# STUDIES ON PSEUDOURIDINE MONOPHOSPHATE SYNTHETASE OF AGROBACTERIUM TUMEFACIENS

by

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# PREFACE

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I am grateful to Dr. Myron Jacobson who contributed a number of valuable suggestions regarding this work. I am again indebted to Mr. A. Christopher Skjold for his helpful criticism during the course of this work.

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# INTRODUCTION

The unusual structure of pseudouridine has inspired a number of investigations into its biosynthesis. Since in pseudouridine the usual nitrogen-carbon glycosidic bond has been replaced by a carbon-carbon bond, most of the investigations have focused on how this bond is formed. Three different mechanisms have been proposed by several laboratories.

The first mechanism was suggested by Hall and Allen (1), who postulated that 1,5-diribosyluracil is an intermediate in the process that interconverts uridine and pseudouridine (reactions 5 and 6, Fig. 1). Subsequently, Lis and Lis isolated a substance which was presumed to be 1,5-diribosyluracil from commercial uridine (2). The biosynthesis of 1,5-diribosyluracil-5'-phosphate with a crude enzyme preparation from Escherichia coli W3301, uridine, and ribose-5-phosphate was also reported in support of this mechanism (3,4). However, neither the identification of the substance nor its participation in a metabolic pathway is yet firmly established. The "diribosyluracil" reported by Lis and Lis was later found to be different from the synthetic 1,5-diribosyluracil (5,6).

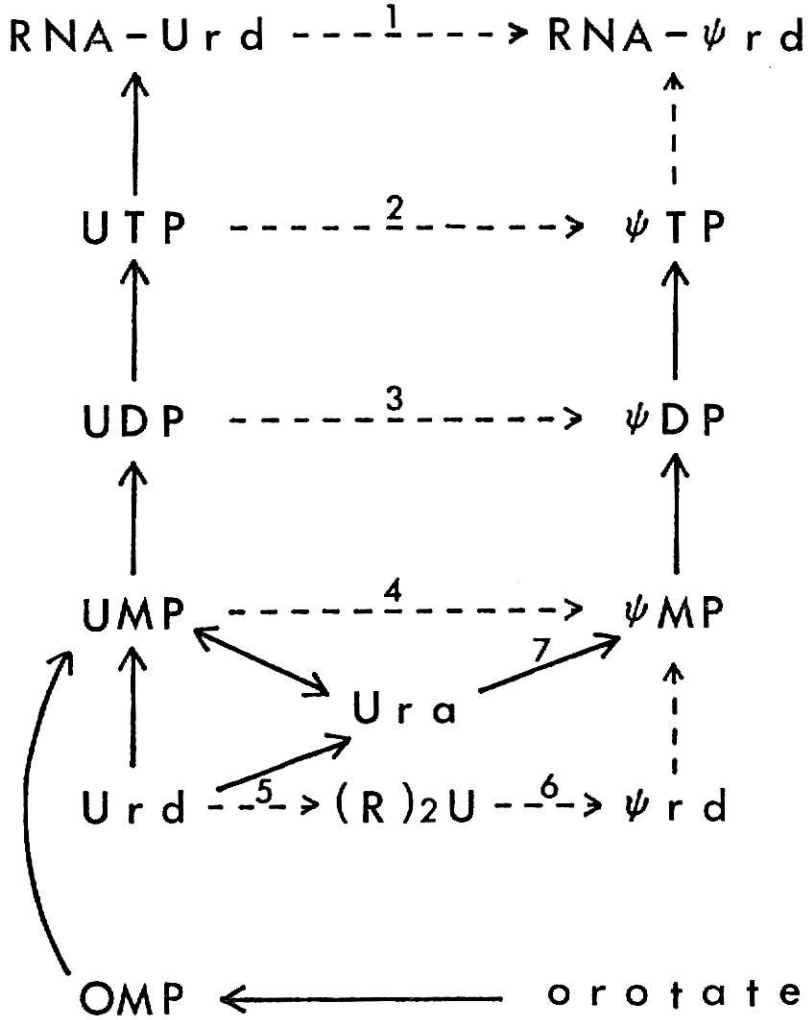
Robbins and Kinsey (7) declared the second mechanism on the basis of their study demonstrating that after mixing doubly labeled 5-fluorouridine and unlabeled uridine in the growth medium of E. coli, only unlabeled ribose appears in the pseudouridine of tRNA, and none of the 5-fluorouridine is utilized for pseudouridine

Figure 1

Proposed alternate pathways of pseudouridine biosynthesis. Broken arrows indicate unknown reactions. $(R)_2U$ represents 1,5-diribosyluracil.

**THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.**

**THIS IS AS RECEIVED FROM CUSTOMER.**


RNA-Urd
1
RNA-ψrd
UTP
2
ψTP
UDP
3
ψDP
UMP
4
ψMP
7
Ura
Urd
5
(R)2U
6
ψrd
OMP
orotate

formation. They concluded that the migration of ribose from N1 to C5 occurs as an intramolecular rearrangement (reactions 2,3, or 4, Fig. 1), although it is possible that 5-fluorouridine can not donate ribose to form pseudouridine.

The third mechanism states that the C-C bond is formed enzymatically by a direct condensation of uracil with ribose-5-phosphate (reaction 7, Fig. 1). The enzyme catalyzing this reaction has been isolated from both Tetrahymena pyriformis (8) and Agrobacterium tumefaciens (9) and tentatively designated as ΨMP synthetase.

As to the biosynthesis of polynucleotide pseudouridine, two alternative hypotheses have been proposed. In the first mechanism, the rearrangement hypothesis (10,11,12), pseudouridine of RNA is visualized as forming as a result of the migration of the ribose of uridine after the formation of polymeric nucleic acid. In the second mechanism, the direct incorporation hypothesis (8,9,13,14), pseudouridine is considered to be synthesized in the mononucleotide form and is incorporated into RNA from ΨTP by RNA polymerase.

The rearrangement hypothesis is strongly supported by the studies of Weiss and Legault-Demare (10). They performed experiments with intact and lysed spheroplasts from E. coli and indicated that in the presence of DNase or actinomycin D, a significant increase in pseudouridine content was observed in a preparation of labeled RNA made, in vitro, with RNA polymerase. The involvement of RNA as an intermediate in the conversion of uridine to pseudouridine was suggested by them. Jacobson (15) found that

an exogenous supply of pseudouridine was utilized very poorly by E. coli. When label supplied as pseudouridine is incorporated into RNA, most of the label appeared in uridine and cytidine of RNA. So, he concluded that pseudouridine of RNA is formed by a mechanism other than by direct incorporation. The difficulty in visualizing how tRNA containing several modified bases in addition to pseudouridine could be derived by complementary alignment with DNA also lends support to the rearrangement hypothesis. All of the defined cellular modifications of nucleosides in tRNA occur at the polynucleotide level: the cases of methylation to form ribothymidine and other methylated purine and pyrimidine nucleosides (16), introduction of sulfur to form 4-thiouridine (17,18), and deamination of adenosine to form inosine (19). By analogy the biosynthesis of pseudouridine in tRNA probably occurs at the polynucleotide level.

However, the discovery of free pseudouridine in the culture medium of A. tumefaciens (13) favors the direct incorporation hypothesis. The isolation of ΨMP synthetase (8,9,20) catalyzing the formation of ΨMP from the condensation of uracil and ribose-5-phosphate identifies this enzymatic pathway for the formation of pseudouridine. Goldberg and Rabinowitz (21,22) also demonstrated the phosphorylation of ΨMP to ΨDP and ΨTP by an enzyme extracted from E. coli. ΨTP than can be incorporated into RNA by RNA polymerase (23).

The question as to whether pseudouridine is formed as a monomer or arises at the polynucleotide level is still not soundly

resolved, although a number of observations have been reported to explain one of these two hypotheses, and the body of evidence, direct and indirect favors the formation of pseudouridine in RNA at the polynucleotide level.

Observations from three laboratories have demonstrated the possible presence of ΨMP synthetase in different organisms. First, Breitman (24,25) reported that pyrimidine auxotrophs of E. coli were capable of utilizing pseudouridine as their sole pyrimidine source and also proposed a direct correlation between ΨMP synthetase activity and the ability of pyrimidine auxotrophs of E. coli to grow on pseudouridine. Second, ΨMP synthetase was isolated from T. pyriformis by Heinrikson and Goldwasser (8,20). Third, Suzuki and Hochster (14) elicited the extracellular accumulation of pseudouridine after adding uracil to the synthetic medium of A. tumefaciens.

This thesis confirms the existence of ΨMP synthetase in A. tumefaciens and the following enzymatic pathway for the formation of pseudouridine and ΨMP as postulated by Suzuki and Hochster (9):

$$\text{uracil} + \text{ribose-5-phosphate} \xrightarrow{E1} \Psi\text{MP} \xrightarrow{E2} \text{pseudouridine} + P_i$$

where E1 is regarded as ΨMP synthetase and E2 as a 5'-nucleotidase.

The results of Suzuki and Hochster (14) imply that the conversion of uracil to pseudouridine is dependent on the presence of pyrimidines or pyrimidine ribosides in the medium. However, the results described in this work show that the existence of

ΨMP synthetase is independent of the presence of uracil in the medium and is present throughout the growth curve of the organism.

An independent method of purification of the ΨMP synthetase was developed, and the partially purified enzyme was used in studies of the effects of analogs of uracil on the enzymatic reaction producing ΨMP.

Attempts were also made to determine the equilibrium constant of the reaction for possible insight into the function of ΨMP synthetase.

# EXPERIMENTAL PROCEDURE

## Materials

A. tumefaciens, strain $IIBNV_6$ (nontumorogenic), was kindly provided by Dr. R. M. Hochster of the Canada Department of Agriculture, Ottawa, Canada. Uracil, cytosine, thymine, 5-carboxy-2-thiouracil, isocytosine, 5-iodouracil, 5-hydroxyuracil (isobarbituric acid), 5-hydroxyuridine, orotic acid, isoorotic acid, 2-thiouracil, 2-thiocytosine, 2,4-dihydro-5-thiopyrimidine, 2,4-dichloro-6-methylpyrimidine, and the sodium salt of ribose-5-phosphate were purchased from Sigma Chemical Company. 5-Fluorouracil was obtained from Hoffmann-La Roche Inc. Showdomycin (3-$\beta$-D-ribofuranosylmaleimide) was obtained from P-L Biochemicals, Inc. Chloramphenicol was obtained from Parke, Davis Company. 2-$^{14}$C-Uracil was purchased from Schwarz BioResearch. DEAE-Cellulose, type 20, was purchased from Carl Schleicher and Schuell Company. Alkaline phosphatase (E. coli) (grade BAPF) was obtained from Worthington Biochemical Corporation. All other chemicals used were the best available grades.

## Methods

Growth. For enzyme purification, A. tumefaciens was maintained and grown according to the method of Suzuki and Hochster (14). For determination of the effect of uracil on $\Psi$MP synthetase activity at different points of the growth curve, A. tumefaciens was grown independently in uracil-supplemented M9 medium (26)

which contained 0.5% uracil and 2% glucose, and non-uracil medium containing the same components except that uracil was omitted. A similar medium containing 1% yeast extract was used for one experiment.

Preparation of cell-free extract (Fraction I). The cell-free extract containing crude enzyme was obtained by following the methods of Suzuki and Hochster (9). The cells of A. tumefaciens were harvested by centrifugation at 5,860 x g for 25 min after incubation for 20 hr at 26° with shaking. The pellet was washed twice with cold 0.9% NaCl and once with 0.01 M Tris-Cl (pH 8.5) containing $MgCl_2$ ($8.3 \times 10^{-4}$ M) and mercaptoethanol ($5 \times 10^{-3}$ M) (Tris buffer). After centrifugation at 12,100 x g for 20 min, the cells were suspended in 2 vol. of Tris buffer. The resultant cell suspension was subjected to sonication in a Raytheon 10-kc sonic oscillator for 20 min at 5 to 7° at a power output of 1 amp. The supernatant solution obtained by centrifugation at 12,100 x g was the crude extract.

All subsequent steps were done at 0 to 5°.

Streptomycin sulfate precipitation (Fraction II). One-tenth volume of a 10% streptomycin sulfate solution was added dropwise to the cell-free extract containing 32 mg of protein per ml. The mixture was stirred for 20 min and then centrifuged for 20 min at 12,100 x g. The precipitate was discarded.

Ammonium sulfate precipitation (Fraction III). Finely divided solid $(NH_4)_2SO_4$, 12 g, containing 0.24 g $(NH_4)_2CO_3$ was added slowly with stirring to 80 ml of the supernatant solution

from the streptomycin treatment to bring the solution to 0.2 of saturation. The precipitate was removed by centrifugation at 12,100 x g. An additional 9 g of $(NH_4)_2SO_4$ and 0.18 g of $(NH_4)_2CO_3$ were added to attain 0.35 of saturation. The precipitate was collected by centrifugation and resuspended in 58 ml of Tris buffer.

DEAE-cellulose fractionation (Fraction IV). Thirty-four milliliters of fraction III containing 505 mg of protein was adjusted to 0.15 M in KCl and applied to a 7.8 g column of DEAE-cellulose (12 x 2.2 cm) previously washed with 0.1 N NaOH and 0.1 N HCl and equilibrated with Tris buffer containing 0. 15 M KCl. The column was then washed with 100 ml of Tris buffer containing 0.15 M KCl. Separation of proteins was achieved by stepwise elution with 45 ml of Tris buffer containing 0.15, 0.20, 0.25, and 0.30 M KCl. ΨMP synthetase activity eluted from the column when the KCl concentration reached 0.25 M.

Second DEAE-cellulose fractionation (Fraction V). The 0.25 M KCl eluate containing 28.8 mg protein was diluted to 0.15 M with Tris buffer. The diluted eluate was applied to a 440 mg DEAE-cellulose column (5 x 1 cm) which was previously washed and equilibrated as before. The enzyme was eluted stepwise with 2.5 ml of Tris buffer containing 0.15, 0.20, 0.25, and 0.30 M KCl.

Enzyme assays. The standard assay mixture contained the following in a final volume of 0.5 ml: $^{14}$C-uracil, 0.05 ml (1 μmole, 150,000 cpm); ribose-5-phosphate, 0.1 ml (4 μmole); Tris buffer, 0.1 ml (50 μmoles), pH 8.5; $MgCl_2$, 0.05 ml (1 μmole) and 0.2 ml

of enzyme preparation. For various assays the volume of the assay mixture varied from 0.1 ml to 0.5 ml.

The assay mixtures were incubated for 30 or 60 min (the time of incubation was adjusted to give not more than 25% conversion of substrate to product) at 30° with shaking. The reaction was terminated by cooling in an ice-bath and spotting 20 µl of the reaction mixture on Whatman 3MM paper (23 x 23 cm) approximately 3 cm from the bottom edge. Ascending paper chromatography was carried out in n-propanol/ethyl acetate/water (70/10/20). Carrier uracil (10 µg) and pseudouridine (10 µg) were added before chromatography. Compounds were identified by examining chromatograms in short wave ultraviolet light in a Chromato-Vue viewing chamber (UV Products, San Gabriel, California).

The ultraviolet absorbing areas were located, cut out, and put into scintillation vials. Five milliliters of toluene-base scintillation fluid was added, and radioactivity was determined in a Beckman LS-200B Liquid scintillation spectrometer. Scintillation fluid consisted of 5 g of Packard Permablend II (98% 2,5-diphenyl-oxazole and 2% 1,4-bis-2-(5-phenyloxazolybenzene)) per liter of toluene.

Protein determination. Protein content of enzyme preparations was determined by the colorimetric method of Lowry et al. (27) using crystalline bovine serum albumin as standard.

Preparation of $^{14}$C-ΨMP. A 2 ml standard assay was incubated for 6 hr at 26° with partially purified enzyme. Protein was removed by centrifugation after heating the assay mixture in

boiling water for 30 sec. The supernatant solution was taken to dryness by lyophilization. The residue was dissolved in 0.25 ml of water and streaked on Whatman 3MM paper (4.7 x 13.5 cm). After chromatography in n-propanol/ethyl acetate/water (70/10/20) by the descending method, the strip of dried chromatogram with an $R_f$ value corresponding to that of $\Psi_{MP}$ was cut out and eluted with 0.5 ml water. The eluate was concentrated to dryness in vacuo. The residue was dissolved in 0.5 ml of water.

# RESULTS

Effect of growth conditions on ΨMP synthetase. As shown in Table I, ΨMP synthetase is found in either the early log or stationary phase of the growth curve of A. tumefaciens (Fig. 2). The data also show that the presence of uracil in the culture medium does not affect the specific activity of ΨMP synthetase.

Enzyme purification. The purification of ΨMP synthetase is summarized in Table II. An approximately 32-fold purification was achieved with this procedure. The 5'-nucleotidase is eluted from the DEAE-cellulose column when the concentration of KCl reaches 0.20 M, while ΨMP synthetase is eluted from the DEAE-cellulose column by 0.25 M KCl.

ΨMP as the product of the reaction. To determine the purity of the $^{14}$C-ΨMP isolated and subsequently used in equilibrium studies, an aliquot was spotted on Whatman 3MM paper and chromatographed. There was no significant amount of radioactivity in the uracil or pseudouridine region of the chromatogram (Table III).

Isolated ΨMP was treated with alkaline phosphatase. Aliquots, 5 µl, of the reaction mixture were chromatographed in two solvent systems. Only a single spot of radioactivity, with an $R_f$ value identical with that of authentic pseudouridine, was detected (Table III).

Enzyme kinetic study and inhibition analyses. The $K_m$ and $V_{max}$ are $1.2 \times 10^{-3}$ M and $1.43 \times 10^{-6}$ M/min for uracil as shown in Fig. 3. The $K_m$ for uracil obtained by Suzuki and Hochster (9)

Table I

The effect of growth conditions on ΨMP synthetase

| Growth time, hours | M9 medium | | M9 medium + uracil | |
|---|---|---|---|---|
| | Specific activity x $10^4$ | Protein concentration | Specific activity x $10^4$ | Protein concentration |
| | µmole/min/mg | mg/ml | µmole/min/mg | mg/ml |
| 6 | 12.4 | 19.2 | 11.2 | 20.5 |
| 6 | | | 10.6[a] | 20.9[a] |
| 6 | 14.4[b] | 24.6[b] | 11.5[b] | 22.3[b] |
| 20 | 5.7 | 50.3 | 4.5 | 41.3 |
| 20 | | | 11.0[b] | 32.0[b] |

[a]Cells harvested and washed in the presence of chloramphenicol (100 µg/ml).

[b]Medium containing 1% yeast extract.

Figure 2

Growth curve of A. tumefaciens grown on a uracil-supplied M9 medium (●). The growth of A. tumefaciens was inhibited by adding chloramphenicol (100 µg/ml) after 6 hr (▲) of growth.

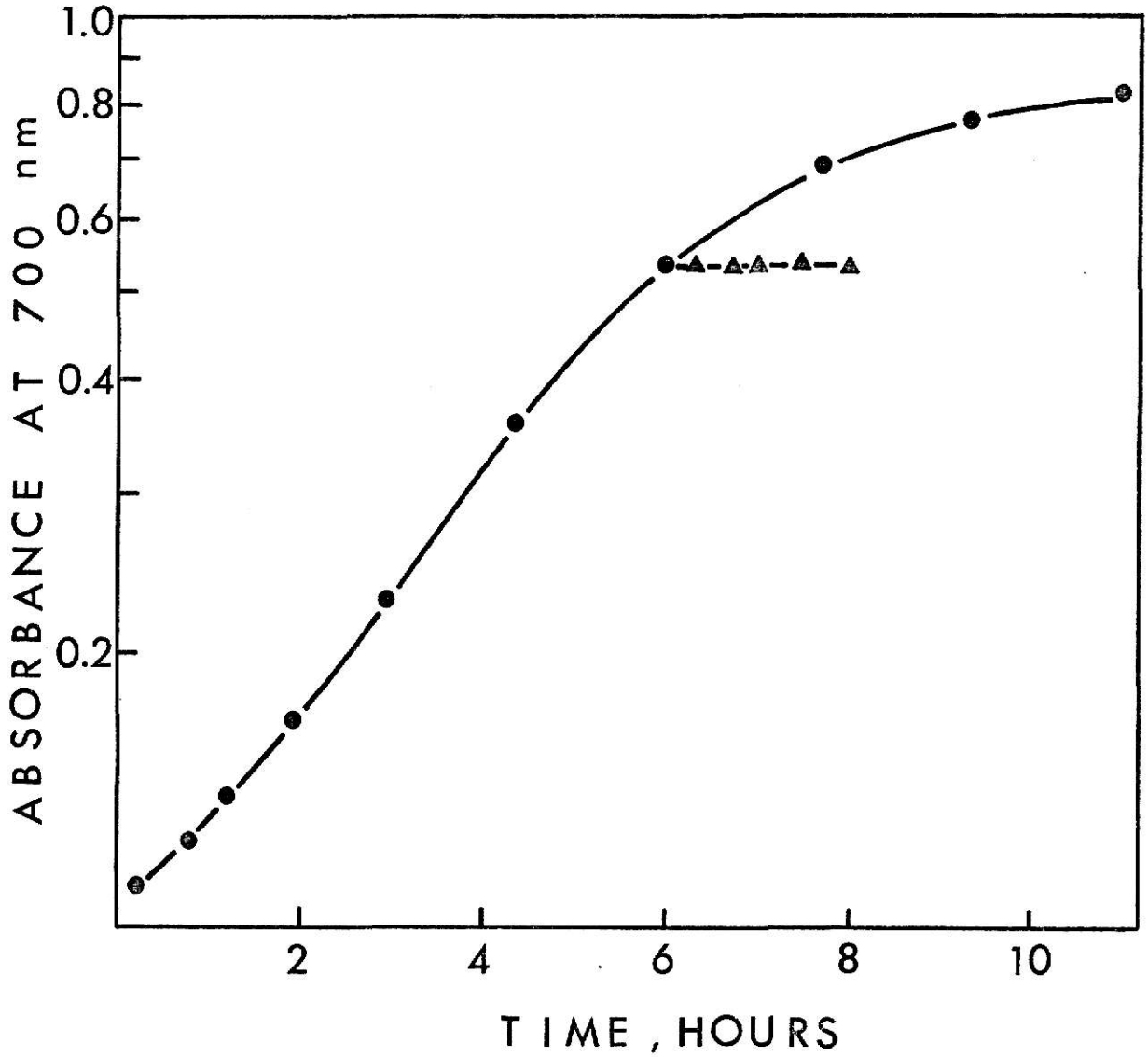

1.0
0.8
0.6
0.4
0.2
ABSORBANCE AT 700 nm
2
4
6
8
10
TIME, HOURS

Table II

Purification of ΨMP synthetase from Agrobacterium tumefaciens

| Fraction | Purification step | Total volume | Protein | Specific activity x $10^4$ | Total activity | Yield |
|---|---|---|---|---|---|---|
| | | ml | mg/ml | μmole/min/mg | μmole/min | % |
| I | Cell-free preparation | 85 | 32.0 | 11.0 | 3.00 | 100 |
| II | Streptomycin sulfate (10%) | 83 | 28.4 | 14.1 | 3.34 | 111 |
| III | 0.2-0.35 of saturation $(NH_4)_2SO_4$ | 77 | 14.8 | 34.3 | 3.92 | 131 |
| IV | DEAE-cellulose fractionation | 90 | 0.44 | 339 | 1.34 | 45 |
| V | Second DEAE-cellulose fractionation (concentration) | 5.0 | 0.70 | 357 | 0.125 | 4 |

## Table III

### Purity of isolated $^{14}$C-ΨMP and the product of dephosphorylation

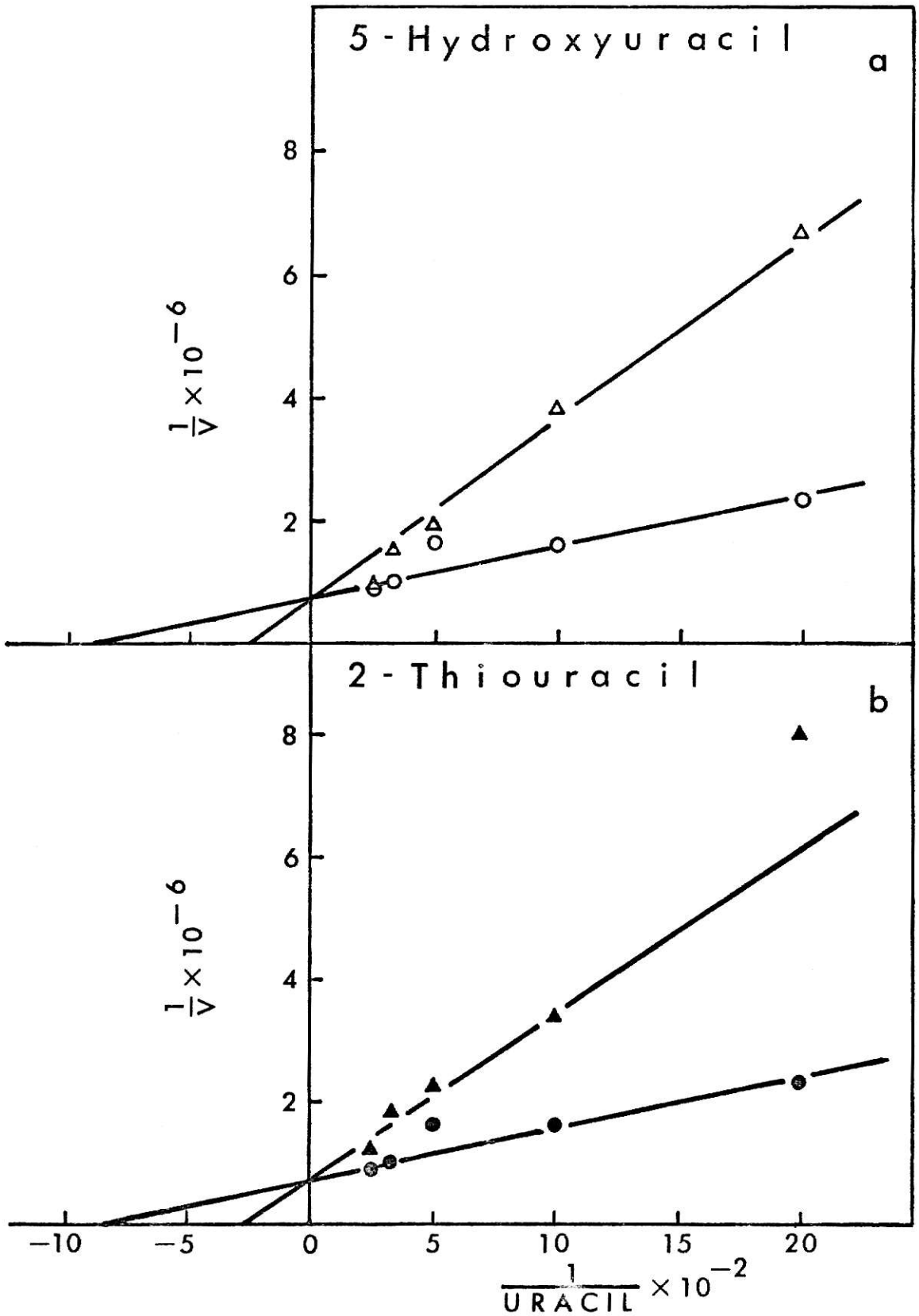
Purity of isolated $^{14}$C-ΨMP was determined by chromatographing in solvent A 20 µl of a mixture containing 10 µl of ΨMP (24,000 cpm), 50 µl of 0.5 M Tris (pH 8.5), 25 µl of 0.02 M $MgCl_2$, and 165 µl of water. The treatment of $^{14}$C-ΨMP with alkaline phosphatase was carried out by mixing 5 µl of alkaline phosphatase (100 µg/ml), 2 µl of of $^{14}$C-ΨMP, 1 µl of 0.5 M Tris (pH 8.5) and 5 µl of water. The mixture was incubated for 16 hr at 37°. Five microliters of the mixture was chromatographed in solvents A and B. Solvent A: n-propanol/ethyl acetate/water (70/10/20). Solvent B: t-butyl alcohol/methyl ethyl ketone/water/$NH_4OH$ (40/30/20/10).

| Compound | $^{14}$C-ΨMP chromatographed in solvent A | | $^{14}$C-ΨMP treated with alkaline phosphatase and chromatographed | | | |
|---|---|---|---|---|---|---|
| | | | Solvent A | | Solvent B | |
| | cpm[a] | $R_f$ | cpm[a] | $R_f$ | cpm[a] | $R_f$ |
| ΨMP | 1837 | 0.02 | 17 | 0.02 | 8 | 0.02 |
| Ψrd | 20 | 0.30 | 1840 | 0.30 | 1770 | 0.31 |
| Ura | 16 | 0.52 | 6 | 0.52 | 10 | 0.42 |
| Cyd | - | - | - | 0.32 | - | 0.49 |
| Urd | - | - | - | 0.42 | - | 0.32 |

[a]Cpm shown in the table are corrected for background.

Figure 3

Plots (Lineweaver-Burk) for ΨMP synthetase (a) in the absence (○) or presence (△) of 2 mM 5-hydroxyuracil and (b) in the absence (●) or presence (▲) of 3 mM 2-thiouracil. The concentrations of uracil were 0.5, 1.0, 2.0, 3.0, and 4.0 mM.


5 - Hydroxyuracil
a
8
6
4
2
$\frac{1}{V} \times 10^{-6}$
2 - Thiouracil
b
8
6
4
2
$\frac{1}{V} \times 10^{-6}$
−10
−5
0
5
10
15
20
$\frac{1}{\text{URACIL}} \times 10^{-2}$

is $6.7 \times 10^{-4}$ M. The ΨMP synthetase from T. pyriformis (8) has a $K_m$ of $7.1 \times 10^{-5}$ M for uracil.

In an attempt to investigate the interaction of uracil with the enzyme, a study of inhibition of ΨMP synthetase by uracil analogs was undertaken. The concentration ratio of analog to uracil was 10 to 1 under the standard assay conditions. During an incubation for 60 min with enzyme fraction IV, orotic acid, isoorotic acid, and thymine failed to inhibit the enzymic reaction. Isocytosine and 2,4-dihydro-5-thiopyrimidine inhibit to about 5%. 5-Carboxy-2-thiouracil, 5-hydroxyuridine, 5-fluorouracil, 5-iodouracil, 2,4-dichloro-6-methyl-pyrimidine, cytosine, and showdomycin inhibit with values ranging from 11 to 18%. 2-Thiocytosine shows 22% inhibition. 5-Hydroxyuracil and 2-thiouracil inhibit the enzymic reaction by 100% and 99%, respectively.

The strong inhibition by 2-thiouracil and 5-hydroxyuracil raised two questions: are these compounds contaminated with uracil, and, if not, is uracil produced from the analogs during the enzymatic reaction. The results obtained to provide answers to these questions are shown in Table IV. Obviously, neither the compounds nor the reaction is contaminated with uracil, although new compounds do appear during the reaction with 2-thiouracil, 5-hydroxyuracil, and 2-thiocytosine.

A study of the type of inhibitions was carried out by assaying with 2-thiouracil and 5-hydroxyuracil in the presence of different uracil concentrations ranging from $0.5 \times 10^{-3}$ M to $4.0 \times 10^{-3}$ M. As shown in Fig. III, the presence of the inhibitors resulted in

Table IV

Determination of the purity of inhibitors and new compounds formed during the enzymatic reaction

For chromatography, 0.5 μmoles of 2-thiouracil, 5-hydroxyuracil, and 2-thiocytosine were used. The enzymatic reactions with inhibitors were carried out as the standard assay except that uracil was omitted. An aliquot containing 0.4 μmoles of inhibitor was used for chromatography. Four solvent systems were used for chromatography: A, n-propanol/ethyl acetate/water (70/10/20); B, upper phase from a mixture of s-butyl alcohol and water; C, t-butyl alcohol/methyl ethyl ketone/water/$NH_4OH$ (40/30/20/10); D, upper phase from a mixture of water, s-butyl alcohol, t-butyl alcohol (48.4/43/8.6).

| Compound | Solvent system | | | |
|---|---|---|---|---|
| | A | B | C | D |
| | $R_f$ | | | |
| Uracil | 0.52 | 0.54 | 0.42 | 0.64 |
| 2-Thiouracil | 0.64 | 0.68 | 0.77 | 0.70 |
| 2-Thiouracil + enzyme | 0.64<br>0.49 | 0.64<br>0.42 | 0.77 | 0.70<br>0.52 |
| 5-Hydroxyuracil | 0.44 | - | 0.37<br>0.02 | - |
| 5-Hydroxyuracil + enzyme | 0.36<br>0.46<br>0.02 | - | 0.34<br>0.02 | - |
| 2-Thiocytosine | 0.45 | 0.73<br>0.44 | - | 0.84<br>0.53 |
| 2-Thiocytosine + enzyme | 0.85<br>0.47 | 0.73<br>0.44 | - | 0.84<br>0.53 |

an increase in the apparent $K_m$ without reducing the $V_{max}$. It therefore appears that each of these inhibitors is competitive. The $K_i$ values for 2-thiouracil and 5-hydroxyuracil are $1.3 \times 10^{-3}$ M and $0.9 \times 10^{-3}$ M.

Equilibrium constant of the enzymic reaction. Attempts to determine the apparent equilibrium constant for the reaction catalyzed by ΨMP synthetase were made by initiating the reaction in either direction. A value of $0.8 \times 10^{3}$ $M^{-1}$ for the apparent equilibrium constant of the forward reaction was obtained with 30-fold purified enzyme (fraction IV, Table II) under standard assay conditions. However, the value changed to $1.0 \times 10^{3}$ $M^{-1}$ when a lower initial concentration (2 mM) of ribose-5-phosphate was used. In addition, the reverse reaction was carried out with isolated $^{14}$C-ΨMP. The apparent equilibrium constants, $0.14 \times 10^{-3}$ M and $0.10 \times 10^{-3}$ M were obtained by using $0.75 \times 10^{-3}$ and $1.32 \times 10^{-3}$ M of $^{14}$C-ΨMP. The details of these assays are presented in the legends to Figs. 4 and 5. A reasonably stoichiometric relationship was observed between the amounts of products formed and the disappearance of reactants in both the forward and the reverse reactions (Table V).

Figure 4

Rate of ΨMP formation. Standard assays were carried out with 2 mM ribose-5-phosphate (a) and 8 mM ribose-5-phosphate (b). Symbols used are: uracil, (●); ΨMP, (▲); pseudouridine, (■).

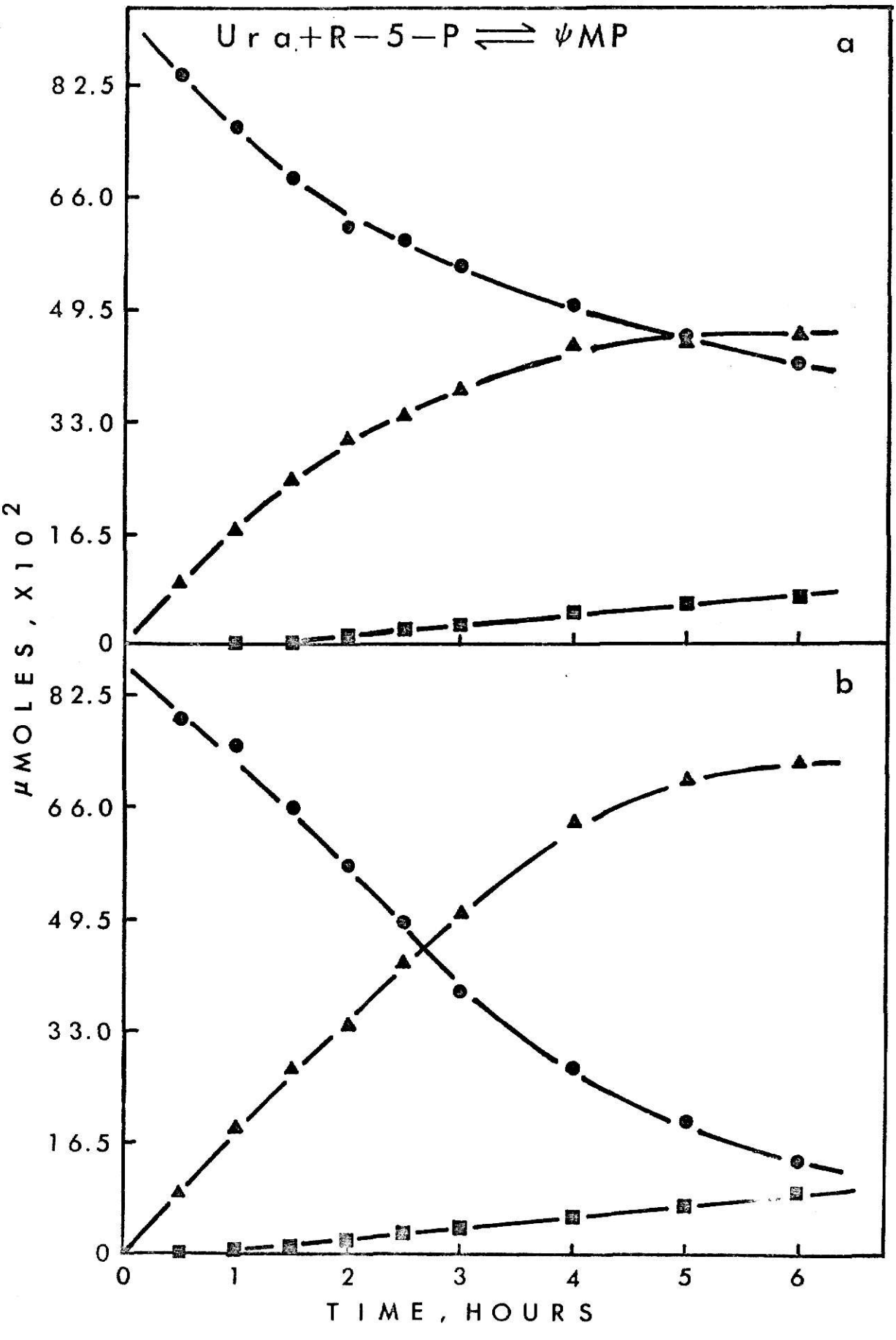

Ura+R-5-P ⇌ ψMP
a
b
82.5
66.0
49.5
33.0
16.5
0
μMOLES, X10²
0
1
2
3
4
5
6
TIME, HOURS

Figure 5

Rate of uracil formation. Contents of reaction mixtures are as follows: $^{14}$C-ΨMP, 1.32 mM (a) or 0.75 mM (b); Tris, 0.1 M, pH 8.5; $MgCl_2$, 2 mM; and water to 0.25 ml total volume. Symbols are used as follows: uracil, (●); ΨMP, (▲); pseudouridine, (■).

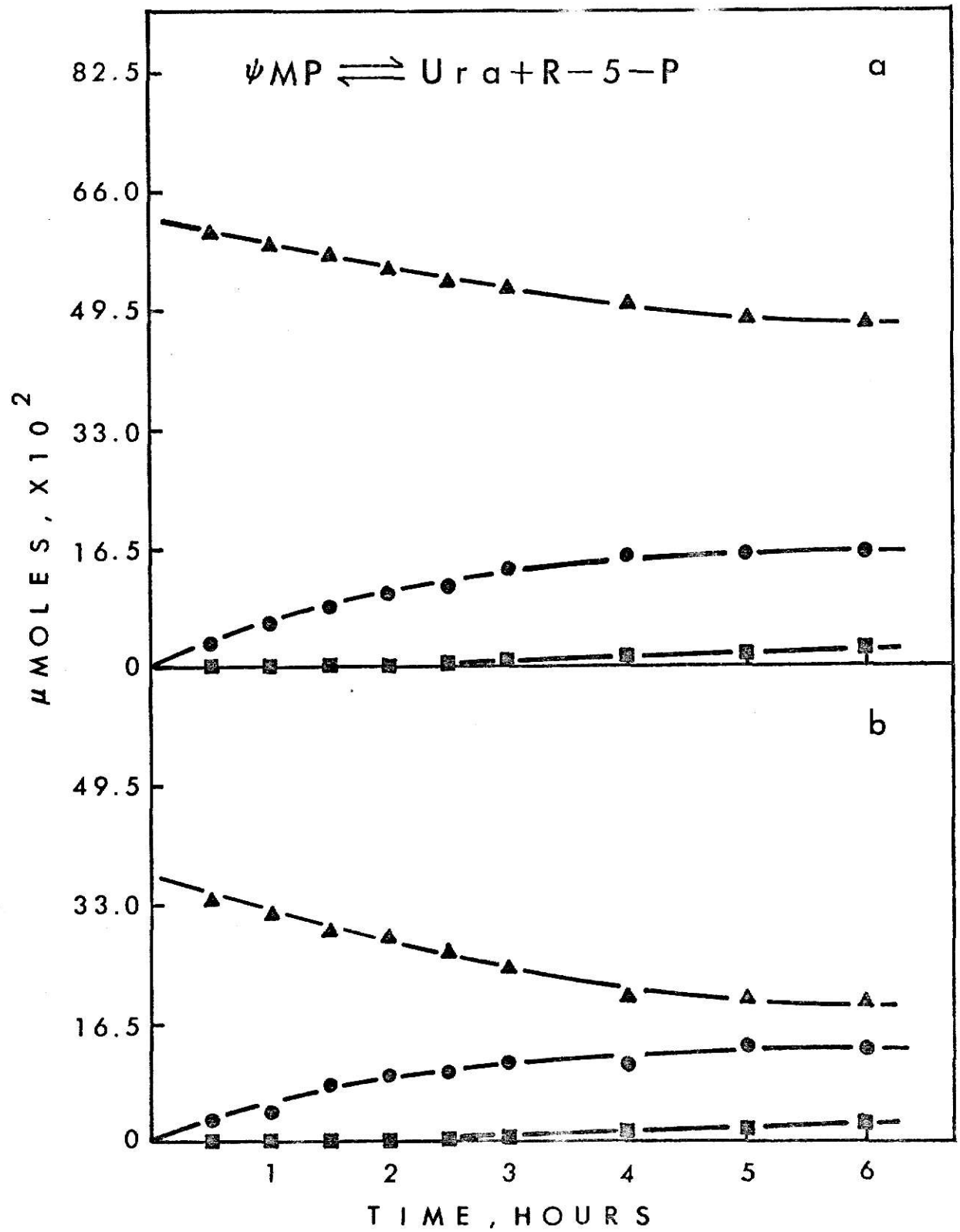

ψMP ⇌ Ura+R−5−P
a
b
82.5
66.0
49.5
33.0
16.5
0
49.5
33.0
16.5
0
μMOLES, X10²
1
2
3
4
5
6
TIME, HOURS

Table V

The stoichiometric relationship between uracil, pseudouridylic acid, and pseudouridine.

The forward reactions were carried out under the standard assay conditions. The reverse reactions were carried out in assays containing 1.32 mM or 0.75 mM of $^{14}$C-ΨMP, 0.1 M Tris (pH 8.5), 2 mM $MgCl_2$, 25 µl of enzyme, and water to bring the reaction mixture to 0.25 ml total volume. Enzyme fraction IV was used in these reactions.

| Direction of the reaction | Concentrations ( x $10^3$ M) at indicated times | | | | | |
|---|---|---|---|---|---|---|
| | 0 hr | | | 6 hr | | |
| | Ura | ΨMP | Ψrd | Ura | ΨMP | Ψrd |
| Forward reaction (R-5-P, 8 mM) | 1.86 | 0 | 0 | 0.27 | 1.45 | 0.19 |
| Forward reaction (R-5-P, 2 mM) | 1.86 | 0 | 0 | 0.80 | 0.91 | 0.14 |
| Reverse reaction | 0 | 0.75 | 0 | 0.25 | 0.45 | 0.05 |
| Reverse reaction | 0 | 1.32 | 0 | 0.31 | 0.96 | 0.05 |

# DISCUSSION

Studies by Suzuki and Hochster (14) showed that when A. tumefaciens is grown on a synthetic medium, the addition of uracil results in the extracellular accumulation of pseudouridine. Breitman and Scher (25) described pyrimidineless strains of E. coli capable of utilizing pseudouridine as their sole pyrimidine source. Breitman (24) further reported that a direct correlation exists between the ΨMP synthetase activity and the ability of pyrimidine auxotrophs of E. coli to grow on pseudouridine. Therefore, the speculated roles of ΨMP synthetase are as follows: (1) the enzyme catalyzes the formation of ΨMP (and indirectly, pseudouridine) from uracil and ribose-5-phosphate; (2) the enzyme catalyzes the degradation of ΨMP formed intracellularly or arising from exogenous pseudouridine.

From the results of the activity of ΨMP synthetase under various growth conditions (Table I), the primary role of ΨMP synthetase appears to be to catalyze the formation of ΨMP from uracil. The results of these experiments do not favor degradation as the role of this enzyme since uracil has no effect on the activity of ΨMP synthetase. The level of enzyme activity extracted from cells harvested and washed with chloramphenicol further indicates that the enzyme activity from cells grown in uracil-containing medium is not a result of derepression during the collection of cells. Almost the same activity is present in the rich medium (containing 1% yeast extract). Therefore, ΨMP

synthetase appears to be synthesized constituitively in this organism.

If the role of ΨMP synthetase is to catalyze the degradation of ΨMP, the enzymic reaction might be expected to favor the reaction in the reverse direction. However, the data on the apparent equilibrium constant, $0.8 \times 10^3$ to $1.0 \times 10^4$ $M^{-1}$, indicate that the reaction favors the formation of ΨMP in vitro. Since the culture medium of A. tumefaciens grown on uracil is a good source of pseudouridine, the equilibrium data probably accurately reflect the same situation in vivo, although some allowance should made for the effect of the 5'-nucleotidase.

As shown in Table I, the crude enzyme extracts from cells at 20 hr of growth have lower specific activities. It is reasonable that relatively more protein is found in the stationary phase with a corresponding reduction of the specific activities.

ΨMP synthetase was isolated from T. pyriformis by Heinrikson and Goldwasser (8) and from A. tumefaciens by Suzuki and Hochster (9). My work is based on that of the latter workers. However, a new purification procedure is reported here. The main advantage of this method is the elimination of the use of acid, alkali, heat, and acetone treatments which were used in the purification by Suzuki and Hochster (9). They reported that ΨMP was gradually produced relative to pseudouridine as purification proceeded. However, after following their procedure, there was a gross loss of activity at preparation II by adding 1 N NaOH. Crude enzyme

extracts were treated with heat and acetone, independently. No formation of ΨMP was observed, although production of pseudouridine was decreased by 84% and 73%, respectively.

The use of arsenate or fluoride for inhibiting 5'-nucleotidase was attempted, but the results were unsuccessful. In the presence of 0.02 M $Na_2HAsO_4$ and 0.1 N NaF, the formation of pseudouridine was only 7.5% and 11.8% of the normal level; there was no corresponding increase in the amount of ΨMP formed. Apparently, ΨMP synthetase was drastically inhibited while 5'-nucleotidase was slightly inhibited by arsenate and fluoride.

The purification procedure gives a distinct separation of ΨMP synthetase and 5'-nucleotidase at the step with DEAE-cellulose. It is a quick way to obtain purified ΨMP synthetase. Pseudouridine is shown as the only product in the enzyme assay with enzyme fractions I, II, and III. The product of the enzyme assay with fraction IV was isolated and identified as ΨMP. When this isolated ΨMP was treated with alkaline phosphatase, pseudouridine was the only product (Table III).

The study of inhibition by uracil analogs shows that 2-thiouracil and 5-hydroxyuracil are two efficient inhibitors. The fact that 5-hydroxyuracil completely inhibited the reaction, while 5-hydroxyuridine inhibited the reaction by 15%, is further evidence that a free pyrimidine, not a nucleoside, is the precursor of ΨMP. Heinrikson and Goldwasser (8) reported that 2-thiouracil and isocytosine interfere in the reaction of ΨMP synthetase from T. pyriformis with uracil.

It is surprising that 5-hydroxyuracil is one of the efficient inhibitors, especially since 5-fluorouracil is much less inhibitory. If a substituent in the 5 position were to influence inhibition by competing with uracil, the smaller fluorine would seem to be a better candidate than the relatively bulky hydroxyl group.

5-Hydroxyuracil has the hydroxyl substituent on the pyrimidine ring where the ribose would be attached by ΨMP synthetase; therefore, the formation of a nucleotide with a C-C bond on the 5 position is not possible. However, a nucleotide, 5-ribosyl-2-thiouracil-5'-monophosphate, could be formed during the competitive inhibition by 2-thiouracil. In fact, Heinrikson and Goldwasser (8) reported that the nucleotides of 2-thiouracil and isocytosine are formed in the reaction of ΨMP synthetase from T. pyriformis.

## SUMMARY

Pseudouridine monophosphate (ΨMP) synthetase, an enzyme catalyzing the formation of the carbon-carbon bond of ΨMP between uracil and ribose-5-phosphate, was isolated from Agrobacterium tumefaciens. A 32-fold purified enzyme was obtained by fractionation with $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose, which gives a distinct separation of ΨMP synthetase and 5'-nucleotidase. ΨMP is the product of the enzymic assay with enzyme obtained by chromatography on DEAE-cellulose; however, pseudouridine is the product of the reaction with relatively crude preparations since 5'-nucleotidase is a contaminate.

The Michaelis constant for uracil is $1.2 \times 10^{-3}$ M. Data on the apparent equilibrium constant, $0.8 \times 10^{3}$ to $1.0 \times 10^{4}$ $M^{-1}$, indicate that the reaction favors the formation of ΨMP in vitro. The data probably accurately reflect the same situation in vivo, since the culture medium of A. tumefaciens grown on uracil is a good source of pseudouridine. The enzyme appears to be constitutive, since the absence or presence of uracil has no effect on the level of enzyme activity. Further, the enzyme is present throughout the growth curve of the organism.

A study of inhibition by uracil analogs shows that 2-thiouracil and 5-hydroxyuracil are efficient inhibitors. The $K_i$ values for 2-thiouracil and 5-hydroxyuracil are $1.3 \times 10^{-3}$ and 0.9nx $10^{-3}$ M, respectively. The fact that 5-hydroxyuracil completely inhibited the reaction, while 5-hydroxyuridine inhibited

the reaction by 15%, is further evidence that a free pyrimidine, not a nucleoside, is the precursor of ΨMP.

REFERENCES


1. Hall, J. B., and Allen, F. W., Biochim. Biophys. Acta 45, 163 (1960).

2. Lis, A. W., and Lis, E. W., Biochim. Biophys. Acta 61, 799 (1962).

3. Lis, A. W., and Lis, E. W., Life Science 4, 2187 (1965).

4. Pollak, J. K., and Arnstein, H. R. V., Biochim. Biophys. Acta 55, 798 (1962).

5. Brown, D. M., Burdon, M. G., and Slachter, R. P., Chem. commun. 77 (1965).

6. Dlugajczyk, A., and Eiler, J. J., Biochim. Biophys. Acta 119, 11 (1966).

7. Robbins, P. W., and Kinsey, B. M., Fed. Proc. 22, 229 (1963).

8. Heinrikson, R. L., and Goldwasser, E., J. Biol. Chem. 239, 1177 (1964).

9. Suzuki, T., and Hochster, R. M., Can. J. Biochem. 44, 259 (1966).

10. Weiss, S. B., and Legault-Demare, J., Science 145, 548 (1964).

11. Kusama, K., Prescott, D. M., Froholm, L. O., and Cohn, W. E., J. Biol. Chem. 241, 17 (1966).

12. Ginsberg, T., and Davis, F. F., J. Biol. Chem. 243, 6300 (1968).

13. Suzuki, T., and Hochster, R. M., Can. J. Microbiol. 10, 867 (1964).

14. Suzuki, T., and Hochater, R. M., Can. J. Microbiol. 12, 271 (1966).

15. Jacobson, M. K., Ph. D. Dissertation, Kansas State University, (1969).

16. Srinivasan, P. R., and Borek, E., Science 145, 548 (1964).

17. Hayward, R. S., and Weiss, S. B., Proc. Nat'l, Acad. Sci. U. S. A. 55, 1161 (1966).

18. Lipsett, M. N., and Peterkofsky, A., Proc. Nat'l. Acad. Sci.

U. S. A. 55, 1169 (1966).

19. Kammen, H. O., and Spengler, S. J., Biochim. Biophys. Acta 213, 352 (1970).

20. Heinrikson, R. L., and Goldwasser, E., J. Biol. Chem. 238, 485 (1963).

21. Goldberg, I. H., and Rabinowitz, M., Biochim. Biophys. Acta 54, 202 (1961).

22. Ko, S. D., Rabinowitz, M., and Goldberg, I. H., Biochemistry 3, 1840 (1964).

23. Slapikoff, S., and Berg, P., Biochemistry 6, 3654 (1967).

24. Breitman, T. R., J. Bacteriol. 103, 263 (1970).

25. Breitman, T. R., and Scher, C. D., J. Bacteriol. 96, 1873 (1968).

26. Anderson, E. H., Proc. Nat'l. Acad. Sci. U. S. A. 32, 102 (1946).

27. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

# STUDIES ON PSEUDOURIDINE MONOPHOSPHATE SYNTHETASE OF AGROBACTERIUM TUMEFACIENS

by

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Pseudouridine monophosphate (ΨMP) synthetase, an enzyme catalyzing the formation of the carbon-carbon bond of ΨMP between uracil and ribose-5-phosphate, was isolated from Agrobacterium tumefaciens. A 32-fold purified enzyme was obtained by fractionation with $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose, which gives a distinct separation of ΨMP synthetase and 5'-nucleotidase. ΨMP is the product of the enzymic assay with enzyme obtained by chromatography on DEAE-cellulose; however, pseudouridine is the product of the reaction with relatively crude preparations since 5'-nucleotidase is a contaminate.

The Michaelis constant for uracil is $1.2 \times 10^{-3}$ M. Data on the apparent equilibrium constant, $0.8 \times 10^{3}$ to $1.0 \times 10^{4}$ $M^{-1}$, indicate that the reaction favors the formation of ΨMP in vitro. The data probably accurately reflect the same situation in vivo, since the culture medium of A. tumefaciens grown on uracil is a good source of pseudouridine. The enzyme appears to be constitutive since the absence or presence of uracil has no effect on the level of enzyme activity. Further, the enzyme is present throughout the growth curve of the organism.

A study of inhibition by uracil analogs shows that 2-thiouracil and 5-hydroxyuracil are efficient inhibitors. The $K_i$ values for 2-thiouracil and 5-hydroxyuracil are $1.3 \times 10^{-3}$ and $0.9 \times 10^{-3}$ M, respectively. The fact that 5-hydroxyuracil completely inhibited the reaction, while 5-hydroxyuridine inhibited the reaction by 15%, is further evidence that a free pyrimidine, not a nucleoside, is the precursor of ΨMP.

# VITA

Ying-chun Liu was born in Mukden, China, on January 15, 1946, the daughter of Jeu and Yen C Liu. She graduated from Taipei First Girls' Middle School in June, 1964. She entered Tunghai University, Taiwan, Rep. of China, in September of that year. Subsequently, she enrolled in National Taiwan University in September, 1965, and graduated in June, 1968, with a Bachelor of Science in Agronomy. In September, 1968, she entered the Graduate School of Kansas State University where she served as a Graduate Research Assistant in the Department of Agronomy until January of 1969. She became a graduate student in the Department of Biochemistry in February, 1969 and served as a Graduate Research Assistant from May, 1969 through September, 1970.