Biochemical Pharmacology, Vol. 34, No. 1, pp. 101–105, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

SALVAGE OF URIDINE IN THE MOUSE

EFFECT OF URIDINE PHOSPHORYLASE PRETREATMENT*

JAMES D. MOYER† and J. FRANK HENDERSON‡
Cancer Research Group (McEachern Laboratory) and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

(*Received* 29 *November* 1983; *accepted* 24 *May* 1984)

Abstract—Salvage of circulating nucleosides provides an alternative to *de novo* synthesis of nucleotides and may modify response to antimetabolites. We have investigated treatment with uridine phosphorylase as a means of inhibiting salvage of uridine *in vivo*. Examination of the metabolism of intravenous [^{3}H] uridine in mice revealed that 30–40% was salvaged by conversion to uracil nucleotides and the remainder was catabolized. In contrast, <0.3% of intravenous [^{3}H]uracil was salvaged. Addition of partially purified bacterial uridine phosphorylase to plasma produced a rapid phosphorolysis of uridine. *In vivo*, 1.5 hr after intravenous injection of 9 units of uridine phosphorylase, plasma activity (1.3 units/ml) was 65-fold greater than that of control mice. Pretreatment with uridine phosphorylase prior to administration of [^{3}H]uridine produced a marked (65–92%) but incomplete inhibition of salvage of uridine in all tissues examined. The dose required to produce 50% inhibition of uridine salvage at 1 hr was 2 to 2.5 units/mouse. The inhibition of nucleoside salvage by this approach may permit an evaluation of the role of nucleoside salvage in the supply of cellular nucleotides and the effects of concurrent inhibition of *de novo* and salvage pathways for nucleotide synthesis.

Cellular requirements for pyrimidine nucleotides can be provided by synthesis *de novo*, or by phosphoribosylation of preformed pyrimidine bases and phosphorylation of pyrimidine nucleosides (salvage synthesis). Several experimental antitumor agents act by inhibiting pyrimidine synthesis *de novo*: 6-azauridine, pyrazofurin, and *N*-(phosphonacetyl)-L-aspartate. The effectiveness of these drugs *in vivo* may be limited by the continued operation of the alternative salvage route to pyrimidine nucleotides. Indeed, provision of pyrimidine nucleosides has been shown to protect cells from the effects of inhibitors of synthesis *de novo* both *in vitro* [1–3] and *in vivo* [4]. Thus, a concurrent inhibition of both *de novo* and salvage pathways to pyrimidine nucleotides may prove more effective in cancer chemotherapy.

A number of approaches to inhibition of pyrimidine nucleoside salvage are possible including the use of inhibitors of the nucleoside transporter [5] or of uridine kinase [6]. To date, however, effective inhibition of uridine salvage *in vivo* has not been demonstrated. We have taken a different approach to preventing uridine salvage, an approach which is analogous to the use of asparaginase to prevent use of circulating asparagine. The present report gives our results on the use of *Escherichia coli* uridine phosphorylase to reduce the utilization of uridine present in plasma.

* Supported by the National Cancer Institute of Canada and by the Medical Research Council. J. D. M. was supported by a Fellowship from the Alberta Heritage Foundation for Medical Research.

† Current address: National Cancer Institute, Building 37, Room 5E26, Bethesda, MD 20205, U.S.A.

‡ To whom correspondence should be addressed.

MATERIALS AND METHODS

[5-^{3}H]Uridine and [5-^{3}H]uracil were purchased from Moravek Biochemicals (Brea, CA), at specific activities of 25 Ci/mmole, and lyophilized before use to remove any residual ^3H_2O.

Uridine phosphorylase was purified from lyophilized *E. coli* B2 (Sigma Chemical Co., St. Louis, MO) as described by Leer *et al.* [7] through the first DEAE step. The enzyme used for these studies had a specific activity of 15 units/mg protein when assayed at 37° by the procedure of Razzell and Khorana [8]. One unit is the amount of enzyme which converts 1 μmole/min of uridine to uracil. This assay was also used to measure plasma uridine phosphorylase.

The phosphorolysis of [^{3}H]uridine in plasma was measured by separating uridine and uracil by thin-layer chromatography on cellulose plates eluted with water-saturated *n*-butanol. The R_f values were 0.39 (uracil) and 0.26 (uridine). Neutralized perchloric acid extracts (10 μl) were chromatographed together with uracil and uridine carriers. The spots corresponding to the carriers were located under ultraviolet light and scraped from the plate; radioactivity was determined by liquid scintillation counting.

To measure the distribution of radioactivity after injection of [^{3}H]uridine, tissues were rapidly removed and frozen in liquid nitrogen until processed further. The remaining organs along with the skin were also frozen and processed as the "carcass". The tissues were weighed and homogenized in 8–10 vol. of cold 0.3 M perchloric acid using a motor-driven glass–Teflon homogenizer. The frozen "carcass" was pulverized and then homogenized with a Polytron homogenizer. The acid-soluble fraction was neutralized with a (1:3) solution of tricaprylyl tertiary amine (Alamine 336) in 1,1,2-trichlorotrifluoro-

ethane [9] and then stored at −20° until analyzed by chromatography. The acid-insoluble fraction was washed twice with cold 0.3 M perchloric acid, then suspended in 0.3 M potassium hydroxide, and incubated at 37° overnight. These conditions produce a quantitative hydrolysis of RNA to acid-soluble nucleoside monophosphates [10]. The sample was then chilled, made 0.6 M in perchloric acid, and the precipitate removed by centrifugation; radioactivity in the soluble fraction was determined as a measure of label in RNA.

The nucleotides in the neutralized acid-soluble extracts were hydrolyzed to 5′-monophosphates by incubation at 37° for 10 min with an equal volume of 0.05 units/ml phosphodiesterase (Type VII, Sigma) in 0.05 M 2-amino-2-(hydroxymethyl-1),3-propanediol(Tris)-acetate (pH 7.8) and 2 mM magnesium chloride. The samples were then placed in a boiling water bath for 1 min and then chilled to 0°; protein was removed by centrifugation. Control experiments indicated that this procedure gives >98% conversion of nucleoside triphosphates to the corresponding 5′-monophosphates. Radiolabeled uracil nucleotides (as 5′-UMP) were determined by chromatography of the hydrolyzed extracts on a Whatman SAX column, 25 cm × 4.6 mm. The column was eluted at 2 ml/min with 0.03 M ammonium phosphate adjusted to pH 3.3 with phosphoric acid. Under these conditions, UMP had a retention time of 4.0 min and was clearly resolved from all other detectable radioactive or ultraviolet absorbing compounds. Fractions (1 ml) were collected, and radioactivity was measured by liquid scintillation counting at an efficiency of 28%.

The radiolabel present as unmetabolized uridine was determined by reversed-phase liquid chromatography. A 15 cm × 3.9 mm, 5 μM particle-size C_{18} column (Waters Associates, Milford, MA) was eluted at 1.0 ml/min with 0.2 M ammonium phosphate, pH 5.1. Uridine and uracil had retention times of 5.7 and 2.8 min respectively. Fractions (1 ml) were collected, and radioactivity was determined by liquid scintillation counting.

RESULTS

Plasma uridine phosphorylase. Mouse plasma has very low uridine phosphorylase activity (Fig. 1). Incubation of trace amounts of [^{3}H]uridine in normal plasma gave <20% conversion to uracil after 5 min at 37°. However, addition of *E. coli* uridine phosphorylase to the plasma resulted in a rapid conversion of uridine to uracil (Fig. 1). At a uridine phosphorylase addition of 1.0 units/ml, >90% of the uridine was converted to uracil in 15 sec. No further conversion of uracil in plasma to other catabolites was observed when the incubation mixture was analyzed by either thin-layer chromatography or liquid chromatography.

Following administration of *E. coli* uridine phosphorylase to mice by either intravenous or by intraperitoneal injection, high activities were found in the plasma (Fig. 2). The activity was somewhat higher initially following intravenous injection, but the levels remained high for a longer time following intraperitoneal injection. Subcutaneous injections

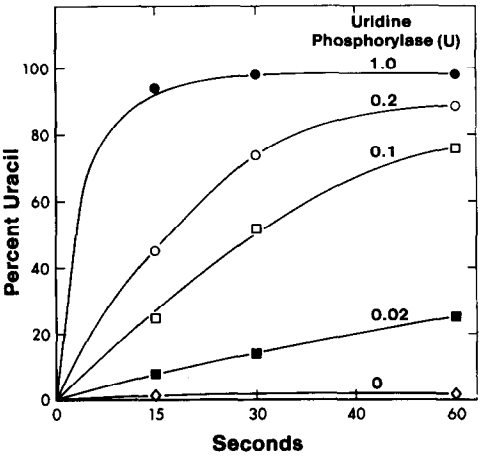

Fig. 1. Phosphorolysis of uridine in mouse plasma. Mouse plasma (1 ml) was supplemented with the indicated amount of *E. coli* uridine phosphorylase and 0.05 μM [^{3}H]uridine. Incubation was at 37°. At the indicated times, a portion was removed, perchloric acid extracts were prepared, and uracil and uridine were determined by thin-layer chromatography as described under Materials and Methods.

were found to give much lower plasma levels of activity (data not shown). Clearance of the activity following intravenous injection was first order with a half-life of 4–5 hr (Fig. 2). The effect of repeated injections, where immune response may be more of a factor, has not been determined.

Disposition of [^{3}H]uridine and [^{3}H]uracil in the mouse. Analysis of plasma samples by high performance ligand chromatography (HPLC) after

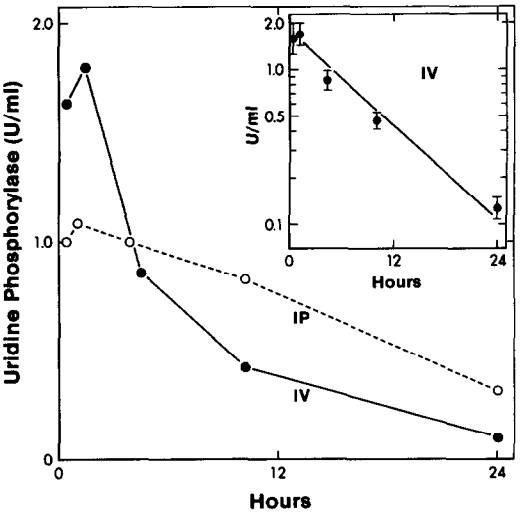

Fig. 2. Clearance of uridine phosphorylase from the circulation. ICR mice received 5 units uridine phosphorylase by the tail vein or by intraperitoneal injection as indicated. Blood samples were obtained from the retro-orbital axis with heparinized micropipettes. Uridine phosphorylase activity in the plasma was determined spectrophotometrically as described under Materials and Methods. Each value is the mean of determinations from four to eight mice. The inset gives the mean ± S.D. of the data from the intravenous injection as a semi-log replot.

Table 1. Salvage of uridine and uracil by mouse tissues*

Tissue	Nucleotide formation (% of total radiolabel injected)	
	Uridine	Uracil
Liver	6.0	0.014
Kidney	2.1	0.061
Lung	0.37	0.021
Spleen	0.19	0
"Carcass"	21.0	0
Total	29.7	0.096

* Male ICR mice (25 g) received 20 μCi of [^{3}H]uracil or [^{3}H]uridine by intravenous injection. After 30 min the mice were killed, rapidly dissected, and frozen in liquid nitrogen. "Carcass" refers to all the remaining tissues including skin after removal of liver, kidney, lung and spleen. Extracts were prepared, and labeled nucleotides were determined as given under Materials and Methods. The average overall recovery of injected radioactivity was 88% for uracil and 89% for uridine. Values are the means from four mice (for uridine) and three mice (for uracil), and are typical of results from at least two experiments.

intravenous injection of [^{3}H]uridine into untreated mice indicated that 30 min after infection virtually all the radiolabel present was associated with catabolites and >4% was present as uridine. Thus, 30 min was chosen as an appropriate time to evaluate the extent of uridine salvage.

Examination of acid-soluble extracts of mouse tissues following injection of [^{3}H]uridine revealed that both anabolism and catabolism were quantitatively important (Tables 1 and 2). In all tissues examined except erythrocytes, labeled uracil nucleotides were easily detected and accounted for 25–54% of the total acid soluble radioactivity (Table 2). The kidneys and liver were particularly effective in producing uracil nucleotides from circulating uridine. Radiolabeled water, released during the catabolism of uridine, was also a major component of the total acid-soluble fraction. A further analysis of the catabolites was not performed.

By performing a "balance-sheet" accounting of the radiolabel we calculated that approximately 30% of the circulating uridine was salvaged as uridine nucleotides (Table 1). Subsequent studies (not shown) have indicated that an additional 10% of the label may be present in an acid-soluble form 30 min after injection.

In contrast to the results with uridine, uracil was very poorly utilized (Table 1). Measurable uracil nucleotide formation was only observed in liver, kidney and lung. In the carcass, uracil nucleotides were not seen although levels as low as 0.3% of the total radioactivity could be detected. Thus, overall salvage of uracil was a minimum of 0.096% (Table 1) and a maximum of 0.3% based on the detection limits of this study. Uridine is, therefore, approximately 100-fold more efficiently salvaged than uracil (30 vs $\leq$0.3%).

Effect of uridine phosphorylase. Pretreatment of mice with 9 units of *E. coli* uridine phosphorylase produced large reductions in the salvage of uridine (Table 2) as measured 1 hr after enzyme administration. Uracil nucleotide formation was 78–93% lower in all tissues examined. In the most active tissues, such as liver and kidney, the total radioactivity in the tissues was also reduced. In contrast, the levels of catabolites, including ^3H_2O, were generally higher in mice pretreated with uridine phosphorylase. The rate of phosphorolysis of uridine in plasma was also determined for the mice studied in this experiment (Table 2). At the time of dissection, plasma samples were obtained, and 1 μCi of [^{3}H]uridine was added to a 100-μl aliquot of plasma. These plasma samples were incubated at 37° and the conversion of the added uridine to uracil was measured. Plasma from mice treated with uridine phosphorylase converted 98% of the uridine to uracil in 1 min, whereas plasma from the controls only phosphorolyzed 3–11% in 1 min.

Although plasma uridine phosphorylase activity increased in direct proportion to the dose (Fig. 3), inhibition of uridine salvage reached a maximum value at 10–25 units per mouse. At this dose 95–92% reduction of uracil nucleotide formation was seen for liver, kidney and spleen (Fig. 4). The dose–response

Table 2. Effect of uridine phosphorylase pretreatment on the salvage of circulating uridine*

	Saline controls			Uridine phosphorylase		
	Total	Uracil nucleotides	^3H_2O	Total	Uracil nucleotides	^3H_2O
Kidney	330 ± 60	170 ± 30	42 ± 9	160 ± 38	14 ± 5.0	37 ± 8.9
Liver	210 ± 29	110 ± 13	38 ± 3.8	120 ± 22	8.9 ± 4.0	39 ± 3.0
Lung	140 ± 25	66 ± 12	37 ± 11	110 ± 44	24 ± 31	58 ± 17
Plasma	62 ± 10	ND†	41 ± 12	74 ± 13	ND	45 ± 14
Duodenum	130 ± 3.8	66 ± 25	26 ± 8	75 ± 18	10 ± 10	32 ± 9.3
Spleen	130 ± 5.8	50 ± 3.6	57 ± 13	83 ± 25	6.7 ± 2.9	54 ± 19
"Carcass"	86 ± 9.3	22 ± 4.9	27 ± 8.7	84 ± 7.8	7.0 ± 3.5	38 ± 7.8

* ICR mice (25 g) received saline or 9 units of uridine phosphorylase by intravenous injection. After 1 hr each mouse received 10 μCi of [^{3}H]uridine by intravenous injection. Thirty minutes later the mouse was killed and tissues were prepared as described under Materials and Methods. All values are means ± S.E.M. for three (controls) or four (treated) mice, expressed as 10^{-3} × cpm/g tissue wet weight.

† ND = not determined.

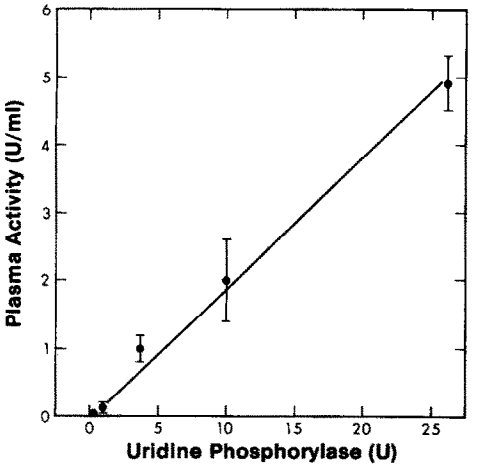

Fig. 3. Dose–response relationship for plasma uridine phosphorylase levels. ICR mice received the indicated dose of uridine phosphorylase by intraperitoneal injection 1.5 hr before determination of plasma activity. The values given are the mean ± S.D. for four mice.

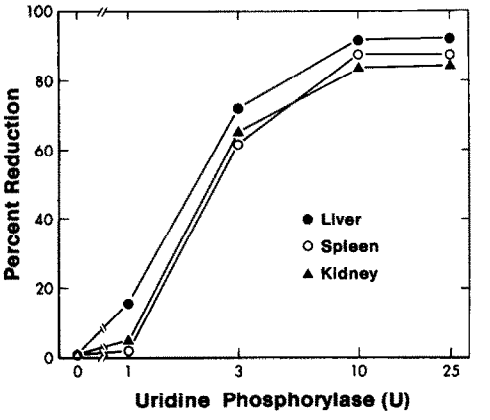

Fig. 4. Relation of enzyme dose to reduction of uridine salvage. ICR mice received the indicated dose of uridine phosphorylase by intraperitoneal injection 1 hr prior to intravenous injection of 20 μCi of [^{3}H]uridine. Uracil nucleotides in liver (●), spleen (○) and kidney (▲) were determined as described under Materials and Methods. The mean control (no enzyme) values for labeled uracil nucleotides were 320 × 10^3, 190 × 10^3, and 470 × 10^3 cpm/g wet weight in liver, spleen and kidney respectively. The values indicated on the chart are the means of measurements on four mice.

curve for all three tissues was essentially identical. A similar decrease in the radiolabel incorporated into tissue RNA at 30 min was observed (data not shown). The ED_{50} as assessed at 1 hr after administration of enzyme was 2 to 2.5 units/mouse.

DISCUSSION

Uridine is present in micromolar concentrations in plasma of mice, rats, and humans [11, 12] and is rapidly cleared from the circulation with a half-life of several minutes in rats [12] and mice [13]. Uridine is an efficient precursor of uracil nucleotides in the mouse in contrast to uracil which is almost entirely degraded (Tables 1 and 2). Although the current report focuses on the salvage of uridine and uracil by normal tissue, tumors also appear to more effectively utilize uridine rather than uracil. For example, uridine but not uracil reverses the effect of an inhibitor of *de novo* pyrimidine synthesis in hepatoma cells [14]. Uracil is also <10% as efficient as uridine as a precursor of uracil nucleotides in L1210 tumors grown subcutaneously.* The results reported here extend earlier reports that uracil is incorporated into uracil nucleotides of various murine tissues, although most (>94%) is degraded *in vivo* [15–17]. These results also suggested to us that injection of uridine phosphorylase may serve to convert plasma from a conduit to a sink for uridine by producing a rapid cleavage of uridine to the poorly urilized uracil. This approach has proven workable: prior treatment with uridine phosphorylase results in a marked reduction in salvage of uridine (Table 2, Fig. 4).

Although the efficiency of uridine salvage is greatly reduced as indicated by greatly decreased nucleotide formation, the steady-state concentrations of uridine in plasma were not measured in the study. The very rapid phosphorolysis of uridine in animals pretreated with uridine phosphorylase makes this technically difficult, since phosphorolysis would be very extensive in even the brief period following collection of blood and preceding the inactivation of the enzyme. The possibility remains, therefore, that an increased contribution of uridine to the circulation compensates for the increased rate of phosphorolysis, resulting in little or no change in steady-state concentrations.

Some tumours as well as intestinal mucosa have been shown to have lowered pools of pyrimidine nucleotides following administration of *N*-(phosphoacetyl)-L-aspartate [18, 19]. The effect of this drug on UTP and CTP pools of Lewis lung or L1210 tumors was marked within only 3 hr of treatment. This suggests that turnover of these pools is very rapid and that the inhibition of salvage produced here will be adequate to assess the contribution of salvage to nucleotide pools *in vivo*. Uridine phosphorylase treatment may also be of use in increasing or extending the anti-tumor action of the previously identified inhibitors of pyrimidine synthesis *de novo*; the relevant studies are in progress.

In addition to uridine, other nucleosides such as cytidine, deoxycytidine, and thymidine can be salvaged and thereby reduce dependence on pyrimidine synthesis *de novo*. The present approach has focused on uridine as a source of pyrimidines because it is present in highest concentrations in human plasma. The use of enzymes is not limited to uridine phosphorylase, however, and others such as thymidine phosphorylase may find use as biochemical tools, alone or in combination with drugs, such as methotrexate or 5-fluorodeoxyuridine, that interfere with the synthesis of thymine nucleotides.

* J. D. Moyer *et al.*, unpublished results.

REFERENCES

1. E. A. Swyrd, S. S. Seaver and G. R. Stark, *J. biol. Chem.* **249**, 6945 (1974).
2. P. G. W. Plagemann and M. Behrens, *Cancer Res.* **36**, 3807 (1976).
3. E. C. Cadman, D. E. Dix and R. E. Handschumacher, *Cancer Res,* **38**, 682 (1978).
4. R. K. Johnson, *Biochem. Pharmac.* **26**, 81 (1977).
5. A. R. P. Paterson, N. Kolassa and C. E. Cass, *Pharmac. Ther.* **12**, 515 (1981).
6. N. K. Ahmed and A. D. Welch, *Cancer Res.* **39**, 3102 (1979).
7. J. C. Leer, K. Hammer-Jespersen and M. Schwartz, *Eur. J. Biochem.* **75**, 217 (1977).
8. W. E. Razzell and H. G. Khorana, *Biochim. biophys. Acta* **28**, 562 (1958).
9. J. X. Khym, *Clin. Chem.* **21**, 1245 (1975).
10. H. N. Munro and A. Fleck, *Meth. biochem. Analysis* **14**, 113 (1966).
11. J. M. Karle, L. W. Anderson, D. D, Dietrick and R. L. Cysyk, *Analyt. Biochem.* **109**, 41 (1980).
12. J. D. Moyer, J. T. Oliver and R. E. Handschumacher, *Cancer Res.* **41**, 3010 (1981).
13. T. Yngner, I. Petersen and L. Lewan, *Int. J. Biochem.* **8**, 395 (1977).
14. E. Olah, M. S. Lui, D. Y. Tzeng and G. Weber, *Cancer Res.* **40**, 2869 (1980).
15. R. J. Rutman, A. Cantarow and K. E. Paschkis, *Cancer Res.* **14**, 119 (1954).
16. N. K. Chaudhuri, B. J. Montag and C. Heidelberger, *Cancer Res.* **18**, 318 (1957).
17. C. Heidelberg, K. C. Leibman, E. Harbers and P. M. Bhargava, *Cancer Res.* **17**, 399 (1957).
18. J. D. Moyer, P. A. Smith, E. J. Levy and R. E. Handschumacher, *Cancer Res.* **42**, 4525 (1982).
19. T. W. Kensler, G. Mutter, J. G. Hankerson, L. J. Reck, C. Harley, N. Han, B. Ardalan, R. L. Cysyk, R. K. Johnson, H. N. Jayaram and D. A. Cooney, *Cancer Res.* **41**, 894 (1981).