Metabolism of Pyrimidine Nucleotides in a Microorganism

III. Enzymatic Production of Ribose-5-Phosphate from Uridine-5'-Monophosphate by Pseudomonas oleovorans

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A study was made to develop a new method for the production of ribose-5-phosphate (R-5-P) from uridine-5'-monophosphate (UMP) by the action of nucleotide-\(N\)-ribosidase of Pseudomonas oleovorans, and a suitable medium for the formation of nucleotide-\(N\)-ribosidase was established. For the enzymatic conversion of UMP to R-5-P, a cell suspension was employed as the enzyme source. Although degradation of R-5-P, the desired product, occurred during the course of the enzyme reaction, it was prevented by the addition of an appropriate amount of zinc ion and resulted in a stoichiometric conversion of UMP to R-5-P and uracil. Accumulated R-5-P was readily isolated by ion-exchange chromatography of the bacteria-free reaction mixture. Yield of isolated R-5-P was about 60% of the theoretical recovery.

Several reports have appeared concerning the methods for the production of R-5-P. Based on the particular principle involved, these methods can be classified into two types: (i) chemical hydrolysis of the \(N\)-ribosidic linkage of purine nucleotides (2, 3) and (ii) phosphorylation of ribose by ribokinase (1, 4). Very little is known about the utilization of pyrimidine nucleotides for the production of ribose-5-phosphate (R-5-P). Pyrimidine nucleotides, such as uridine-5'-monophosphate (UMP) and cytidine-5'-monophosphate (CMP), are readily available as waste by-products of purine nucleotide production from ribonucleic acid. Thus, a procedure which results in an efficient conversion of pyrimidine nucleotides to R-5-P is likely to be a very desirable method for the production of R-5-P.

Our previous work (5) showed that a cell-free extract of Pseudomonas oleovorans catalyzes the hydrolysis of the \(N\)-ribosidic linkage of pyrimidine nucleotides producing R-5-P and the corresponding base. The present paper deals with the development of an enzymatic method for the efficient preparation of R-5-P from UMP, which utilizes cells of P. oleovorans.

MATERIALS AND METHODS

Reagents. With the exception of UMP, all reagents were obtained from commercial sources. UMP was obtained from Yamasa Shoyu Co., Ltd., Choshi, Japan. Organism. P. oleovorans ATCC 1086 was used and was precultivated in bouillon-peptone medium containing 1% meat extract, 1% peptone, 0.5% NaCl, and 0.3% yeast extract (pH adjusted to 7.0 with an aqueous solution of NaOH), at 28 C for 24 hr with reciprocal shaking (140 rev/min, 8-cm stroke). To 100 ml of medium in 500-ml shaking flasks, 5 ml of precultivated broth was added, and the cultivations were carried out at 28 C for 36 hr with reciprocal shaking (140 rev/min, 8-cm stroke). The cells were harvested by centrifugation, washed with saline, and suspended in 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.0. The suspension was appropriately diluted with the same buffer and used for the enzyme reaction.

Assay of enzyme activity. The activity of nucleotide-\(N\)-ribosidase was assayed by estimating the formed reducing sugar. The reaction mixture containing 25 \(\mu\)moles of NaF, 500 \(\mu\)moles of Tris-hydrochloride buffer (pH 7.0), and cell suspension (5.5 mg, dry weight) in a total volume of 2.5 ml was incubated at 37 C for 4 hr. After the incubation, the reaction mixture was heated in a boiling water bath for 3 min. The denatured cells were removed by centrifugation, and the supernatant fluid was assayed for reducing sugar. The enzyme activity was expressed as micromoles of reducing sugar formed in 1 ml of reaction mixture in a 1-hr reaction.
RESULTS AND DISCUSSION

(i) Cultural conditions for formation of the enzyme. To establish optimal conditions for the formation of the enzyme, the following investigations were carried out.

Effect of carbon source. The utilization of various carbon sources, such as sugars and organic acids, was examined. Among those tested, fumaric and citric acids were found to be most favorable for both growth and enzyme formation (Table 1). Fumarate was chosen as the main carbon source for the subsequent experiments.

Effect of concentration of fumaric acid. As shown in Table 2, 2% of fumaric acid (as disodium salt) was the best concentration for the formation of the enzyme. Concentrations greater than 2% stimulated growth but not enzyme formation.

Effect of nitrogen source. As shown in Table 3, bacterial growth and enzyme formation was affected by the nitrogen source employed. Peptone and meat extract were most effective on both growth and enzyme formation. Moreover, it was observed that ammonium chloride and urea were effective stimulators of enzyme formation and had less effect on bacterial growth than did peptone.

Effect of concentration of peptone. The optimal concentration of peptone for both enzyme for-

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**Table 2. Effect of concentration of fumaric acid on enzyme formation**

| Conc of fumarate (Na2) (%) | Growth (mg/ml) | Enzyme activitya |
|---------------------------|---------------|------------------|
|                           | per mg of cells | per ml of medium |
| 0                         | 1.71          | 0.07             |
| 0.5                       | 2.29          | 0.07             |
| 1.0                       | 2.80          | 0.25             |
| 1.5                       | 3.68          | 0.38             |
| 2.0                       | 4.60          | 0.50             |
| 2.5                       | 5.13          | 0.39             |
| 3.0                       | 5.09          | 0.34             |

* In addition to fumaric acid, all media contained 1.5% peptone, 0.2% yeast extract, 0.01% K2HPO4, 0.01% KH2PO4, and 0.03% MgSO4·7H2O (pH adjusted to 7.0).

**Table 3. Effect of nitrogen sources on enzyme formation**

| Additions to medium | Growth (mg/ml) | Enzyme activitya |
|---------------------|---------------|------------------|
|                     | per mg of cells | per ml of medium |
| (NH4)2CO3 (0.53%)   | 0.08          | 0.24             |
| (NH4)2SO4 (0.62%)   | 0.34          | 0.24             |
| NH4Cl (0.50%)       | 0.29          | 0.45             |
| (NH4)2HPO4 (0.62%)  | 0.44          | 0.25             |
| (NH4)H2PO4 (1.07%)  | 0.57          | 0.21             |
| NH4NO3 (0.75%)      | 0.38          | 0.16             |
| NaNO3 (0.79%)       | 1.71          | 0.14             |
| Urea (0.28%)        | 1.65          | 0.43             |
| Peptone (0.90%)     | 3.30          | 0.33             |
| Corn steep liquor   | 3.00          | 0.27             |
| Yeast extract (0.45%) | 2.30        | 0.12             |
| Meat extract (7.10%) | 3.70          | 0.32             |

* In addition to the above nitrogen sources, all media contained 2.0% fumarate(Na2), 0.2% yeast extract, 0.01% K2HPO4, 0.01% KH2PO4, and 0.03% MgSO4·7H2O (pH adjusted to 7.0). Concentrations of the respective nitrogen sources were equivalent to 1.0% casein hydrolysate on a nitrogen basis.

**Table 1. Effect of carbon sources on enzyme formation**

| Additions to medium | Growth (mg/ml) | Enzyme activitya |
|---------------------|---------------|------------------|
|                     | per mg of cells | per ml of medium |
| Glucose (2.0%)      | 3.69          | 0.16             |
| Sucrose (1.9%)      | 1.64          | 0.13             |
| Glycerol (2.1%)     | 2.77          | 0.13             |
| Citrate(Na2)·2H2O (2.9%) | 3.93      | 0.27          |
| Tartrate(Na2)·2H2O (2.2%) | 1.01      | 0.11          |
| Acetate(Na) (2.7%)  | 2.58          | 0.13             |
| Succinate(Na2)·3H2O (4.5%) | 2.00      | 0.11          |
| Fumarate(Na2) (2.0%) | 3.52          | 0.12             |
| None                | 1.79          | 0.19             |

* In addition to the above carbon sources, all media contained 1.0% casein hydrolysate, 0.2% yeast extract, 0.01% K2HPO4, 0.01% KH2PO4, and 0.03% MgSO4·7H2O on a carbon basis.

b Dry cell weight.

c Micromoles of reducing sugar formed per hour.

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Analytical methods. Nucleic acid-related compounds were determined by methods previously reported (5). Reducing sugar was determined by the method of Somogyi (7), and R-5-P by the method of Wilson (8).

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ammonium chloride was found to be favorable when either 1 or 1.5% peptone was added.

**Effect of complex nutrients.** A series of complex nutrients, such as yeast extract, meat extract, and corn steep liquor (CSL), was tested for effect on enzyme formation. Only CSL gave a favorable result, although yeast extract did stimulate growth. The highest enzyme activity was obtained with the addition of 5% CSL (Table 6).

**Effect of initial pH.** From the above experiments, a favorable medium for the formation of the enzyme was devised: 2% fumaric acid (as disodium salt), 1.5% peptone, 5% CSL, 0.03% MgSO₄·7H₂O, 0.01% KH₂PO₄, and 0.2% yeast extract. When the microorganism was cultured in this medium, the pH of the medium increased. As indicated in Fig. 1 and 2, the optimum initial pH was found to be near 7.0, and this pH was used in the remaining experiments.

**Time course of enzyme formation.** As shown in Fig. 3, the maximum formation of the enzyme was obtained after 48 hr of cultivation and gradually declined thereafter. The 48-hr-old cells were used in the subsequent experiments.

(ii) **Enzymatic preparation of R-5-P: effect of**

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**Table 4. Effect of concentration of peptone on enzyme formation**

| Peptone concn (%) | Growth (mg²/ml) | Enzyme activity<sup>c</sup> per mg of cells | Enzyme activity<sup>c</sup> per ml of medium |
|-------------------|-----------------|---------------------------------------------|---------------------------------------------|
| 0.01              | 0.12            |                                             |                                             |
| 0.05              | 0.26            |                                             |                                             |
| 0.10              | 0.43            |                                             |                                             |
| 0.50              | 2.00            | 0.02                                        | 0.04                                        |
| 0.75              | 2.63            | 0.16                                        | 0.24                                        |
| 1.00              | 5.24            | 0.21                                        | 1.10                                        |
| 1.25              | 4.00            | 0.27                                        | 1.08                                        |
| 1.50              | 4.60            | 0.30                                        | 2.30                                        |
| 1.75              | 4.83            | 0.30                                        | 1.45                                        |
| 2.00              | 3.00            | 0.16                                        | 0.48                                        |

<sup>a</sup> In addition to peptone, all media contained 2.0% fumarate(Na₂), 0.2% yeast extract, 0.01% K₂HPO₄, 0.01% KH₂PO₄, and 0.03% MgSO₄·7H₂O (pH adjusted to 7.0).

<sup>b</sup> Dry cell weight.

<sup>c</sup> Micromoles of reducing sugar formed per hour.

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**Table 5. Effect of NH₄Cl on enzyme formation**

| Additions to medium (%) | Growth (mg²/ml) | Enzyme activity<sup>c</sup> per mg of cells | Enzyme activity<sup>c</sup> per ml of medium |
|-------------------------|-----------------|---------------------------------------------|---------------------------------------------|
| Peptone                 | NH₄Cl           |                                             |                                             |
| 1.0                     | 0               | 3.50                                        | 0.40                                        | 1.40                                        |
| 1.0                     | 0.25            | 3.73                                        | 0.51                                        | 1.90                                        |
| 1.0                     | 0.50            | 3.30                                        | 0.46                                        | 1.52                                        |
| 1.0                     | 1.00            | 3.42                                        | 0.45                                        | 1.54                                        |
| 1.0                     | 1.25            | 3.00                                        | 0.40                                        | 1.20                                        |
| 1.5                     | 0               | 4.40                                        | 0.47                                        | 2.07                                        |
| 1.5                     | 0.25            | 4.30                                        | 0.44                                        | 1.90                                        |
| 1.5                     | 0.50            | 3.89                                        | 0.44                                        | 1.72                                        |
| 1.5                     | 1.00            | 4.18                                        | 0.67                                        | 2.80                                        |
| 1.5                     | 1.25            | 4.20                                        | 0.50                                        | 2.10                                        |

<sup>a</sup> In addition to peptone and NH₄Cl, all media contained 2.0% fumarate(Na₂), 0.2% yeast extract, 0.01% K₂HPO₄, 0.01% KH₂PO₄, and 0.03% MgSO₄·7H₂O (pH adjusted to 7.0).

<sup>b</sup> Dry cell weight.

<sup>c</sup> Micromoles of reducing sugar formed per hour.

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**Table 6. Effect of corn steep liquor on enzyme formation**

| Additions to medium | Growth (mg²/ml) | Enzyme activity<sup>c</sup> per mg of cells | Enzyme activity<sup>c</sup> per ml of medium |
|---------------------|-----------------|---------------------------------------------|---------------------------------------------|
| Peptone             | CSL (%)         |                                             |                                             |
| 0                   | 1.0             | 1.57                                        | 0.07                                        | 0.11                                        |
| 0                   | 3.0             | 3.88                                        | 0.16                                        | 0.62                                        |
| 0                   | 5.0             | 4.94                                        | 0.16                                        | 0.79                                        |
| 0                   | 6.0             | 4.71                                        | 0.35                                        | 1.65                                        |
| 0.5                 | 1.0             | 2.83                                        | 0.18                                        | 0.51                                        |
| 0.5                 | 3.0             | 5.25                                        | 0.28                                        | 1.47                                        |
| 0.5                 | 5.0             | 4.91                                        | 0.35                                        | 1.72                                        |
| 0.5                 | 6.0             | 2.31                                        | 0.74                                        | 1.71                                        |
| 1.0                 | 1.0             | 4.13                                        | 0.24                                        | 0.99                                        |
| 1.0                 | 3.0             | 5.94                                        | 0.32                                        | 1.90                                        |
| 1.0                 | 5.0             | 5.43                                        | 0.40                                        | 2.17                                        |
| 1.0                 | 6.0             | 4.25                                        | 0.51                                        | 2.17                                        |
| 1.5                 | 1.0             | 4.47                                        | 0.30                                        | 1.34                                        |
| 1.5                 | 3.0             | 6.00                                        | 0.42                                        | 2.52                                        |
| 1.5                 | 5.0             | 6.22                                        | 0.58                                        | 3.61                                        |
| 1.5                 | 6.0             | 6.45                                        | 0.47                                        | 3.03                                        |

<sup>a</sup> In addition to peptone and corn steep liquor (CSL) all media contained 2.0% fumarate(Na₂), 0.2% yeast extract, 0.01% K₂HPO₄, 0.01% KH₂PO₄, and 0.03% MgSO₄·7H₂O (pH adjusted to 7.0).

<sup>b</sup> Dry cell weight.

<sup>c</sup> Micromoles of reducing sugar formed per hour.
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**FIG. 1.** Effect of initial pH on enzyme formation. The microorganism was cultured in medium containing 2.0% fumaric acid disodium salt, 1.5% peptone, 5.0% corn steep liquor, 0.03% MgSO₄·7H₂O, 0.2% yeast extract, 1.0% NH₄Cl, 0.01% KH₂PO₄, and 0.01% K₂HPO₄ (pH 7.0). Cultivation was carried out at 30°C for 24 hr. Symbols: (△) enzyme activity per milligram of dry cells, (○) enzyme activity per milliliter of medium, (●) growth, (△) final pH.

**FIG. 2.** Effect of initial pH on enzyme formation. Cultivation was carried out at 30°C for 48 hr. See legend to Fig. 1 for other details and for symbols.

**TABLE 7.** Effect of concentration of ZnCl₂ on ribose-5-phosphate formationa

| ZnCl₂ concn (μmoles/ml) | UMP degradation (μmoles/ml) | Formed (μmoles/ml) | Reducing sugarb |
|-------------------------|-----------------------------|--------------------|----------------|
|                         |                             | R-5-P              |                 |
| 0                       | 36.0                        | 0                  | 36.0            |
| 0.69                    | 36.5                        | 10.8               | 36.0            |
| 1.38                    | 36.5                        | 16.9               | 37.2            |
| 2.08                    | 35.8                        | 26.9               | 38.4            |
| 2.77                    | 36.3                        | 33.8               | 36.6            |
| 3.46                    | 36.9                        | 36.2               | 36.1            |
| 5.00                    | 36.7                        | 36.4               | 37.0            |

a Reaction mixture contained 400 μmoles of uridine-5'-monophosphate (UMP), 500 μmoles of NaF, 1.0 mmole of Tris-hydrochloride buffer (pH 7.0), 56 mg (dry weight) of cells, and indicated amounts of ZnCl₂ in a total volume of 10 ml. Reactions were carried out at 37°C for 4 hr.
b Calculated as ribose-5-phosphate (R-5-P).

degradation to an unidentified phosphate ester. Studies designed to prevent the degradation of R-5-P revealed that zinc ion prevented degradation of R-5-P without inhibiting nucleotide-N-ribosidase activity. As shown in Table 7, addition of 3.5 μmoles of ZnCl₂ per ml completely prevented degradation of R-5-P and increased the accumulation of R-5-P.

**Effect of reaction temperature.** It was also found that degradation of R-5-P in the reaction mixture...
was affected by temperature. For example, even in the presence of ample ZnCl₂, 30% of the formed R-5-P was degraded after 10 hr when the reaction was carried out at 37 C. However, no degradation was observed at 32 C (Fig. 4).

**Effect of increasing concentration of substrate.** When the concentration of UMP was increased above 40 µmoles per ml, the reaction rate decreased (Fig. 5).

**FIG. 4.** Effect of reaction time on R-5-P formation from UMP. Reactions were carried out as described in footnote a of Table 7, except that reaction temperature was changed as indicated. Reducing sugar was calculated as R-5-P. Symbols: (△) uracil, (□) R-5-P, (●) reducing sugar.

**FIG. 5.** Effect of UMP concentration on UMP degradation. Reactions were carried out as described in footnote a of Table 7, except that UMP concentration and reaction temperature were changed as indicated. Symbols: (□) at 37 C, (●) at 32 C, (△) at 28 C.

The preparation of R-5-P from UMP was carried out under the optimum condition established by the above experiments, i.e., 43.1 mmoles of UMP-Na₂, 50 mmoles of NaF, 3.5 mmoles of ZnCl₂, and 5.6 g (dry weight) of cells in a total volume of 1 liter (pH adjusted to 7.0) with incubation at 32 C. After 10 hr, the reaction mixture
was centrifuged and the clear supernatant fluid was subjected to the treatment described in Fig. 6. The yield of R-5-P (as barium salt) was about 60% (27 mmoles) of the theoretical recovery.

**Physical and chemical properties of the isolated material.** The absorption spectrum and the infrared spectrum of the isolated material were almost identical to those of authentic R-5-P (Fig. 7 and 8) and the physical and chemical properties of the isolated material agree with those of authentic R-5-P (Table 8). These data thus indicate that the isolated material was indeed R-5-P. The isolated material contained 47.2% R-5-P [estimated by the method of Wilson (8)], 23.7% water, and 28.1% ash; no other sugar was detected by paper chromatography.

**Fig. 7.** Absorption spectra of isolated sample and ribosyl compounds in carbazole-H$_2$SO$_4$ test. (- - - - ) ribose, (-----) R-5-P, (------) R-1-P, (-----) sample.

**Fig. 8.** Infrared spectra of authentic R-5-P (A) and isolated sample (B).

| Property                        | Isolated material | Authentic material$^a$ |
|---------------------------------|-------------------|------------------------|
| Total phosphate/ribose          | 0.98              | 0.98                   |
| Inorganic phosphate             | Trace             | Trace                  |
| Reducing sugar/ribose           | 1.02              | 0.99                   |
| Acid-labile phosphate$^b$       | 0                 | 0                      |
| Carbazol reaction E$_{260}$/E$_{190}$ | 0.70          | 0.69                   |

$^a$ Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

$^b$ Phosphate liberated after incubation in 0.5 N H$_2$SO$_4$ at 37 C for 15 min.

$^c$ Solvent system: A, 2-propanol-2-butanol-15 N NH$_4$OH (40:20:1); B, 2-propanol-trichloroacetic acid-15 N NH$_4$OH-water (75:5:3:25); C, isobutyric acid-15 N NH$_4$OH (10:6); D, 1-butanol-propionic acid–water (100:50:7).

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