# Deoxycytidine Kinase

## I. Distribution in Normal and Neoplastic Tissues and Interrelationships of Deoxycytidine and 1-β-D-Arabinofuranosylcytosine Phosphorylation

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SUMMARY

Deoxycytidine kinase is confined primarily to lymphoid tissues in the rat and mouse. Its activity varies widely, with highest levels in the thymus. A similar range of activities was found among the tumors examined. There is not an exact correlation of kinase activity with the cell proliferation rate. In crude tissue extracts the phosphorylation of cytosine arabinoside (araC) is inhibited to a variable and greater extent than the phosphorylation of deoxycytidine (CdR), and this inhibition is largely removed by dialysis of the preparations.

Partially purified CdR kinase from calf thymus phosphorylates CdR, araC, GdR, and AdR, with CdR being the kinetically preferred substrate. Each of these nucleosides will competitively inhibit the phosphorylation of any of the others. Enzymatic activity is also inhibited by a number of nucleotides at physiological concentrations, and in each case the phosphorylation of araC is more sensitive to the inhibition than that of CdR. dTTP, dUTP, and UTP, which themselves inhibit phosphate transfer from ATP, nonetheless will partially reverse the very potent inhibition of dCTP.

The preferential inhibition of araC phosphorylation in extracts is therefore tentatively explained in terms of complex interactions of a number of nucleosides and nucleotides with the enzyme, all of which tend to select for phosphorylation of CdR.

### INTRODUCTION

1-β-D-Arabinofuranosylcytosine inhibits the growth of a variety of neoplasms of both animals (1–3) and humans (4–8). In cell culture araC[2] inhibits the intracellular multiplication of DNA viruses (9–12). AraC also decreases the synthesis of DNA in mammalian cells (5, 9, 11, 13–15), exerting its effect primarily during the S phase of the cell cycle (16–18). The inhibitory effect of araC on tumor and viral systems and DNA synthesis could be prevented or reversed by CdR, if administered early enough (5, 11, 15, 18–20). AraC and araCTP inhibited the DNA polymerase activity of tumor extracts (21) and of partially purified mammalian enzyme (22), respectively, and these inhibitions were reversed by dCTP.

Very small amounts of araC are incorporated into DNA and RNA *in vivo* (5, 11, 23), but isolated enzyme systems from both bacterial (24) and mammalian (22) sources failed to incorporate araCTP.

It has been proposed (20) that araC

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[2] The abbreviations used are: araC, 1-β-D-arabinofuranosylcytosine; araCMP, 1-β-D-arabinofuranosylcytosine 5'-monophosphate; araCTP, 1-β-D-arabinofuranosylcytosine 5'-triphosphate; araU, 1-β-D-arabinofuranosyluracil; araUMP, 1-β-D-arabinofuranosyluracil 5'-monophosphate; CdR, deoxycytidine; CR, cytidine; UdR, deoxyuridine; UR, uridine; TdR, thymidine; AdR, deoxyadenosine; AR, adenosine; GdR, deoxyguanosine; GR, guanosine.

inhibits the formation of dCDP from CDP, but this has not been confirmed either in cell cultures (15) or when the effect of phosphorylated derivatives of araC on CDP reductase was tested *in vitro* (25).

Although the primary mode or modes of action of araC are thus still uncertain, it is generally accepted that phosphorylation of araC is an essential prerequisite to its activity. From studies of mutant leukemic cell lines resistant to araC, it was concluded that the enzyme which phosphorylates araC is CdR kinase (26, 27). This has been confirmed using partially purified CdR kinase (28–31). CdR inhibits the phosphorylation of araC (27–30), and it is proposed that this is the mechanism of its reversal of the inhibitory effects of araC. The phosphorylation of CdR to dCTP will also potentiate the feedback inhibition of CdR kinase by dCTP (15, 31–33). There may also be some competition during uptake of the nucleosides into the cell (5, 34).

In this study we show that CdR kinase is found primarily in tissues of lymphoid origin, and it is suggested that the relatively high levels of this enzyme in lymphoid neoplasms may account for the clinical effectiveness of araC in the treatment of acute leukemia. This success notwithstanding, we find that the enzyme has a great tendency to select the natural substrate, CdR, rather than araC, and a variety of nucleotides besides dCTP may serve to regulate this process.

## MATERIALS AND METHODS

### *Enzyme Preparations*

*CdR kinase.* Calf thymus glands were obtained at a local abattoir from freshly killed animals, and the CdR kinase was partially purified.[3] This preparation contains insignificant levels of phosphatase and no deaminase activity. Weanling rats were supplied by Harlan Industries, Inc., and $CF_1$ mice were purchased from Carworth Farms. Novikoff hepatoma was of a strain described previously (35). All other tumors were kindly provided by R. Folk, Battelle Memorial Institute, Columbus. The L5178Y and L1210 leukemias were drawn from the peritoneal cavity with heparinized syringes, and any necrotic tissue was dissected from the other tumors, all of which were solid. Lymphocytes were collected by thoracic duct cannulation.[4] Blood was collected by cardiac puncture of ether-anesthetized animals, using heparinized syringes. The animals were then decapitated and the tissues removed. The epithelium was scraped away from segments of small intestine. For lymph node preparations, only the cervical nodes were utilized.

The blood, bone marrow, lymphocytes, and L5178Y and L1210 leukemias were centrifuged for 5 min at $30,000 \times g$. The precipitated cells were washed once with 0.9% NaCl solution and then suspended in 3 volumes (w/v) of 0.154 M KCl. Other tissues were washed in 0.154 M KCl. The intestinal epithelium was centrifuged for 5 min at $30,000 \times g$ to precipitate the fragments. Muscle tissue was minced with scissors and passed through the fine grid of a hand tissue press (Harvard Apparatus Company).

The whole blood was sonicated for 20 sec at full power using a Bronwill Biosonic I sonicator. The lymph nodes and bone marrow were homogenized with Potter-Elvejhem homogenizers in a minimal volume of 0.154 M KCl. All other tissues were homogenized in 3 volumes (w/v) of 0.154 M KCl. The homogenates and sonic extract were centrifuged for 30 min at $30,000 \times g$, and the supernatant fluid was decanted, rapidly frozen in Dry Ice–acetone, and stored at —30° until required for assay.

Dialysis of supernatant fractions was carried out against 1000 volumes of 0.154 M KCl for 8 hr with a change of dialyzing solution after 4 hr.

*Escherichia coli cytosine nucleoside deaminase. E coli* $B_{12}$ cells in late log phase were harvested by centrifugation, washed once with buffer (0.05 M Tris-HCl, pH

[3] D. H. Ives and J. P. Durham, manuscript in preparation.

[4] Obtained from Dr. M. Whitehouse.

$7.5_{25}$),[5] then mixed with an equal volume (w/v) of buffer and disrupted in a French press at 15,000 psi. The suspension was centrifuged for 20 min at 30,000 × *g*, and the supernatant fraction was further centrifuged for 1 hr at 100,000 × *g*. The resulting supernatant was diluted with 5 volumes of buffer, and the CdR deaminase was partially purified by the method of Cohen (36). Final specific activity was 5.3 μmoles of CdR deaminated per 30 min per milligram of protein.

*Phosphotransferases.* Malt phosphotransferase was prepared by a method modified from that of Brawerman and Chargaff (37). Malt diastase (400 g) was suspended in 1200 ml of 0.154 M KCl and homogenized in a Waring Blendor for 30 sec at high speed. The homogenate was centrifuged for 30 min at 15,000 × *g*, yielding a supernatant fraction (938 ml) to which 227.5 g of $(NH_4)_2SO_4$ were added with stirring over a 20-min period. After standing for a further 20 min, the mixture was centrifuged for 20 min at 15,000 × *g*. A further 62.5 g of $(NH_4)_2SO_4$ were added to the supernatant fluid (995 ml) under the same conditions, and the solution was recentrifuged. The precipitate was taken up in 1 mM phosphate, pH 6.5 (430 ml).

Chick embryo extract was prepared by the method of Maley and Maley (38).

### *Enzyme Assays*

*CdR kinase and deaminase in extracts.* Phosphorylation and deamination were followed simultaneously, using the same assay conditions for both CdR and araC as substrates. In all enzyme assays Eppendorf automatic pipettes were used whenever possible, and all assays were carried out in duplicate. An aliquot of extract was chosen to give close to but not greater than 20% conversion of substrate. Up to 75 μl were added to 25 μl of reaction mixture containing 1 μmole of ATP, 0.5 μmole of $MgCl_2$, 1.5 μmoles of NaF, 4 μmoles of Tris-HCl (pH $8.0_{37}$), and 2 mμmoles of CdR or araC, plus 0.154 M KCl to a final volume of 100 μl in a 2-ml conical glass centrifuge tube. After incubation for 30 min at 37°, the reaction was stopped by heating for 2 min at 100°. Denatured protein was centrifuged to the bottom of the tube (10 min at 1500 × *g*). Reaction products were analyzed and quantitated in one of two ways.

1. Strip method: The total assay mixture is spotted on a 1 × 22.5 inch strip of DEAE-cellulose paper held on a glass frame by Teflon clips. The strips are developed to within 1 inch of the end of the paper by descending chromatography, using 0.03 N formic acid as the solvent. When an extract had significant levels of both CdR kinase and CdR deaminase, so that both dCTP and UdR nucleotides might be formed, the assay volume was doubled. Half the assay mixture is treated as above, and the other half is spotted on a second strip of DEAE-cellulose paper and developed with 4 N formic acid containing 0.1 N ammonium

TABLE 1

*Ion exchange paper chromatography of nucleosides and nucleoside phosphates*

The nucleosides (or their phosphates) were spotted on 1-inch strips of Whatman DE81 and developed by descending chromatography to within 1 inch of the bottom of the strip (approximately 5 hr).

| | $R_F$ value | |
|---|---|---|
| Nucleoside (phosphate) | 0.03 N formic acid | 4 N formic acid + 0.1 N ammonium formate |
| CdR | 0.82 | 0.92 |
| araC | 0.81 | 0.92 |
| CR | 0.81 | |
| TdR | 0.70 | |
| UdR | 0.59 | |
| araU | 0.58 | |
| UR | 0.53 | |
| araCMP | 0.26 | 0.90 |
| dCMP | 0.21 | 0.90 |
| CMP | 0.19 | 0.87 |
| dUMP | <0.1 | 0.65 |
| UMP | <0.1 | 0.62 |
| araUMP | 0.10 | 0.68 |
| dTMP | 0 | 0.58 |
| araCTP | 0 | 0.28 |
| dCTP | 0 | 0.24 |
| dUTP | 0 | 0.07 |
| dTTP | 0 | 0.04 |

[5] Subscript indicates the temperature at which the pH was measured.

formate (Table 1). For this method, 0.05 $\mu$Ci of CdR-2-$^{14}$C or 0.625 $\mu$Ci of $^{3}$H-araC was used per assay. The developed strips are air-dried, and the $^{14}$C-CdR assays are quantitated on a Vanguard 880 ADS integrating strip scanner (efficiency, 20%).

The product peaks for $^{3}$H-araC assays were located on the strip scanner, but the very low tritium efficiency (approximately 1%) and the absorption of the weak tritium radiation within the paper made another method of quantitation desirable. Therefore substrate and product peaks were cut out of the strips, placed in 20-ml liquid scintillation vials, and eluted with 1.5 ml of standard eluent; 15 ml of scintillation solvent (39) were added, and the samples were counted in a Beckman LS100 liquid scintillation counter.

2. Disc assay: The method of Ives *et al.* (39) is followed. One-half microcurie of $^{3}$H-CdR or $^{3}$H-araC was used per assay (when the araC concentration was 0.002 mM, 0.25 $\mu$Ci was used). After heating, 200 $\mu$l of $H_2O$ are added, the tubes are centrifuged, and 50-$\mu$l aliquots are placed on three 17-mm discs of SB-2 paper. Only phosphorylated products are retained upon washing, retention of the deamination product being less than 0.5%.

*CdR kinase kinetics and general properties of the calf thymus enzyme.* These were investigated using the disc assay. The basic incubation mixture (80 $\mu$l) contained 0.8 $\mu$mole of ATP, 0.8 $\mu$mole of $MgCl_2$, 3.2 $\mu$moles of Tris-HCl (pH $8.0_{37}$), 1.6 m$\mu$moles of $^{3}$H-CdR or $^{3}$H-araC (0.4 $\mu$Ci), 0.08 mg of bovine serum albumin, and 0.5–5 $\mu$g of calf thymus enzyme protein. Duplicate assays were always run. After incubation for 30 min at 37° and stopping the reaction by heating for 2 min at 100°, 150 $\mu$l of $H_2O$ were added and the tubes were centrifuged briefly to collect any condensate on the upper part of the tube.

$ATP\text{-}Mg^{2-}$ concentrations were calculated by a method detailed elsewhere.[8]

*TdR kinase.* TdR kinase was assayed by the disc method as previously described (39).

*Phosphotransferase activity.* Chick embryo extract (25–70 $\mu$l; 9 mg of protein per milliliter) was added to 20 $\mu$l of a reaction mixture containing 0.8 $\mu$mole of dUMP, 0.4 $\mu$mole of $MgCl_2$, 1 $\mu$mole of NaF, 2 m$\mu$moles of substrate (0.04 $\mu$Ci of 2-$^{14}$C-CR; 0.05 $\mu$Ci of 2-$^{14}$C-UdR, 2-$^{14}$C-TdR, 2-$^{14}$C-CdR, or 2-$^{14}$C-UR; 0.625 $\mu$Ci of $^{3}$H-araC or $^{3}$H-araU), 10 $\mu$l of 1 M Tris-HCl (pH $8.0_{37}$), and $H_2O$ to 100 $\mu$l. Incubation was conducted for 1 hr at 37°.

Malt diastase extract (25 $\mu$l; 4.14 mg of protein per milliliter) was added to 20 $\mu$l of the above reaction mixture, 10 $\mu$l of 1 M Tris-maleate (pH $6.0_{37}$), and 45 $\mu$l of $H_2O$. Incubation was conducted for 30 min at 37°.

The concentration of fluoride in the assay inhibits phosphotransferase activity less than 5% while inhibiting phosphatase activity more than 80%. Duplicate assays were run for both enzymes. The reactions were stopped by heating for 2 min at 100° and analyzed by the strip method.

*Materials*

AraC was a gift from G. W. Camiener of the Upjohn Company. Other unlabeled nucleosides and nucleotides were obtained from P-L Biochemicals or Sigma Chemical Company. 2-$^{14}$C-CdR (26.6 mCi/mmole), 2-$^{14}$C-CR (20 mCi/mmole), $^{3}$H-GdR (4.8 Ci/mmole), and $^{3}$H-araC (1.34 Ci/mmole) were supplied by Schwarz BioResearch. $^{3}$H-CdR (10.5 Ci/mmole), 2-$^{14}$C-UdR (28.0 mCi/mmole), 2-$^{14}$C-UR (30.2 mCi/mmole), and 2-$^{14}$C-TdR (30 mCi/mmole) were products of New England Nuclear Corporation. Uniformly labeled $^{14}$C-AdR (274 mCi/mmole) was purchased from Amersham-Searle.

The purity of the $^{3}$H-araC was checked by descending chromatography for 72 hr on Whatman No. 1 paper, using 1-butanol–formic acid–$H_2O$ (77:10:13) as the solvent; the sample was repurified on the same system if the purity was less than 98%.

Malt diastase was obtained from Nutritional Biochemicals Corporation. Dithiothreitol was supplied by Calbiochem. Crystalline bovine serum albumin was a product of Pentex, Inc. DEAE-substituted paper sheets (Whatman DE81) and Amberlite ion exchange resin-loaded paper sheets

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TABLE 2

*CdR kinase and related enzyme activities in rat tissues*

Each enzyme activity was determined separately on the tissues from five individual animals, and the activities were averaged. CdR kinase and deaminase activities were assayed by the strip method. CdR/araC phosphorylation ratios (using pooled extracts) and TdR kinase were determined by the disc assay (see the text for details).

| Rat tissue | CdR kinase | | | | | TdR kinase[b] | TdR/CdR ratio | CdR deaminase[b] |
|---|---|---|---|---|---|---|---|---|
| | Relative activity[a] | Range of activities[b] | CdR/araC ratio | | Dialyzed/ undialyzed[c] | | | |
| | | | Undialyzed | Dialyzed | | | | |
| Thymus | 1.0 | 1.75–2.71 | 4.0 | 2 | 0.8 | 7.7 | 3.5 | 0 |
| Bone marrow | 0.75 | 1.16–1.96 | 5.8 | 2.3 | 0.9 | 41 | 25 | 0 |
| Lymph nodes | 0.36 | 0.57–1.01 | 5.9 | 1.7 | 0.9 | 3.9 | 4.9 | 0 |
| Spleen | 0.16 | 0.29–0.44 | 16 | 2.1 | 1.2 | 1.8 | 5.2 | 0 |
| Intestine[d] | 0.10 | 0.07–0.36 | 5.7 | 3.8 | 0.9 | 0.22 | 1.0 | 0.38 |
| Lung[d] | 0.04 | 0.04–0.13 | 20 | 7.2 | 1.3 | 0.38 | 4.0 | 0 |
| Testis[d] | 0.01 | 0.02–0.04 | 17 | 4.2 | 1.4 | 0.18 | 6.2 | 0.01 |
| Kidney[d] | 0.01 | 0.02–0.05 | 21 | 14 | 1.4 | 0.06 | 1.9 | 0 |
| Liver | 0.005 | 0.00–0.02 | | | | 0.46 | ~50 | 0 |
| Brain | 0.001 | 0.00–0.01 | | | | 0.08 | ~30 | 0 |
| Muscle | 0 | | | | | 0.09 | | 0 |
| Whole blood | 0 | | | | | 0.03 | | 0 |
| Lymphocytes[d] | 0.01 | 0.03–0.07 | >20 | >20 | 1.1 | | | 0 |
| 28-hr regenerating liver[d] | 0.12 | 0.20–0.32[e] | 18 | 2.4 | 4.9 | 18.8 | ~70 | 0 |

[a] Relative to rat thymus (2.22 nanomoles of dCMP per milligram of protein per 30 min).

[b] Nanomoles of product per milligram of protein per 30 min.

[c] Ratio of CdR kinase activities, with CdR as substrate, in the dialyzed and the undialyzed extracts.

[d] The concentrations of CdR and araC were 0.002 mM when the CdR/ araC phosphorylation ratio was determined.

[e] Determinations on extracts from three animals only.

TABLE 3

*CdR kinase and related enzyme activities in mouse tissues*

Results are the average of two experiments, each with the pooled tissues from three animals. Other conditions were the same as in Table 2.

| Mouse tissue | CdR kinase | | | | TdR kinase[c] | TdR/CdR ratio | CdR deaminase[c] |
|---|---|---|---|---|---|---|---|
| | Relative activity[a] | CdR/araC ratio | | Dialyzed/ undialyzed[b] | | | |
| | | Undialyzed | Dialyzed | | | | |
| Thymus | 0.86 | 1.7 | 1.3 | 1.0 | 11.5 | 6.3 | 0 |
| Spleen | 0.83 | 4.9 | 2.4 | 0.9 | 12.4 | 6.9 | 0.20 |
| Lymph nodes | 0.28 | 5.3 | 2.2 | 0.9 | 12.5 | 20 | 0.24 |
| Testis[d] | 0.05 | 7.9 | 7.4 | 0.8 | 0.33 | 7 | 0.04 |
| Intestine[d] | 0.04 | 6.8 | 3.4 | 0.9 | 1.4 | 35 | 4.44 |
| Lung[d] | 0.02 | 4.9 | 4.7 | 1.0 | 0.19 | 10 | 0.14 |
| Liver | 0 | | | | 0.09 | | 0.12 |
| Kidney | 0 | | | | 0.05 | | 10.6 |
| Brain | 0 | | | | 0.12 | | 0.55 |
| Muscle | 0 | | | | 0 | | 0 |
| Blood | 0 | | | | 0 | | 0 |

[a] Relative to rat thymus.

[b] Ratio of CdR kinase activities, with CdR as substrate, in the dialyzed and the undialyzed extracts.

[c] Nanomoles per milligram of protein per 30 min.

[d] The concentrations of CdR and araC were 0.002 mM when the CdR/araC phosphorylation ratio was determined.

(grade SB-2) were purchased from H. Reeve Angel Company. The DEAE-cellulose paper was washed extensively with 0.001 N formic acid prior to use.

*Enzymatic synthesis of $^3$H-araU.* $^3$H-araC (20 $\mu$Ci), unlabeled araC (49 m$\mu$moles), Tris-HCl, pH $7.5_{37}$ (15 $\mu$moles), were incubated with 2 $\mu$l of the partially purified *E. coli* cytosine nucleoside deaminase (total volume, 0.15 ml) for 2 hr at 37°. After heating for 3 min at 100°, the mixture was spotted on a $2 \times 22.5$ inch strip of DEAE-cellulose paper and developed with 0.03 N formic acid. The solution was lyophilized to dryness and the residue was taken up in 64 $\mu$l of water.

## RESULTS

### *Tissue Levels of CdR Kinase and Related Enzyme Activities*

*Rat and mouse.* The tissue distributions of several enzymes of nucleoside metabolism have been determined for both the rat (Table 2) and the mouse (Table 3). CdR kinase is confined primarily to tissues of lymphoid origin. However, the level of activity in circulating rat lymphocytes appears to be very low, and whole blood has no measurable activity. Only the spleen shows a clearly significant species difference in CdR kinase activity between the rat and the mouse.

The ratio of TdR kinase to CdR kinase activities (TdR/CdR kinase ratio) shows a wide variability, perhaps indicating divergent roles for these two enzymes, which have been considered as involved primarily in salvage mechanisms (40, 41). The TdR/CdR kinase ratio for individual tissues shows some differences between the rat and the mouse, but only the very large difference for the intestine can be considered statistically significant.

Cytosine nucleoside deaminase (CdR deaminase) is found in consistently measurable levels only in the intestine and the testis of the rat. While it has been supposed that this enzyme is confined to the kidney in the mouse (4, 42), we find, in agreement with Creasey (43), appreciable activity in a number of mouse tissues.

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TABLE 4

*CdR kinase and related enzyme activities in some rat and mouse tumors*

Determinations were made on the pooled tumor tissues of five to eight animals. Other conditions were the same as in Table 2.

| Tumor | CdR kinase | | | | TdR kinase[c] | TdR/CdR ratio | CdR deaminase[c] | UdR/araU[d] |
|---|---|---|---|---|---|---|---|---|
| | Relative activity[a] | CdR/araC ratio | | Dialyzed/ undialyzed[b] | | | | |
| | | Undialyzed | Dialyzed | | | | | |
| Rat | | | | | | | | |
| Carcinosarcoma—Walker 256 | 0.17 | 13 | 1.4 | 1.0 | 23 | 60 | 0 | |
| Lymphosarcoma—Lymphoma 8 | 0.52 | 1.8 | 1.3 | 1.0 | 2.5 | 2.1 | 0 | |
| Hepatoma—Novikoff[e] | 0.07 | 1.7 | 1.8 | 0.9 | 4.4 | 28 | 0.06 | 2.5 |
| Mouse | | | | | | | | |
| Adenocarcinoma 755 | 0.23 | 2.4 | 2.2 | 1.0 | 3.9 | 7.4 | 0.14 | 2.4 |
| Lymphocytic Leukemia L1210 | 1.5 | 2.1 | 1.9 | 0.9 | 4.7 | 1.5 | 0 | |
| Lymphocytic Leukemia L5178Y | 0.59 | 1.0 | 0.9 | 0.8 | 8.2 | 6.3 | 0 | |
| Hepatoma 129 | 0.33 | 1.4 | | | 6.3 | 8.5 | 0.19 | 2.1 |
| Fibrosarcoma 180 | 0.69 | 1.6 | | | 8.7 | 5.7 | 1.7 | 3.0 |
| Solid Friend virus leukemia | 0.33 | 2.2 | | | 6.5 | 8.8 | 1.0 | 4.3 |

[a] Relative to rat thymus.

[b] Ratio of CdR kinase activities, with CdR as substrate, in the dialyzed and the undialyzed extracts.

[c] Nanomoles of product per milligram of protein per 30 min.

[d] Ratio of CdR deaminase activity with CdR and araC as substrates.

[e] The concentrations of CdR and araC were 0.002 mM when the CdR/araC phosphorylation ratio was determined.

Activity is extremely high in the kidney, and large amounts are also present in the intestine. Camiener and Smith (44) did not find deaminase activity in mouse liver, but, like Creasey, we find that this tissue contains low levels of the enzyme.

The rates of phosphorylation of CdR and araC were measured in both undialyzed and dialyzed extracts. The results are presented as the following ratio.

$$\frac{\text{Rate of phosphorylation of CdR}}{\text{Rate of phosphorylation of araC}} = \text{CdR/araC ratio}$$

Undialyzed rat tissue homogenates show highly variable CdR/araC phosphorylation ratios, that for spleen being especially high among the lymphoid tissues. On dialysis the ratios tend to approach a value of 2.

Dialysis causes little change in the rate of CdR phosphorylation (dialyzed/undialyzed ratio of CdR phosphorylation), although there does tend to be some loss of activity for tissues in which the undialyzed CdR/araC phosphorylation ratio is low (less than 8). In contrast, undialyzed tissues with higher CdR/araC phosphorylation ratios show slight increases in the rate of CdR phosphorylation upon dialysis. The decrease in the CdR/araC phosphorylation ratio upon dialysis may thus be due to the removal of molecules in the extract which preferentially inhibit the phosphorylation of araC. In the mouse, the undialyzed tissue extracts have lower CdR/araC phosphorylation ratios than in the rat, and this is especially significant for the spleen. Dialysis again tends to reduce the ratio.

*Tumor tissues.* Of the tumor extracts tested, only lymphocytic Leukemia L1210 showed a higher level of activity of CdR kinase than normal rat thymus (Table 4), but all contained measurable levels of activity. The undialyzed tumor extracts, except for Walker carcinoma, were characterized by very low CdR/araC phosphorylation ratios, and dialysis produced a significant change in the ratio only in the Walker carcinoma, reducing its ratio to the level of the other tumor extracts.

*Phosphotransferase activity.* Nucleoside phosphotransferase activity has been demonstrated in chick embryo (38), fowl leukemic myeloblasts (45), human placenta (46), bacteria (47), and plants (48, 49). Attempts to demonstrate a similar activity in calf thymus and several of the tumors have been unsuccessful. However, as this enzyme activity might be significant under certain conditions, the phosphorylation of nucleosides was investigated using both chick embryo and malt enzyme preparations. Table 5 shows that both araC and araU are extremely poor acceptors for both enzyme systems, so that even if substantial levels of phosphotransferase are present it is unlikely that araC will be phosphorylated to any significant extent. That the phosphorylation is a phosphotransferase and not a kinase reaction is further suggested by the lack of feedback control on the reaction by triphosphates. *E. coli* mutants lacking CdR deaminase[6] will not incorporate CdR into DNA,[7,8] so that the phosphotransferase system of this organism does not appear to supply deoxynucleotides as precursors for DNA synthesis. Thus the presence of phosphotransferase activity in a tissue does not itself indicate that trans-

TABLE 5

*Phosphate acceptor specificity of nucleosides in phosphotransferase reactions*

Conditions are specified in the text.

| Nucleoside | Specific activity[a] | |
|---|---|---|
| | Chick embryo | Malt diastase |
| CR | 0.009 | 2.22 |
| araC | 0.065 | 0.48 |
| UR | 0.107 | 4.08 |
| araU | 0.045 | 0.46 |
| CdR | 0.73 | 3.34 |
| UdR | 1.00 | 6.10 |
| TdR | 0.86 | 6.24 |
| CdR + 2.5 mM dCTP | 0.65 | 3.36 |
| UR + 5 mM UTP | | 4.54 |
| TdR + 5 mM dTTP | | 4.6 |

[a] Nanomoles of monophosphate per milligram of protein per 30 min.

[6] A gift from J. C. Gerhart.

[7] *E. coli* $B_{12}$ contains no CdR kinase.

[8] D. H. Ives and W. Snyder, unpublished observations.

TABLE 6
*Specificity of phosphate acceptors with calf thymus enzyme*

For conditions, see MATERIALS AND METHODS; however, 1.6 mμmoles of the following nucleosides were used as indicated: $^3$H-TdR, $^3$H-GdR, and $^3$H-araU, 0.5 μCi; $^{14}$C-AdR, $^{14}$C-UdR, and $^{14}$C-UR, 0.04 μCi; $^{14}$C-CR, 0.03 μCi.

| Substrate | Relative activity |
|---|---|
| CdR | 1[a] |
| GdR | 0.41 |
| AdR | 0.36 |
| TdR | <0.002 |
| UdR | <0.002 |
| araC | 1.23 |
| CR | <0.01 |
| UR | <0.002 |
| araU | <0.002 |

[a] 366 nanomoles/mg of protein per 30 min.

phosphorylation will be a significant anabolic pathway *in vivo*.

*Calf Thymus Enzyme*

*Phosphate acceptors.* The partially purified CdR kinase was able to utilize CdR, araC, GdR, and AdR as phosphate acceptors (Table 6). All these activities appear to be associated with one enzyme.[3] Recent studies on a subline of leukemia resistant to araC also indicate that GdR is phosphorylated by CdR kinase *in vivo* (50). The activity toward GdR and AdR as phosphate acceptors is susceptible to inactivation under the normal conditions of enzyme isolation, and the ratio of the activities toward CdR and araC as acceptors is slightly variable from preparation to preparation of the enzyme. The values in Table 6 are maximum observed activities relative to CdR phosphorylation. Partially inactivated enzyme is reactivated by incubation with sulfhydryl reagents and nucleoside triphosphates, but it has not been possible to stabilize completely the relative activities of the enzyme toward the various acceptors. As a result, the kinetic constants have been found to vary slightly as different enzyme preparations are used. However, the values of the kinetic constants relative to each do not change.

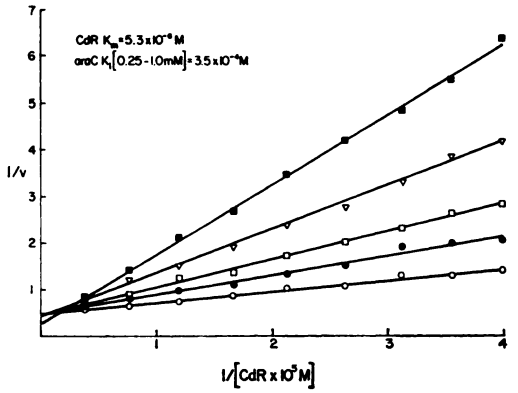


FIG. 1. *Inhibition of CdR phosphorylation by araC*
Inhibitor levels were: ○, none; ●, 0.25 mM araC; □, 0.5 mM araC; ▽, 1.0 mM araC; ■, 2.5 mM araC. Other conditions were the same as in MATERIALS AND METHODS, except that the CdR concentration was varied as indicated.

CdR kinase exhibits complex kinetics with CdR as substrate. Lineweaver-Burk (51) plots yield nonlinear plots over certain concentration ranges,[3] but at CdR concentrations of less than $2 \times 10^{-5}$ M the $K_m$ is $5.3 \times 10^{-6}$ M (Fig. 1). In contrast, with araC as the phosphate acceptor, $1/v$ against $1/S$ plots are linear over all concentration ranges employed (0.015–1.5 mM), and the $K_m$ is $4.1 \times 10^{-5}$ M (Fig. 2).

The inhibition of araC phosphorylation by other nucleosides is shown in Table 7. While TdR, UdR, and GR are not sub-

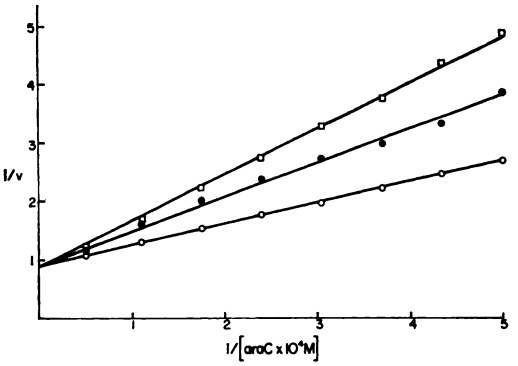


FIG. 2. *Inhibition of araC phosphorylation by GdR*
Inhibitor levels were: ○, none; ●, 2 mM GdR; □, 4 mM GdR. Other conditions were the same as in MATERIALS AND METHODS, except that the araC concentration was varied as indicated.

TABLE 7
*Inhibition of araC phosphorylation by other nucleosides*
For conditions, see MATERIALS AND METHODS.

| Nucleoside added | Inhibition |
|---|---|
| | % |
| 0.8 μmole of TdR | 15 |
| 0.8 μmole of UdR | 18 |
| 0.8 μmole of UR | 0 |
| 0.8 μmole of CR | 76 |
| 0.08 μmole of AR | 0 |
| 0.08 μmole of GR | 6 |
| 0.08 μmole of GdR | 25 |
| 0.08 μmole of AdR | 91 |
| 0.0008 μmole of CdR | 95 |
| 0.08 μmole of CdR | 100 |

strates, these compounds nonetheless act as poor inhibitors of araC phosphorylation at very high concentrations. CR, which is also not a substrate, is a stronger inhibitor, but again only at high concentrations. CdR, GdR, and AdR, which are phosphate acceptors, are more potent inhibitors. CdR and araC are competitive inhibitors of each other's phosphorylation: $K_i$ for araC = $3.5 \times 10^{-4}$ M (Fig. 1); $K_i$ for CdR = $1.7 \times 10^{-6}$ M (Fig. 3). At very high concentrations of araC, however, the inhibition of CdR phosphorylation is no longer purely competitive.

In contrast to a previous report that GdR did not affect CdR phosphorylation (29), we found GdR to be a competitive inhibitor of araC phosphorylation (Fig. 2), with a $K_i$ for GdR of $3.4 \times 10^{-3}$ M. AdR also acts as a competitive inhibitor (not shown).

The kinetic constants for CdR and araC indicate that in the presence of both substrates CdR will be preferentially utilized. Figure 4 shows that this is indeed the case. The CdR is selectively phosphorylated and, as its concentration is depleted, the rate of araC phosphorylation increases toward a steady rate that is decreased as the level of CdR is increased, probably because of inhibition by dCMP.

Maley and Maley (33) first showed that dCTP was a feedback inhibitor of CdR kinase, and this was confirmed in this laboratory using a more purified enzyme

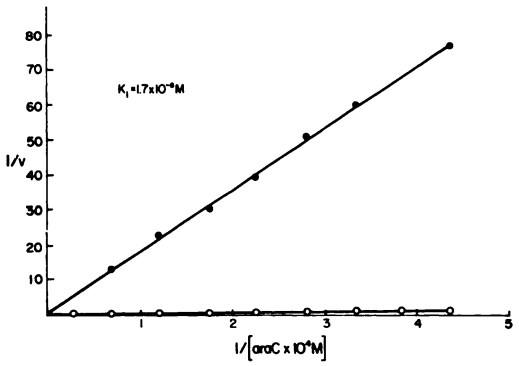


FIG. 3. *Inhibition of araC phosphorylation by CdR*
Inhibitor levels were: ○, none; ●, 0.1 mM CdR. Other conditions were the same as in MATERIALS AND METHODS, except that the araC concentration was varied as indicated.

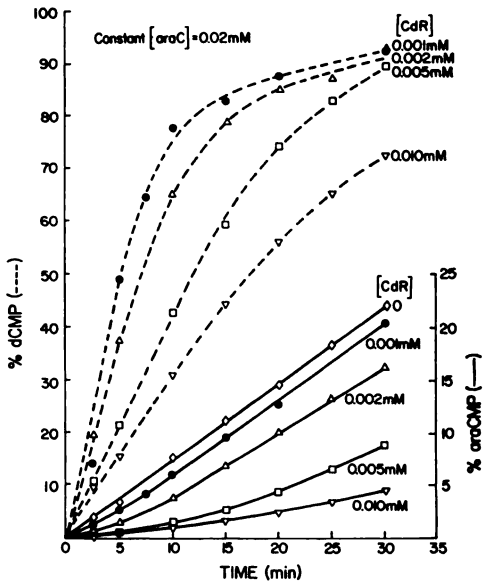


FIG. 4. *Relative rates of phosphorylation of CdR and araC in the presence of both substrates*
The concentration of araC was held constant at 0.02 mM, and the CdR concentration was varied. The phosphorylation of both CdR and araC was followed by setting up duplicate tubes, one with $^3$H-CdR and the other with $^3$H-araC. The assay volume was 0.4 ml. Aliquots of 50 μl were removed at each time point and heated at 100° for 2 min, and 150 μl of $H_2O$ were added. Other conditions were the same as in MATERIALS AND METHODS, but the larger volume was taken into account. % dCMP and % araCMP are the percentages of nucleoside substrates phosphorylated.

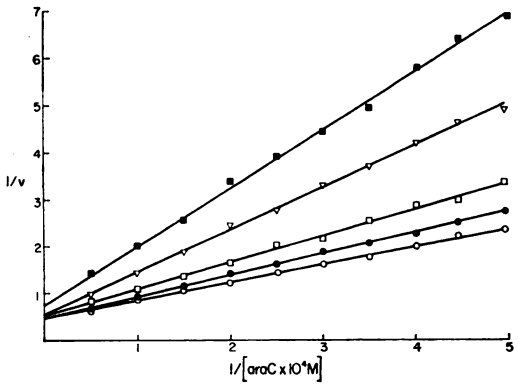


FIG. 5. *Inhibition of araC phosphorylation by dCTP*

Inhibitor levels were: ○, none; ●, 0.001 mM dCTP-$Mg^{2-}$; □, 0.0025 mM dCTP-$Mg^{2-}$; ▽, 0.005 mM dCTP-$Mg^{2-}$; ■, 0.0075 mM dCTP-$Mg^{2-}$. Other conditions were the same as in MATERIALS AND METHODS, except that the araC concentration was varied as indicated.

(32). The effect of dCTP on the phosphorylation of araC is shown in Fig. 5. At low concentrations of dCTP the inhibition is simple competitive, with a $K_i$ of $5.4 \times 10^{-6}$ M. As the concentration of dCTP is raised, the inhibition pattern changes to mixed inhibition, with an increasing noncompetitive element.

dTTP and dUTP are phosphate donors to araC (see below). However, in the presence of 10 mM ATP, they act as inhibitors of the phosphorylation of araC (Fig. 6). The inhibition is "uncompetitive," although the divergence of the inhibited plots from the control line indicates that the inhibition is not simple uncompetitive. We had previously demonstrated that dTTP reverses the inhibition of CdR phosphorylation by dCTP (32). Although dTTP and dUTP are less effective phosphate donors in araC phosphorylation, they still partially reverse the inhibition of araC phosphorylation by dCTP at low araC concentrations (Fig. 7). Furthermore, in the presence of dCTP the inhibition by dTTP and dUTP is now simple uncompetitive.

*Phosphate donors.* CdR kinase can utilize a number of nucleoside triphosphates as phosphate donors. The abilities of nucleoside phosphates to donate a phosphate group to araC in both the presence and absence of sulfhydryl reagent are shown in Table 8. In contrast to other reports (31, 52), ATP, rather than UTP, was found to be the best phosphate donor under the conditions employed in our assay. However, we also found UTP to be the best phosphate donor at low nucleoside triphosphate concentrations. dUTP, which was almost inactive in the system of Grindey *et al.* (52), was the next best donor to ATP in the

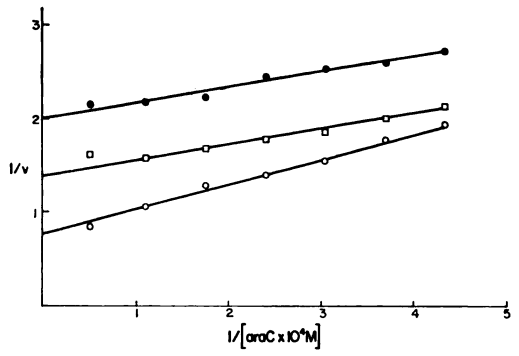


FIG. 6. *Inhibition of araC phosphorylation by dUTP and dTTP*

Inhibitor levels were: ○, none; ●, 5 mM dTTP-$Mg^{2-}$; □, 4 mM dUTP-$Mg^{2-}$. Other conditions were the same as in MATERIALS AND METHODS, except that the araC concentration was varied as indicated.

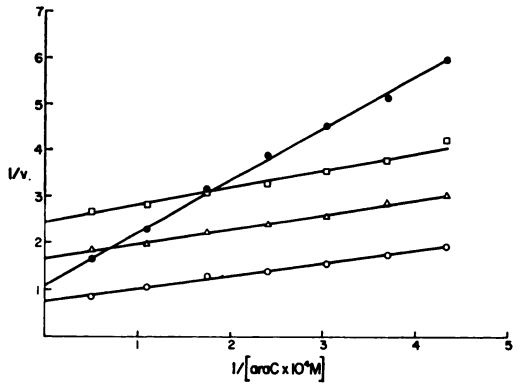


FIG. 7. *Inhibition of araC phosphorylation by dCTP, and effect of dTTP and dUTP on this inhibition*

Inhibitor levels were: ○, none; ●, 0.01 mM dCTP-$Mg^{2-}$; △, 0.01 mM dCTP-$Mg^{2-}$ + 4 mM dUTP-$Mg^{2-}$; □, 0.01 mM dCTP-$Mg^{2-}$ + 5 mM dTTP-$Mg^{2-}$. Other conditions were the same as in MATERIALS AND METHODS, except that the araC concentration was varied as indicated.

TABLE 8

*Phosphate donor specificity in araC phosphorylation*

The enzyme with dithiothreitol was first incubated for 1 hr at 22° in the presence of 50 mM dithiothreitol and 1 mg of bovine serum albumin per milliliter. The final assay concentration of dithiothreitol was 12.5 mM. Other conditions were the same as in MATERIALS AND METHODS, except that the ATP was replaced by the indicated concentrations of other nucleoside phosphates, and $MgCl_2$ was present in all cases in the same concentration as the nucleoside phosphate.

| Phosphate donor | Relative activity | |
|---|---|---|
| | − Dithiothreitol | + Dithiothreitol |
| 0.8 μmole of ATP | 100 | 100[a] |
| 0.8 μmole of dATP | 11 | 11 |
| 0.8 μmole of GTP | 84 | 90 |
| 0.8 μmole of dGTP | 26 | 22 |
| 0.8 μmole of CTP | 20 | 20 |
| 0.8 μmole of dCTP | <1 | <1 |
| 0.8 μmole of UTP | 76 | 35 |
| 0.8 μmole of dUTP | 95 | 58 |
| 0.8 μmole of dTTP | 53 | 31 |
| 0.2 μmole of CTP | | 25 |
| 0.2 μmole of dATP | | 17 |
| 0.08 μmole of UDP | 2.3 | 1.1 |
| 0.8 μmole of UDP | 1.5 | |
| 0.08 μmole of dTDP | 3.3 | 2.0 |
| 0.8 μmole of dTDP | 2.2 | |
| 0.8 μmole of ATP + 0.0004 μmole of UDP | 114 | 97 |
| 0.8 μmole of ATP + 0.004 μmole of UDP | 97 | |
| 0.8 μmole of ATP + 0.04 μmole of UDP | 37 | |
| 0.8 μmole of ATP + 0.8 μmole of dTTP | 63 | |

[a] 137% relative to the activity without dithiothreitol.

absence of sulfhydryl reagent, while UDP and dTDP gave essentially no activity. dATP, dGTP, dTTP, dUTP, and UTP all show a lower donor activity relative to ATP when araC is the acceptor rather than CdR, while CTP and GTP show approximately the same donor activity with either acceptor.[3] The presence of dithiothreitol significantly decreases the relative donor activity of UTP, dUTP, and dTTP but has little effect on other donors. Some special property for these donors is further indicated by the fact that they alone significantly reverse the inhibition by dCTP of the phosphorylation of araC (Table 9). dATP is a very poor donor and also inhibits phosphorylation by ATP. This would support the proposal that dATP could exert part of its inhibitory effect on DNA synthesis by inhibiting the phosphorylation of DNA precursors (53).

Lineweaver-Burk plots with ATP-$Mg^{2-}$ as the variable substrate yield biphasic curves. When araC is "saturating" there is a clear break in the curve (Fig. 8), with apparent $K_m$ values for the low and high ATP-$Mg^{2-}$ sections of $1.0 \times 10^{-4}$ M and $1.9 \times 10^{-4}$ M, respectively. Lowering the araC concentration accentuates the biphasic nature of the plot (not shown). dCTP, dTTP at concentrations where it is not a significant phosphate donor, and thymidylyl diphosphonate, which cannot donate phosphate by virtue of a methylene bridge, all act as simple competitive inhibitors against the "high $K_m$" portion of the ATP-$Mg^{2-}$ curve (Figs. 8 and 9). The apparent $K_i$ values for dCTP, dTTP, and thymidylyl diphosphonate are $5.7 \times 10^{-7}$ M, $4.7 \times 10^{-6}$ M, and $1.4 \times 10^{-3}$ M, respectively. The presence of the inhibitor straightens out the reciprocal ATP-$Mg^{2-}$ plot if araC is satu-

TABLE 9

*Reversal by nucleoside triphosphates of inhibition of araC phosphorylation by dCTP*

dCTP (3.2 mμmoles) was added to the normal incubation mixture as described in MATERIALS AND METHODS. Other nucleoside triphosphates (0.64 μmole) and $MgCl_2$ (0.64 μmole) were added as indicated. ATP (0.8 μmole) was always present.

| $Mg^{2+}$-nucleoside triphosphate | Relative activity |
|---|---|
| Control (dCTP, ATP) | 1[a] |
| +UTP | 2.27 |
| +dUTP | 2.83 |
| +dTTP | 1.96 |
| +GTP | 1.30 |
| +dGTP | 1.03 |
| +CTP | 0.94 |

[a] Absolute activity was 11.3% of that without dCTP.

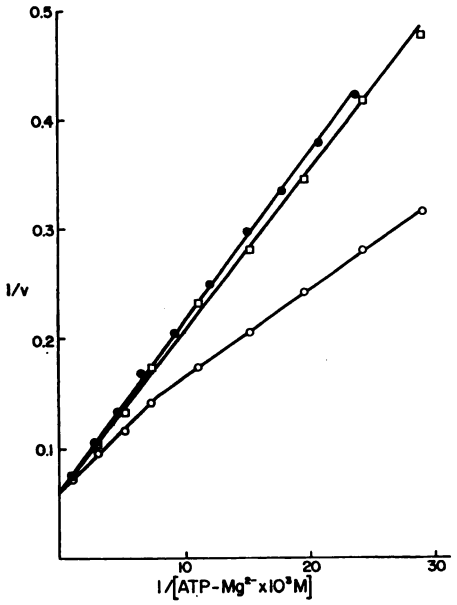


FIG. 8. *Effect of ATP-$Mg^{2+}$ concentration on the inhibition produced by dTTP and thymidylyl diphosphonate with araC as the phosphate acceptor*

Inhibitor levels were: ○, none; ●, 0.5 mM thymidylyl diphosphonate; □, 0.00125 mM dTTP. The araC concentration was 1.875 mM. Other conditions were the same as in MATERIALS AND METHODS, except that the total nucleoside phosphate and $MgCl_2$ were varied in a 1:1 ratio and the ATP-$Mg^{2+}$ concentrations were calculated.

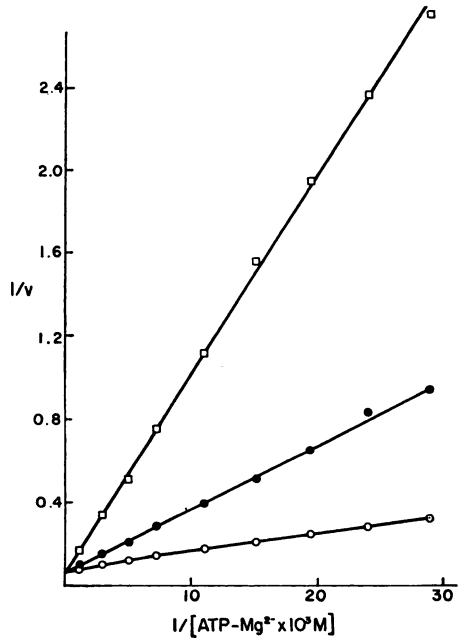


FIG. 9. *Effect of ATP-$Mg^{2+}$ concentration on the inhibition produced by dCTP with araC as the phosphate acceptor*

Inhibitor levels were: ○, none; ●, 0.001 mM dCTP; □, 0.004 mM dCTP. The araC concentration was 1.875 mM. Other conditions were the same as in MATERIALS AND METHODS, except that the total nucleoside phosphates and $MgCl_2$ were varied in 1:1 ratio and the ATP-$Mg^{2+}$ concentrations were calculated.

rating, but if it is not, then all the inhibitor plots are also nonlinear.

UDP shows unusual effects on araC phosphorylation (ATP as donor). Low concentrations can be slightly activating, but higher concentrations are inhibitory (Table 8).

## DISCUSSION

It has been proposed that nucleoside kinases are "salvage" enzymes (40, 41, 54). This may well be the primary role of CdR kinase in cell metabolism. Mammals have significant levels of CdR in the blood, and in the rat this deoxynucleoside is present at a concentration of about 0.04 mM (55, 56). On the other hand, blood levels of TdR are very low and thymine is utilized only very poorly. The persistence of CdR presumably results from the inability of pyrimidine phosphorylases to utilize this compound

(57, 58). Thus most cells will have a constant supply of CdR, which can act as a precursor both of dTTP, through either CdR or dCMP deaminase (59), and of dCTP.

Both CdR and TdR kinases are found only in tissues containing some dividing cells. However the TdR/CdR kinase ratio can vary considerably from tissue to tissue. There are also temporal differences in the induction patterns between the two kinases, both in spleen cells after phytohemagglutinin stimulation[9] and in rat thymus after irradiation.[10] In the spleen TdR kinase reaches its highest activity some hours after the rate of DNA synthesis has begun to decline, while CdR kinase activity reaches a maximum at about the same time as the incorporation of $^3$H-TdR into DNA is at its highest level.

This is circumstantial evidence that CdR and TdR may have differing roles in cell metabolism. Grav (41) has suggested that TdR kinase is not primarily a salvage enzyme but that "some other mechanism may allow TdR kinase to participate in a *de novo* pathway for the synthesis of dTTP." This is not to say that the enzyme will not reutilize TdR produced by DNA breakdown within the cell or organ (54).

The highest levels of CdR kinase are found in lymphohematopoietic organs and tumors derived from them. Other tissues containing dividing cells have lower levels of the enzyme (Tables 2–4). This distinction between lymphoid and nonlymphoid tissues and the tumors derived from each of them becomes more striking when we correlate the CdR kinase levels with available values of the mitotic index. (Obviously a single value of the mitotic index is only a very approximate measure of a tissue's cell multiplication rate, as it is subject to animal strain and age as well as circadian variations, and to problems of defining which cells are in mitosis.) If we divide the specific activity of CdR kinase by the mitotic index for that tissue, we obtain a value called the *activity index*. This is an expression of the level of CdR kinase per dividing cell (Table 10). It can be seen that the activity index is higher in all lymphoid tissues than in any nonlymphoid tissue and that the activity indices of the most rapidly dividing nonlymphoid tissues (intestine, regenerating liver, Walker 256 carcinosarcoma) are all very low. Thus, although CdR kinase is confined to tissues with some dividing cells, there is not a direct correlation between the enzyme level and the cell proliferation rate.

It has often been supposed (e.g., ref. 84) that the level of TdR kinase closely parallels the cell multiplication rate. However, the data in Table 10 show that the same arguments used above for CdR kinase can be applied to this enzyme, except that the levels of TdR kinase in liver and regenerating liver are much closer to those of lymphoid tissues.

Since the glycosidic bond of cytosine nucleosides is resistant to cleavage, deamination to the noncytotoxic araU (85) might be expected to be not only a major catabolic pathway but also a detoxification mechanism. AraC is rapidly deaminated *in vivo* in the human (86), in mouse kidney homogenates (44), and by partially purified cytosine nucleoside deaminase (42). Nonetheless, araC has been found to be far more toxic to the mouse than to the rat, which contains very little deaminase (44). The rat can excrete considerable amounts of CdR in the urine (87, 88), but even if araC is very efficiently removed from the blood by this mechanism it is most unlikely that the rate of removal would approach that of deamination by animals with high levels of deaminase (86). This seems to indicate that the rate of phosphorylation to araCMP, thereby removing araC from the sphere of action of the nucleoside deaminase or the excretion mechanism, is the determining factor in the cytotoxicity of araC. Mouse tissues generally have lower CdR/araC phosphorylation ratios than rat tissues; this is especially pronounced in the spleen, which also has a

[9] D. H. Ives and H. Qavi, manuscript in preparation.

[10] D. H. Ives and D. Campbell, unpublished observations.

TABLE 10
*Relationship of levels of CdR and TdR kinases to the cell proliferation rate of rat and mouse tissues*

The CdR and TdR kinase levels are those of Tables 2–4. The mitotic indices are values from the indicated references.

| Tissue | Mitotic index[a] | Specific activity of kinase/mitotic index | | References |
|---|---|---|---|---|
| | | CdR | TdR | |
| | % | | | |
| Rat | | | | |
| Thymus | 0.27 | 8.2 | 29 | 60–62 |
| Bone marrow | 0.62 | 2.7 | 66 | 61–64 |
| Lymph nodes | 0.13 | 6.1 | 30 | 60, 61 |
| Spleen | 0.06 | 5.8 | 30 | 60 |
| 28-hr regenerating liver | 3.5 | 0.07 | 5.4 | 65, 66 |
| Intestine | 3.5 | 0.07 | 0.06 | 67, 68 |
| Testis | 0.8 | 0.04 | 0.23 | 69 |
| Lung | 0.17 | 0.56 | 2.2 | 70 |
| Kidney | 0.06 | 0.48 | 1.0 | 71, 72 |
| Liver | 0.04 | 0.23 | 12 | 73 |
| Muscle | 0.000 | | | 74 |
| Walker 256 carcinosarcoma | 2.5 | 0.15 | 9.2 | 63 |
| Mouse | | | | |
| Thymus | 0.29 | 6.6 | 40 | 75, 76 |
| Lymph nodes | 0.12 | 5.2 | 104 | 77 |
| Spleen | 0.10 | 18.4 | 124 | 78 |
| Intestine | 1.9 | 0.05 | 0.74 | 77, 79, 80 |
| Lung | 0.06 | 0.73 | 3.2 | 81 |
| Sarcoma 180 | 1.7 | 0.90 | 5.1 | 82 |
| L1210 leukemia | 0.9 | 3.7 | 5.2 | 64, 83 |

[a] When the stathmokinetic method was used, a mitotic time of 25 min was assumed (63).

very much higher level of the enzyme in the mouse. Mouse tissues might therefore be expected to phosphorylate araC more effectively than rat tissues. If this premise is correct, the presence of deaminase in some of the tumors tested will be of less significance to araC activity than CdR kinase levels and CdR/araC phosphorylation ratios. Among the tumors investigated, araC was previously reported to be ineffective against Walker carcinosarcoma and Novikoff hepatoma (3). The former has a very high undialyzed extract CdR/araC phosphorylation ratio, and the latter a very low CdR kinase level (Table 4). AraC was effective to varying degrees against all the other tumors (3), and all have at least moderate CdR kinase levels and low CdR/araC phosphorylation ratios, although there is no direct correlation between effectiveness and optimum dosage of araC and these two cellular parameters.

CdR kinase partly purified from calf thymus apparently catalyzes the phosphorylation of a number of deoxynucleosides. However, the various nucleosides show important differences in their interaction with the enzyme. Varying the phosphate acceptor, with saturating ATP as the donor, produces complex kinetics only when CdR is the substrate. The $K_m$ for CdR is lower than that for araC and much lower than that for GdR and AdR, indicating that the enzyme has the highest affinity for CdR. The phosphate acceptors all appear to inhibit each other's phosphorylation competitively. The apparent greater affinity for CdR is reflected by the observation that low levels of this substrate significantly decrease the rate of

araC phosphorylation and almost entirely prevent the phosphorylation of AdR and GdR. On the other hand, AdR and GdR will inhibit araC phosphorylation, but only poorly, and do not significantly inhibit CdR phosphorylation.

Inhibition by dCTP is complex against both araC and CdR, but araC is more sensitive to its inhibitory effects. dTTP, dUTP, and UTP are less effective in reversing inhibition by dCTP when araC rather than CdR is the substrate.

The activity of CdR kinase with a variety of phosphate donors is not fully understood. The data for relative activity at any one concentration of either phosphate donor or acceptor, or both, must be carefully interpreted. Both CTP and dATP, with optima of 2.5 and 5 mM, respectively, inhibit at higher concentrations, and dTTP, dUTP, and UTP become less effective phosphate donors relative to ATP as the CdR or araC concentration is increased. Most of the other nucleoside triphosphates appear to have a greater affinity for CdR kinase than ATP, so that in the presence of ATP and a competing triphosphate donor the rate of phosphorylation will be close to that observed with the competing triphosphate alone.

The kinetic studies show that the $K_m$ for ATP is higher and the $K_i$ for dCTP (against variable ATP) lower when araC is the phosphate acceptor rather than CdR. Again, dTTP, dUTP, and UTP are less effective in reversing the inhibition by dCTP when araC is the phosphate acceptor.

Higher concentrations of UDP have been found to inhibit araC phosphorylation, and dTDP and CDP are also inhibitors, but not as effective as UDP.[3]

The kinetic data presented here do not allow one to deduce a kinetic scheme for the enzyme. However, these and other data[3] indicate that the mechanism is complex.

Thus the utilization of araC is limited by relative concentrations of several classes of compounds, each acting in different ways and each tending to select for the normal substrate CdR. Approximate levels of a number of the nucleosides and nucleotides affecting CdR kinase have been reported. In rat thymus these are: dTDP, 0.03 mM; dTTP, 0.04 mM (89); dCTP, 0.008 mM (90, 91); UTP + GTP, 0.6 mM; ATP, 2 mM (91); and CdR, 0.1 mM (55). The UDP level in the thymus has not, to our knowledge, been reported, but in rabbit bone marrow it is 0.15 mM (92). All these levels are high enough to affect CdR kinase activity significantly. Partial resistance to araC in a mammalian cell line, apparently resulting from elevated dCTP levels within the cell, has been observed (93).

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