Effects of Uracil Derivatives on Phosphorylation of Arabinosylcytosine

G. B. GRINDEY,[1] L. D. SASLAW, AND V. S. WARAVDEKAR

Microbiological Associates, Inc., Bethesda, Maryland 20016

(Received July 24, 1967)

SUMMARY

The probable mechanism underlying the effect of uridine in potentiating the biological activity of an antileukemic agent, arabinosylcytosine, is described. Only uridine, among other nucleosides, was capable of increasing the radioactivity in spleen after concurrent administration with ara-C-^{3}H. Uridine did not augment the radioactivity in spleen when given in conjunction with other labeled nucleosides. Enzymatic studies with a dialyzed splenic supernatant from BDF_1 mice bearing advanced Leukemia L1210 disclosed that ara-C-^{3}H was phosphorylated with uridine 5′-triphosphate at twice the initial rate as compared to adenosine 5′-triphosphate at equinolar concentrations. The presence of an enzymatic system in spleen was indicated with which uridine 5′-triphosphate is the active phosphate donor. This system is capable of phosphorylating deoxycytidine, but not cytidine. The effects of uridine on the biological activity of ara-C are thus considered a consequence of increased phosphorylation of ara-C in the presence of uridine 5′-triphosphate.

INTRODUCTION

Previous studies (1, 2) have shown that Urd,[2] administered in conjunction with a suboptimal dose of ara-C, resulted in prolonged survival of BDF_1 mice bearing Leukemia L1210 in comparison with administration of the suboptimal dose of ara-C alone. No potentiation occurred when Urd was replaced by dThd, ara-U, or several other uracil metabolites. In normal BDF_1 mice, inclusion of Urd with an optimal dose of ara-C in the daily regimen resulted in toxicity (2). Urd also enhanced the toxicity of a toxic dose of ara-C. In normal mice, an increase of 117% in radioactivity of spleen was observed 60 min after concurrent administration of Urd and ara-C-^{3}H as compared with ara-C-^{3}H alone (2). Lesser increases were also observed in marrow, and to a slight degree in thymus, but not in blood, liver, kidney, or brain.

Studies of other investigators, on interactions of ara-C with the metabolism of uracil derivatives, do not provide evidence for a distinct role for Urd in sparing either the antileukemic action or toxicity of ara-C. While Urd, ara-U, and dThd each inhibited deamination of ara-C in a cul-

[1] Present address: Department of Experimental Therapeutics, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, New York 14203.

[2] The following abbreviations have been used: Urd, uridine; ara-C, 1-β-D-arabinofuranosylcytosine; dThd, thymidine; ara-U, 1-β-D-arabinofuranosyluracil; PEP, phosphoenolpyruvate; Cyd, cytidine; dCyd, deoxycytidine; ATP, adenosine 5′-triphosphate, disodium salt; UTP, uridine 5′-triphosphate; dUrd, deoxyuridine; ara-CMP, 1-β-D-arabinofuranosylcytosine 5′-monophosphate; ara-CDP, 1-β-D-arabinofuranosylcytosine 5′-diphosphate; ara-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; Ado, adenosine; Guo, guanosine, dGuo, deoxyguanosine; U, uracil; dAdo, deoxyadenosine; UDPG, uridine diphosphoglucose; dUMP, deoxyuridylic acid; UMP, uridylic acid; UDP, uridine 5′-diphosphate; TTP, thymidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; dATP, deoxyadenosine 5′-triphosphate; dCTP, deoxycytidine 5′-triphosphate; dUTP, deoxyuridine 5′-triphosphate; CDP, cytidine 5′-diphosphate.

Mol. Pharmacol. 4, 96–103 (1968)

ture of carcinoma cells contaminated with mycoplasmata (3), the latter two compounds failed to spare the antileukemic activity of ara-C (2). Furthermore, chromatographic fractionation of spleens following administration of ara-C and Urd indicated that repression, or inhibition, of deamination was unlikely as the major mechanism by which Urd exerted a sparing action on utilization of ara-C in mice. No distinct variations in the patterns of pulse labeling with kidney and blood were noted with the use of both regimens which might have conceivably indicated relative differences in the degree of deamination.

More recently, Yushok (4) reported that Urd promoted phosphorylation of hexoses and decreased respiration in ascites cells. The significance of phosphorylation of ara-C for therapeutic activity was suggested by Chu and Fischer (5), who observed a markedly decreased conversion of ara-C to the corresponding 5′-phosphate esters in a subline of L5178Y cells resistant to ara-C.

These considerations led us to study relationships between uracil derivatives and phosphorylation of ara-C.

MATERIALS AND METHODS

1-β-D-Arabinofuranosylcytosine hydrochloride and ara-U were provided by the Cancer Chemotherapy National Service Center. Nucleosides, and nucleotides as sodium salts, were purchased from P-L Biochemicals, Inc. ATP, PEP, and Tris buffer were purchased from Sigma Chemical Co. Ara-C-^{3}H and Cyd-^{3}H were purchased from Schwarz BioResearch Inc. and dCyd-^{3}H was purchased from New England Nuclear Corp. The AG 50W-X8 (Cl^-, 200–400 mesh) and AG 1-X8 (Cl^-, 200–400 mesh) resins were purchased from Bio-Rad laboratories. Chromatographic columns were monitored at 280 mμ with a Gilson Medical Electronics absorption meter. All inorganic chemicals were purchased from J. T. Baker Chemical Co. All chemicals used for determination of radioactivity were purchased from Packard Instrument Co., Inc. except for dioxane, which was purchased from Fisher Scientific Co.

Male BDF_1 mice (19–25 g) obtained both from Laboratory Supply Co. and from the colony at Microbiological Associates, Inc., were maintained on Purina Laboratory Chow and water ad libitum. For the study on the effect of Urd on localization of labeled nucleosides (20 mg/kg of body weight) in spleen, an aqueous solution containing either 2 μC of the ^{14}C-labeled nucleoside or 5 μC of the ^{3}H-labeled nucleoside was injected subcutaneously in the right scapular region in normal male BDF_1 mice. A second group of mice was similarly injected except that Urd (480 mg/kg) was administered subcutaneously in the left scapular region immediately before injection of labeled nucleoside. Mice were sacrificed by cervical dislocation after 45 min, at which time maximal localization of ara-C occurs (2). Spleens were processed for determination of radioactivity by the method described previously (2).

In the study on the effects of various nucleosides and nucleotides on the localization of ara-C-^{3}H in spleen, equimolar concentrations of nucleosides and nucleotides comparable to Urd (480 mg/kg) were injected as described above. After 45 min, normal mice were sacrificed by cervical dislocation and radioactivity was determined in spleen. In the study on the localization of ara-C-^{3}H with increasing splenomegaly, BDF_1 mice bearing either Leukemia L1210 or a resistant subline (AW-75) of Leukemia L1210 (6) were used. For studying Leukemia L1210, each mouse was inoculated subcutaneously in the right inguinal region with 6.2 million splenic cells obtained from DBA/2 mice 7 days after inoculation with Leukemia L1210. The resistant subline (AW-75) was obtained from CDBA mice 7 days after inoculation with the AW-75 subline and each mouse was inoculated subcutaneously with 5.7 million cells in the right inguinal region. Each subsequent day, three mice from each leukemic group were injected subcutaneously in the right scapular region with ara-C (20 mg/kg) containing 5 μC of ara-C-^{3}H. After 45 min, the mice were sacrificed by cervical dislocation and radioactivity in spleen was determined.

Phosphorylation of ara-C-^{3}H *in vitro* was studied using the supernatant (2000 *g* for 10 min) of a 30% homogenate (0.25 M sucrose, Potter-Elvehjem homogenizer with Teflon pestle) of pooled spleens from BDF_1 mice bearing advanced (7 days) Leukemia L1210. Incubation mixtures (1.5 ml, pH 6.4) contained: ATP, 2.25 μmoles; PEP, 11.25 μmoles; NaF, 15 μmoles; $MgCl_2$, 3.5 μmoles; ara-C-^{3}H (2 μC) 7.5×10^{-3} μmole; 0.2 ml supernatant (4.48 mg protein); and 0.05 *M* Tris acid maleate buffer. After incubation at 37° for varying intervals, the mixture was heated for 2 min in boiling water. After centrifugation, the supernatant was chromatographed on columns (1×8 cm) of AG 1-X2 (Cl^-, 200–400 mesh). Ara-C was eluted by 50 ml of distilled water, ara-CMP by 50 ml of 0.005 N HCl, and di- and triphosphates by 150 ml of 0.1 N HCl. The elution scheme was devised with the use of appropriate reference standards. Radioactivity was determined in 0.5 ml of each fraction, and corrections for quenching were found to be unnecessary when the solvent system of Tye and Engel (7) was used. Further enzymatic studies were conducted following recentrifugation (10^5 *g* for 1 hr) and dialysis of the resulting supernatant in 7 liters of tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, changed twice during 72 hr. The Beckman Model L-2 ultracentrifuge with the Type 40 rotor was used for the high speed centrifugation. Each supernatant was stored at −15° until used. Protein determinations were done by the method of Lowry *et al.* (8).

RESULTS

As expected from the previous study (2), localization of ara-C *in vivo* was increased by Urd (Table 1). However Urd did not increase the localization of the other naturally occurring nucleosides. The uptake of Cyd-^{14}C and dUrd-^{14}C was decreased by 30% after concurrent administration of Urd. Since Urd increased localization neither of dCyd-^{3}H nor of other labeled nucleosides, in spleen, the effect of Urd appeared to be restricted to ara-C.

When other nucleosides were substituted for Urd at equimolar quantities, none of the other naturally occurring nucleosides increased the localization of radioactivity in spleen to the same extent as Urd (Table 2). Several of the other uracil derivatives increased the localization of radioactivity when given in conjunction with labeled ara-C, but generally not to the same extent as Urd. On the other hand, UTP, when administered in conjunction with labeled ara-C, produced an even greater increase in the localization of radioactivity in spleen than Urd. These results indicated that UTP is probably involved in the effect of Urd in increasing the localization of ara-C-^{3}H in spleen.

When the extent of labeling in spleen of untreated BDF_1 mice bearing Leukemia L1210 was studied, the concentration of radioactivity in the spleen tripled, after injection of ara-C-^{3}H, with increasing splenomegaly during the two days preceding death of the remaining mice (Fig. 1). In an analogous study, with mice bearing a subline resistant to the action of ara-C, the AW-75 subline of Leukemia L1210 (6), the concentration of radioactivity in the spleen did not increase with the advent of splenomegaly. Chu and Fischer (5) reported that a clone of L5178Y cells, resistant to growth inhibition by ara-C, was defective in the conversion of ara-C and dCyd to the corresponding 5′-phosphate esters. Chromatographic fractionation of the radioactive constituents in spleens, 45 min after administration of ara-C-^{3}H to mice bearing 7-day-old Leukemia L1210, using Reichard's column (9) as described previously (2), disclosed that 60% of the recovered radioactivity was attributable to ara-CMP. A similar study disclosed that only 14% was attributable to ara-CMP in spleen from BDF_1 mice bearing a 10-day-old AW-75 resistant subline of Leukemia L1210. Another study indicated that Urd was no longer capable of increasing the localization of radioactivity in spleen of mice bearing the AW-75 resistant subline of Leukemia L1210. Thus, the possibility that the effects *in vivo* of Urd and UTP in increasing the radioactivity in the spleen

TABLE 1
Effects of Urd on localization of nucleosides in normal spleen

Nucleoside	Without Urd (cpm/mg spleen)	With Urd (cpm/mg spleen)	Percent change in cpm/mg spleen
ara-C-^{3}H	88[a] (90, 85)	155 (150, 159)	+76
dCyd-^{3}H	207 (198, 216)	226 (197, 254)	+9
Cyd-^{14}C	380 (390, 370)	261 (248, 273)	−31
dThd-^{14}C	158 (151, 165)	167 (158, 176)	+3
dUrd-^{14}C	326 (332, 320)	234 (203, 265)	−28

[a] Averages (and individual values) are shown for two experiments as described in Materials and Methods

may be related to phosphorylation of ara-C was evaluated.

To study phosphorylation of ara-C-^{3}H, conditions for maximum phosphorylation of ara-C-^{3}H utilizing ATP as the phosphate donor were sought. The primary conditions, as described by Chu and Fischer (5), were modified by inclusion of PEP to regenerate ATP and addition of NaF to inhibit ATPases and phosphatases. Deamination of ara-C-^{3}H was negligible in the incubation system as evidenced by the use of methods described previously (2).

Phosphorylation studies with the low-speed supernatant showed that ara-C-^{3}H was phosphorylated in the presence of ATP over a wide pH range. Throughout the range from pH 5.2 to pH 8.5, the percentage of conversion varied between 20 and 25% tritiated nucleotides without evidence of a distinct optimum. In these studies, when ATP was used as the phosphate donor, the tritiated nucleotides formed were primarily ara-CDP and ara-CTP as characterized by column chromatography. The rate of phosphorylation of ara-C-^{3}H was linear during the first 30 min, and by 60 min a maximum of 50% conversion to nucleotide occurred (Fig. 2). With substitution of UTP for ATP, ara-C-^{3}H was phosphorylated at twice the initial rate as compared with ATP, and by 60 min the maximum conversion to nucleotide (primarily ara-CMP) was increased to 75%. Phosphorylation of dCyd-^{3}H at 45 min was slightly enhanced in the presence of UTP over that observed with ATP (Fig. 2). Cyd-^{3}H was phosphorylated to the same extent as dCyd-^{3}H in the presence of ATP, but only to a slight extent in the presence of UTP.

When other nucleotides were substituted for ATP, ara-C-^{3}H was phosphorylated to varying extents; TTP and UDP acted almost as well as ATP (Table 3). GTP and ITP served as phosphate donors to some extent, while CTP, dUTP, dATP, and dCTP showed little or no capability. The presence of Mg^{2+} in the incubation mixture was a requirement for phosphorylation with UTP and ATP. In the case of ATP, inclusion of PEP in the incubation mixture aided phosphorylation of ara-C-^{3}H, probably by regeneration of ATP, whereas in the case of UTP, PEP had very

TABLE 2
Effects of various nucleosides and nucleotides on the localization of ara-C-^{3}H in normal spleen

Compound	Percent change in cpm/mg spleen	Compound	Percent change in cpm/mg spleen	Compound	Percent change in cpm/mg spleen
UTP	163[a] (127, 198)	UMP	91 (89, 93)	ara-U	62 (49, 75)
Urd	147 (143, 150)	UDPG	78 (49, 107)	dGuo	39 (18, 59)
dUrd	131 (122, 140)	dAdo	71 (51, 91)	Guo	38 (36, 40)
UDP	120 (82, 157)	dCyd	71 (66, 75)	dThd	0 (−7, 7)
dUMP	106 (105, 107)	Cyd	66 (42, 89)	U	−6 (−30, 18)

[a] Averages (and individual values) are shown for two experiments as described in Materials and Methods.

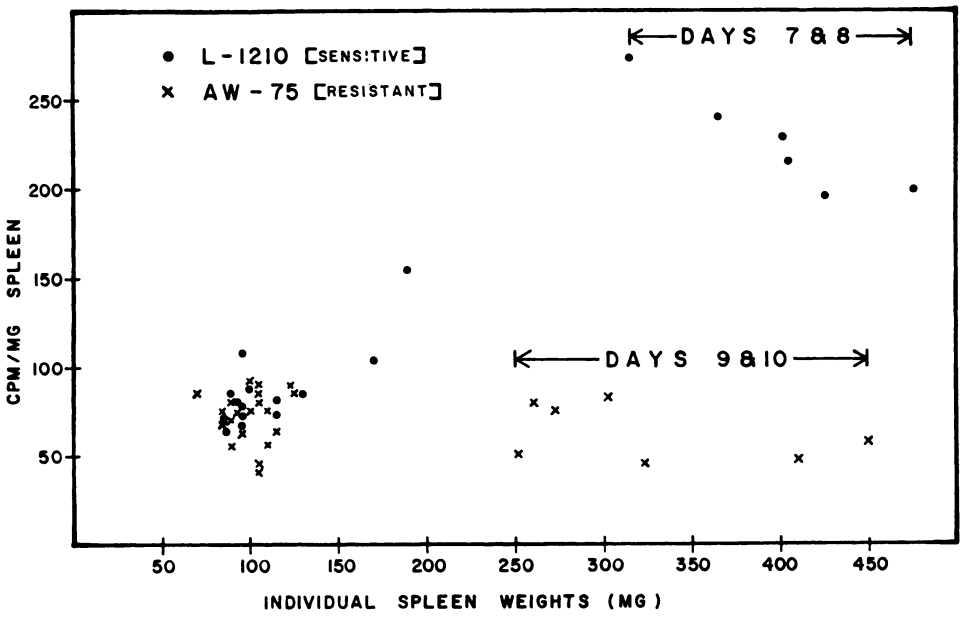

FIG. 1. *Comparison of localization of ara-C-^{3}H in spleen of mice bearing resistant and sensitive sublines of Leukemia L1210*

Each point is a value using one mouse.

little effect. Also, PEP itself was ineffective as a phosphate donor. However, none of these donors were as effective as UTP with which 71% conversion to nucleotide was achieved.

After high-speed centrifugation, followed by dialysis of the supernatant, UTP was still three times more effective than ATP as a phosphate donor to ara-C-^{3}H (Table 4).

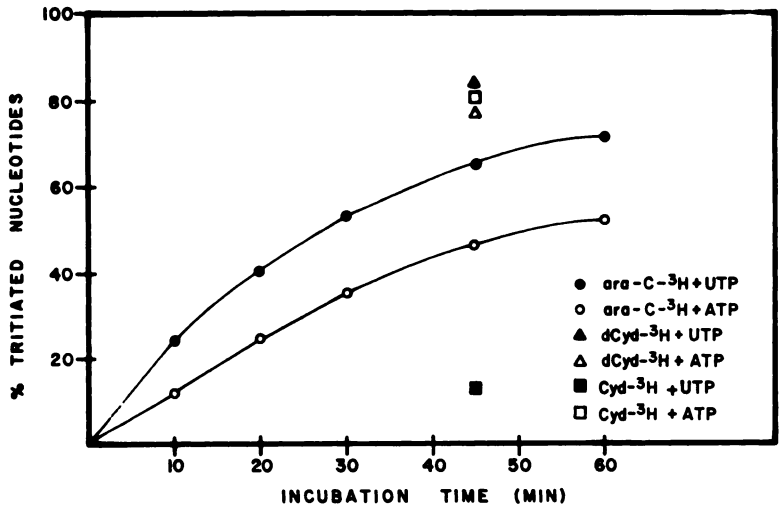

FIG. 2. *Comparison of phosphorylation in the presence of either ATP or UTP*

Incubation mixtures (1.5 ml, pH 6.4) contained: nucleotide, 2.25 μmoles; PEP, 11.25 μmoles; NaF, 15 μmoles; $MgCl_2$, 3.5 μmoles; labeled nucleoside (2 μC), 7.5×10^{-3} μmole; Tris acid maleate, 50 μmoles; and 0.2 ml of supernatant (4.48 mg protein). Incubation was for varying intervals at 37°.

TABLE 3

Phosphorylation of ara-C-^{3}H with various nucleotides

Incubation mixtures (1.5 ml, pH 6.4) contained: nucleotide, 2.25 μmoles; PEP, 11.25 μmoles; NaF, 15 μmoles; $MgCl_2$, 3.5 μmoles; ara-C-^{3}H (2 μC), 7.5×10^{-3} μmole; Tris acid maleate, 50 μmoles; and 0.2 ml of supernatant (4.48 mg protein). Incubation was for 45 min at 37°.

Nucleotide added	Percent tritiated nucleotides formed	Nucleotide added	Percent tritiated nucleotides formed
+UTP	71	+dATP	3
+ATP	48	+dCTP	1
+TTP	44	+dUTP	4
+GTP	35	None	3
+ITP	24	+UTP, −PEP	62
+UDP	43	+ATP, −PEP	26
+CTP	10	+UTP, $-MgCl_2$	15
+UDPG	5	+ATP, $-MgCl_2$	6

TABLE 4

Phosphorylation of ara-C-^{3}H by dialyzed and undialyzed supernatants

Incubation mixtures (1.5 ml, pH 6.4) contained: ara-C-^{3}H (2 μC), 7.5×10^{-3} μmole; $MgCl_2$, 3.5 μmoles; Tris acid maleate, 50 μmoles; NaF, 15 μmoles; and 0.2 ml of supernatant (2.08 mg of protein). The concentrations of the indicated phosphate donors were UTP, 2.25 μmoles; ATP, 2.25 μmoles; PEP, 11.25 μmoles. Incubation was for 45 min at 37°.

Phosphate donor in incubation mixture	Percent tritiated nucleotides formed	
	Crude supernatant, 2000 *g*	Dialyzed supernatant, 10^5 *g*
UTP, PEP	72	69
ATP, PEP	48	31
UTP	62	60[a]
ATP	26	23[a]

[a] NaF omitted.

DISCUSSION

Urd *in vivo* selectively increased the localization of ara-C-^{3}H in normal spleen (Table 1). The results with Cyd and dUrd indicate that Urd may be a precursor of these two nucleosides via salvage pathways. Furthermore, increased localization also occurred with several uracil derivatives (Table 2). In the phosphorylation studies *in vitro*, UTP increased phosphorylation of ara-C-^{3}H. Anderson and Brockman (10) have observed that phosphorylation of Urd per se to produce UMP is a rate-limiting step in the sequence Urd→UMP→UDP→UTP. It is considered likely that Urd is more readily converted to UDP and UTP *in vivo* than *in vitro* and that the phosphorylation of ara-C-^{3}H is facilitated primarily by the triphosphorylated derivative of Urd rather than by Urd directly. The effect of UTP on increasing the phosphorylation of ara-C-^{3}H was studied in leukemic spleen because of the high kinase activity. However, a low speed supernatant of normal spleen also utilized UTP and ATP in phosphorylation of ara-C and dCyd, but possessed only one-fifth of the enzymatic activity found in leukemic spleen.

The actual mechanism of stimulation of phosphorylation of ara-C-^{3}H by UTP can only be clarified by purification of the enzyme. However, several possibilities can be eliminated by considering the data. Since PEP alone in the incubation mixture was inactive in phosphorylation of ara-C-^{3}H, and UTP was still effective with the dialyzed supernatant, it is obvious that UTP acted without regenerating endogenous ATP. Also, since UTP was the sole phosphorylated compound added with the dialyzed supernatant in the incubation mixture, it appears that UTP was the direct phosphate donor to ara-C-^{3}H (Table 4). As Cyd-^{3}H and dCyd-^{3}H were each

converted to 80% nucleotide in the incubation mixture containing ATP, the possibility that ATP was unavailable for further phosphorylation of ara-C-^{3}H was eliminated. UTP was a more effective phosphate donor than ATP for ara-C-^{3}H, the rate of phosphorylation and the quantity of nucleotide being significantly increased with UTP. Kinetic study of the purified enzyme is necessary to delineate the mechanism.

Since ara-C has not yet been isolated as a natural constituent (11), it is likely that a specific enzymatic system for phosphorylation of ara-C does not exist and that ara-C is an unnatural substrate for an existing enzymatic system. As dCyd-^{3}H, but not Cyd-^{3}H, was phosphorylated in the presence of UTP (Fig. 2), dCyd kinase is probably the enzyme that phosphorylates ara-C. This observation is substantiated by the finding of Chu and Fischer (5) that extracts of cells resistant to the action of ara-C were defective in the conversion of ara-C and dCyd to the corresponding 5′-phosphate esters. Studies by Schnebrecker and Urshel (12) also substantiate the observation that ara-C is phosphorylated by dCyd kinase. The failure of the AW-75 subline to respond to treatment is ascribed to an insufficiency of phosphorylated derivatives of ara-C. Several investigators (5, 12–14) have indicated the importance of phosphorylation for the therapeutic action. The regimen comprising Urd and ara-C (2) not only provided increased quantities of phosphorylated derivatives of ara-C, but also elevated the concentration of the free agent in spleen. Kessel (14) has emphasized the desirability of providing for increased phosphorylation during therapy with ara-C.

While the present study was in progress, a novel role in phosphorylation was reported for UTP in comparison with ATP. Nakamura and Sugino (15) have observed that UTP was 66% more active than ATP as a phosphate donor when dCDP was used as a substrate with a 2800-fold purified calf thymus nucleoside diphosphokinase. Studies in our laboratory, with a 120-fold purified dCyd kinase from spleens of mice bearing Leukemia L1210, have similarly disclosed greater activity with UTP than ATP.

With respect to the therapeutic efficacy of ara-C, Chu and Fischer (16) demonstrated that nucleotides of ara-C inhibited CDP reductase and that other ribonucleotide reductases are similarly affected (17). However, as dThd also inhibits CDP reductase (18), though neither exhibiting antileukemic activity nor sparing the action of ara-C (2), the inhibition of reductases may not entirely account for the antileukemic activity of ara-C. The therapeutic advantage provided by ara-C toward Leukemia L1210 is considered to be partly due to the comparatively selective splenic uptake (2). While phosphorylation of ara-C is apparently mandatory, the relationship of increased levels of nucleotides of ara-C to the therapeutic activity have not yet been completely elucidated.

ACKNOWLEDGMENTS

This investigation was supported by Contract PH 43-64-911 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health. Part of this material appears in abstract form in *Proc. Am. Assoc. Cancer Res.* 8, 24 (1967) and in a thesis by G. B. Grindey in partial fulfillment of the requirements for the M.S. degree in the Department of Biochemistry, The George Washington University, 1967. Abbreviations of chemical compounds are in conformity with the IUPAC requirements.

REFERENCES

1. L. D. Saslaw, R. Tomchick, G. B. Grindey, I. Kline and V. S. Waravdekar, *Proc. Am. Assoc. Cancer Res.* 7, 62 (1966).
2. L. D. Saslaw, G. B. Grindey and V. S. Waravdekar, *Cancer Res.* in press.
3. C. G. Smith, H. H. Buskirk and W. L. Lummis, *Proc. Am. Assoc. Cancer Res.* 6, 60 (1965).
4. W. D. Yushok, *Proc. Am. Assoc. Cancer Res.* 7, 79 (1966).
5. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* 14, 333 (1965).
6. R. L. Dixon and R. H. Adamson, *Cancer Chemotherapy Rept.* 48, 11 (1965).
7. R. Tye and J. D. Engel, *Anal. Chem.* 37, 1225 (1965).

8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. P. Reichard, *Acta Chem. Scand.* **12**, 2048 (1958).
10. E. P. Anderson and R. W. Brockman, *Biochim. Biophys. Acta* **91**, 380 (1964).
11. S. S. Cohen, *Progr. Nucleic Acid Res.* **5**, 1 (1966).
12. A. W. Schrecker and M. J. Urshel, *Proc. Am. Assoc. Cancer Res.* **8**, 58 (1967).
13. A. W. Schrecker, J. A. R. Mead and M. J. Urshel, *Biochem. Pharmacol.* **15**, 1443 (1966).
14. D. Kessel, *Proc. Am. Assoc. Cancer Res.* **8**, 36 (1967).
15. H. Nakamura and Y. Sugino, *J. Biol. Chem.* **241**, 4917 (1966).
16. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **11**, 423 (1962).
17. A. P. Kimball, B. Bowman, P. S. Bush, J. Herriot and G. A. LePage, *Cancer Res.* **26**, 1337 (1966).
18. E. D. Whittle, *Biochim. Biophys. Acta* **14**, 44 (1966).