL STUDIES OF A NEW ANTITUMOR AGENT, O^2,2′-CYCLOCYTIDINE*

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Abstract—Cyclocytidine, O^2,2′-cyclocytidine (cyclo-C), structurally related to 1-β-D-arabinofuranosyl cytosine (ara-C), inhibits the incorporation of ^{3}H-thymidine into DNA of L1210 leukemia cells both *in vivo* and *in vitro*, and human normal marrow cells and leukemic cells *in vitro*; but it has no effect on uridine or L-valine incorporation. The inhibition is proportional to the dose, and on an equimolar basis *in vivo*, cyclo-C shows a lesser but longer lasting effect than ara-C. Cyclo-C has no effect on thymidine incorporation into DNA of L1210 leukemic cells resistant to ara-C. Cyclo-C is stable in 0·1 M Tris buffer, pH 7·0, at 37° for the incubated 4-hr period, but at pH 9·0 for 1hr, > 90 per cent is hydrolyzed to ara-C. When cyclo-C was incubated at 37° for 60 min with plasma from various species, the supernatant from boiled human plasma, or Eagle's minimum essential medium, the only product found was ara-C. Ara-C was found in dog's plasma and urine 2 hr after the i.v. injection of ^{14}C-cyclo-C and in mouse urine 1 hr after the injection (i.p.). The above results suggest that cyclo-C is hydrolyzed to ara-C and may thus serve as a reservoir of ara-C. Intermittent treatment with cyclo-C may, therefore, replace the current clinical practice of 5-day continous intravenous infusion of ara-C.

1-β-D-ARABINOFURANOSYL cytosine (ara-C) is effective against acute myelogenous leukemia[1,2] and lymphoma[3] in man, but it is a schedule-dependent agent. The plasma half-time of ara-C is short because it is rapidly deaminated to a biologically inactive compound, 1-β-D-arabinofuranosyl uracil (ara-U).[4] O^2,2′-cyclocytidine (cyclo-C), a cytidine analog, is structurally related to ara-C. Mice bearing L1210 leukemia were treated once daily for 5 days, and cyclo-C was found to be more effective than ara-C.[5,6] Some of the tumor-bearing mice survived for over 45 days after daily treatment with cyclo-C, whereas none survived with ara-C therapy.[5,6] The present studies were, therefore, undertaken to evaluate the effect of cyclo-C, as compared to ara-C, on thymidine (TdR), uridine or L-valine incorporation into macromolecules *in vivo* in mouse L1210 leukemic cells and *in vitro* in human normal bone marrow cells and leukemic cells. The hydrolysis of cyclo-C to ara-C was also studied *in vitro* and *in vivo*. Preliminary results were presented elsewhere.[7]

MATERIALS AND METHODS

Ara-C (NSC 63878), cyclo-C hydrochloride (NSC 145668), 2-^{14}C-ara-C (sp. act. 10 mCi/m-mole) and 2-^{14} C-cyclo-C (sp. act. 60 mCi/m-mole) were supplied by the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute. Methyl-^{3}H-TdR (sp. act. 17–20 Ci/m-mole), 5-^{3}H-uridine

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(sp. act. > 20 Ci/m-mole) and ^{3}H-L-valine (sp. act. 7 Ci/m-mole) were purchased from Schwarz Bio-Research Inc., Orangeburg, N.Y. The radiochemical purity was 98–99 per cent for the precursors, 75–97 per cent for cyclo-C and 98 per cent for ara-C. Eagle's minimum essential medium (MEM), fetal calf serum, glutamine, penicillin and streptomycin were obtained from Grand Island Biological Co., Grand Island, N.Y.

L1210 leukemic cell lines sensitive and resistant to ara-C were a gift from Mr. I. Wodinsky, A. D. Little, Inc., Cambridge, Mass. Male DBA/2 mice (Texas Inbred Mouse Co.) were 3–6 months old and weighed 25 ± 4 g. Six days prior to treatment, mice were inoculated, i.p., with 1×10^6 L1210 leukemic cells. The mice were injected, i.p., with equimolar (0·0054, 0·054 and 0·54 m-mole/kg) ara-C or cyclo-C, and sacrificed at different time intervals. Duplicate groups, each consisting of two mice, were used at each interval. Six culture tubes were subsequently set up for each group; at each interval the results from twelve culture tubes were averaged. Each culture was adjusted to a concentration of 2×10^6 cells/ml in 2 ml of Eagle's MEM with 5 per cent fetal calf serum containing 1·3 mM of L-glutamine, 200 μg of dihydrostreptomycin, 200 units of penicillin, and 4 μCi of tritiated TdR, uridine or L-valine. The precursor incorporation was no more than 5 per cent of the total amount used. Thus, the amount of precursor used in the culture was not a limiting factor. Preliminary studies of each 2-ml culture consisting of 0·5, 1, 2 or 4×10^6 cells/ml showed that the precursor uptake was linear up to 2×10^6 cells/ml. Thus, 2×10^6 cells/ml was chosen for routine experiments. The culture was incubated at 37 for 1 hr with the pH maintained at 7·2 with a mixture of 5 per cent CO_2 and 95 per cent air. Incubations of 1 or 2 hr showed the same results; therefore, a 1-hr incubation was routinely performed.

Human leukemic cells were obtained by leukapheresis with an IBM blood cell separator, and normal bone marrow cells were prepared from ribs obtained surgically. With the exception of increasing the number of cells to 2×10^6 cells/ml, all of the other procedures are the same as previously described.[8] The incubation was interrupted by washing the cells twice with 10 ml of 0·9 per cent NaCl solution. After decanting the last wash, the cells were suspended in 0·2 ml of 0·9 per cent NaCl solution, lysed with 0·5 per cent sodium dodecyl sulfate, precipitated with 5 per cent cold trichloroacetic acid, and filtered through a Millipore filter with a 0·45 μm pore size. Depending on the precursor used, the radioactivity on the filter, representing its incorporation into macromolecules, was measured by a Packard liquid scintillation spectrometer with 10 ml of a counting solution (4 g 2,5-diphenyloxazole, PPO, 0·2 g 1,4-bis-[2-(5-phenyloxazolyl)]-benzene, POPOP, and toluene to make 1 liter). The amount of DNA, RNA and protein was determined by previously described methods.[9-11]

Cyclo-C, 1×10^{-4} M (0·1 μCi of ^{14}C-cyclo-C), was incubated at 37 for various time intervals in 1 ml of Eagle's MEM or plasma from normal human subjects, DBA/2 mice or mongrel dogs. Control samples of 1×10^{-4} M cyclo-C in 0·1 M Tris buffer, pH 7·0, were also incubated for the same time intervals. At the end of each incubation period, ethanol was added to each sample to a final concentration of 66 per cent, and Tris buffer, pH 7·0, was also added to a final concentration of 0·1 M. After a brief centrifugation, the supernatant and appropriate markers were separately applied to the same spot on Whatman No. 1 paper which was developed for 7 hr

with descending flow in a solvent system containing isopropanol–H_2O–ethyl acetate, v/v, 22·8:12·2:65. The R_f values are 0·06, 0·11, 0·15 and 0·33 for cyclo-C, ara-C, cyclouridine (cyclo-U) and ara-U respectively. For routine use, however, paper chromatograms were developed overnight (18 hr). The labeled cyclo-C and ara-C were also separated by paper electrophoresis for 2 hr in 0·014 M barbitone acetate buffer, pH 6·8, with 10 mA constant current. Ara-C and cyclo-C moved 0·5 and 6·5 cm, respectively, from the origin toward the anode. The fluorescent spots were identified under ultraviolet light, cut out, eluted with 1 ml of 4 per cent perchloric acid, and counted in a Packard liquid scintillation spectrometer in 10 ml of 10 per cent BBS counting solution (100 ml of Beckman Bio-Solv, 4 g of PPO, 0·2 g of POPOP and toluene to make 1 liter). Quenching, if any, was corrected by channel ratios with an automatic external standard. The relative counting efficiency was 20–30 per cent for tritium and 80–95 per cent for ^{14}C.

Male mongrel dogs, 11–15 kg, were lightly anesthetized with pentobarbital. Drug administration (20 mg/kg, 30 μCi of ^{14}C-cyclo-C, i.v.) and blood sampling were performed in opposite femoral veins. Heparin was used as an anticoagulant. Urine was collected with an indwelling catheter. The dog plasma and urine samples were taken 1 hr after drug administration. DBA/2 mice were given, i.v. ^{14}C-cyclo-C (113 mg/kg, 3μCi) and housed in plastic metabolism cages which allowed the collection of urine (2 hr) free of fecal materials. Tris buffer, pH 7·0, and 100 per cent ethanol were added to the samples to a final concentration of 0·1 M and 66 per cent respectively. The drug and its metabolite(s) were analyzed as described above.

RESULTS AND DISCUSSION

Studies in vivo

Mice, 6 days after transplantation with L1210 leukemic cells sensitive to ara-C, were injected, i.p., with cyclo-C or ara-C, at doses of 0·0054, 0·054 or 0·54 m-mole/kg. The control mice had saline injected. The mice were sacrificed at designated time intervals, and the effect of the drug on TdR incorporation into L1210 leukemic cells was expressed as per cent activity of the control samples. The effect of ara-C on TdR incorporation (Fig. 1a) was also compared with that of cyclo-C (Fig. 1b). With ara-C or cyclo-C (0·54 m-mole/kg), TdR incorporation was still inhibited at 24 hr after the drug injection. A 10-fold lower concentration of ara-C, 0·054 m-mole/kg, inhibited TdR incorporation as studied at 2, 6 and 10 hr, but the incorporation recovered to normal range by 16 hr. After treatment with an equimolar dose (0·054 m-mole/kg) of cyclo-C, TdR incorporation at 2, 6 and 10 hr was not inhibited as much as with ara-C, and by 16 hr, 30 per cent inhibition was still observed. After a further reduction in the dose (0·0054 m-mole/kg), cyclo-C again showed a lesser effect than ara-C. However, by 16 hr TdR incorporation had recovered to normal range in both cases. The incorporation of TdR recovered to over 100 per cent, which might be expected since ara-C partially synchronizes the cell population.[12–14] By the time the drug is inactivated and/or excreted, more cells will have progressed into S phase and be ready to use accumulated nucleosides to synthesize DNA. Similar findings were reported in HeLa S-3 cells,[12] murine B16 melanoma and Ehrlich ascites tumour,[13] and rat small intestine.[14]

A similar experiment was performed with 0·054 m-mole/kg of ara-C or cyclo-C

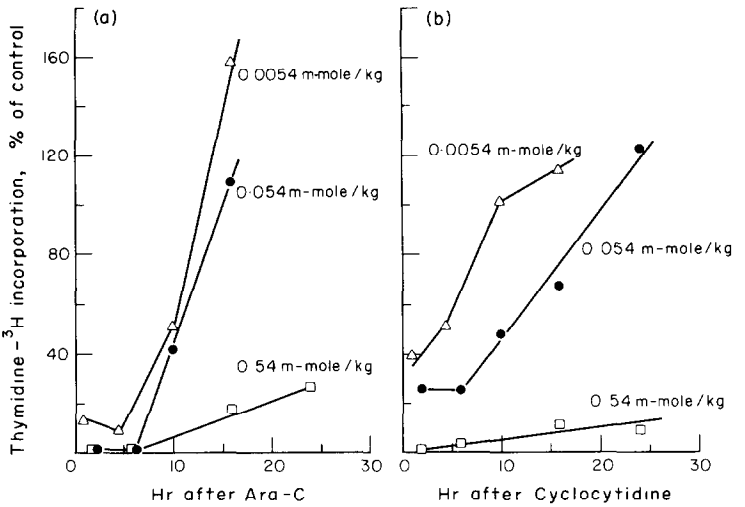

FIG. 1. Comparison of the inhibitory effect of ara-C (a) and cyclo-C (b) on ^{3}H-TdR incorporation. Mice, 6 days after transplantation with L1210 leukemic cells sensitive to ara-C, were injected, i.p., with various doses of ara-C or cyclo-C. The effect of drugs on the TdR incorporation in L1210 leukemic cells was expressed as per cent of control vs time in hr after the drug. The experiment was performed in 2 consecutive days, and controls were included for each day (experiments of 2, 6 and 10 hr were conducted on the first day, and 16 and 24 hr the second day. The mean cpm ± S.D. for the first day control were 184,585 ± 10,486 for duplicate groups of six culture tubes each, and for the second day control 94,170 ± 8,540 and 101,992 ± 7,279.

on mice bearing L1210 cells resistant to ara-C. No inhibitory effect on TdR incorporation was observed 1, 5, 17 and 24 hr after treatment with either drug. The resistant cells are known to lack kinase activity.[15]

Studies in vitro

Effect on TdR, uridine or L*-valine incorporation.* Normal human marrow cells were incubated with Eagle's MEM containing ara-C or cyclo-C in the presence of labeled precursors. Controls without drugs were included. The results showed that 10 μg/ml of ara-C or cyclo-C completely inhibited TdR incorporation at 1, 6, 16 and 24 hr of incubation, whereas no effect was seen on uridine or L-valine incorporation (Fig. 2). Similar findings were also obtained with human or mouse leukemic cells; therefore, the TdR incorporation was the only parameter studied thereafter. During revision of this paper, a report has appeared stating that cyclo-C specifically inhibits DNA but not RNA or protein syntheses of L5178Y leukemic cells in culture.[16] However, no data were presented to differentiate whether cyclo-C affects the uptake of precursors and/or the synthetic process. It was reported that ara-C inhibited DNA, but not RNA or protein synthesis in a growing suspension culture of KB cells; thus giant cells were formed.[17] However, our culture was non-growing, and the amount of incorporated labeled uridine and L-valine into acid insoluble fraction was negligible in comparison with the labeled precursors present in cultures. In addition, quantitative determinations of DNA, RNA and protein in CML cells showed no change after up to 24 hr of incubation with the drug. This suggests that the incorporated labeled precursors reflect the turnover of these macromolecules.

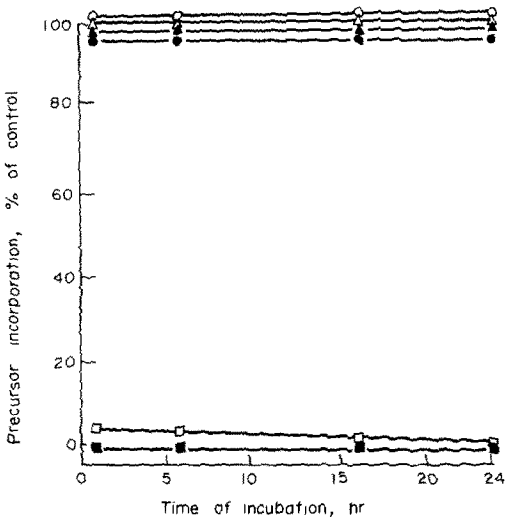

FIG. 2. Effect of cyclo-C or ara-C on TdR, uridine or L-valine incorporation of normal human marrow cells *in vitro*. The cells were incubated with no drug, 10 μg/ml of cyclo-C or 10 μg/ml of ara-C in Eagle's MEM. The open symbols were the effect of cyclo-C, and closed symbols, ara-C. At 1, 6, 16 and 24 hr of incubation, the mean cpm ± S. D. for controls of TdR incorporation (□■) were 4,685 ± 237, 7,556 ± 302, 10,328 ± 516 and 13,381 ± 1,208; uridine incorporation (△▲) were 505 ± 61, 615 ± 49, 709 ± 42 and 835 ± 88; and L-valine incorporation (○●) were 170 ± 18, 355 ± 14, 406 ± 20 and 575 ± 14.

Dose response. Leukemic cells from patients with chronic myelogenous leukemia (CML) were incubated *in vitro* with various concentrations of ara-C or cyclo-C for 1, 4 and 20 hr. The inhibition of TdR incorporation was expressed as per cent of control activity vs the concentration (Fig. 3). For cyclo-C, the ID_{50} (the concentration required for 50 per cent inhibition of TdR incorporation) was determined to be 2·2, 0·7 and 0·3 μg/ml for 1, 4, and 20 hr of incubation respectively. On the

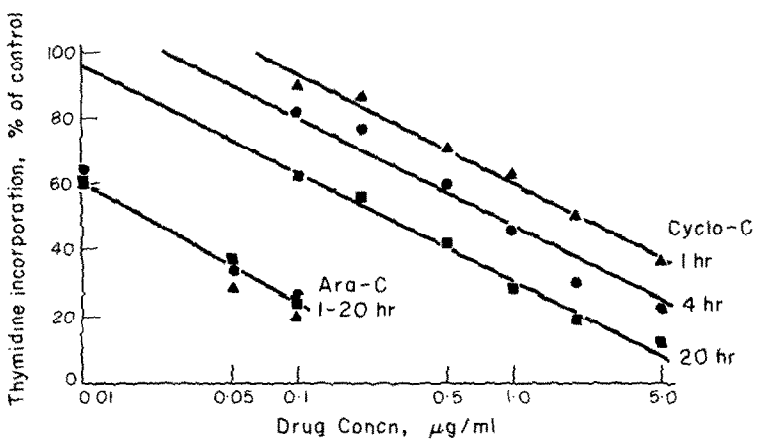

FIG. 3. Response of chronic myelogenous leukemic cells to various concentrations of cyclo-C and ara-C *in vitro*. The effect of drugs on TdR incorporation was studied at 1, 4 or 20 hr of incubation. ID_{50}, the dose required for 50 per cent inhibition on TdR incorporation, was calculated from the results. The mean cpm ± S.D. for controls were 7,571 ± 560, 23,501 ± 2,500 and 49,698 ± 1,585 at 1, 4 and 20 hr of incubation respectively.

other hand, the ID_{50} for ara-C was approximately 0·02 μg/ml regardless of time of incubation. Similar findings have been reported for L5178Y cells.[16]

Reversal of drug effects by washing cells. In order to determine whether or not the effect of cyclo-C or ara-C on the inhibition of TdR incorporation may be reversed by washing the cells to remove the drug, the following experiment was performed. CML cells or L1210 leukemic cells were preincubated for 1 or 3 hr in Eagle's MEM containing no drug, 1 μg/ml of cyclo-C or 1 μg/ml of ara-C in groups of twelve tubes each. After the preincubation, six tubes from each group were washed twice with 10 ml of sterile saline, and 2 ml of Eagle's MEM was then added to each washed tube.

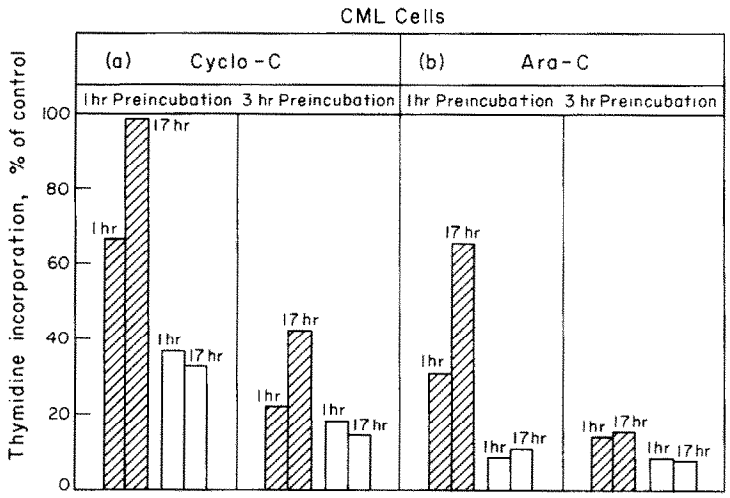

FIG. 4. Reversal of drug effects in chronic myelogenous leukemic cells by washing *in vitro*. Cells were washed with saline after 1-hr or 3-hr preincubation with 1 μg/ml of cyclo-C (a) or ara-C (b) in Eagle's MEM. The effect on TdR incorporation was compared with unwashed samples at 1 or 17 hr of reincubation. The symbols of striped and clear bars represent washed and unwashed samples. At 1- and 17-hr reincubation, the mean cpm ± S. D. for 1-hr preincubation controls were 4,315 ± 302 and 16,953 ± 2,005 (washed samples), and 5,343 ± 362 and 24,172 ± 2,198 (unwashed samples). For 3-hr preincubation controls they were 5,027 ± 319 and 15,136 ± 178 (washed samples), and 2,336 ± 84 and 9,666 ± 518 (unwashed samples).

All of the samples (washed and unwashed) were reincubated in Eagle's MEM for 1 or 17 hr with 4 μCi of ^{3}H-TdR. A typical example for CML cells is shown in Fig. 4a and 4b, and for L1210 leukemic cells in Fig. 5a and 5b (cells are preincubated for 1 and 3 hr with (a) for cyclo-C and (b) for ara-C). After incubating CML cells with cyclo-C or ara-C without washing, the inhibitory effect is more pronounced with ara-C. Washing cells after preincubating with drugs for 1 hr reversed the inhibitory effect on TdR incorporation. In fact, after 17 hr of reincubation, the effect of cyclo-C was completely reversed by washing (Fig. 4a). With a longer preincubation (3 hr), however, washing only partially reversed the effect of cyclo-C after 17 hr of reincubation, and had no effect after 1 hr of reincubation. Washing of the cells would remove more nucleosides than nucleotides because of the relative impermeability of cell membranes to nucleotides. Since cyclo-C is hydrolyzed to ara-C before phosphorylation,[16,18,19] one might expect that the rate of ara-C nucleotide formation

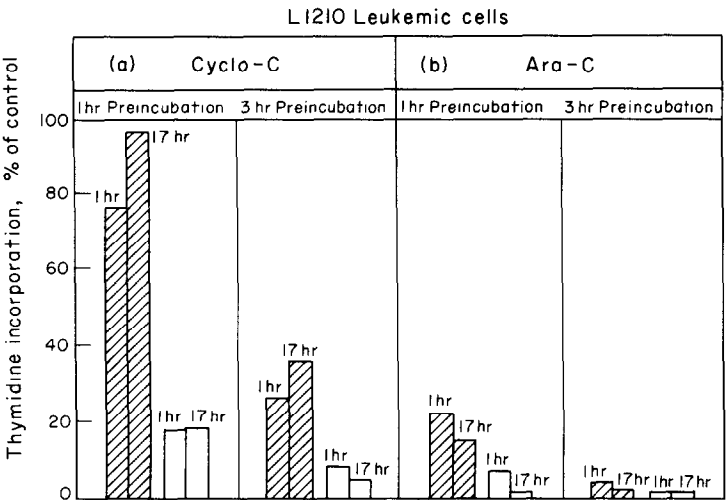

FIG. 5. Reversal of drug effects in L1210 leukemic cells by washing *in vitro*. Cells were preincubated at 37° for 1hr and 3 hr with 1 μg/ml of cyclo-C (a) or ara-C (b) washed with saline twice, and then reincubated 1 and 17 hr with Eagle's MEM containing ^{3}H-TdR. The effect on TdR incorporation was compared with unwashed samples. The symbols of striped and clear bars represent washed and unwashed samples. At 1- and 17-hr reincubation, the mean cpm ± S. D. for 1-hr preincubation controls were 16,994 ± 693 and 103,000 ± 9,230 (washed samples), and 21,523 ± 1,449 and 114,928 ± 4,458 (unwashed samples). For 3-hr preincubation controls, they were 9,023 ± 934 and 20,823 ± 1,087 (washed samples) and 7,542 ± 517 and 31,681 ± 2,745 (unwashed samples).

arising from hydrolyzed cyclo-C would be slower than from ara-C directly. This hypothesis is supported by the data of Fig. 4a, showing that the cyclo-C effect is more reversible than that of ara-C. Similar results were obtained by washing L1210 leukemic cells after preincubation with cyclo-C or ara-C (Fig. 5a and 5b). After 1 hr of preincubation with ara-C, washing of cells resulted in a greater reversal of inhibition of TdR incorporation in CML cells than in L1210 cells (Figs. 5b and 4b). If cyclo-C is hydrolyzed to ara-C for its effect,[16,18,19] then ara-C deaminase and kinase activities are also the determinants of the effect. The L1210 leukemic cells have a high kinase but no deaminase activity, whereas CML cells have a moderate kinase but relatively high deaminase activities.[15,19,20] This would explain why the drugs are more effective in L1210 leukemic cells than CML cells *in vitro*.

In water, cyclo-C showed almost complete hydrolysis to ara-C when it was applied on paper and dried with hot air. However, this could be prevented by drying with cool air. In plasma or MEM, hydrolysis of cyclo-C took place even after this precaution was taken. Experiments were then conducted to devise some means of stabilizing the drug for routine laboratory uses. Since the biochemical studies were performed at 37°, the drug was incubated at 37° with 0·1 M Tris buffer at pH 7·0 to 9·0. As shown in Table 1, experiment A, 40 and 92 per cent of the drug were hydrolyzed to ara-C in 0·1 M Tris buffer, pH 8·0 and 9·0 respectively, after 60 min of incubation. However, incubating cyclo-C at 37° up to 4 hr in 0·1 M Tris buffer at pH 7·0 did not hydrolyze the drug. Cyclo-C is stable in H_2O or 0·1 M Tris buffer, pH 7·0, with or without incubation. The 10 per cent of ara-C must be the impurity originally present in the cyclo-C. When cyclo-C was incubated at 37° for 60 min with Eagle's MEM or human plasma, ara-C (> 90 per cent) was the only product formed. As shown in Table 1, experiment

TABLE 1. STABILITY OF CYCLO-C*

Expt.	Incubation medium for cyclo-C	Duration of incubation at 37° (min)	Adjustment of pH of incubation mixtures†	% Ara-C
A	H_2O	0	−	10
	H_2O	60	−	10
	H_2O	60	+	10
	Tris buffer pH 7·0, 0·1 M	0	−	10
	Tris buffer pH 7·0, 0·1 M	60, 120, 200 or 240	−	10
	Tris buffer pH 8·0, 0·1 M	60	−	40
	Tris buffer pH 9·0, 0·1 M	60	−	92
B	Eagle's MEM	0	+	10
	Eagle's MEM	60	+	42
	Eagle's MEM	60	−	92
	Human plasma	0	+	10
	Human plasma	60	+	64
	Human plasma	60	−	93
	Supernatant of boiled human plasma	60	+	66

* Concentration of cyclo-C was 1×10^{-4} M (0·1 μCi of ^{14}C-cyclo-C). Human plasma was boiled for 5 min in a boiling water bath, and the supernatant was used after centrifugation. The pH of H_2O, MEM and human plasma was 6·0, 7·2 and 7·3 respectively. The cpm in controls were 10,474 ± 419.

† After the incubation, the pH of the reaction mixture was (+) or was not (−) adjusted to 7·0 with Tris buffer (final concentration 0·1 M).

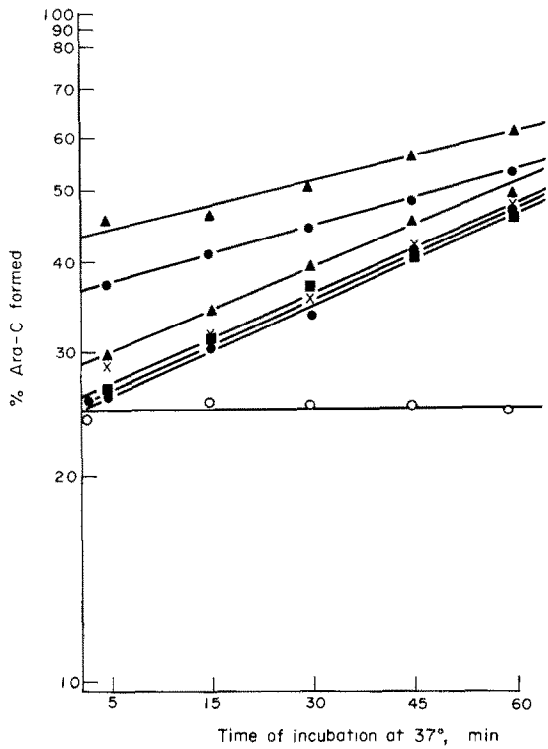

FIG. 6. Rate of cyclo-C hydrolysis at 37° in Eagle's MEM and plasma from various species. The cyclo-C concentration was 1×10^{-4} M (0·1 μCi of ^{14}C-cyclo-C). The mean cpm ± S. D. for controls were 20,249 ± 709. The symbols represent the following: (▲) two different dog plasma samples; (●) two normal human plasma samples; (■) mouse plasma samples; (×) Eagle's MEM; and (○) controls with 0·1 M Tris buffer at pH 7·0.

B, after cyclo-C incubation with various medium, adjusting the pH of the incubation mixture to 7·0 with Tris buffer (final concentration 0·1 M) produces a different cyclo-C hydrolysis rate from unadjusted samples. With the pH adjusted, the hydrolysis rate is decreased to 42 and 64 per cent in MEM and human plasma respectively. Incubating cyclo-C with the supernatant of boiled human plasma (5 min in a boiling water bath) also shows 66 per cent of cyclo-C being hydrolyzed to ara-C (Table 1). Experimentally, the large amount of protein precipitated due to heat coagulation precluded

TABLE 2. PRESENCE OF ARA-C IN DOG PLASMA AND DOG AND MOUSE URINE AFTER ADMINISTRATION OF CYCLO-C

Sample*	Cyclo-C	Ara-C
Dog plasma	14 μg/ml	9 μg ml
Dog urine, cumulative excretion	13%	8%
Mouse urine, cumulative excretion	33%	5%

* Cyclo-C was given to dogs, 20 mg/kg (i.v.), and mice, 113 mg/kg (i.p.). Dog plasma and urine samples were taken 1 hr after the drug, and the mouse urine sample was taken after 2 hr. The mean cpm ± S. D. were 492 ± 20 for dog plasma, 6,634 ± 158 for dog urine and 38,560 ± 3,607 for mouse urine.

the quantitative comparison of hydrolysis before and after boiling. However, the hydrolysis rate remained approximately the same after boiling, suggesting that the conversion of cyclo-C to ara-C is probably mediated by chemical reaction.

The rate of cyclo-C hydrolysis to ara-C was studied in plasma samples from dog, man and mouse for various time periods up to 60 min at 37°. The results are shown in Fig. 6. Controls in 0·1 M Tris buffer, pH 7·0, are included.

After injecting cyclo-C into dogs, i.v., or mice, i.p., ara-C was found in dog plasma and urine samples (1 hr) and in mouse urine samples (2 hr) (Table 2).

Studies in our laboratory found that cyclo-C is not phosphorylated.[19] The above findings suggest that cyclo-C releases ara-C, and may serve as a reservoir.

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