The Synthesis of 5-Fluorouridine 5′-Phosphate by a Pyrimidine Phosphoribosyltransferase of Mammalian Origin. I. Some Properties of the Enzyme from P1534J Mouse Leukemic Cells*

Philip Reyes†

ABSTRACT: Cell-free extracts of P1534J mouse leukemic cells catalyze the synthesis of 5-fluorouridine 5′-phosphate from 5-fluorouracil and 5-phosphoribosyl 1-pyrophosphate. The product was identified by its migration during paper chromatography in three different solvent systems. This enzymatic reaction requires the presence of a divalent cation and is inhibited by inorganic pyrophosphate but not by orthophosphate. These results suggest that the synthesis of 5-fluorouridine 5′-phosphate is catalyzed by a pyrimidine phosphoribosyltransferase. The enzyme exhibits a pH optimum of 10 and shows maximal activity in the presence of Mg^{2+} although Mn^{2+}, Co^{2+}, and Ca^{2+} support substantial reaction rates. Neither Hg^{2+}, nor Zn^{2+}, nor Cu^{2+} is able to substitute for Mg^{2+}. The K_m values are 83 μM for 5-fluorouracil and 120 μM for 5-phosphoribosyl 1-pyrophosphate. Evidence indicating that the enzyme can also utilize uracil and possibly orotic acid as substrates for synthesis of the corresponding ribonucleotide is presented.

The carcinostatic effect of 5-fluorouracil is generally believed to be due to the inhibition of thymidylate synthetase by 5-fluoro-2′-deoxyuridine 5′-phosphate (see the recent review by Heidelberger, 1965). Thus, 5-fluorouracil must first be metabolized to the nucleotide level before it can exert its carcinostatic effect. Heretofore this metabolic transformation has been considered to proceed in large measure *via* the ribonucleoside intermediate, 5-fluorouridine, which is then phosphorylated to yield 5-fluorouridine 5′-phosphate.[1] Presumably, these reactions are catalyzed by uridine phosphorylase and uridine kinase, respectively (Sköld, 1960).

On the other hand, little chemotherapeutic importance has been ascribed previously to the pyrimidine phosphoribosyltransferase catalyzed condensation of 5-fluorouracil with 5-phosphoribosyl 1-pyrophosphate to yield F-UMP directly (Kasbekar and Greenberg, 1963). It has recently been observed, however, that extracts from a wide spectrum of transplantable mouse leukemic cells contain an enzyme that catalyzes such a reaction.[2] Furthermore, a statistically significant correlation was found between the specific activity of this enzyme in the various extracts and the 5-fluorouracil-promoted increase in survival of mice bearing these leukemic cells. It therefore appears very probable that F-UMP synthesis from 5-fluorouracil may occur predominantly *via* this pathway in tumor tissues.

Because of the possible cancer chemotherapeutic importance of this pathway, it was considered highly desirable to examine the properties and characteristics of the enzyme responsible for this reaction. Some of these are now reported for the enzyme present in extracts of P1534J mouse leukemic cells.

Experimental Procedure

Materials. 5-Phosphoribosyl 1-pyrophosphate was purchased from Sigma Chemical Co. as the dimagnesium salt and converted into the Na^+ salt by treatment with Dowex 50 Na^+, followed by filtration. 5-Fluorouracil-2-^{14}C (22 μCi/μmole), obtained from Calbiochem, was mixed with unlabeled 5-fluorouracil (Calbiochem) to give a specific activity of 5.2 μCi/μmole. Ribose 1-phosphate was purchased from Sigma Chemical Co.; ATP and UMP from P-L Biochemicals; Whatman DEAE-cellulose paper (DE 81) from Reeve Angel; and the various purine and pyrimidine bases from Calbiochem. Tumor-bearing mice were kindly provided by Isidore Wodinsky, Arthur D. Little, Inc.

Preparation of Enzyme Extracts. Solid tumors were excised 6–7 days after subcutaneous inoculation of DBA_2 mice with P1534J leukemic cells. The excised tumors were minced quickly, suspended in approximately three volumes of ice-cold 10 mM Tris buffer (pH 7.5), and homogenized with a ground-glass tissue grinder. The homogenate was centrifuged at 30,000*g* for 30 min. Small aliquots of the resulting supernatant fraction were stored at −20° and served directly as the source of the enzyme. The protein content was determined by the method of Lowry *et al.* (1951).

* From the Laboratories of Pharmacology, The Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115. *Received November 4, 1968.* This Investigation was supported in part by the U. S. Public Health Service Grant C-6516, and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health.

† Address reprint requests to the author at Children's Cancer Research Foundation, Boston, Mass. 02115.

[1] Abbreviations used are: FU, 5-fluorouracil; F-UMP, 5-fluorouridine 5′-phosphate.

[2] P. Reyes and T. C. Hall, in preparation.

Enzyme Assay. Unless otherwise indicated, the standard assay mixture contained the following components: 5-phosphoribosyl 1-pyrophosphate, 2.0 mM; 5-fluorouracil-2-^{14}C, 0.5 mM (5.2 μCi/μmole); $MgCl_2$, 2 mM; Tris-HCl buffer, pH 7.5, 60 mM; and enzyme extract, in a total volume of 45–50 μl. Reactions were initiated by the addition of enzyme extract and after an incubation period of 30 min at 38° were terminated by chilling the assay mixtures in an ice–water bath and adding 5 μl of cold 65% trichloroacetic acid. After the removal of precipitated protein by centrifugation, an 8-μl aliquot of the supernatant fraction was applied to DEAE-cellulose paper strips (formate form). Development of the paper with distilled water completely separated the residual 5-fluorouracil from the radioactive product which remained at the origin.[3] A 2.3-cm disk was punched from the origin of the dried paper strip and the radioactivity in the product was measured by liquid scintillation counting in 2,5-bis[2-(5-*t*-butylbenzoxazolyl)]thiophene phosphor solution.[4] Blanks consisted of reaction mixtures to which trichloroacetic acid was added prior to the addition of enzyme extract. Enzyme activities are expressed as micromicromoles of F-UMP formed per hour.[5] The reaction rate was proportional to enzyme concentration over a fivefold concentration range and was constant for a minimum of 45 min at 38°.

Results

Requirements for Enzyme Activity. The data presented in Table I summarize the requirements for F-UMP synthesis. The reaction is dependent upon 5-phosphoribosyl 1-pyrophosphate (expt 1) and enzyme extract (expt 3). The near-maximal activity observed in the absence of added Mg^{2+} (expt 1) very probably reflects the presence of endogenous Mg^{2+} in the assay mixtures.[6] In the absence of added Mg^{2+}, however, the addition of 2 mM EDTA produced a 95% inhibition of enzyme activity. No such inhibition was produced when the same concentration of EDTA was added to the complete system (containing 2 mM added Mg^{2+}). This latter finding indicates that a divalent cation is essential for enzyme activity. Table I (expt 2) also shows that in the absence of added ATP, ribose 1-phosphate could not substitute for 5-phosphoribosyl 1-pyrophosphate. In the presence of ATP, however, a low but reproducible level of activity was detected with ribose 1-phosphate. In all likelihood, this latter activity was due to the uridine phosphorylase–uridine kinase system (Sköld, 1960).

TABLE I: Requirements for 5-Fluorouridine 5′-Phosphate Formation.[a]

Conditions of Incubation	F-UMP Formed ($\mu\mu$moles/hr)
1. Complete system	1070
−5-Phosphoribosyl 1-pyrophosphate	14
$-Mg^{2+}$	924
$-Mg^{2+}$ + EDTA	55
+EDTA	873
2. Complete system	1230
−5-Phosphoribosyl 1-pyrophosphate + ribose 1-phosphate	26
−5-Phosphoribosyl 1-pyrophosphate + ribose 1-phosphate + ATP	278
3. Complete system	1540
−Enzyme extract	0

[a] The assay conditions were those noted under Experimental Procedure except for the following additions, where indicated: EDTA, 2 mM, ribose 1-phosphate, 2 mM; and ATP, 14 mM. The protein content of expt 1, 2, and 3 was 230, 270, and 300 μg, respectively.

Paper Chromatographic Characterization of the Radioactive Product. The R_F values obtained upon paper chromatography of a purified sample of the radioactive product are seen in Table II along with the corresponding values for related compounds. It is clear that the radioactive product behaved chromatographically like UMP in all three solvent systems employed. In other experiments (not shown) hydrolysis of the purified radioactive product with 70% perchloric acid for 90 min at 100° was found to yield a new substance that cochromatographed with authentic 5-fluorouracil. In view of the above requirement for 5-phosphoribosyl 1-pyrophosphate and the observation that 5-fluorouracil and its derivatives possess paper chromatographic properties very similar to those of the corresponding uracil compounds (Brockman *et al.*,

[3] The specificity of the DEAE-cellulose paper toward nucleotides was confirmed by experiments with uracil, FU, uridine, 5-fluorouridine, and UMP. Only the latter was retained at the origin subsequent to irrigation of the paper with distilled water (P. Reyes, unpublished data).

[4] 2,5-Bis[2-(5-*t*-butylbenzoxazolyl)]thiophene phosphor solution was prepared as follows: 12 g of 2,5-bis[2-(5-*t*-butylbenzoxazolyl]thiophene (Packard Instrument Co.) and 240 g of naphthalene were dissolved in 1800 ml of toluene and 1200 ml of methyl Cellosolve. All measurements of radioactivity were made with a Nuclear-Chicago scintillation spectrometer.

[5] Phosphatase degradation of F-UMP was examined by incubating approximately 0.5 μmole of the radioactive product, previously purified by paper chromatography (Table II), with the enzyme extract for 30 min at 38°. A maximum of 6–8% was degraded during the incubation. In view of the possible sparing action by 5-phosphoribosyl 1-pyrophosphate, which was absent from these incubations, this value was considered to represent the upper limits of phosphatase activity. For this reason, phosphatase degradation of F-UMP during the actual enzyme assays was judged not to be significant (P. Reyes, unpublished experiments).

[6] The further finding that significant enzyme activity was observed in the absence of added Mg^{2+} even when the cell-free extract had been passed through a column of Sephadex G-25, indicates that part of the endogenous Mg^{2+} was contributed by 5-phosphoribosyl 1-pyrophosphate (P. Reyes, unpublished data).

TABLE II: Paper Chromatographic Mobility of Purified Radioactive Product and Related Compounds.[a]

Compound	R_F Value		
	Solvent System 1	Solvent System 2	Solvent System 3
Reaction product	0.27	0.43	0.32
UMP	0.24	0.46	0.29
Uridine	0.49	0.70	0.56
5-Fluorouracil	0.64	0.72	0.69

[a] The radioactive product was purified as follows. A number of reaction mixtures were pooled, neutralized with KOH, centrifuged to remove insoluble material, and lyophilized to dryness. The residue was taken up in a small volume of distilled water and streaked on Whatman No. 1 paper. The paper was developed with solvent system 1 which consisted of 1-butanol–acetic acid–water (100:40:60, v/v) and the area corresponding to a reference sample of unlabeled UMP was cut out and eluted with 2 ml of distilled water. Liquid scintillation counting of 0.5-cm sections of the chromatogram confirmed that a peak of radioactivity was associated with the area corresponding to UMP. After lyophilization of the eluate, the residue was dissolved in a small volume of distilled water. This solution served as the source of purified radioactive product. Mobilities were determined using Whatman No. 1 paper. Unlabeled reference compounds were detected under ultraviolet light while the radioactive product was monitored by counting 0.5-cm sections of the chromatogram as above. Solvent system 2 contained 1-propanol–NH_4OH–water (55:35:10, v/v) while solvent system 3 contained isobutyric acid–1 N NH_4-OH–0.1 N EDTA (100:60:1.6, v/v).

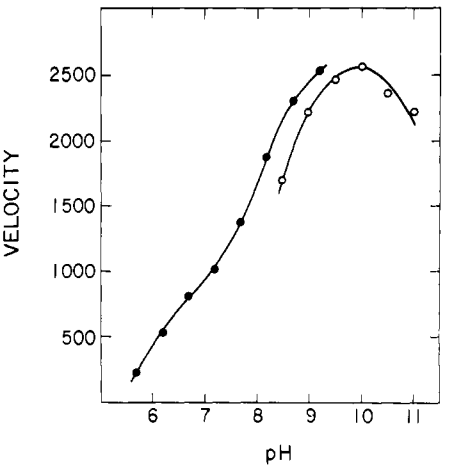

FIGURE 1: Effect of pH on the rate of 5-fluorouridine 5'-phosphate formation. The reaction mixtures contained either Tris–maleate (●—●) or glycine–NaOH (○—○), both at a concentration of 50 mM, and 300 μg of protein. All other conditions were identical with those described under Experimental Procedure.

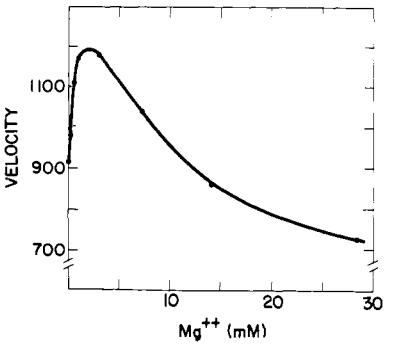

FIGURE 2: Effect of added Mg^{2+} concentration on the rate of 5-fluorouridine 5'-phosphate formation. Mg^{2+} was added as the chloride salt to reaction mixtures containing 270 μg of protein. All other conditions as described under Experimental Procedure.

TABLE III: Inhibition of 5-Fluorouridine 5'-Phosphate Formation by Inorganic Pyrophosphate.[a]

Additions	F-UMP Formed (μμmoles/hr)	
	2 mM Mg^{2+}	21 mM Mg^{2+}
None	1070	798
Orthophosphate	970	895
Pyrophosphate	32	498

[a] The assay conditions were those described under Experimental Procedure except for the addition of 15 mM orthophosphate or pyrophosphate, where indicated. The protein content was 230 μg.

1960), the product was considered to be identical with F-UMP.

Inhibition by Inorganic Pyrophosphate. Table III illustrates that the presence of 15 mM inorganic pyrophosphate produced a 97% inhibition of enzyme activity whereas an equivalent concentration of inorganic orthophosphate affected the enzyme only slightly. That the inhibition by pyrophosphate was not simply a result of the removal of essential Mg^{2+} due to formation of the pyrophosphate complex (Bock, 1960) was indicated by the fact that excess Mg^{2+} (21 mM) only partially prevented this inhibition. Accordingly, the effect of pyrophosphate is interpreted as representing product inhibition of the enzyme.

The results of the above studies, showing that the formation of F-UMP from 5-fluorouracil is not only dependent upon 5-phosphoribosyl 1-pyrophosphate, Mg^{2+}, and enzyme extract but also specifically inhibited by inorganic pyrophosphate, are consistent with the expected properties of a pyrimidine phosphoribosyltransferase (Lieberman *et al.*, 1955; Crawford *et al.*, 1957; Hatfield and Wyngaarden, 1964).

The remainder of this report will describe some additional properties of the enzyme.

pH Optimum. Figure 1 illustrates the effect of pH

TABLE IV: Effect of Divalent Cations on the Rate of 5-Fluorouridine 5'-Phosphate Formation.[a]

Additions	F-UMP Formed ($\mu\mu$moles/hr)
None	52
Mg^{2+}	1190
Mn^{2+}	434
Co^{2+}	298
Ca^{2+}	284
Hg^{2+}	10
Zn^{2+}	10
Cu^{2+}	4

[a] Cations at a final concentration of 6 mM were added to initiate the reaction after endogenous divalent metals had been depleted with 2 mM EDTA. The protein content was 270 μg and the other conditions were those noted under Experimental Procedure.

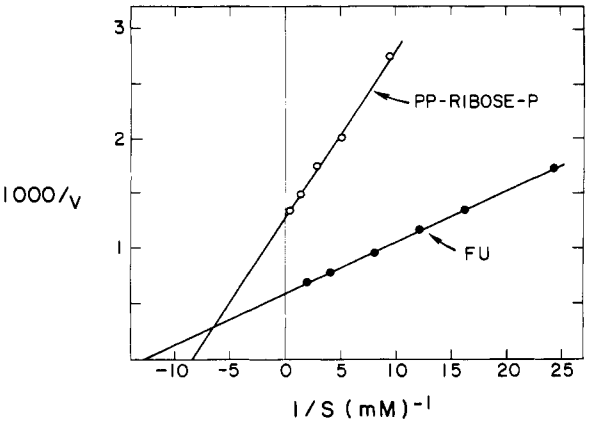

FIGURE 3: Double-reciprocal plot of reaction rate *vs.* substrate concentration. When 5-phosphoribosyl 1-pyrophosphate served as the variable substrate, 5-fluorouracil was held constant at 0.8 mM in a reaction mixture containing 200 μg of protein. When 5-fluorouracil concentration was varied, 5-phosphoribosyl 1-pyrophosphate was held constant at 2.0 mM in a reaction mixture containing 340 μg of protein. All other conditions as described under Experimental Procedure.

on the rate of F-UMP formation. An unusually high pH optimum at 10 and a shoulder at pH 6.5 were reproducible findings. However, all routine assays were conducted at pH 7.5 in order to reflect physiological values more closely.

Cation Requirement. The effect of added Mg^{2+} concentration on enzyme activity is seen in Figure 2. Approximately 2 mM Mg^{2+} gave maximal activity while higher concentrations were inhibitory to the enzyme. On the basis of these results, 2 mM Mg^{2+} was routinely added to all assay mixtures, unless otherwise noted.

The ability of a variety of divalent cations to substitute for Mg^{2+} was examined and the results of a typical experiment are shown in Table IV. In these experiments, the various cations were tested for their ability to restore enzyme activity to assay mixtures (lacking added $MgCl_2$) which had been treated with 2 mM EDTA. Such treatment was necessary since it was believed that Mg^{2+} had not been completely removed from 5-phosphoribosyl 1-pyrophosphate (see above, Requirements for Enzyme Activity). Maximal restoration of enzymy activity was provided by Mg^{2+} although Mn^{2+}, Co^{2+}, and Ca^{2+} each produced substantial activity. Hg^{2+}, Zn^{2+}, or Cu^{2+} was not able to restore enzyme activity.

Kinetic Properties. Double-reciprocal plots for 5-fluorouracil and 5-phosphoribosyl 1-pyrophosphate are presented in Figure 3. The apparent Michaelis constants evaluated from these plots (Lineweaver and Burk, 1934) are 83 μM for 5-fluorouracil and 120 μM for 5-phosphoribosyl 1-pyrophosphate. The latter value is well below the endogenous level of 5-phosphoribosyl 1-pyrophosphate (0.6 mM) reported by Henderson and Khoo (1965) for Ehrlich ascites tumor cells.

Comparative Studies with 5-Fluorouracil and Uracil as Substrates. Experiments were conducted to compare the relative abilities of radioactive 5-fluorouracil and uracil to serve as substrates for the synthesis of the corresponding ribonucleotide. Table V shows that at equal concentrations of these two substrates the enzyme activity with 5-fluorouracil was far greater than that with uracil. Thus, at pH 7.5 (expt 1) the ratio of activities was 20 while at pH 10 the ratio was approximately 5–6 (expt 2 and 3, no unlabeled base present). A pH of 10 was chosen because of the low activity with uracil at pH 7.5 and because other experiments (not shown) had demonstrated that the pH optimum with uracil lies between 10.5 and 11. Table V also shows that nonradioactive uracil and orotic acid each produced an apparent inhibition of enzyme activity when 5-fluorouracil served as substrate. A similar effect was produced by 5-fluorouracil and orotic acid when uracil was the substrate. This apparent inhibitory effect appears to be specific for the above compounds since none of the other bases tested produced any significant effect on the rate of the reaction. The observation that increasing the concentration of 5-phosphoribosyl 1-pyrophosphate to 4 mM did not prevent this apparent inhibition (P. Reyes, unpublished data) supports the view that the reduced enzyme activity was not due to depletion of 5-phosphoribosyl 1-pyrophosphate by other pyrimidine phosphoribosyltransferases in the cell-free extract. The possible relation of the above findings to the substrate specificity of the enzyme is discussed below.

Discussion

Studies reported in this paper have shown that extracts of P1534J mouse leukemic cells catalyze the synthesis of 5-fluorouridine 5'-phosphate from 5-fluorouracil. This enzymatic reaction requires 5-phosphoribosyl 1-pyrophosphate and a divalent cation and is strongly inhibited by inorganic pyrophosphate. Inorganic orthophosphate does not inhibit the enzyme. It is concluded, on the basis of these results, that the synthesis of F-UMP is catalyzed by a pyrimidine phos-

TABLE V: Effect of Nonradioactive Pyrimidine and Purine Bases on Enzyme Activity with 5-Fluorouracil or Uracil as Substrate.[a]

Expt	pH of Reaction Mixture	Nonradioactive Base	5-Fluorouracil $\mu\mu$moles of F-UMP Formed/hr	Uracil $\mu\mu$moles of UMP Formed/hr
1	7.5	None	1200	60
2	10.0	None	2250	467
		5-Fluorouracil		24
		Uracil	304	
		Orotic acid	27	24
		Thymine	2250	464
		Cytosine	2310	430
3	10.0	None	2660	435
		Adenine	2680	444
		Guanine	2630	467
		Xanthine	2640	412
		Uric acid	2680	426

[a] The reaction mixtures in expt 1 contained the following components: 5-phosphoribosyl 1-pyrophosphate, 2.0 mM; either 5-fluorouracil-2-^{14}C, 0.5 mM (5.2 μCi/μmole), or uracil-2-^{14}C, 0.5 mM (5.8 μCi/μmole); $MgCl_2$, 2 mM; Tris-HCl buffer, pH 7.5, 60 mM; and enzyme extract, 250 μg of protein. The conditions for expt 2 and 3 were the same except for the replacement of Tris-HCl by glycine–NaOH buffer, pH 10, 50 mM, and the inclusion of nonradioactive pyrimidine or purine bases, 4 mM, where indicated.

phoribosyltransferase. Studies to be reported elsewhere[7] have shown that a similar enzyme is present in extracts of a wide spectrum of transplantable mouse leukemic cells. Moreover, since the specific activity of the enzyme in these extracts was found to correlate with the 5-fluorouracil-promoted increase in survival of mice bearing these leukemic cells, it appears very likely that this reaction may represent the major pathway for the synthesis of F-UMP.

The identity of the enzyme responsible for the synthesis of this UMP analog remains to be determined. Only a limited number of pyrimidine phosphoribosyltransferases have been described in the literature. Orotic acid phosphoribosyltransferase (Lieberman *et al.*, 1955) is now known to play an essential role in pyrimidine biosynthesis *de novo* in higher animals and in a variety of microorganisms; it, therefore, is widely distributed in nature. Crawford *et al.* (1957) reported that a partially purified enzyme from *Lactobacillus bifidus* catalyzed the condensation of uracil with 5-phosphoribosyl 1-pyrophosphate to yield UMP. A similar reaction has been noted to occur in extracts of Ehrlich ascites tumor cells (Reichard and Sköld, 1957).

In this light, one possible interpretation of the apparent inhibitory effects produced by nonradioactive 5-fluorouracil, uracil, and orotic acid (Table V) is that such effects represent competition by these compounds with the radioactive substrates (5-fluorouracil or uracil) for binding sites on the enzyme. A corollary of this interpretation is that uracil and possibly orotic acid are the normal substrates for the enzyme. Additional evidence that a single enzyme utilizes 5-fluorouracil and uracil was the finding that the ratio of enzyme activity with these two compounds at pH 7.5 was constant at approximately 20 in extracts of 8 different lines of mouse leukemic cells (P. Reyes, unpublished data).[8]

Consistent with the concept of a single enzyme with the above substrate specificity is the report by Hatfield and Wyngaarden (1964). These authors obtained a highly purified enzyme from beef erythrocytes that catalyzed the condensation of several pyrimidines (including 5-fluorouracil, uracil, and orotic acid), xanthine, and uric acid with 5-phosphoribosyl 1-pyrophosphate to yield the corresponding ribonucleotide. The relative activities toward these substrates remained constant during purification of the enzyme. Since the nucleotide products produced from xanthine and uric acid were the N^3-ribosylphosphate derivatives, this enzyme was judged to be a 2,4-diketopyrimidine phosphoribosyltransferase also capable of acting upon uric acid and xanthine. In this regard, the observation by Kasbekar *et al.* (1964) that a 600-fold-purified enzyme from calf thymus similarly converted orotic acid and 5-fluorouracil into the corresponding ribonucleotide supports the possibility that a pyrimidine

[7] P. Reyes and T. C. Hall, in preparation.

[8] The unusually high pH optima observed with 5-fluorouracil and uracil in the present study indicate that the anionic form of the pyrimidine bases may be the true substrate (Hatfield and Wyngaarden, 1964). Hence, the preferential utilization of 5-fluorouracil over uracil may simply reflect the known differences (Gottschling and Heidelberger, 1963) in the pK_a values for 5-fluorouracil (8.15) and uracil (9.45).

phosphoribosyltransferase of relatively broad substrate specificity may exist in mammals.

Furthermore, since the present enzyme was found to be virtually absent from L1210 mouse leukemic cells resistant to 5-fluorouracil (P. Reyes, unpublished studies), it would appear that its presence is not essential for nucleic acid synthesis. Accordingly, it is probably distinct from orotic acid phosphoribosyltransferase. Its normal function may be the catalysis of a "salvage" pathway (Kornberg, 1957) for the utilization of preformed uracil. Using a similar if not identical line of L1210 cells, Goldberg *et al.* (1966) recently concluded that the presence of a salvage pathway for uracil is a determinant of tumor sensitivity to 5-fluorouracil. The report (Kasbekar and Greenberg, 1963) that cell-free extracts from a 5-fluorouracil-resistant variant of Gardner lymphosarcoma catalyzed the condensation of orotic acid, but not that of 5-fluorouracil, with 5-phosphoribosyl 1-pyrophosphate, reinforces this view.

Further studies involving the purification of the present enzyme are planned. It is hoped that the determination of the relative activity toward a number of possible substrates during the purification procedure as well as the study of comparative enzymatic, kinetic, and physical properties will aid in identifying the enzyme.

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