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ISOLATION AND ANTILEUKEMIC ACTIVITY, IN MICE, OF 1-β-D-ARABINOFURANOSYLCYTOSINE 5′-PHOSPHATE PRESENT IN SYNTHETIC 5′-CYTIDYLIC ACID

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Abstract—Cytidine 5′-monophosphate (CMP), prepared by phosphorylation of 2′,3′-O-benzylidenecytidine with polyphosphoric acid, contained about 11 per cent of a by-product which produced an increase in the survival time of mice inoculated with leukemia L1210. This antileukemic effect resembled that of arabinosylcytosine (ara-C), since it could be reversed by deoxycytidine and was absent in a subline of L1210 rendered resistant to ara-C. The by-product was separated from CMP by anion-exchange chromatography in the presence of borate, and identified as arabinosylcytosine 5′-monophosphate (ara-CMP) by the isolation of ara-C after treatment with 5′-nucleotidase. The rate of dephosphorylation of ara-CMP was 13 per cent of that of CMP.

DURING an investigation of the antileukemic activity in mice of 1-β-D-arabinofuranosylcytosine (ara-C), and of its reversal by deoxycytidine (CdR) and related metabolites, it was observed that some commercial samples of cytidine 5′-monophosphate (CMP) exhibited antitumor activity similar to that of ara-C. Further investigation of this unexpected finding disclosed that only CMP prepared by the Michelson procedure[1] (phosphorylation of 2′,3′-O-benzylidene- or isopropylidenecytidine with polyphosphoric acid[2] and subsequent hydrolysis) possessed antileukemic activity, whereas CMP obtained by isolation from RNA did not. The biological activity of the synthetic CMP, as well as its behavior during preliminary chromatographic experiments, suggested that it contained, as a minor constituent, an epimeric substance, such as 1-β-D-arabinofuranosylcytosine 5′-monophosphate (ara-CMP) or the corresponding xylosyl derivative. The former alternative appeared more probable in view of the finding of Walwick *et al.*[3] that phosphorylation of cytidine (CR) with polyphosphoric acid yielded, in addition to the 2′,5′-diphosphate and 3′,5′-diphosphate of the original nucleoside, the 3′,5′-diphosphate of ara-C. The hypothesis was confirmed by the isolation and characterization of ara-CMP from samples of synthetic CMP. The present report details these findings.

MATERIALS AND METHODS

Unless specified otherwise, the CMP used in the present study was prepared, according to the Michelson procedure,[1] by phosphorylation of 2′,3′-O-benzylidenecytidine and subsequent hydrolysis, and was purchased from Calbiochem (lot 30452).

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CMP prepared by isolation from RNA was obtained from Schwarz Bioresearch, Inc. and from P-L Biochemicals. Arabinosylcytosine was supplied as the hydrochloride by Riker Laboratories and obtained through Dr. Robert R. Engle of the Cancer Chemotherapy National Service Center. Cytidine, deoxycytidine (as the hydrochloride), deoxycytidine 5′-monophosphate (dCMP), and cytidine 3′(2′)-monophosphate were obtained from Calbiochem.

Dowex 1-X8 (200–400 mesh) was obtained in the chloride form and washed in a large column with N NaOH, with water, with 2 N HCl, and with water until the effluent was neutral. It was converted to the bicarbonate form by passing 2 M NH_4HCO_3 (adjusted to pH 9 with concentrated NH_4OH) until the effluent was chloride-free, washed with freshly redistilled water and with 0·01 M NH_4HCO_3 (pH 9), and suspended in this solution. The borate form[4] was prepared analogously by treatment with 0·8 M potassium tetraborate; the formate form was prepared with 3 M sodium formate or purchased from Bio-Rad Laboratories. Dowex 50W-X8 (H^+) (200–400 mesh) was also obtained from Bio-Rad.

Chromaflex columns (Kontes Glass Co.) and a Gilson Medical Electronics model VL volumetric fractionator with model UV-280IF recording absorption meter were used for column chromatography. Fractions were evaporated *in vacuo* (aspirator pump) by means of a Büchi rotary evaporator. Descending paper chromatography on Whatman No. 1 filter paper was carried out with Reichard's solvent[5] (12 ml 5 M ammonium acetate pH 9·5; 48 ml saturated $Na_2B_4O_7$; 0·3 ml 0·5 M EDTA.Na_3; and 132 ml ethanol) or with a modification of this mixture (methanol instead of ethanol).

5′-Nucleotidase, prepared from bull seminal plasma by treatment with protamine sulfate and heating at 60° for 20 min,[6] was kindly donated by Dr. Leon A. Heppel. Inorganic phosphate (P_i) was determined according to Lowry and Lopez.[7] Ultraviolet and visible absorbances were determined with a Zeiss model PMQ-2 spectrophotometer.

Hybrid male CDBA [(BALB/cAn × DBA/2J)F_1] or BDF_1 [(C57BL/6 × DBA/2)F_1] mice, 10–12 weeks old and weighing 22–28 g, were maintained on Purina laboratory chow pellets and water *ad libitum*. They were inoculated subcutaneously, in the right inguinal region, with the lymphoid leukemia L1210, as described previously.[8] Each experimental group consisted of eight animals. The nucleosides and nucleotides were dissolved in isotonic saline or in 2% $NaHCO_3$ and injected s.c. into the axillary region (0·01 ml/g body weight). Treatment was usually begun on day 4 or 5 after tumor inoculation and continued daily until the death of the animals. A subline of L1210 resistant to ara-C (L1210/ara-C) was obtained within one transfer generation by treating mice carrying the parent line daily from day 4 with 40 mg ara-C/kg. Median survival time (M.S.T.) of the treated animals was 49·5 days (three out of eight tumor-free survivors on day 61), and that of the untreated controls 8 days. On day 28, an animal was selected which exhibited a palpable local tumor,* and transfer was carried out by s.c. inoculation of a saline suspension of the spleen, which was considerably infiltrated with leukemic cells. The tumor, after this transfer, was fully resistant to daily treatment (from day 2) with 40 mg ara-C/kg (M.S.T. of both treated and control mice, 9·5 days). It was maintained by serial weekly transfer in mice that were treated daily from day 1 with the same dose of ara-C.

* The majority of the treated animals exhibited no local tumor after day 20.

RESULTS

It has been reported that the antineoplastic effect of ara-C could be reversed by CdR both *in vitro*[9] and *in vivo*.[10] In order to investigate the effect of CdR during more prolonged treatment with ara-C than in previous studies, and to find out whether CR or CMP had a similar effect, mice bearing leukemia L1210 were treated daily with 20 mg ara-C/kg alone or in combination with 80 mg of the metabolites/kg until day 58 or until the mice had died (Table 1). As a control, animals were also treated

TABLE 1. EFFECT OF CYTOSINE DERIVATIVES AGAINST MOUSE LEUKEMIA L1210

Compound A	Dose (mg/kg)	Compound B	Dose (mg/kg)	Median survival time (days)
				8·0
		ara-C	20	>58*
CdR	80	ara-C	20	12·0
CR	80	ara-C	20	>58
CMP	80	ara-C	20	>58
CdR	80			8·5
CR	80			8·5
CMP	80			17·0

Treatment was initiated on day 4 after tumor inoculation and continued daily until the death of the animals. Arabinosylcytosine hydrochloride (ara-C), deoxycytidine hydrochloride (CdR), cytidine (CR), and cytidine 5′-monophosphate (CMP) (by phosphorylation; Calbiochem lot 30452) were dissolved in isotonic saline and administered s.c. Combination treatments were given by injecting solutions containing both compounds. Each group consisted of 8 CDBA mice.

* The experiment was discontinued on day 58, at which time 7 of the 8 animals were still alive.

with each of the metabolites alone. The experiment showed that the antileukemic effect of ara-C was partially reversed by CdR but not by CR or CMP.

The unexpected observation was made in this experiment (Table 1) that CMP, by itself, produced a significant increase in the survival time of the leukemic mice. In an attempt to confirm this finding, CMP from a different source was administered to leukemic mice at daily dose levels as high as 320 mg/kg. This time, the compound was without any effect. The CMP that was used in the first experiment had been prepared by phosphorylation of 2′,3′-O-benzylidenecytidine and subsequent hydrolysis[1] (Calbiochem, lot 30452), while CMP isolated from RNA (Schwarz Bioresearch) was used in the second experiment. A direct comparison of the two samples confirmed the antileukemic activity of the synthetic CMP (M.S.T. 22 days with a daily dose of 80 mg/kg) and the lack of activity of the material prepared by isolation (M.S.T. of both treated and untreated mice, 9 days).

There seemed to be two possible explanations for the different behavior of the two samples of CMP, namely, (1) that CMP possessed an intrinsic antileukemic effect, which was abolished by some impurity present in the material isolated from RNA; (2) that the CMP prepared by phosphorylation contained a by-product with antileukemic activity. The first alternative appeared unlikely because CR had no antitumor effect (Table 1) and because it would be expected that, in order to enter leukemic cells,

CMP would first have to be dephosphorylated. The second alternative was shown to be correct by chemical studies and by additional biological experiments.

No impurities, such as dCMP, could be detected by ion-exchange chromatography on Dowex-50 (H^+)[5] and elution with acetic and hydrochloric acid in either the synthetic CMP or the CMP isolated from RNA. However, the synthetic CMP (Calbiochem

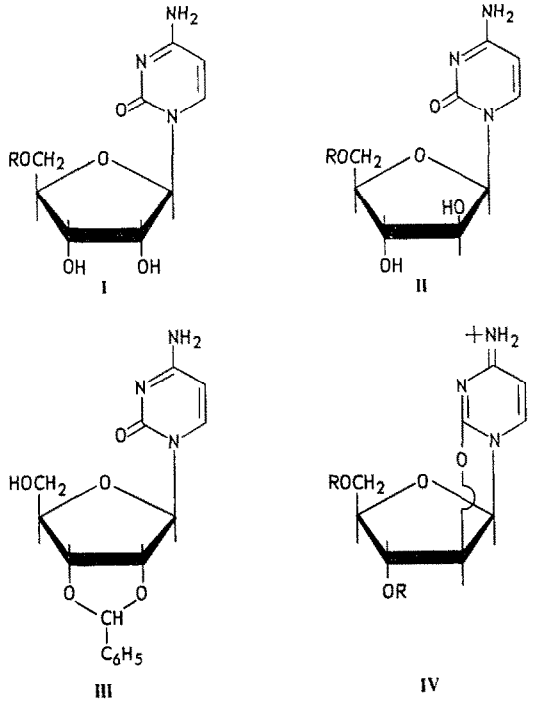

FIG. 1. Structures of cytosine derivatives. I (R = H), cytidine (CR); I (R = PO_3H_2), cytidine 5′-monophosphate (CMP); II (R = H), 1-β-D-arabinofuranosylcytosine (ara-C); II (R = PO_3H_2), 1-β-D-arabinofuranosylcytosine 5′-monophosphate (ara-CMP); III, 2′,3′-O-benzylidenecytidine; IV (R = H), O^2,2′-cyclocytidine; IV (R = PO_3H_2), O^2,2′-cyclocytidine 3′,5′-diphosphate.

lot 30452 or 540671), when subjected to paper chromatography with Reichard's borate system,[5] was resolved into two components. The major spot (R_f 0·04) corresponded to the single spot detected with CMP isolated from RNA. The minor component (R_f 0·14) had a mobility slightly lower than dCMP (R_f 0·17). This suggested that the impurity might be an epimer of CMP, in which the 2′- and 3′-hydroxyl groups were *trans* and, therefore, unable to form a borate complex. Paper chromatography of the synthetic CMP was repeated in both Reichard's system and a modified mixture (methanol instead of ethanol), in which nucleotides had increased mobilities. Elution of the spots with 0·1 N HCl and determination of the ultraviolet absorbance (after subtraction of a paper blank) showed that the impurity had the same absorption spectrum as pure CMP (λ_{max} 280 mμ) and that it constituted 10·7% of the total amount (in Calbiochem lot 30452).

Of the possible *trans*-isomers of CMP, either 1-β-D-arabinofuranosylcytosine 5′-monophosphate (ara-CMP) (Fig. 1) or the corresponding xylosyl derivative could

have been formed by inversion at the 2′- or 3′-position, respectively, during the phosphorylation of 2′,3′-O-benzylidenecytidine and subsequent removal of the benzylidene group. Formation of the arabinosyl derivative seemed more probable because phosphorylation of CR with polyphosphoric acid yielded, in addition to the expected diphosphates of CR, the 3′,5′-diphosphate of ara-C via the 3′,5′-diphosphate of O^2,2′-cyclocytidine[3] (Fig. 1).

TABLE 2. COMPARATIVE EFFECTS OF ARABINOSYLCYTOSINE AND SYNTHETIC CYTIDYLATE AGAINST SENSITIVE AND RESISTANT LEUKEMIA L1210

Tumor	Compound	Dose (mg/kg)	Median survival time (days)
L1210	None		8·0
	ara-C	20	25·0
	ara-C	10	24·0
	ara-C	5	24·0
	ara-C	2·5	13·0
	CMP	240	23·5
	CMP	120	24·0
	CMP	60	12·0
	CMP	30	10·0
L1210/ara-C	None		9·0
	ara-C	60	9·0
	ara-C	40	9·0
	ara-C	20	9·0
	CMP	320	9·0
	CMP	160	9·0
	CMP	80	9·0

L1210/ara-C was the subline of leukemia L1210 made resistant to arabinosylcytosine hydrochloride (ara-C) (see Materials and Methods). Treatment was initiated on day 5 after tumor inoculation and continued daily until the death of the animals. Ara-C was dissolved in isotonic saline, and cytidine 5′-monophosphate (CMP) (by phosphorylation, Calbiochem lot 30452) in 2% $NaHCO_3$, and administered s.c. The CMP contained 10·7% ara-CMP. L1210 was inoculated into BDF_1, and L1210/ara-C into CDBA mice.

The hypothesis that the antileukemic activity of the synthetic CMP was caused by the presence of ara-CMP was supported by further biological studies. Table 2 illustrates an experiment which was based on the assumption that the antileukemic effect of ara-CMP present to the extent of about 10 per cent in CMP would duplicate the effect of an equimolecular dose of ara-C. Indeed, the effect of such corresponding doses on the survival time of mice bearing leukemia L1210 was about the same. On the other hand, the subline of L1210 resistant to ara-C was fully cross-resistant to the synthetic CMP. Table 3 shows that the antileukemic activity of the synthetic CMP was reversed by the simultaneous administration of CdR in the same manner as the activity of ara-C.

Isolation of ara-CMP

The by-product present in synthetic CMP was conclusively identified as ara-CMP after its isolation by chromatography on Dowex-1 (bicarbonate) and elution with a mixture of aqueous NH_4HCO_3 (adjusted to pH 9 with NH_4OH) and H_3BO_3. A

resin column (20 × 340 mm) was equilibrated with 0·01 M NH_4HCO_3–0·01 M H_3BO_3 and treated with 1·751 g (5 m-moles) CMP · 1·5 H_2O (Calbiochem lot 30452) in 50 ml 0·2 M H_3BO_3 and 1 ml concentrated NH_4OH. Stepwise elution with 0·01 M (200 ml), 0·05 M (1500 ml), and 0·075 M (2500–3000 ml) NH_4HCO_3 containing 0·01 M H_3BO_3 removed ara-CMP at the top concentration in a peak volume of about

TABLE 3. EFFECT OF SYNTHETIC CYTIDYLATE AND DEOXYCYTIDINE AGAINST MOUSE LEUKEMIA L1210

Compound	Dose (mg/kg)	Median survival time (days)
None		9·0
CMP	160	26·5
CMP	80	21·5
CMP + CdR	80 + 80	10·0

Cytidine 5′-monophosphate (CMP) (prepared by phosphorylation; Calbiochem lot 30452) and deoxycytidine hydrochloride (CdR) were dissolved in 2% $NaHCO_3$ and injected s.c. Combination treatment was administered by injecting a solution containing both CMP and CdR. Daily treatment was initiated on day 5 after tumor inoculation. Each group consisted of 8 CDBA mice.

1700 ml. The amount of ara-CMP, as determined by the absorbance at 280 mμ (pH 2), varied from 0·53 to 0·57m-mole (10·6–11·4%) in several runs. Paper chromatography (see Materials and Methods) demonstrated the absence of CMP. The latter was eluted by increasing the NH_4HCO_3 concentration to 0·09 M or by omitting H_3BO_3. The eluate containing ara-CMP was evaporated to dryness *in vacuo* below 40°, and the residue freed from ammonium borate by heating it three times with 30 ml methanol and re-evaporating each time.[4] Ara-CMP was obtained as colorless needles by dissolving the residue in 5 ml warm H_2O, acidifying with 1 ml N formic acid, adding 12 ml ethanol and chilling. The yield of air-dried trihydrate was 170–185 mg (9·0–9·8% from synthetic CMP); m.p. 135° (foaming), unchanged after recrystallization from water:ethanol (1:2). *Anal.* Calcd. for $C_9H_{14}N_3O_8P \cdot 3H_2O$: H_2O, 14·33. Found: H_2O, 14·17, 14·52.

The anhydrous compound was obtained by drying at 110°/0·05 mm; darkening at 178°, decomposition at 183–187° before and after recrystallization; $[\alpha]^{25}_{5,890}$ + 112·7°, $[\alpha]^{25}_{5,000}$ + 179·8°, $[\alpha]^{25}_{4,360}$ + 283·9° (*c* 1·2, H_2O). Ultraviolet spectra: in 0·01 N HCl λ_{max} 280 mμ (ε 13,040), 213 mμ (ε 10,140), λ_{min} 241 mμ (ε 1,484); in 0·01 N NaOH λ_{max} 272·5 mμ (ε 9,240), λ_{min} 249 mμ (ε 5,550). The spectra were not significantly different from those of CMP. *Anal.* Calcd. for $C_9H_{14}N_3O_8P$: C, 33·45; H, 4·37; N, 13·00; P, 9·58. Found: C, 33·48; H, 4·31; N, 13·17; P, 9·55.

Cardeilhac and Cohen[11] have prepared ara-CMP from ara-C by both chemical and enzymatic phosphorylation but did not report physical constants of the pure compound.

Ara-CMP could also be separated from CMP by chromatography on Dowex-1 (formate) and elution with 0·15 M ammonium formate (pH 9) containing 0·01 M H_3BO_3, or by chromatography on Dowex-1 (borate)[4] and elution with 0·075 M

ammonium tetraborate (pH 8·9). However, removal of salts from the eluates proved to be less practical.

Dephosphorylation of ara-CMP

Figure 2 shows that the rate of dephosphorylation of ara-CMP by the 5′-nucleotidase of bull seminal plasma[6] was about 13 per cent of the rate of hydrolysis of CMP

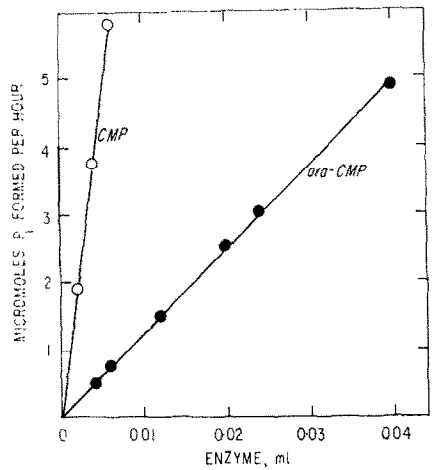

FIG. 2. Rates of hydrolysis of cytidine 5′-monophosphate (CMP) (○——○) and of 1-β-D-arabinofuranosylcytosine 5′-monophosphate (ara-CMP) (●——●) by 5′-nucleotidase prepared[6] from bull seminal plasma. Mixtures containing 3 μmoles nucleotide, 100 μmoles Tris buffer (pH 8·4), 10 μmoles $MgCl_2$, and the appropriately diluted enzyme in a total volume of 1 ml were incubated at 37° for 15 min. The reaction was stopped with 1 ml 10% TCA, and inorganic phosphate (P_i) determined according to Lowry and Lopez.[7]

The same enzyme, even at high concentrations, was without any effect on cytidine 3′(2′)-monophosphate. The specificity of the enzyme for 5′-phosphate esters proves that ara-CMP is indeed a 5′-phosphate. The K_m for ara-CMP, as determined from Fig. 3, was 9×10^{-4}, as compared to the K_m of 2×10^{-4} for CMP reported by Heppel and Hilmoe.[6]

The structure of ara-CMP was proved by its conversion to ara-C as follows. An aqueous solution of 97 mg (300 μmoles) of ara-CMP was adjusted to pH 8·5 with NH_4OH and incubated with 3 ml 0·1 M $MgCl_2$, 3 ml 1 M NH_4HCO_3 (pH 8·5), and 1·5 ml 5′-nucleotidase in a total volume of 30 ml at 37° for 2·5 hr. The mixture was heated at 100° for 5 min, filtered from $MgNH_4PO_4$, evaporated *in vacuo*, dissolved in 5 ml water, and applied to a column (12 × 80 mm) of Dowex 50W-X8 (H^+). The column was washed with water (50 ml), 0·1 N HCl (100 ml), and water (50 ml); then ara-C was eluted with 200 ml of 1 N NH_4OH. The eluate was evaporated *in vacuo*, and the residue was dissolved in 10 ml of 0·01 M NH_4HCO_3 (pH 9) and applied to Dowex 1-X8 (bicarbonate) (12 × 160 mm). Ara-C was eluted with 140 ml of 0·01 M NH_4HCO_3 (pH 9) in 98 per cent yield, as determined by u.v. absorbance. After evaporation *in vacuo*, ara-C was crystallized from ethanol–ether; yield 61 mg (84%), m.p. 212·5–213·5° (dec.). Recrystallization from ethanol afforded small,

colorless hexagonal prisms, m.p. 214–215° (dec.) (reported m.p. 212–213·5°,[3] 212–213°[12]); $[\alpha]_{5,890}^{25}$ + 152·9°, $[\alpha]_{5,000}^{25}$ + 241·9°, $[\alpha]_{4,360}^{25}$ + 376·5° (*c* 1·02, H_2O) (reported $[\alpha]_D$ in H_2O: + 158°,[3] + 153°[12]). The mixed m.p. with an authentic sample, m.p. 214–215° (dec.) (prepared from the hydrochloride by chromatography on Dowex-1 (bicarbonate) and crystallization from ethanol), showed no depression; and both samples gave identical i.r. spectra and the same R_f values on paper chromatography.

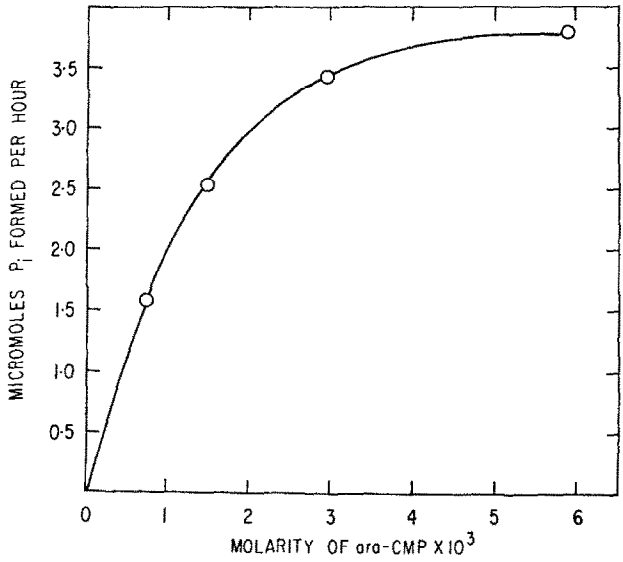

FIG. 3. Rate of hydrolysis of 1-β-D-arabinofuranosylcytosine 5′-monophosphate (ara-CMP) by bull semen 5′-nucleotidase as a function of substrate molarity. The assay was performed as in Fig. 2 with 0·025 ml enzyme and varying concentrations of ara-CMP.

Table 4 shows the R_f values of ara-CMP, ara-C, and related compounds.

Ara-C could be separated from CR by chromatography on Dowex 1-X8 (borate)[4] and elution with water; CR, which forms a borate complex, required elution with at least 0·02 M ammonium tetraborate.

TABLE 4. R_f VALUES OF CYTOSINE NUCLEOSIDES AND NUCLEOTIDES

Compound	R_f	
	A	B
CMP	0·04	0·27
ara-CMP	0·14	0·39
dCMP	0·17	0 45
CR	0 22	0·57
ara-C	0·62	0·68
CdR	0·67	0·72

Descending paper chromatography. Solvent A: Reichard's mixture[5] (see Materials and Methods); Solvent B: same as A, except methanol instead of ethanol. As noted by Reichard,[5] R_u values varied in different runs.

DISCUSSION

The formation of ara-CMP as a by-product during the phosphorylation of 2′,3′-O-benzylidenecytidine (Fig. 1) and subsequent acid hydrolysis is formally analogous to the formation of the 3′,5′-diphosphate of ara-C during the phosphorylation of CR, reported by Walwick *et al.*[3] However, these authors have shown that O^2,2′-cyclocytidine 3′,5′-diphosphate was an intermediate in the latter case. Formation of such a cyclocytidine derivative cannot occur with the intact 2′,3′-O-benzylidene compound, while complete removal of the benzylidene group during phosphorylation would lead to the 3′,5′-diphosphate rather than the 5′-monophosphate. Formation of an intermediary 3′,5′-cyclic phosphate is also excluded because it would yield mainly the 3′- rather than the 5′-phosphate on acid hydrolysis.[13] It therefore seems likely that the reaction proceeds by some concerted mechanism in which an O^2,2′-cyclic intermediate is formed, with the benzaldehyde residue still attached to the 3′-position.

It may be noted that Chambers *et al.*[14] have reported no evidence for the formation of ara-C derivatives in their detailed study of the products obtained by the action of polyphosphoric acid on either free cytidine or its 2′,3′-O-isopropylidene derivative. Most of their chromatographic procedures would not have produced separation of ribosyl and arabinosyl derivatives, except electrophoresis in borate, and in this case ara-CMP could have been present in the 2′- and 3′-phosphate fraction. The present finding illustrates the difficulty of proving the absence of unknown impurities in a compound: CMP and ara-CMP differ physically only by their optical rotation and their mobility in systems containing borate; most standard tests of ultraviolet absorption, elemental analysis, and chromatographic behavior would not differentiate between them. The undetected presence of the biologically active ara-CMP in a supposedly homogeneous sample of CMP, which in turn serves as the starting material for the synthesis of cytidine di- and triphosphate, could certainly put in question results of studies in molecular biology in which the di- or triphosphate is used.

Chu and Fischer[15] have shown that ara-C is rapidly phosphorylated after its uptake by leukemic cells. However, since intact nucleotides generally do not enter cells, it is likely that ara-CMP enters the cells as the nucleoside, which then is rephosphorylated. Such a mechanism would be consistent with the very similar antileukemic effectiveness of ara-CMP and ara-C. Studies are in progress on the biological effects of purified ara-CMP and further clarification of the mechanism by which ara-CMP is formed from 2′,3′-O-benzylidenecytidine.

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