Some Observations on the Phosphorylation of Cytosine Arabinoside

DAVID KESSEL

Laboratories of Pharmacology, Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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SUMMARY

Phosphorylation of cytosine arabinoside, a requisite for drug action, was catalyzed by deoxycytidine kinase. This reaction was strongly inhibited by deoxycytidine and by deoxycytidine nucleotides. An examination of kinetic data indicates the K_i for deoxycytidine was 40-fold smaller than the K_m for cytosine arabinoside. This difference apparently explains one site of the reversal of cytosine arabinoside toxicity by deoxycytidine.

Cytosine arabinoside is a synthetic nucleoside with antineoplastic properties. Phosphorylation of araC[1] to nucleotides is a requisite for action of the drug (1); impaired phosphorylation is associated with insensitivity to araC in human (2) and animal (1, 3, 4) leukemias. Both araC phosphorylation (1, 3) and toxicity (5) are inhibited by CdR.

The mode of araC action was initially believed related to an interference with cytosine ribotide reduction (1), or to incorporation of araC into cell DNA (1, 6, 7). More recent reports (8, 9) have indicated competition between dCTP and araCTP for DNA polymerase. There are, therefore, at least two possible sites of reversal of araC toxicity by CdR, i.e., competition for nucleoside kinase (1, 3) and competition for DNA polymerase.

To assess the first above-mentioned possibility, an examination of the conversion of ^{3}H-araC[2] to the 5′ phosphate was undertaken. For this purpose, a preparation of CdR kinase, purified 150-fold from L1210 mouse leukemia cells, was used.[3] During all stages of purification, a constant ratio of CdR kinase to araC kinase activity was found.

Phosphorylation of araC was measured by the DEAE disc method (10, 11). The enzyme preparation employed was free from deoxycytidylate kinase, araC deaminase, and araC phosphorylase. The only product formed from araC, characterized by chromatography (4), was araCMP. The reaction required a divalent cation (Mg^{2+} and Mn^{2+} were optimal), and

[1] Abbreviations: araC, cytosine arabinoside; araCMP, araC monophosphate; araCTP, araC triphosphate; CdR, deoxycytidine; dCMP, CdR monophosphate; dCDP, CdR diphosphate; dCTP, CdR triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

[2] Data to be published elsewhere. A K_m of 11 μM for CdR was found, with competitive inhibition by araC ($K_i = 500$ μM) observed.

[3] Purchased from Schwarz BioResearch. The compound was labeled with tritium (5 C/mmole) predominantly in the 5 position of the pyrimidine ring, and was purified by paper chromatography in *n*-butanol–formic acid–water (77:10:13) before use.

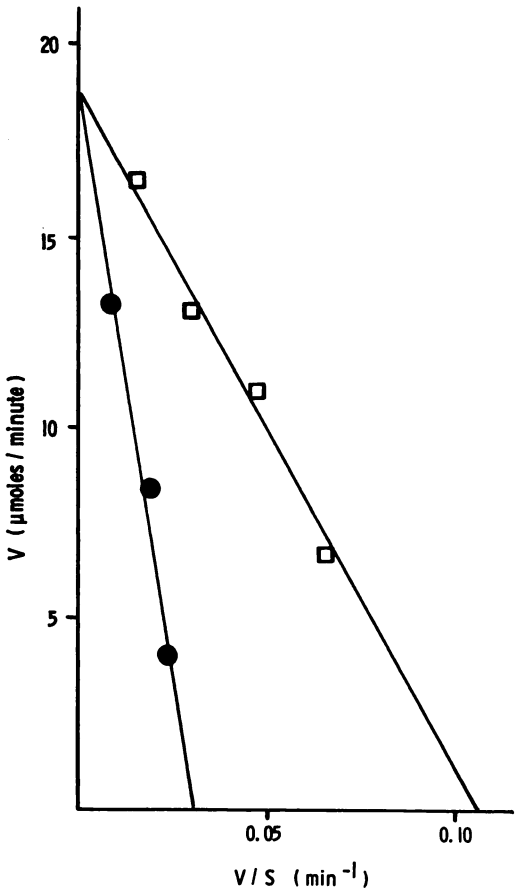

FIG. 1. *Inhibition of araC phosphorylation*

Inhibitor levels were (□) none, (●) 10 μM CdR. The complete system (50 μl) contained 150 mμmoles of MgATP, 3 μmoles of TES buffer at pH 7.0, 5 μg of purified enzyme, and 5–1000 μM ^{3}H-CdR.

a phosphate donor. Either ATP or UTP could be employed, as discussed below. The purified enzyme showed a broad pH optimum, ranging from 6 to 10. The apparent K_m for araC was 175 μM; inhibition by CdR ($K_i = 4$ μM) was competitive (Fig. 1). Competitive inhibition of araC phosphorylation by dCMP ($K_i = 4$ μM), and by dCDP and dCTP ($K_i = 2$ μM) was also found. Inhibition by araCMP was weaker ($K_i = 140$ μM).

A recent report indicated that UTP served as a more effective P donor for araC phosphorylation than did ATP (12). This finding could be used to argue against the concept that both CdR and araC are phosphorylated by a single enzyme. Since the apparent affinity of CdR kinase for araC and CdR, as estimated from comparative K_m and K_i values, differed, it seemed important to study the relative effectiveness of UTP and ATP at different substrate levels. UTP was the better P donor only if the *nucleoside* substrate level was low, relative to that required for CdR kinase saturation (Table 1). The basis of

TABLE 1

Phosphorylation of araC and CdR with ATP and UTP as P Donors

The complete system (50 μl) contained 150 mμmoles of MgATP or MgUTP, 3 μmoles of TES at pH 7.0, ^{3}H-araC or ^{14}C-CdR, and 5 μg of enzyme. Incubations were for 10 min at 37°.

Substrate	Level (μM)	Nucleotide 5'monophosphate formed (μμmoles)	
		With MgATP	With MgUTP
araC	50	50	90
	100	85	120
	5000	340	225
CdR	5	125	140
	100	350	300
	200	360	200

the UTP effect is therefore a concentration-dependent phenomenon. In other studies,[2] we found that high levels of araC or CdR noncompetitively inhibited transfer of the terminal phosphate from UTP to the substrate by CdR kinase.

Recent data (9, 13) have shown that virus infection of rabbit kidney cells results in a considerable increase in the intracellular dCTP pool, and in a decreased cell capacity for araC phosphorylation. The finding that dCTP inhibits araC phosphorylation offers an explanation for this report.

Studies on CdR kinase from L1210 mouse leukemia cells,[2] rabbit kidney cells (13), and calf thymus and mouse and rat tissues (14) all indicate a higher K_m for araC than for CdR. The ready reversal of araC toxicity by CdR in normal and tumor cells is explained, in part, by a block

of araC phosphorylation. The only other site of such reversal presently described is the competition for DNA polymerase noted above. The likelihood of competition between araC and CdR for entry into cells seems remote.[4]

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REFERENCES

1. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **14**, 333 (1965).
2. D. Kessel, *Proc. Am. Assoc. Cancer Res.* **8**, 36 (1967).
3. A. W. Schrecker and M. J. Urshel, *Proc. Am. Assoc. Cancer Res.* **8**, 58 (1967).
4. D. Kessel, T. C. Hall and I. Wodinsky, *Science* **156**, 1240 (1967).
5. J. S. Evans and G. D. Mengel, *Biochem. Pharmacol.* **13**, 898 (1964).
6. W. A. Creasey, R. J. Papac, M. E. Markiw, P. Calabresi and A. D. Welch, *Biochem. Pharmacol.* **15**, 1417 (1966).
7. S. Silagi, *Cancer Res.* **25**, 1446 (1965).
8. J. J. Furth and S. S. Cohen, *Proc. Am. Assoc. Cancer Res.* **9**, 23 (1968).
9. T. Ben-Porat, M. Brown and A. S. Kaplan, *Mol. Pharmacol.* **4**, 139 (1968).
10. F. J. Bollum and V. R. Potter, *J. Biol. Chem.* **233**, 478 (1958).
11. T. R. Breitman, *Biochim. Biophys. Acta* **67**, 153 (1963).
12. G. B. Grindey, L. O. Saslaw and V. S. Waravdekar, *Mol. Pharmacol.* **4**, 96 (1968).
13. A. S. Kaplan, M. Brown and T. Ben-Porat, *Mol. Pharmacol.* **4**, 131 (1968).
14. J. P. Durham and D. H. Ives, *Federation Proc.* **27**, 538 (1968).

[4] A study of araC transport in a sub-line of the L1210 mouse leukemia lacking CdR kinase, to be published elsewhere, indicated that the drug transport process was not saturated, at 37°, until nucleoside levels were far in excess of those employed therapeutically.

The Nature of Inhibitor Binding Sites in Butyrylcholinesterase

WILLIAM P. PURCELL AND JAMES G. BEASLEY

Department of Medicinal Chemistry, University of Tennessee College of Pharmacy, Memphis, Tennessee 38103

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SUMMARY

Evidence suggesting the presence of a specific "hydrophobic" binding site in horse serum butyrylcholinesterase (BuChE) is presented. The data indicate that the site exhibits definite size limitations. Assuming van der Waals forces to be dominant in the binding of the alkyl side chain of some N^1-alkylnipecotamide homologs to BuChE, an enzyme-alkyl chain distance of the complex of 4.1 A can be calculated for a C_4–C_{10} chain.

The controversy regarding the existence of an "anionic" site in butyrylcholinesterase (BuChE; acylcholine acylhydrolase, EC 3.1.1.8) has recently been discussed in a paper by Augustinsson (1) in which he proposed that this site is a "nonesteratic" site. The dominant factor in enzyme-substrate or enzyme-inhibitor complex formation is attributed to van der Waals forces (1).

Belleau (2, 3), in developing his macromolecular perturbation theory (MPT) of