Ribose-transfer Activity from Uridine to 5-Fluorouracil in Ehrlich Ascites Tumor Cells

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Anabolism of 5-fluorouracil (5-FU) in the presence of uracil was examined using the cell-free extract of Ehrlich ascites tumor cells. FU-nucleoside formation from 5-FU with ribose 1-phosphate (R-1-P) or 2'-deoxyribose 1-phosphate was not readily inhibited even by the addition of uracil at 100 times higher concentration than 5-FU. FU-nucleotide formation from 5-FU with R-1-P and adenosine 5'-triphosphate or with 5-phosphoribosyl 1-pyrophosphate was slightly reduced as the concentration of uracil was increased. It was also found that 5-fluorouridine (5-FUR) was produced by "nucleoside *N*-ribosyltransferase," transferring a ribose moiety from uridine (UR) to 5-FU directly. This activity might play a role in the preferential formation of 5-FUR. However, 5-fluoro-2'-deoxyuridine was not produced by directly transferring a deoxyribose moiety. On the basis of several column chromatographies and characterization of kinetics, pH dependency, and response to inhibitors, the enzyme protein of the ribosyltransferase could not be distinguished from that of the phosphorylase.

Key words: Nucleoside *N*-ribosyltransferase — 5-Fluorouracil — Uridine — Uridine phosphorylase — Thymidine phosphorylase

5-Fluorouracil (5-FU⁴) has been widely used for cancer treatment since its synthesis as an analog of uracil,¹⁾ and is known to be an antimetabolite which inhibits DNA²⁻⁷⁾ and RNA⁸⁻¹¹⁾ synthesis. But, surprisingly, previous studies showed that co-administration of uracil with 5-FU increased the antitumor activity of 5-FU *in vivo*.¹²⁾ Ikenaka *et al.*¹³⁾ demonstrated that the addition of uracil inhibited the degradation of 5-FU in liver slices, whereas uracil did not compete in FU-nucleotide formation with 5-FU, as, for example, the presence of 2,500 μ M uracil (1,000 times the concentration of 5-FU) with 2.5 μ M 5-FU did not inhibit FU-nucleotide synthesis in Yoshida sarcoma cells.

It has been thought that the anabolism of 5-FU toward FU-nucleotides includes three pathways: (1) catalyzed by uridine (UR) kinase¹⁴⁾ with adenosine 5'-triphosphate (ATP) and 5-fluorouridine (5-FUR) formed from ribose

1-phosphate (R-1-P) and 5-FU by UR phosphorylase [EC 2.3.2.3],¹⁵⁾ (2) catalyzed by 2'-deoxythymidine (TdR) kinase¹⁶⁾ with ATP and 5-fluoro-2'-deoxyuridine (5-FUdR) formed from 2'-deoxyribose 1-phosphate (dR-1-P) and 5-FU by TdR phosphorylase [EC 2.3.2.4],¹⁷⁾ and (3) with 5-phosphoribosyl 1-pyrophosphate (PRPP) and 5-FU catalyzed by phosphoribosyltransferase [EC 2.4.2.10].¹⁸⁾ However, these reactions alone do not appear to account for the fact¹³⁾ that uracil did not compete with 5-FU in FU-nucleotide formation. The possibility must be entertained that other pathways may exist by which 5-FU could be anabolized preferentially compared to uracil.

In this regard, De Verdier and Potter¹⁹⁾ reported the transfer reaction of a ribose moiety from one nucleoside to another base, and showed that 5-FU had a strong inhibitory effect on ribose-exchange from uridine to uracil in the supernatant of Dunning LC 18 hepatoma. Formation of ribothymidine by the ribose-transfer reaction from UR to thymine with the cell-free extract of Ehrlich ascites tumor cells was also demonstrated by Yano and Tamemasa.²⁰⁾ They reported that the enzyme "nucleoside *N*-ribosyltransferase" [EC 2.4.2.5] catalyzed this ribose-transfer reaction, although the enzyme protein has not yet been identified.

In this study, we tried to determine if the transfer reaction of a pentose moiety also exists from UR or 2'-deoxyuridine (UdR) to 5-FU, using the cell-free extract of Ehrlich ascites tumor cells, and we discuss the possibility that this reaction may play a role in preferential 5-FU anabolism toward FU-nucleotides even in the presence of uracil.

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⁴ The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUR, 5-fluorouridine; 5-FUdR, 5-fluoro-2'-deoxyuridine; UR, uridine; UdR, 2'-deoxyuridine; TdR, 2'-deoxythymidine; FUMP, 5-fluorouridine 5'-monophosphate; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; FU-nucleotides, nucleotides containing 5-FU as a base; FU-nucleosides, nucleosides containing 5-FU as a base; R-1-P, ribose 1-phosphate; dR-1-P, 2'-deoxyribose 1-phosphate; ATP, adenosine 5'-triphosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; Pi, inorganic phosphate ion; BAU, 5-benzylacetyluridine [5-benzyl-1-(2'-hydroxyethoxymethyl)uracil]; BTU, 6-benzyl-2-thiouracil; 6-AT, 6-aminothymine; 2-ME, 2-mercaptoethanol; PCA, perchloric acid; TLC, thin layer chromatography; BSA, bovine serum albumin.

MATERIALS AND METHODS

Materials [6-³H]5-FU (1.30 Ci/mmol), [6-³H]uracil (30.0 Ci/mmol), [methyl-³H]thymine (41.0 Ci/mmol) and [methyl-³H]thymidine (TdR) (43.0 Ci/mmol) were purchased from Amersham Corp., Buckinghamshire; [6-³H]uridine (UR) (27.1 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Authentic 5-FU, 5-FUR, 5-FUdR, 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) and 6-benzyl-2-thiouracil (BTU) were obtained from Sigma Chemical Co., St. Louis, MO and other bases, nucleosides and nucleotides were products of Yamasa Co., Tokyo. DEAE-Sephacel and Sephadex G-25 were products of Pharmacia Fine Chemicals, Uppsala. Ultrogel AcA 34 was obtained from LKB-Produkter AB, Bromma, Sweden. Silica gel (Kieselgel 60 F₂₅₄, precoated, 20×20 mm, thickness 0.2 mm) and cellulose (Cellulose F, precoated, 20×20 mm, thickness 0.1 mm) thin layer chromatography (TLC) plates were obtained from E. Merck, Darmstadt, FRG. 5-Benzylacetyluridine [5-benzyl-1-(2'-hydroxyethoxymethyl)uracil] (BAU) was a generous gift from Dr. J. W. Darnowski of Yale University. All of the other chemicals were of analytical grade.

Tumor cells Ehrlich ascites tumors were maintained in the peritoneal cavity of male dd/Y strain mice (Funabashi Farm, Funabashi) weighing 20–25 g, 6 weeks old, by weekly serial transplantation of 2×10⁷ tumor cells (0.2 ml of ascites fluid). These mice were killed 7 days after tumor inoculation, and the collected ascites tumor cells were immediately used for experiments as described below.

Enzyme preparations (1) Homogenate; The collected ascites tumor cells were homogenized in 9 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 5 mM 2-mercaptoethanol (2-ME) by sonication for 2 min at 4°C, and then used for the experiments immediately.

(2) Ammonium sulfate (AS:30-60) fraction; The tumor cells were homogenized in 4 volumes of 50 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 10 mM 2-ME as described above, and centrifuged at 105,000g for 60 min. Crystalline ammonium sulfate was added to the obtained supernatant to make a 30% saturated solution. After centrifugation at 15,000g for 15 min, ammonium sulfate was added to the supernatant up to 60% saturation. The finally obtained pellet was dissolved in a minimum amount of 50 mM Tris-HCl buffer (pH 7.2) containing 5 mM 2-ME. A Sephadex G-25 (fine) column (1.8×16 cm) was employed for desalting of this enzyme, which was then used immediately as standard enzyme preparation (AS:30-60 fraction). All of the above procedures were performed at 4°C.

Assay of 5-FU metabolism in the whole cell homogenate

(1) To investigate the effect of uracil on 5-FUR formation from 5-FU, an incubation mixture containing 5 μM [6-³H]5-FU (0.325 μCi), 1 mM R-1-P, various concentrations (0, 5 μM, 50 μM, 500 μM) of uracil, and the homogenate as described above was prepared in a final volume of 50 μl.

(2) To investigate the effect of uracil on 5-FUdR formation from 5-FU, the same mixture was used but with 1 mM dR-1-P instead of R-1-P.

(3) To examine the effect of uracil on FU-nucleotide synthesis from 5-FU, a mixture containing 5 μM [6-³H]5-FU (0.325 μCi), 1 mM R-1-P or dR-1-P, 5 mM ATP-MgCl₂, various concentrations (0, 5 μM, 50 μM, 500 μM) of uracil, and the homogenate was prepared in a final volume of 50 μl.

(4) A mixture containing 5 μM [6-³H]5-FU (0.325 μCi), 1 mM PRPP, various concentrations (0, 5 μM, 50 μM, 500 μM) of uracil, and the homogenate in a final volume of 50 μl was prepared in order to examine the effect of uracil on synthesis of FU-nucleotides from 5-FU and PRPP.

Each mixture was incubated for 15 min at 37°C, and the 0.5 N perchloric acid (PCA)-soluble fraction of the mixture was neutralized with KOH. Together with authentic markers (5-FU, 5-FUR, and 5-FUdR), an aliquot of the neutralized supernatant was applied to a TLC plate of silica gel (Kieselgel 60 F₂₅₄), and developed with a mixture of chloroform, acetone, and water (25:50:2, v/v/v) as described previously.²¹⁾ In this development, FU-nucleotides and degradation products of 5-FU remained at the origin.²¹⁾ Therefore, in order to measure the amount of FU-nucleotides, a TLC plate of cellulose (Cellulose F) and a solvent system composed of *n*-propanol, ammonia, and water (6:3:1, v/v/v) were used as described previously.²¹⁾ The metabolites on the plate were identified from their R_f values,²¹⁾ after detection under an ultraviolet lamp. Each spot was scraped off the plate and the radioactivity was measured as described previously.²²⁾

The amount of each metabolite of 5-FU was calculated and expressed as percent of the original amount of 5-FU.

Enzyme assays (1) 5-FUR-forming ribosyltransferase; The assay mixture contained 5 μM [6-³H]5-FU (0.65 μCi), 1 mM UR, 50 mM Tris-malate buffer (pH 7.0), and the enzyme preparation in a final volume of 100 μl. For measuring the formation of UR, 5 μM [6-³H]uracil (0.60 μCi) was used in place of [6-³H]5-FU.

(2) 5-FUdR-forming deoxyribosyltransferase; The conditions were the same as described above except that 1 mM unlabeled UdR was used in place of UR.

(3) 5-FUR-forming phosphorylase; The assay mixture contained 5 μM [6-³H]5-FU (0.65 μCi), 5 mM R-1-P, 50 mM Tris-malate buffer (pH 7.5), and the enzyme prepa-

ration in a final volume of 100 μ l. For measuring the formation of UR, 5 μ M [$6\text{-}^3\text{H}$]uracil (0.60 μ Ci) was used instead of [$6\text{-}^3\text{H}$]5-FU.

(4) 5-FUDr-forming phosphorylase; The assay mixture contained 5 μ M [$6\text{-}^3\text{H}$]5-FU (0.65 μ Ci), 5 mM dR-1-P, 50 mM Tris-malate buffer (pH 6.0), and the enzyme preparation in a final volume of 100 μ l. For measuring the formation of UdR, 5 μ M [$6\text{-}^3\text{H}$]uracil (0.60 μ Ci) was used in place of [$6\text{-}^3\text{H}$]5-FU.

All mixtures were incubated at 37°C for 5 min. Then the radioactivities of the produced [$6\text{-}^3\text{H}$]5-FUR and [$6\text{-}^3\text{H}$]5-FUDr were measured with the aid of authentic markers as described above. The radioactivities of [$6\text{-}^3\text{H}$]UR and [$6\text{-}^3\text{H}$]UdR were measured under the same conditions as above, except that the markers used were uracil, UR, and UdR, the *R_f* values of which were described previously.²¹⁾

All of the enzyme activities were expressed as nmol/min/mg of protein. When inhibitors of phosphorylase (BAU and BTU) were used, BAU and BTU were dissolved in ethanol at final concentrations of 0.50% and 0.75%, respectively.

DEAE-Sephacel column chromatography A DEAE-Sephacel column (1.1 $\times$ 30 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.2) containing 5 mM 2-ME. After application of the enzyme preparation, chromatography was performed with 80 ml of the same buffer and then with a linear concentration gradient (0–0.5 M) of KCl in the same buffer, at a flow rate of 14 ml/h. Fractions of 2 ml of the eluate were collected and each fraction was assayed for the enzyme activities as described above.

Gel filtration An Ultrogel AcA 34 column (1.5 $\times$ 50 cm) and the elution buffer containing 0.14 M NaCl and 5 mM 2-ME in 50 mM Tris-HCl buffer (pH 7.2) were used for gel filtration chromatography. The enzyme preparation (1.0 ml) was applied to this column, and fractions of 1.0 ml of the eluate were collected at a flow rate of 14 ml/h. The enzyme activities in each fraction were assayed as described above. Chymotrypsinogen A, BSA, ovalbumin, and aldolase were used as markers for estimating the molecular weight.

Protein determination The amount of protein was determined by the method of Lowry *et al.*²³⁾ using bovine serum albumin (BSA; Nacalai Tesque, Inc., Kyoto) as the standard.

RESULTS

Effect of uracil on 5-FU metabolism in the whole cell homogenate of Ehrlich ascites tumor cells Fig. 1A shows that the addition of uracil up to the concentration of 500 μ M did not inhibit 5-FUR formation from 5 μ M 5-FU and 1 mM R-1-P by the homogenate, but rather tended to

cause an increase up to that concentration (100 times higher concentration than 5-FU). Little 5-FUDr or FU-nucleotides was detected at any concentration of uracil. Similarly, Fig. 1B shows the effect of uracil on the fate of 5 μ M 5-FU and 1 mM dR-1-P. In this case, the addition of uracil up to 50 μ M caused almost no decrease of 5-FUDr formation, although a slight decrease of the production of 5-FUDr was observed at 500 μ M uracil.

Fig. 1C shows the effect of uracil on the metabolism of 5 μ M 5-FU, with 1 mM R-1-P and 5 mM ATP. Production of FU-nucleotides (mainly FUMP) decreased with increase in the concentration of uracil, correlating with the increased amounts of both residual 5-FU and produced 5-FUR. A similar effect of uracil on the metabolism of 5 μ M 5-FU, with 1 mM dR-1-P and 5 mM ATP, was observed. On the contrary, as shown in Fig. 1D, uracil up to 500 μ M hardly inhibited FU-nucleotide synthesis from 5 μ M 5-FU and 1 mM PRPP.

In the examples shown in Fig. 1B and 1C, the reaction products (5-FUDr and FU-nucleotides) amounted to 60 and 80% of the initial reactant (5-FU), respectively. Since these reactions were linear during the assay period, the inhibitory effect of uracil could be reasonably evaluated. In all the experiments described above, degradation of 5-FU scarcely occurred in the homogenate.

Synthesis of 5-FUR from 5-FU with UR Production of 5-FUR by ribose transfer from UR to 5-FU was detected using the AS:30-60 fraction of Ehrlich ascites tumor cells. The amount of 5-FUR produced depended on the concentration of 5-FU in the presence of 10 mM UR, as shown in Fig. 2. Ribose transfer from UR to uracil was also determined in the same manner, although 5-FU showed a higher affinity than uracil in the reaction, as will be mentioned below. Since there was an insignificant amount of inorganic phosphate ion (Pi) in this assay mixture, this reaction was expected to be catalyzed by "nucleoside *N*-ribosyltransferase." In fact, it was preliminarily confirmed that 10 mM UR in the assay without 5-FU/uracil could not be degraded into R-1-P and uracil by this AS:30-60 fraction unless Pi was added. In the presence of Pi, 5-FUR as well as UR would be produced by the following two reactions catalyzed by nucleoside phosphorylase; (1) the formation of R-1-P from UR and Pi, and then (2) 5-FUR/UR formation from 5-FU/uracil and the formed R-1-P. This ribosyltransferase activity was found in the 30–60% saturated ammonium sulfate fraction, but not in the 0–30% or over 60% fraction.

On the other hand, nucleoside deoxyribosyltransferase [EC 2.4.2.6] activity from UdR to 5-FU was not detected in this cell fraction (Fig. 4).

Partial purification of nucleoside *N*-ribosyltransferase Both 5-FUR-forming (from 5-FU with R-1-P), and 5-FUDr-forming (from 5-FU with dR-1-P) phosphorylase activities were also detected in the AS:30-60 frac-

Fig. 1. Effect of uracil on 5-FU metabolism with R-1-P (A), dR-1-P (B), R-1-P and ATP-MgCl₂ (C) and PRPP (D). Incubation of 5 μ M [6-³H]5-FU, 1 mM R-1-P (A), 1 mM dR-1-P (B), 1 mM R-1-P and 5 mM ATP-MgCl₂ (C), 1 mM PRPP (D), and uracil at the indicated concentrations with the cell homogenate was carried out at 37°C for 15 min. Metabolites of 5-FU were assayed as described under "Materials and Methods." The radioactivity of the initial [6-³H]5-FU was taken as 100% (control), and the radioactivity of each formed metabolite was expressed as percent of the control. The results are representative of several separate experiments. 5-FU (Δ), 5-FUR ($\circ$), 5-FUdR ($\bullet$), FU-nucleotides ($\square$).

tion. The profile of DEAE-Sephacel column chromatography of the ammonium sulfate fraction is shown in Fig. 3A. 5-FUR-forming ribosyltransferase activity (from 5-FU with UR) was found in a single peak together with two phosphorylase activities.

The fractions having the enzyme activities were then pooled (Fr. No. 56–66 in Fig. 3A), concentrated by ultrafiltration and subjected to Ultrogel AcA 34 column chromatography. Fig. 3B shows that ribosyltransferase activity was found again in a single peak with two phosphorylase activities. Thus, the molecular weight of each of these three enzymes was estimated to be about 44,000 Da.

These three activities were also co-eluted in Phenyl-Sepharose CL-4B column chromatography or disc polyacrylamide gel electrophoresis (data not shown).

When uracil was used in place of 5-FU as a substrate, the ribose moiety was also transferred from UR to uracil, but deoxyribose was not transferred from UdR to uracil. UR or UdR was produced from uracil with R-1-P or dR-1-P by phosphorylase, respectively. Moreover the above three enzyme activities were also detected in the same single peak in DEAE-Sephacel and AcA 34 column chromatographies, as shown in Fig. 3A and 3B, together

with UR/TdR-degrading (producing uracil/thymine from UR/TdR with Pi) and TdR-forming (from thymine with dR-1-P) phosphorylase activities (data not shown).

Kinetic studies of the enzymes synthesizing nucleosides K_m values of ribosyltransferase and phosphorylases (forming 5-FUR/5-FUdR) were determined from Lineweaver-Burk double-reciprocal plots,²⁴⁾ with 5-FU or uracil as a substrate, using the AS:30-60 fraction. As described in Table I, the K_m values of 5-FUR/UR-forming phosphorylase and ribosyltransferase for 5-FU were apparently 10 times smaller than those for uracil except those of 5-FUdR/UdR-forming phosphorylase, the K_m values of which for 5-FU and uracil were similar, 0.35×10^{-3} M and 0.50×10^{-3} M (with 10 mM dR-1-P), respectively.

Optimum pH The rates of formation of 5-FUR and 5-FUdR were examined in 50 mM Tris-malate buffer with a pH range of 5.0–9.0, using the AS:30-60 fraction. As shown in Fig. 4, 5-FUR-forming ribosyltransferase had its optimum pH at pH 6.5, showing a rather broad peak of activity ranging from pH 5.5 to 7.0 (left side), while 5-FUdR-forming deoxyribosyltransferase did not show activity at any pH value (right side). The optimum pH of

Fig. 2. Kinetics of the activity of ribose transfer from UR to 5-FU or uracil. Assay was performed using the AS:30-60 fraction of the cells as described under "Materials and Methods," except that 5-FU or uracil was used at the indicated concentrations in the presence of 10 mM UR. The initial velocity (v) of the reaction was expressed as the amount of formed 5-FUR or UR (nmol/min/mg of protein). Lineweaver-Burk double-reciprocal plots were drawn using the data of 5-FUR/UR-forming activity versus various concentrations of 5-FU (●) or uracil (△).

5-FUR-forming phosphorylase was at pH 7.5, with a shoulder near pH 6.0 (left side), but the optimum pH of 5-FUR-forming phosphorylase was at pH 6.0 in a single peak (right side). The pH-activity profile of 5-FUR-forming phosphorylase was somewhat similar to that of 5-FUR-forming ribosyltransferase, although the latter showed a slightly broader peak.

Inhibition of pyrimidine nucleoside phosphorylase by several inhibitors 5-Benzylcyclouridine (BAU), a pyrimidine acyclonucleoside, was shown to be a potent inhibitor of UR-degrading phosphorylase.²⁵⁻²⁸ Fig. 5 shows that the activities of 5-FUR-forming ribosyltransferase and 5-FUR-forming phosphorylase were inhibited dose-dependently by BAU to the same extent. At the concentration of 5 μ M, both activities were less than 10% of the control, while the activity of 5-FUR-forming phosphorylase was less inhibited, with about 80% remaining at 5 μ M BAU.

The effect of 6-benzyl-2-thiouracil (BTU), an analog of uracil, which was demonstrated to be an inhibitor of TdR-degrading phosphorylase in the mouse liver,²⁹ was also investigated. As shown in Fig. 6, the inhibitory effect of BTU on the above three enzyme activities seemed to be negligible. However, the activity of 5-FUR-forming phosphorylase was slightly more inhibited at 50–100 μ M BTU. A higher concentration of BTU than 100 μ M could not be tested because of its solubility in ethanol.

Fig. 3. Elution patterns of 5-FUR-forming ribosyltransferase, 5-FUR-forming phosphorylase and 5-FUR-forming phosphorylase in DEAE-Sephacel column chromatography of AS:30-60 fraction (A) and Ultrogel AcA 34 column chromatography of the pooled fractions having enzyme activities after DEAE-Sephacel column chromatography (B). Chromatography and assay of each enzyme activity were performed as described under "Materials and Methods." 5-FUR-forming ribosyltransferase (○; nmol/min/ml), 5-FUR-forming phosphorylase (△; nmol $\times 10^{-1}$ /min/ml), 5-FUR-forming phosphorylase (▲; nmol $\times 10^{-1}$ /min/ml).

When uracil was used in place of 5-FU as a substrate, the modes of inhibition of the enzyme activities by these two inhibitors were the same as those with 5-FU.

DISCUSSION

This study revealed that uracil did not compete with 5-FU in 5-FUR/5-FUR synthesis (with 5-FU and R-1-P/dR-1-P) in the homogenate of Ehrlich ascites tumor cells (Fig. 1A and 1B). However, the apparent K_m value of UR/5-FUR-forming phosphorylase for 5-FU was 10

Table I. K_m Values for 5-FU and Uracil of 5-FUR-forming Ribosyltransferase, 5-FUR-forming Phosphorylase and 5-FUdR-forming Phosphorylase

	$K_m (\times 10^{-3} M)$	
	5-FU	Uracil
5-FUR or UR-forming ribosyltransferase	0.10	1.00
5-FUR or UR-forming phosphorylase	0.20	2.00
5-FUdR or UdR-forming phosphorylase	0.35	0.50

Fig. 4. Effect of pH on 5-FUR- and 5-FUdR-forming enzyme activities. All of the enzyme assays were done with 50 mM Tris-malate buffer, using the AS:30-60 fraction of the cells as described under "Materials and Methods." 5-FUR-forming ribosyltransferase ($\circ$; nmol $\times$ 10/min/mg of protein), 5-FUR-forming phosphorylase ($\triangle$; nmol/min/mg of protein), 5-FUdR-forming deoxyribosyltransferase ($\bullet$; nmol $\times$ 10/min/mg of protein), 5-FUdR-forming phosphorylase ($\blacktriangle$; nmol/min/mg of protein).

times lower than that for uracil, which is in fair agreement with the previous observation by Ikenaka *et al.*¹³⁾ Moreover, in the case of deoxyribonucleoside synthesis from 5-FU/uracil and dR-1-P, the K_m values for 5-FU and uracil were similar (Table I). Thus, it seems puzzling why even 500 μM uracil could not compete with 5 μM 5-FU in 5-FUR/5-FUdR synthesis.

As for the inhibition by uracil in FU-nucleotide (mainly FUMP) formation from 5-FU, R-1-P, and ATP (Fig. 1C), possibly the formed UR may have competed with the formed 5-FUR in the nucleoside kinase reaction, as suggested by Ikenaka *et al.*¹³⁾ In the light of the inhibitory rates by 50–500 μM uracil in Fig. 1C, the K_m value of the kinase for 5-FUR could be speculated to be at least 10 times lower than that for UR.

Fig. 5. Inhibition of 5-FUR- and 5-FUdR-forming enzyme activities by BAU. Activity in the absence of BAU was taken as 100% (control), and the activities in the presence of BAU at the indicated concentrations were expressed as percent of control. Enzyme assay was done as described under "Materials and Methods." 5-FUR-forming ribosyltransferase ($\circ$), 5-FUR-forming phosphorylase ($\triangle$), 5-FUdR-forming phosphorylase ($\blacktriangle$).

Fig. 6. Inhibition of 5-FUR- and 5-FUdR-forming enzyme activities by BTU. The details are the same as in Fig. 5, except that BTU was used instead of BAU as the inhibitor. 5-FUR-forming ribosyltransferase ($\circ$), 5-FUR-forming phosphorylase ($\triangle$), 5-FUdR-forming phosphorylase ($\blacktriangle$).

The above findings led us to concentrate our investigation on the FU-nucleoside synthesis from 5-FU, especially by "nucleoside *N*-ribosyltransferase." This study seems to be the first to show that this enzyme activity catalyzing 5-FUR synthesis from 5-FU and UR exists in the AS:30-60 fraction of Ehrlich ascites tumor cells (Fig. 2).

In our experimental system which formed 5-FUR from 5-FU and R-1-P (Fig. 1A), it seemed that the more uracil was added, the more UR would be formed with R-1-P

and used for the ribosyltransferase to synthesize even more 5-FUR. Since almost all of the uracil added, up to at least 500 μM , would be converted into UR by phosphorylase, the ribosyltransferase could be little, if at all, influenced by the added uracil. However, since we did not measure the amount of every constituent in the reaction mixture after the reaction, it is not yet clear that this ribosyltransferase does contribute to the competition of 5-FU in 5-FUR formation with uracil added even at 100 times higher concentration than 5-FU (Fig. 1A). But our proposed ribosyltransferase might actually function more effectively than phosphorylase to produce 5-FUR from 5-FU in the cells, because it was previously suggested that a low R-1-P level would be rate-limiting to 5-FU anabolism⁹⁾ and that a certain UR pool could always be sustained in the cells.³⁰⁾

Our preliminary study showed that the K_m values of ribosyltransferase and 5-FUR/UR-forming phosphorylase for 5-FU were 10 times lower than those for uracil in the supernatant of the homogenate as well as in the AS:30-60 fraction. Uracil did not compete markedly with 5-FU in 5-FUR synthesis in the supernatant either, although a slight competition was noted in that case. Some additional mechanism might operate here. Deoxyribosyltransferase activity was not detected in Ehrlich ascites tumor cells (Fig. 4), at least in this context, so it may not contribute.

Both 5-FUR and UR syntheses are mediated by UR phosphorylase.¹³⁻¹⁵⁾ 5-FuDR and UdR inhibited competitively the activity of TdR kinase purified from Ehrlich ascites tumor cells with K_m values which were 10 times higher than that of TdR. This suggests that 5-FU behaves like uracil, not thymine.

In fact, Ehrlich ascites tumor cells revealed a much higher level of UR-degrading phosphorylase activity than TdR-degrading phosphorylase activity, as suggested previously,^{31, 32)} but 5-FuDR and UdR were readily produced *in vitro*, as shown in Fig. 1B. Therefore, it is suggested that the syntheses of 5-FuDR and UdR might also be catalyzed by UR phosphorylase. However, we cannot definitely conclude this, as these enzymes have yet to be separated.

Moreover, the enzyme protein of nucleoside *N*-ribosyltransferase has not been distinguished from that of phosphorylase. Their activities were found in the same fraction not only on DEAE-Sephacel and Ultrogel AcA 34 columns (Fig. 3A and 3B), but also on Phenyl-Sepharose CL-4B column chromatography, suggesting that the ribosyltransferase activity might be another activity of the phosphorylase protein, i.e., pyrimidine nucleoside phosphorylase [EC 2.4.2.2]. In this regard Pinto and Touster³³⁾ showed that two peaks of phosphorolysis and one peak of ribose-transfer activities were

obtained after sucrose density gradient centrifugation of purine nucleoside phosphorylase prepared from Ehrlich ascites tumor cells.

It was reported that the maximum rates of degradation of 5-FUR and 5-FuDR to 5-FU, catalyzed by phosphorylases, occurred at pH 7.4 and 6.4, respectively, in the supernatant of Ehrlich ascites tumor cells.³⁴⁾ Taking this together with the results shown in Fig. 4, UR/5-FUR-forming ribosyltransferase showed the same behavior as deoxyribonucleoside-forming/degrading phosphorylase. Although 5-FuDR-forming deoxyribosyltransferase activity was not observed, TdR-forming deoxyribosyltransferase in various tissues of other animals¹⁷⁾ showed the same behavior as ribonucleoside-forming/degrading phosphorylase in our study.

BAU, a potent inhibitor of UR-degrading phosphorylase,²⁵⁻²⁸⁾ inhibited 5-FUR/UR-degrading phosphorylase, 5-FuDR/UdR-forming/degrading phosphorylase, 5-FUR/UR ribosyltransferase, and TdR-forming/degrading phosphorylase, but hardly inhibited 5-FUR/UR-forming phosphorylase at 0.5–5 μM BAU (Fig. 5 and our preliminary results). BTU, a potent inhibitor of thymidine phosphorylase²⁹⁾ did not inhibit any of these enzyme activities, except that 5-FUR/UR-forming phosphorylase activity was inhibited very slightly at a concentration of 100 μM . It is not clear why only 5-FUR/UR-forming phosphorylase showed different behavior in response to BAU and BTU. It might be speculated that it is due to some interaction of the inhibitor with R-1-P.

In any case, the data on optimum pH and the mode of inhibition are insufficient to prove the identity of ribosyltransferase protein with 5-FUR/UR- or 5-FuDR/UdR-forming phosphorylase protein. It was previously reported that rodent tissues have mainly UR phosphorylase, while human tissues have TdR phosphorylase as pyrimidine nucleoside phosphorylase.^{31, 32, 35)} We also examined human MKN-45³⁶⁾ (gastric cancer) and TE-2³⁷⁾ (esophageal cancer) cells, which were found to contain also 5-FuDR-forming deoxyribosyltransferase activity, differently from Ehrlich ascites tumor cells (data not shown). Therefore, it was suggested that the deoxyribosyltransferase activity co-existed with TdR phosphorylase. However, it is still not certain whether the ribosyltransferase activity of Ehrlich tumor cells might be catalyzed by UR phosphorylase or not.

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