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PHOSPHORYLATION OF SELECTED SUBSTRATES BY SEMIPURIFIED CYTOPLASMIC KINASES OF THREE P815 MURINE LEUKEMIAS EXHIBITING COLLATERAL SENSITIVITY (P815/TG), SENSITIVITY (P815) OR RESISTANCE (P815/ARA-C) TO 1-β-D-ARABINOFURANOSYLCYTOSINE

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Abstract—Partial separation and purification of the kinases phosphorylating 2′-deoxycytidine (EC 2.7.1.74) (D-K), pyrimidine nucleoside-5′-monophosphate (EC 2.7.4.14) (PM-K) and nucleoside-5′-diphosphate (EC 2.7.4.6) (NDP-K) were achieved by high performance liquid chromatography on Micro Pak TSK-gel 3000 SW columns. Using the standard conditions for all three kinases investigated, the following observations were made: a comparison of the D-K activities using deoxycytidine (dCyd) or 1-β-D-arabinofuranosylcytosine (ara-C) as substrate in peak fractions derived from homogenates of murine neoplasms P815, either sensitive (P815) or resistant to ara-C (P815/ara-C) or resistant to 6-thioguanine (P815/TG), revealed comparable specific activities for dCyd and somewhat lower specific activities for ara-C in fractions derived from P815 and P815/TG cells, while substantially reduced specific activities were observed for both substrates in fractions derived from P815/ara-C cells. The 5′-monophosphate of ara-C (ara-CMP) exhibited a higher specific activity than 2′-deoxycytidine-5′-monophosphate (dCMP) in peak fractions with PM-K activity derived from all three cell lines. The 5′-diphosphate of dCyd (dCDP) was phosphorylated to comparable extents by peak fractions with NDP-K activity derived from all three cell lines. The 5′-diphosphate of ara-C (ara-CDP) is a substrate with specific activities comparable to dCDP in peak fractions with NDP-K activity derived from P815/ara-C and P815/TG cell lines, but with somewhat lower specific activities than the dCDP substrate in P815-derived fractions. The ratios of total enzyme activities recovered after injection of a crude P815 cell homogenate were: 1:260:20,000 for D-K (dCyd as substrate), PM-K (dCMP as substrate) and NDP-K (CDP as substrate); their yield was 100% for D-K and NDP-K, and 40% for PM-K activities. The purification achieved ranged from 5 to 33 times, with substantial reductions in the number of bands observed in disc electrophoresis when compared with those in the crude extracts. Experiments evaluating the inhibitory activity of ara-C and its 5′-mono-, di- and triphosphates upon the phosphorylation by these semipurified kinases of dCR and its 5′-phosphates revealed the possibility that ara-CDP and, less so, ara-CTP effectively inhibit the phosphorylation of dCDP to dCTP in the *de novo* biosynthesis of the latter and, thus, provide insufficient amounts of dCTP for DNA synthesis.

In the search for a rapid screening procedure for the kinases responsible for phosphorylation of 2′-deoxycytidine (dCyd)† and 1-β-D-arabinofuranosylcytosine (ara-C) to their respective 5′-mono-, di- and triphosphates in three P815 mouse neoplasms that are sensitive (P815) or resistant to ara-C (P815/ara-C), or resistant to 6-thioguanine (P815/TG), a recently developed resin capable of separating proteins according to their molecular weight was investigated. The present study describes (1) a high performance liquid chromatography (HPLC) technique using Micro Pak TSK-gel 3000 SW columns for simultaneous separation from crude cell homogenates of 2-deoxycytidine kinase (D-K) (EC 2.7.1.74) [as evaluated for conversion of dCyd to dCMP and of ara-C to 1-β-D-arabinofuranosylcytosine 5′-monophosphate (ara-CMP)], of pyrimidine nucleoside-5′-monophosphate kinase (PM-K) (EC 2.7.4.14) [as evaluated for conversion of dCMP to dCDP and of ara-CMP to 1-β-D-arabinofuranosylcytosine 5′-diphosphate (ara-CDP)], and of nucleoside-5′-diphosphate kinase (NDP-K) (EC 2.7.4.6) (as evaluated for conversion of CDP, dCDP and ara-CDP to their respective triphosphates) and (2) experiments evaluating the inhibitory activities of ara-C and its 5′-mono-, di- and triphosphates on the phosphorylation by these semipurified kinases of dCR and its 5′-mono- and diphosphates. Of the three presently available types of TSK SW columns packed with a

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† Abbreviations: dCyd, deoxycytidine; ara-C, 1-β-D-arabinofuranosylcytosine; ara-CMP, 1-β-D-arabinofuranosylcytosine 5′-monophosphate; ara-CDP, 1-β-D-arabinofuranosylcytosine 5′-diphosphate; ara-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; D-K, 2′-deoxycytidine kinase; PM-K, pyrimidine nucleoside-5′-monophosphate kinase; NDP-K, nucleoside-5′-diphosphate kinase; HPLC, high performance liquid chromatography; PC, paper chromatography; and TLC, thin-layer chromatography.

spherical, hydrophilic, porous gel, the 3000 SW, with a molecular weight exclusion of 150,000 and a particle size of 10 ± 2 μM, was chosen because adequate separation could be expected for proteins with molecular weights within our estimated range, according to the specifications of the distributor.* The three P815 mouse neplasms were chosen for this study because D-K had been purified and characterized from P815 and P815/ara-C previously and the line P815/TG had exhibited collateral sensitivity to ara-C [1–4]. A preliminary report was published elsewhere [5].

MATERIALS AND METHODS

Materials. dCyd, dCMP, CTP, dCDP, CDP, CMP, dCTP, ara-CMP, and 1-β-D-arabinofuranosylcytosine 5'-triphosphate (ara-CTP) were purchased from the Sigma Chemical Co. (St. Louis, MO). Ara-CDP was purchased from Terra-Marine Bioresearch (La Jolla, CA), and ara-C was supplied by the Upjohn Co. (Kalamazoo, MI). The [^{14}C]dCyd (45 mCi/mmole), [5,6-^{3}H]ara-C (18 Ci/mmole) and [5,6-^{3}H]ara-CMP (12 Ci/mmole) were purchased from Moravek Biochemicals (City of Industry, CA), and [^{14}C]dCMP (27.9 mCi/mmole) and [^{14}C]CDP (453.0 mCi/mmole) were purchased from the New England Nuclear Corp. (Boston, MA).

Radiolabeled dCDP and ara-CDP were synthesized enzymatically from their respective monophosphates. Ten microcuries of [5,6-^{3}H]ara-CMP or 1 μCi of [^{14}C]dCMP was added to 4 μmoles ATP, 4 μmoles $MgCl_2$, 0.1 mmole Tris buffer (pH 7.4) and 8.8×10^{-2} or 2.2×10^{-2} units of monophosphate kinase (Sigma). The reaction mixtures were incubated at 37° for 1 hr, the protein was precipitated with 6% trichloroacetic acid, and the aqueous phases were lyophilized and reconstituted with 100 μl of H_2O. All these reaction products and all other substrates were subjected to purification by HPLC on a Partisil-10 SAX column, 4.6 × 250 mm (Whatman, Inc., Clifton, NJ). Elution was achieved over 20 min with a linear gradient of 0.005 to 0.4 M potassium phosphate buffer (pH 3.0) at a flow rate of 2 ml/min; the absorbance was monitored at 254 nm.

Thin-layer plates (Polygram Sil G/UV 254) were manufactured by Machery-Nagel (Brinkmann Instruments, Inc., Westbury, NY). Whatman No. 3MM was used for paper chromatography (PC), and DEAE discs were cut from Whatman DE-81 cellulose paper.

The TSK G-3000 SW column (7.5 mm × 500 mm) was purchased from Varian (Palo Alto, CA). All chemicals used for chromatography were analytical grade. The HPLC apparatus used was a Micromeritics model 7000B (Norcross, GA).

Methods for enzyme preparation, separation and assays. P815, P815/ara-C, and P815/TG cells, harvested 5–6 days after inoculation with about 10^6 cells to BDF_1 mice, were homogenized in cold sucrose (final concn. 40%) with ten strokes in a Stir-R model S63 homogenizer (Tri-R-Instruments, Jamaica, NY) and centrifuged at 100,000 *g* for 1 hr as described earlier [2, 6]. Recently, Cheng *et al.* [7] described the characteristics of cytoplasmic and mitochondrial deoxycytidine kinase isozymes prevalent in blast cells of patients with acute myelocytic leukemia (AML). While gel electrophoresis of their cytoplasmic and mitochondrial preparations revealed distinctly different dCyd kinase patterns, crude, cell-free cytosol extracts from our P815 and P815/ara-C cells exhibited only one peak with dCyd kinase activity at comparable R_f positions for each cell type [4]. It is unlikely that our procedure for the cytosol preparation will rupture or "solubilize" mitochondria [7, 8]. From each homogenate, 0.1 ml of supernatant fluid that had been diluted to contain 0.68 mg protein was injected onto the TSK G-3000 column, and the proteins were eluted at room temperature with 0.035 M potassium phosphate buffer (pH 6.8) containing 0.1 mM mercaptoethanol + 20% glycerol. At a flow rate of 0.8 ml/min and a pressure of about 1100 psi, forty-seven fractions of 0.4 ml were collected over 30 min into tubes containing 0.75 mg albumin, 2.2 mM ATP, 2.2 mM $MgCl_2$ and 55 mM Tris (pH 7.8). The absorption was measured at 210 nm, and protein determinations were performed by the method of Bradford [9].

D-K was determined with the disc method of Bresnick and Karjala [10], adapted for our substrates as described earlier [6]. The standard reaction mixtures contained, in a total volume of 0.20 or 0.25 ml, 3 μmoles of NaOH-neutralized ATP, 14.15 μmoles of Tris–HCl (pH 8.0), 5 nmoles of nucleoside substrate (0.05 ml with about 15,000 cpm/assay), 3 μmoles of $MgCl_2$, and 0.05 or 0.10 ml of enzyme solution. The mixtures were incubated at 37° for 30 or 60 min. The enzyme reactions were stopped by cooling the tubes in ice. Aliquots of 0.05 or 0.10 ml of the reaction mixtures were placed on DEAE filter discs and these were washed alternatively with 1 mM ammonium formate, water and 95% ethanol before drying. The radioactivity of the discs was evaluated using Permablend (5.5 g/liter Toluene, Packard Instrument Co., Downer's Grove, IL) as the fluor.

The PM-K assay used was adapted from Orengo and Maness [11] by preincubating 0.025 or 0.050 ml of undiluted sample with 10 μl of 0.05 M dithiothreitol (Cleland's Reagent, Sigma) for 30 min at 37° and subsequent cooling in ice before the addition of 0.05 ml of a solution composed of 40 μl of 240 mM Tris–HCl (pH 7.5), 36 mM ATP-Na and 134 mM $MgCl_2$, and 10 μl of substrate, radioactive dCMP or ara-CMP (0.841 μmole/tube containing approximately 25,000 cpm). After incubation for 60 min at 37°, the reaction mixture was cooled in ice. Because of the instability of the PM-K, it was essential that the individual fractions derived from the HPLC were cooled and processed immediately upon collection. For the calculations of the PM-K activity, the values for both reaction products (5'-di- and triphosphates) were added, taking into account that PM-K and NDP-K activities overlapped in the elution profile.

The NDP-K assay was adapted for our purpose from Ingraham and Ginther [12]. The total volume of 0.08 ml incubation mixture contained 10 mM $MgCl_2$, 2 mM mercaptoethanol and 0.01 ml of 25 mM ATP in 1 M triethanolamine (pH 8.0); 0.01 ml of radioactive CDP, dCDP or ara-CDP (37 nmoles

* Information according to a pamphlet distributed by Varian Associates, Inc.

containing approximately 40,000 cpm); and 5 or 10 μl of undiluted or appropriately diluted enzyme. Before adding the enzyme, the incubation mixture was preincubated for 2 min and, after cooling and subsequent addition of the enzyme, incubated for 10 min at 37°. The tubes were then immersed in ice to stop the reaction.

After precipitation of the proteins according to Khym [13], the reaction products of the PM-K and NDP-K assays in P815 cells were subjected initially to PC on Whatman No. 3MM paper (System: *n*-propanol–tetrahydrofurfurylalcohol–0.08 M citrate buffer (pH 5.6) [20:10:10]), or to thin-layer chromatography (TLC) on silica gel (System: isobutyric acid–2 N ammonium hydroxide [66:34, v/v]) (Fig. 1). Areas from paper and thin-layer chromatograms, marked by co-migration with unlabeled CMP, CDP, CTP, dCMP, and dCTP, were cut into rectangles (2 × 3.8 cm for PC and 0.5 × 2.0 cm for TLC), and their relative radioactivities were evaluated in Permablend. For the comparison of enzyme activities in all three P815 cell lines, HPLC (Partisil-10 SAX, same conditions as previously described for substrate purification) was used exclusively (Figs. 2–4). A comparison of the efficiency of the initially used PC and TLC with the HPLC technique used for the analysis of the reaction products derived from the PM-K and NDP-K assays revealed HPLC to be superior in sensitivity, speed and specificity. Individual samples eluted from the HPLC column were collected directly into scintillation vials to which 8 ml of Hydrofluor (National Diagnostic, Sommerville, NJ) was added. All samples from PC, TLC and HPLC were counted in a Packard Tri-Carb model 3380 scintillation spectrometer (Packard Instrument Co.) with approximate counting efficiencies of 82% for ^{14}C-radioisotopes and 13% for ^{3}H-radioisotopes. Thus, for analytical evaluation of the reaction products, HPLC is preferable but not essential.

The concentrations of ara-C, ara-CMP, ara-CDP and ara-CTP necessary for 50% inhibition of the semipurified kinases that catalyze the phosphorylation of dCR, dCMP and dCDP were established under the standard conditions for the kinase assays described above. A series of concentrations of the respective inhibitors and kinase fractions with highest specific activities derived from the HPLC procedure were used.

Polyacrylamide disc electrophoresis was performed according to Ornstein [14] and Davis [15] at pH 9.5 at 20° in 0.5 × 6 cm tubes. The concentration of acrylamide (Eastman–Kodak, Rochester, NY) was 7%. The current was 3–4 mA/tube. The gels were stained with 0.25% Coomassie Brilliant Blue G (Sigma) in an aqueous solution containing 50% methanol and 10% glacial acetic acid at room temperature overnight.

For molecular weight determination by HPLC, commercial proteins (phosphorylase B, mol. wt 92,000; bovine serum albumin, mol. wt 66,200; ovalbumin, mol. wt 45,000; chymotrypsinogen, mol. wt 25,000; soybean trypsin inhibitor, mol. wt 21,500; and lysozyme, mol. wt 14,400) were run using the standard conditions for elution. The retention times were 10.5, 13.5, 16.0, 20.0, 24.0, and 30.5 min, respectively, compared with 14.0, 15.0, and 17.0 min for the D-K, NDP-K, and PM-K peak activities. A plot of the log of the molecular weight versus retention time yielded a curved line (not shown) from which the molecular weights of the three kinases were extrapolated.

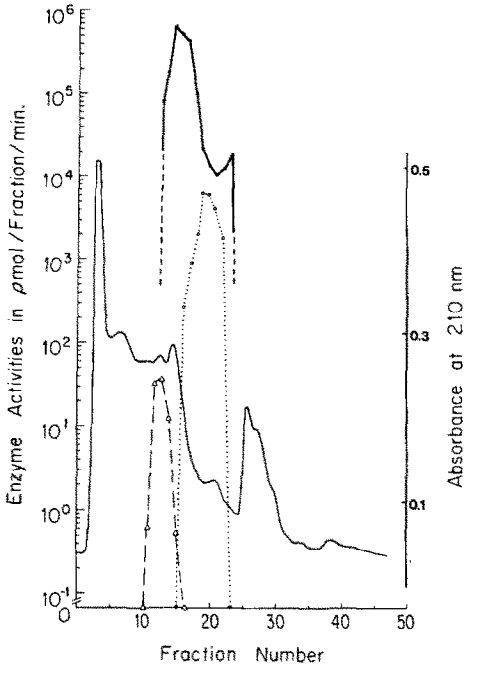

Fig. 1. Separation by HPLC of three kinases derived from cell homogenates of P815 mouse neoplasms. Key: (Δ—Δ) D-K (dCyd as substrate); (○···○) PM-K (dCMP as substrate); (●—●) NDP-K (CDP as substrate); and (———) absorbance at 210 nm. Abscissa: fraction numbers. Ordinates: left, enzyme activities in pmoles per fraction per min; right, absorbance at 210 nm. Analysis of enzymes was by PC and TLC.

RESULTS

Specific activity. The analysis of the fractions derived from P815 cell homogenates revealed that twenty-one fractions (numbers 10–30) contained the activities of all three kinases (Fig. 1). The D-K activity was eluted quantitatively within five fractions and the maximum specific activities for both substrates, dCyd and ara-C, coincided at fraction 13 in all three P815 cell lines (Fig. 2). In these peak fractions the specific activities for dCyd in the P815 and P815/TG-derived samples were substantially higher than that of P815/ara-C [sp. act. 1.05 and 1.08 vs 0.12 nmoles·min^{-1}·(mg protein)$^{-1}$ respectively], while ara-C was less susceptible to phosphorylation than dCyd with P815 and P815/TG fractions and exhibited slightly higher specific activities than dCyd for the P815/ara-C-derived peak sample [sp. act. 0.73 and 0.61 vs 0.25 nmole·min^{-1}·(mg protein)$^{-1}$]. The overall yield of D-K activity of the P815 extract (dCyd as substrate) was 105% of the injected amount.

The evaluation of the PM-K activities by HPLC in the semipurified fractions derived from all three cell lines revealed a 2–3 times higher specific activity

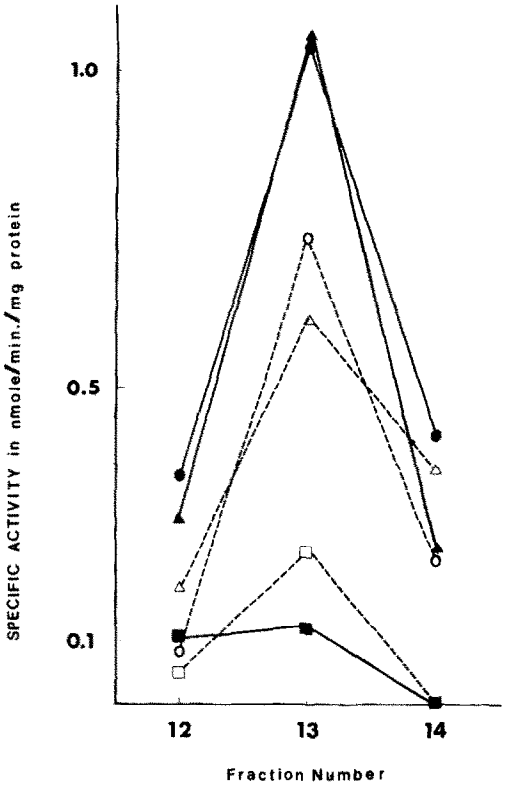

Fig. 2. Specific activities of the three peak fractions with nucleoside kinase activities following separation by HPLC. Symbols: (●—●) dCyd and (○—○) ara-C kinase activities in fractions derived from P815 cell homogenates; (■—■) dCyd and (□---□) ara-C kinase activities in fractions derived from P815/ara-C cell homogenates; and (▲—▲) dCyd and (△---△) ara-C kinase activities in fractions derived from P815/TG cell homogenates. See text for methods. Abscissa: fraction number. Ordinate: nucleoside kinase specific activity in nmoles per min per mg protein.

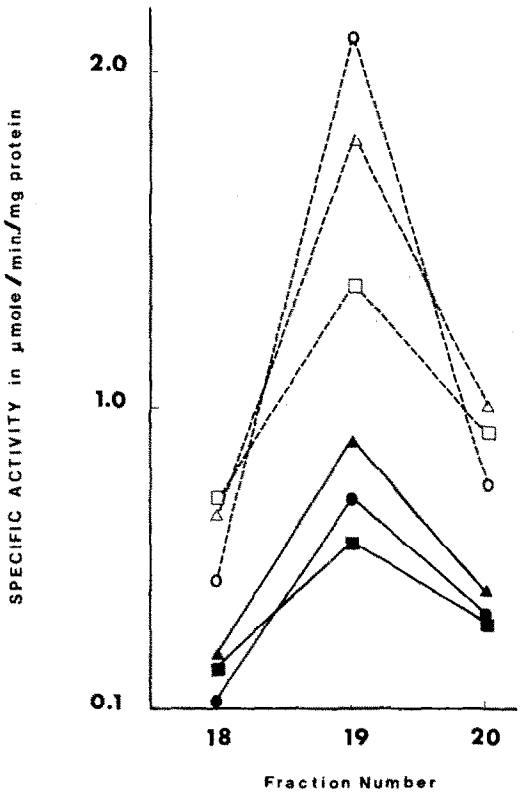

Fig. 3. Specific activities of the three peak fractions with pyrimidine-5′-monophosphate kinase activities following separation by HPLC. Abscissa: fraction number. Ordinate: pyrimidine-5′-monophosphate kinase specific activity in μmoles per min per mg protein. Symbols: (●—●) dCMP and (○---○) ara-CMP kinase activities in fractions derived from P815 cell homogenates; (■—■) dCMP and (□---□) ara-CMP kinase activities in fractions derived from P815/ara-C cell homogenates; and (▲—▲) dCMP and (△---△) ara-CMP kinase activities in fractions derived from P815/TG cell homogenates. See text for methods.

for ara-CMP than for dCMP at peak fraction 19 (Fig. 3). Furthermore, the P815 and P815/TG-derived samples with peak enzyme activity exhibited higher specific activities for ara-CMP phosphorylation than the one observed in the corresponding P815/ara-C sample [sp. act. 2.14 and 1.80 vs 1.38 μmoles·min^{-1}·(mg protein)$^{-1}$]. A similar pattern was observed for the phosphorylation of dCMP with specific activities of 0.74, 0.91, and 0.60 μmole·min^{-1}·(mg protein)$^{-1}$ for the P815, P815/ara-C- and P815/TG-derived samples. When the PM-K activity of fraction 19, derived from P815 cell homogenate, was analyzed by PC (dCMP as substrate), a value of 0.28 μmole·min^{-1}·(mg protein)$^{-1}$ was obtained, whereas the identical reaction product analyzed by HPLC yielded a value of 0.74 μmole·min^{-1}·(mg protein)$^{-1}$. The overall recovery of PM-K activity derived from P815 cell homogenate using dCMP as substrate and PC for analysis of the reaction products was 40% of the injected enzyme activity [fractions 15–23 (Fig. 1)].

Analysis by HPLC of the NDP-K peak activities for dCDP, ara-CDP, and CDP as substrates, revealed, with the exception of ara-CDP phosphorylation in the respective fraction derived from P815 cells, little variation in the three cell lines with specific activities ranging from 2 to 4 μmoles·min^{-1}·(mg protein)$^{-1}$ (Fig. 4). With one exception (ara-CDP phosphorylation in P815/TG), peak activities occurred repeatedly in fraction 15 where the sequences of specific activities for dCDP, ara-CDP, and CDP were as follows for the three cell lines: 2.16, 0.95, and 3.55 μmoles·min^{-1}·(mg protein)$^{-1}$ (P815); 2.99, 2.87, and 4.16 μmoles·min^{-1}·(mg protein)$^{-1}$ (P815/ara-C); and 2.39, 2.64, and 4.15 μmoles·min^{-1}·(mg protein)$^{-1}$ (P815/TG).

Analysis by TLC of NDP-K activities of P815-derived fractions, using CDP as substrate, revealed enzyme activity over 20 fractions with one major peak extending from fractions 12 to 21 and one small peak each at fractions 6–10 and 22 or 23, with specific activities approximately 60 and 13 times smaller, respectively, than the one evaluated for the fraction with maximum activity. The latter was observed at fraction 14 or 15 (Fig. 1) [1.34 μmoles·min^{-1}·(mg protein)$^{-1}$] yielding a 2-fold lower specific activity

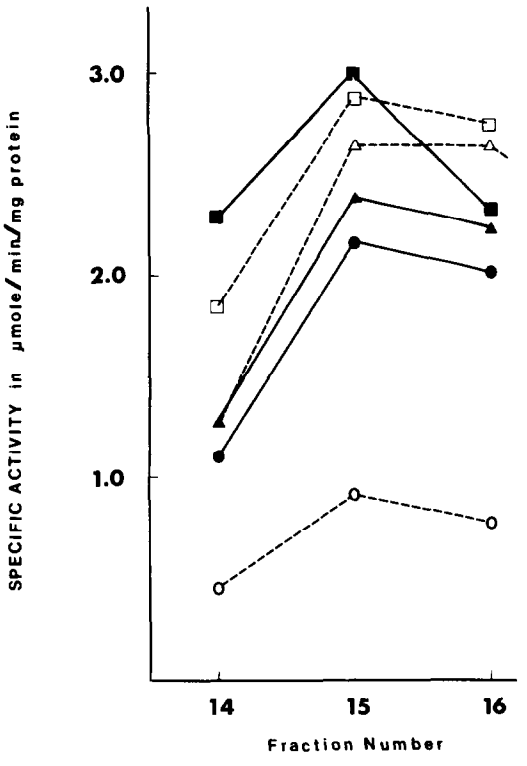

Fig. 4. Specific activities of the three peak fractions with nucleoside-5′-diphosphate kinase activities following separation by HPLC. Abscissa: fraction number. Ordinate: nucleoside-5′-diphosphate kinase specific activity in μmoles per min per mg protein. Symbols: (●—●) dCDP and (○---○) ara-CDP kinase activities in fractions derived from P815 cell homogenates; (■—■) dCDP and (□---□) ara-CDP kinase activities in fractions derived from P815/ara-C homogenates; and (▲—▲) dCDP and (△---△) ara-CDP kinase activities in fractions derived from P815/TG cell homogenates. See text for methods.

than the value revealed by HPLC analysis of the identical fraction [3.55 μmoles·min^{-1}·(mg protein)$^{-1}$]. The overall yield was 132% of the injected amount of enzyme-active material.

In all three cell lines, equal amounts of protein applied to the column gave quantitatively and qualitatively comparable absorbance profiles at 210 nm (not shown). A respresentative recording of the absorption at 210 nm derived from the separation of P815 cell homogenate revealed eight distinct peaks and two shoulders (Fig. 1).

The molecular weights of the D-K, PM-K, and NDP-K, as evaluated from a standard curve of six known proteins, were approximately 58,000, 36,000, and 49,000 daltons respectively.

The comparison of the specific activities (HPLC analysis) for the respective peak fractions derived from these three cell homogenates revealed for dCyd, dCMP, dCDP, and CDP phosphorylation the following relative values: 1:700:2,000:3,200 (P815), 1:6,000:29,900:41,600 (P815/ara-C), and 1:800:2,200:3,800 (P815/TG); for the phosphorylation of ara-C, ara-CMP and ara-CDP the ratios were: 1:3,000:1,400 (P815), 1:6,900:14,400 (P815/ara-C), and 1:3,000:4,400 (P815/TG) respectively.

The optimal purification achieved in the peak fractions for the D-K activity of the three cell lines using dCyd as substrate amounted to 11, 33 and 18 times for P815, P815/ara-C and P815/TG, respectively, while the optimal purification for the PM-K activity using ara-CMP as substrate amounted to 15, 16 and 17 times, respectively, and the purification gained using dCDP as substrate was 5, 9 and 6 times respectively.

HPLC analysis of the reaction products in fraction 14 of the dCyd kinase assay of the P815 cells revealed only dCMP, indicating no overlap with the subsequently eluted PM-K. Due to the overlap with the NDP-K, the reaction products of the PM-K (dCMP as substrate) in fractions 16–19 were triphosphates exclusively, whereas in fraction 20, the predominant product was diphosphate (26.7%, as compared to triphosphate, 2.3%). The triphosphate was always the main reaction product of the NDP-K, while contaminants such as unphosphorylated or deaminated by-products remained constant at 2–3%.

Disc electrophoresis of the P815 crude material separated into more than twenty bands [2], whereas 6.9 μg protein of the peak D-K fraction produced seven visible bands; 4.5 μg protein of the peak PM-K fraction revealed three visible bands and

Table 1. Ratios of ara-C and its 5′-nucleotides versus dCR and its 5′-nucleotides resulting in 50% inhibition of substrate phosphorylation using the semipurified kinases* derived from three P815 cell lines with different susceptibilities to treatment with ara-C

	Ratio of peak fractions (inhibitor:substrate)		
	Cell lines		
Inhibitor:substrate	P815 fraction 13	P815/ara-C fraction 19	P815/TG fraction 15
Ara-C:dCR	60:1	ND†	100:1
Ara-CMP:dCMP	0.5:1	2.2:1	0.5:1
Ara-CDP:dCDP	1.7:1	3.5:1	4.0:1
Ara-CTP:dCDP	17:1	20:1	30:1

* Peak fraction of each preparation.
† Not done because of lack of kinase activity of this enzyme preparation.

8.2 μg protein of the NDP-K peak fraction exhibited six bands.

Ratios of ara-C and its 5'-nucleotides versus dCR and its 5'-nucleotides resulting in 50% inhibition of substrate phosphorylation revealed (Table 1) that large excesses of ara-C were required to inhibit dCR phosphorylation (60:1 and 100:1 for the semipurified D-K preparations of P815 and P815/TG cells respectively). The D-K activity of the P815/ara-C cell enzyme was too low for meaningful evaluation. The ratios of ara-CMP versus dCMP in the inhibitory experiments with semipurified PM-K were 0.5:1 for the P815 and P815/TG-derived preparation and 2.2:1 for the P815/ara-C-derived material. The inhibitory activity of ara-CDP versus dCDP ranged from 1.7:1 (P815) to 4.0:1 (P815/TG), while ratios of end-product inhibition of ara-CTP versus dCDP increased in all three cell lines from 17:1 (P815) to 30:1 (P815/TG).

DISCUSSION

The present study using HPLC for the partial separation and purification of the kinases responsible for the phosphorylation of ara-C to ara-CTP confirms the observation of Kozai and Sugino [16] that three different enzymes are involved in the production of ara-CTP from ara-C. Evaluation of the total enzyme activities eluted from the HPLC column, following the injection of crude P815 cell homogenate, revealed significant increases of the kinases from the D-K to the NDP-K activities. Without taking into consideration the different yields and different substrates, the ratios of total kinases, as evaluated by the disc assay, PC and TLC for dCR, dCMP and CDP phosphorylation, amounted to 1 to 260 to 24,070 respectively (Fig. 1). These large increases of total kinase activity cannot be explained with the data available, but may have been related to the loss of substrate specificity from D-K, with a very narrow specificity as reported earlier [4], to the less specific PM-K [17] and the highly unspecific NDP-K. Hande and Chabner [17] reported that CMP, dCMP, UMP, dUMP and 5'-monophosphates of ara-C, 5-fluorouridine, 5-fluorodeoxyuridine and 1-β-D-arabinofuranosyluracil are substrates for a highly purified pyrimidine monophosphate kinase isolated from human leukemic blast cells [17]. Our observation of a wide range of fractions with NDP-K activities, with a main peak and two minor peaks, indicates heterogeneity of this enzyme for the substrate investigated (CDP). It is possible, but not provable by these very limited experiments, that the NDP-K activities isolated by the new technique represent several isozymes with different molecular weights and different specific activities for different substrates. Heterogeneity of NDP-K was reported earlier for this enzyme isolated from human erythrocytes [18, 19] and rat tissues [20].

The comparison of dCyd and ara-C as substrates for the peak fractions with D-K activities in the P815 and P815/ara-C cells revealed patterns similar to the ones reported earlier for the homogenous D-K [4]. While a complete purification using HPLC was not expected, the purification observed (11 and 33 times, respectively, for dCyd as substrate) is a substantial one and exceeds the purification obtained in any of the single steps observed in the previously reported enzyme isolation [4] with the additional advantage of speed and complete recovery of the applied enzyme activity.

PM-K activity for both dCMP and ara-CMP substrates exhibited comparable values in the respective HPLC fractions derived from all three cell lines. Strikingly, ara-CMP was phosphorylated more efficiently than was dCMP. The purification obtained (4 to 13 times using dCMP substrate) made HPLC a desirable step for efficient purification of PM-K due to its speed, but not necessarily due to the (limited) amount of proteins that could be loaded on the column.

NDP-K activity was comparable at peak fractions for both substrates in all three cell lines except for the phosphorylation of ara-CDP in the peak fractions derived from P815 cells. These findings suggest the possibility of a competitive reaction between ara-CDP and dCDP and may provide another reaction mechanism for ara-C (see below).

While the phosphorylation of ara-C was comparable to that of ara-CMP, in peak fractions derived from P815 and P815/TG cell homogenates, two peak fractions of the P815/TG NDP-K catalyzed the phosphorylation of ara-CDP with significantly higher specific activities than that observed in the peak fraction of the P815 NDP-K separation, indicating the possibility that P815/TG cells may produce substantially more ara-CTP per cell than P815 cells. It is unlikely, however, that the strong collateral sensitivity observed in chemotherapy experiments with this cell line can be explained by this observation, since the D-K activities in both cell lines were comparable, were many-fold smaller than the PM-K and NDP-K activities, and were probably rate limiting in the phosphorylation of ara-C to ara-CTP.

The presently accepted reaction mechanisms for ara-C are the inhibition of DNA polymerase [21], its incorporation into DNA [22, 23] and, under appropriate circumstances, inhibition of ribonucleotide diphosphate reductase [24]. In the *de novo* synthesis of DNA precursors, dCTP is synthesized from dCDP. The pools of dCTP in a variety of cells are small when compared to those of other ribonucleoside phosphates [25]. When peripheral cells of patients with AML were incubated for 45 min in medium containing 2.5 μg of ara-C/ml (= 10.3 nmoles/ml), cellular accumulations of ara-CTP of 2–35 ng/10^6 cells (= 8–140 pmoles/10^6 cells) were reported [26], similar to levels of deoxyribonucleotides reached in pools of mammalian cells in culture [27, 28]. Levels of ara-C, as used by Chou *et al.* [26] in tissue culture, can easily be reached in plasma, although for short times only, after a single dose of 200 mg/m^2 of ara-C to patients [29]. Thus, under favorable circumstances, intracellular levels of ara-CTP can exceed dCTP levels substantially. Our *in vitro* experiments with semipurified NDP-K, although performed at higher levels of substrate and inhibitor concentrations (dCDP, ara-CDP and ara-CTP), made possible a competitive and feedback inhibition of the phosphorylation of dCDP to dCTP even by slight, but more likely by highly excessive, amounts of ara-CDP and ara-CTP. The resulting

insufficient levels of intracellular dCTP may constitute another possible reaction mechanism for ara-C. Indeed, an initial but short-lasting decrease in the pool size of dCTP (contrary to the expanded pool sizes of dATP, dGTP and dTTP) by incubation of mouse embryo cells in the presence of ara-C was reported by Skoog and Nordenskjöld [30].

The technique of enzyme separation by HPLC provided adequate resolution of nucleoside and monophosphate kinases, but it was less adequate in the separation of PM-K from NDP-K. Preliminary studies using two columns with a total length of 100 cm did not markedly improve the separation.

In comparing the molecular weights of the three P815 cell-derived enzymes at their peak activities with previous reports, the value calculated for the D-K compares favorably with the one reported from this laboratory for homogenous material (58,000) [3], whereas the value of PM-K is higher than the one reported for the same enzyme extracted from human leukemic blast cells (36,000 vs 28,000) [17]. The range of molecular weights of the NDP-K possibly representing a number of isozymes exhibits a wider range (34,000–78,000) than the one reported by Cheng *et al.* [18] for several NDP-K isozymes of human erythrocytes (mol. wt 80,000–100,000). This lower range of molecular weight values in our experiments as compared to the ones isolated from erythrocytes is disturbing and might be due to proteolysis or dissociation into subunits, but it was not investigated further.

The rapid separation of proteins and especially enzymes for the study of physical parameters and reaction mechanisms has been a major goal of protein chemists. While the primary aim of this study was the comparison in three P815 cell lines with different responses to ara-C of rapidly semipurified kinases involved in the metabolism of ara-C, the techniques used and the conclusions drawn from these experiments warrant the application of this methodology to studies of metabolism and reaction mechanisms of other drugs.

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