Purine Nucleoside Synthesis, an Efficient Method Employing Nucleoside Phosphorylases†

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ABSTRACT: An improved method for the enzymatic synthesis of purine nucleosides is described. Pyrimidine nucleosides were used as pentosyl donors and two phosphorylases were used as catalysts. One of the enzymes, either uridine phosphorylase (Urd Pase) or thymidine phosphorylase (dThd Pase), catalyzed the phosphorolysis of the pentosyl donor. The other enzyme, purine nucleoside phosphorylase (PN Pase), catalyzed the synthesis of the product nucleoside by utilizing the pentose 1-phosphate ester generated from the phosphorolysis of the pyrimidine nucleoside. Urd Pase, dThd Pase, and PN Pase were separated from each other in extracts of *Escherichia coli* by titration with calcium phosphate gel. Each enzyme was further purified by ion-exchange chromatography. Factors that affect the stability of these catalysts were studied. The pH optima for the stability of Urd Pase, dThd Pase, and PN Pase were 7.6, 6.5, and 7.4, respectively. The order of relative heat stability was Urd Pase > PN Pase > dThd Pase. The stability of each enzyme increased with increasing enzyme concentration. This dependence was strongest with dThd Pase and weakest with Urd Pase. Of the substrates tested, the most potent stabilizers of Urd Pase, dThd Pase, and PN Pase were uridine, 2′-deoxyribose 1-phosphate, and ribose 1-phosphate, respectively. Some general guidelines for optimization of yields are given. In a model reaction, optimal product formation was obtained at low phosphate concentrations. As examples of the efficiency of the method, the 2′-deoxyribonucleoside of 6-(dimethylamino)purine and the ribonucleoside of 2-amino-6-chloropurine were prepared in yields of 81 and 76%, respectively.

Two types of enzymes have been used as catalysts in laboratory-scale syntheses of nucleosides of purines and their analogues: *trans-N*-deoxyribosylases and nucleoside phosphorylases. The *trans-N*-deoxyribosylases seem to be limited in their distribution to the bacterial genus *Lactobacillus* and also in their catalytic use to the synthesis of 2′-deoxyribonucleosides (Holguin et al., 1975; Stout et al., 1976). In contrast, the phosphorylases are widely distributed in nature and have been used for the synthesis of both ribonucleosides and 2′-deoxyribonucleosides. In this respect, they are more versatile catalysts than are the *trans-N*-deoxyribosylases. Syntheses of purine nucleosides have been carried out with purine nucleoside phosphorylase (PN Pase)[1] from both microbial (Sivadjian et al., 1969) and mammalian sources (Krenitsky et al., 1967; Parks et al., 1979). Methods involving a variety of bacterial extracts as catalysts have also been described (Imada et al., 1966; Kamimura et al., 1973).

Despite past efforts and the potential advantages of enzyme catalysts, nucleosides are still largely synthesized by chemical procedures not involving enzymes. This reflects the relative inefficiency of current enzymatic methods and clearly indicates the need for improvement in the enzymatic approach to nucleoside synthesis.

After extensive study, an improved general method for the synthesis of purine nucleosides and their analogues has been developed in this laboratory. This method employs two nucleoside phosphorylases as catalysts with pyrimidine nucleosides as pentosyl donors. Procedures for purifying three nucleoside phosphorylases from *Escherichia coli* are described in this report, together with studies on the factors which affect the stabilities of these catalysts under reaction mixture conditions. Examples demonstrating the efficiency of this synthetic approach are provided.

Experimental Procedures

Materials. Casamino acids (vitamin free) were purchased from Difco Labs; DEAE-cellulose (Microgranular DE52) was purchased from Whatman; ECTEOLA-cellulose was purchased from Bio-Rad Laboratories; crystalline calf spleen PN Pase was purchased from Boehringer Mannheim; bovine serum albumin (Type V) was purchased from Sigma; Sephadex G-25 (coarse) was purchased from Pharmacia; [8-^{14}C]Hyp (4.1 Ci/mol) was purchased from New England Nuclear; thin-layer cellulose plates were purchased from Eastman.

Enzyme Assays. Nucleoside phosphorylase assays were performed by continuous spectrophotometric monitoring as previously described (Krenitsky et al., 1976). One unit of enzyme activity was that amount of enzyme which catalyzed the formation of 1 μmol of product/min under the assay conditions defined. Protein concentrations were determined by the method of Murphy & Kies (1960) and phosphate concentrations by the method of Ames (1966).

Heat Treatments. An appropriate volume of the ammonium sulfate suspension of one of the purified enzyme preparations (Tables I–III) or calf spleen PN Pase was centrifuged and the pellet dissolved in 1 mL of 10 mM Pipes·KOH buffer, pH 7.0. This solution was passed through a Sephadex G-25 column (1.5 × 18 cm) equilibrated and eluted with this buffer at 4 °C. The fractions containing the bulk of the enzyme activity were combined and stored at −73 °C. Aliquots of the desalted enzyme solutions were heat treated for 10 min at the specified temperatures and then were rapidly cooled in an ice bath.

Quantitation of [^{14}C]Nucleoside Formation. Reactions were stopped by drying a 5-μL aliquot spotted on a cellulose thin-layer sheet. Separation of [^{14}C]Hyp from [^{14}C]Ino or [^{14}C]dIno was achieved by developing the 15-cm sheet in water. Hyp had an R_f value of 0.63 and its nucleosides both had values of 0.83. These spots were cut out and the levels

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[1] Abbreviations used: PN Pase, purine nucleoside phosphorylase (EC 2.4.2.1); Urd Pase, uridine phosphorylase (EC 2.4.2.3); dThd Pase, thymidine phosphorylase (EC 2.4.2.4); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Rib-1-P, α-D-ribofuranosyl 1-phosphate; dRib-1-P, α-D-2′-deoxyribofuranosyl 1-phosphate; Ino, inosine; Ado, adenosine; Hyp, hypoxanthine; Ade, adenine.

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Table I: Purification of Urd Pase from *E. coli* B-96

step	volume (mL)	total units	units/mg of protein	x-fold purifcn	% recovery
cell extract	1180	6460	0.16	1	100
calcium phosphate gel eluate	2000	4510	1.24	7.8	70
DEAE-cellulose chromatography	252	3320	4.17	26	51
ECTEOLA-cellulose chromatography	67	2190	10.9	68	34

Table II: Purification of dThd Pase from *E. coli* B-96

step	volume (mL)	total units	units/mg of protein	x-fold purifcn	% recovery
cell extract	1180	76 460	1.9	1	100
calcium phosphate gel eluate	2520	40 500	76.2	8.5	53
DEAE-cellulose chromatography	203	28 100	76	40	37
ECTEOLA-cellulose chromatography	120	24 100	153	81	32

of radioactivity were determined by the liquid scintillation method.

Results

Enzyme Preparations. All operations were performed at room temperature unless otherwise specified.

(1) Preparation of the Cell Extract. The medium for a 550-L culture of *E. coli* B-96/1 (ATCC 13,863) contained the following components: 18.9 g/L $Na_2HPO_4 \cdot 7H_2O$, 6.3 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L $(NH_4)_2SO_4$, 0.5 g/L adenosine, and 8.0 g/L casamino acids. These components were dissolved in water and the resulting solution was autoclaved. Cells were grown at 36 °C with continuous aeration. When the optical density at 600 nm (as measured without dilution) reached 2.7, the culture was centrifuged. The resulting cell paste (2.5 kg) was suspended in 5 L of 5 mM potassium phosphate buffer, pH 8.0. The cells were disrupted by means of a pressure cell. The supernatant fluid obtained upon centrifugation of the disrupted cell suspension was designated "cell extract" and was stored in aliquots at −73 °C.

(2) Titration with Calcium Phosphate Gel. Calcium phosphate gel was prepared as described elsewhere (Keilin & Hartree, 1938) except that deionized water was used throughout the procedure. The dry weight of the gel suspension was 36 mg/mL. Two hundred and ninety milliliters of this gel was slowly added to 1180 mL of cell extract at 3 °C with continuous mechanical stirring. This suspension was stirred for 1 h and then centrifuged at 13000*g* for 10 min at 3 °C. The pellet (I) was discarded. More gel (650 mL) was added to the supernatant fluid and the mixture was stirred for 1 h at 3 °C. This suspension was centrifuged at 4000*g* for 10 min. The resulting gel pellet (II) contained the bulk of the Urd Pase. The elution of this enzyme from the gel and further purification are described below. The supernatant fluid contained the bulk of the dThd Pase and PN Pase. Six hundred milliliters of gel was slowly added to this supernatant fluid and the mixture was stirred for 1 h at 3 °C. The suspension was centrifuged at 4000*g* for 10 min. The resulting gel pellet (III) contained the bulk of the dThd Pase while the supernatant fluid contained the bulk of the PN Pase.

(3) Purification of Urd Pase. Purification of this enzyme is summarized in Table I. Gel pellet II described above was suspended in 1 L of pH 7.4 potassium phosphate buffer (buffer A), 20 mM with respect to phosphate. After gentle mechanical stirring for 1 h, the suspension was centrifuged at 2600*g* for 10 min. The supernatant fluid was saved and the pellet was extracted again with 1 L of 40 mM buffer A. After centrifugation, the resulting supernatant fluid was combined with that from the first extraction. The combined supernatant fluids could be stored at −73 °C without loss of activity for at least 1 month prior to further purification.

Forty-one grams of DEAE-cellulose (DE-52) was added with stirring to the combined supernatant fluids (2 L) from the calcium phosphate elution. The resin was then packed into a column, 6 cm in diameter and 4 cm in height. The column was consecutively washed with 150 mL of 40 mM buffer A, 50 mL of 100 mM buffer A, 170 mL of 170 mM buffer A, and finally 80 mL of 235 mM buffer A. The last two washes were combined and dialyzed for 17 h at 3 °C against 10 L of deionized water.

The dialysate (252 mL) was applied to an ECTEOLA-cellulose column, 5 cm in diameter and 7 cm in height, equilibrated with 20 mM buffer A. After the sample application, the column was washed with 100 mL of 20 mM buffer A and then the enzyme was eluted with a 1.4-L linear gradient of 20–250 mM buffer A. The combined fractions having the highest activity had a phosphate concentration of 47 mM. Solid ammonium sulfate was added to a concentration of 3.0 M. When stored in this form at 3 °C, the enzyme was stable for at least 1 year.

(4) Purification of dThd Pase. Purification of this enzyme is summarized in Table II. Gel pellet III described above was suspended by mechanical stirring for 15 min at 3 °C in 850 mL of pH 8.0 potassium phosphate buffer (buffer B), 5 mM with respect to phosphate, and then was centrifuged at 4000*g* for 10 min at 3 °C. The pellet was extracted again with 950 mL of 10 mM buffer B, with 800 mL of 15 mM buffer B, and finally with 760 mL of 20 mM buffer B. The last three washes were combined. At this stage, the preparation was stored overnight at 3 °C.

DEAE-cellulose (19 g) was added with stirring at 3 °C to the combined washes. The resin was then packed into a column 2.5 cm in diameter and 7.5 cm in height. This column was washed with 100 mL of 50 mM potassium phosphate, pH 6.4, and then with 200 mL of 75 mM potassium phosphate, pH 6.4. The latter wash, containing the bulk of the enzyme activity, was dialyzed against 10 L of 5 mM buffer B at 3 °C for 17 h.

The dialysate (210 mL) was applied to an ECTEOLA-cellulose column, 5 cm in diameter and 8 cm in height, which had been equilibrated with 5 mM buffer B at 25 °C. The column was then washed with 100 mL of the equilibrating buffer. The enzyme was eluted with a 1.6-L linear phosphate gradient of 5–200 mM buffer B. The fractions having the highest activity were combined and had a phosphate concentration of 50 mM. Solid ammonium sulfate was added to a concentration of 3.0 M. When stored in this form at 3 °C, the enzyme was stable for at least 1 year.

Table III: Purification of PN Pase from *E. coli* B-96

step	volume (mL)	total units	units/mg of protein	x-fold purifcn	% recovery
cell extract	1180	7780	0.19	1	100
calcium phosphate gel eluate	1700	6510	1.36	7.2	84
DEAE-cellulose chromatography	465	3890	7.46	39.3	50
ECTEOLA-cellulose chromatography	70	2650	27.7	146	34

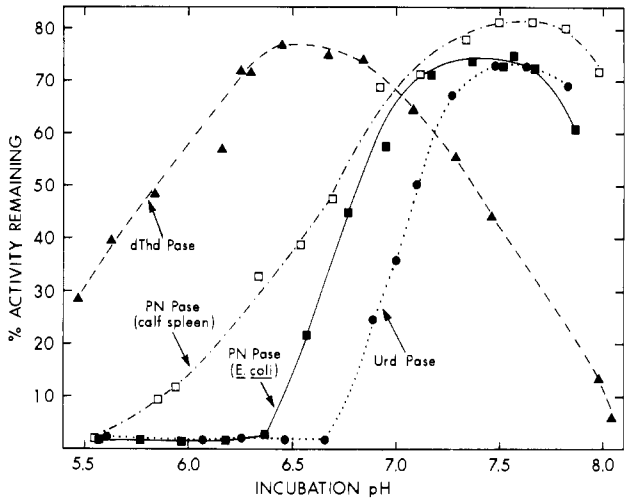


FIGURE 1: Stabilities of some nucleoside phosphorylases as a function of pH. PN Pase from calf spleen (□) was incubated at 35 °C and a protein concentration of 1.6 μg/mL for 10 min. Urd Pase (●), PN Pase (■), and dThd Pase (▲) purified from *E. coli* were incubated at 67, 60, and 48 °C for 10 min and at protein concentrations of 5.8, 0.8, and 1.1 μg/mL, respectively. Incubation mixtures contained 0.2 M Pipes·KOH buffer at the pH value indicated. The pH values at the incubation temperatures were calculated from those measured at 25 °C by using the relationship $\Delta pK_a/°C = -0.009$ (Good et al., 1966).

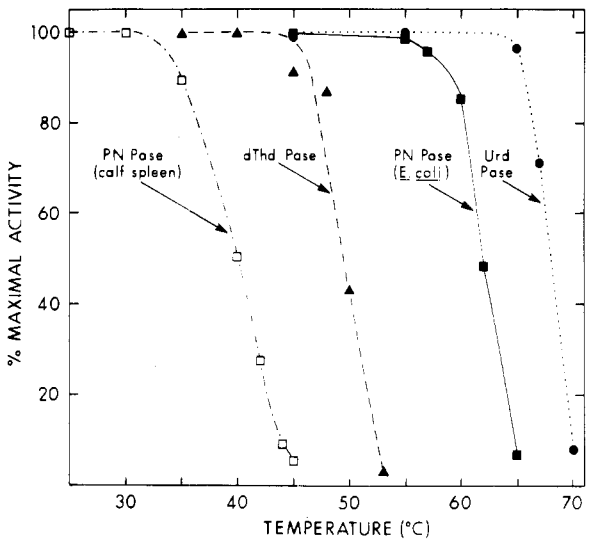


FIGURE 2: Stabilities of some nucleoside phosphorylases as a function of temperature. Incubation conditions were as described in the legend of Figure 1. The pH of incubation for calf spleen PN Pase (□), Urd Pase (●), *E. coli* PN Pase (■), and dThd Pase (▲) was 7.6, 7.8, 7.6, and 6.8, respectively.

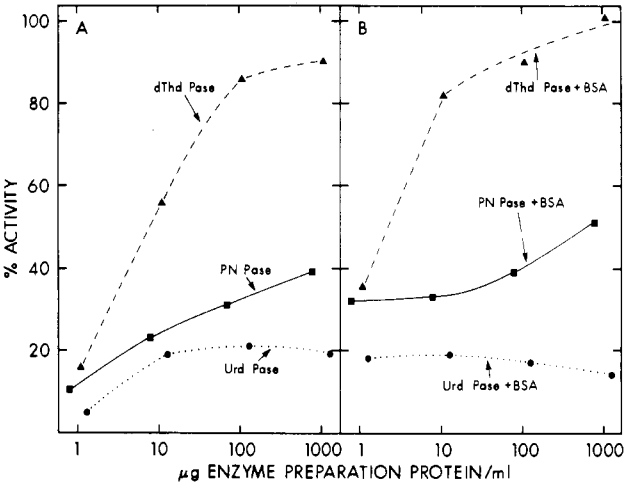


FIGURE 3: Stabilities of nucleoside phosphorylases purified from *E. coli* as a function of the concentration of the enzyme preparation. Urd Pase (●), PN Pase (■), and dThd Pase (▲) were incubated for 10 min at 64, 57, and 50 °C, respectively. The concentration of Pipes·KOH buffer was 0.04 M and the incubation pH for each enzyme was as described in the legend of Figure 2. Incubation conditions for panel B were the same as in panel A except that bovine serum albumin (BSA) was present at 1 mg/mL for Urd Pase and 10 mg/mL for dThd Pase and PN Pase.

(*5*) *Purification of PN Pase.* Purification of this enzyme is summarized in Table III. The final supernatant fluid after the calcium phosphate gel treatment described above contained the bulk of the PN Pase. This material could be stored at −73 °C for at least 20 days before continuation of the procedures as described below. DEAE-cellulose (13 g) was added with stirring at 3 °C to 1700 mL of the calcium phosphate gel supernatant fluid. This resin was then packed into a column 2.5 cm in diameter and 5 cm in height. The column was washed with 25 mL of 25 mM buffer A and then washed consecutively with 100 mL of 50 mM buffer A, 75 mL of 75 mM buffer A, 25 mL of 100 mM buffer A, 265 mL of 100 mM buffer A, and finally 200 mL of 150 mM buffer A. The last two washes were combined and dialyzed against 24 L of deionized H_2O at 3 °C for 17 h.

The dialysate was applied to an ECTEOLA-cellulose column, 5 cm in diameter and 21 cm in height, equilibrated with 10 mM Tris-HCl, pH 8.0 (buffer C), at 25 °C. After the column was washed with 500 mL of 50 mM KCl in buffer C, the enzyme was eluted with 1.6-L linear gradient of 50–500 mM KCl in buffer C. The fractions having the highest specific activity were combined and had a KCl concentration of 170 mM. Solid ammonium sulfate was added to a concentration of 3.0 M. When stored in this form at 3 °C, the enzyme was stable for at least 1 year.

Stability of the Enzyme Catalysts. The effects of pH on the stability of the phosphorylases isolated from *E. coli* are shown in Figure 1. Urd Pase was most stable at pH 7.6, PN Pase at pH 7.4, and dThd Pase at pH 6.5. Also shown in Figure 1 is the pH dependence of the stability of PN Pase from calf spleen, which is similar to that of the *E. coli* enzyme. All heat treatments described below were performed near the pH optima for the stability of each enzyme.

Thermal inactivation curves for the three phosphorylases isolated from *E. coli* and for calf spleen PN Pase are shown in Figure 2. Under the conditions of the heat treatment, the relative stability with the bacterial enzymes was Urd Pase > PN Pase > dThd Pase. Calf spleen PN Pase was much less stable than that from *E. coli*.

The effects of the concentration of each purified enzyme preparation (Tables I–III) on its stability are shown in Figure

Table IV: Effects of Substrates on the Stability of PN Pase from *E. coli*[a]

substrate	% act.[b] remaining	protection[c] constant (mM)
none	<2	
Ino	62	3
Ado	60	3
PO_4(K)	37	5
Hyp	3	
Hyp, PO_4(K)	64	
Ade	6	
Ade, PO_4(K)	61	
Rib-1-P	100	<0.04
dRib-1-P	93	0.2

[a] Heat treatments were as described under Experimental Procedures. The concentration of protein from the purified preparation (Table III) was 120 μg/mL. Treatments were at 60 °C for 10 min in 40 mM Pipes·KOH buffer at pH 7.6 (measured at 25 °C). [b] All substrates were at 4 mM during the heat treatment. [c] Protection constant is defined as that concentration of stabilizer necessary for 50% protection. These values were determined by measuring the stability of the enzyme in the presence of a range of concentrations of substrates.

Table V: Effects of Substrates on the Stabilities of Urd Pase and dThd Pase from *E. coli*[a]

substrate	% act. after 10 min	
	Urd Pase	dThd Pase
none	<2	23
uridine	65	48
thymidine	<2	27
PO_4(K)	<2	87
uracil	<2	51
thymine	<2	17
Rib-1-P	11	87
dRib-1-P	10	116

[a] Heat treatments were as described under Experimental Procedures. The concentration of protein from the purified Urd Pase preparation (Table I) and from the purified dThd Pase preparation (Table II) was 64 and 78 μg/mL, respectively. Substrate concentrations were 18 mM. Urd Pase was treated at 72 °C and dThd Pase at 53 °C in 40 mM Pipes·KOH buffer at pH 7.8 and 6.8, respectively (measured at 25 °C).

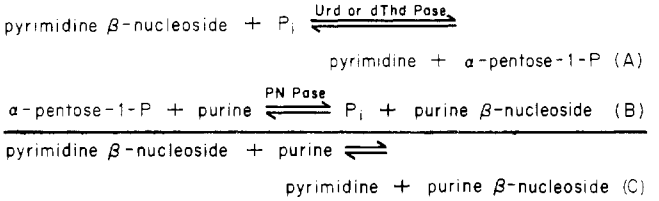
3A. The effect of added bovine serum albumin is shown in Figure 3B. With Urd Pase, the enzyme stability was a function of the concentration of the enzyme preparation only at concentrations below 10 μg/mL. Bovine serum albumin was a suitable substitute. With PN Pase, stability was a function of the concentration of the enzyme preparation at all levels tested. The presence of bovine serum albumin had a greater stabilizing effect at lower enzyme concentrations. However, it did not completely mimic the effect of protein from the enzyme preparation. With dThd Pase, stability was highly dependent on the concentration of the enzyme preparation. Bovine serum albumin had some stabilizing effect at all enzyme concentrations tested.

Table IV shows the effects of substrates on the stability of PN Pase from *E. coli*. The order of stabilizing potency was Rib-1-P > dRib-1-P > Ino = Ado > P_i > Ade > Hyp. Combinations of phosphate and hypoxanthine or adenine protected the enzyme more than was expected from the effects of either alone.

Table V shows the effects of substrates on the stability of Urd Pase. The most potent stabilizer was uridine. About equal protection was seen with Rib-1-P and dRib-1-P.

Table V also shows the effects of substrates on dThd Pase. The most potent stabilizer was dRib-1-P. Next in potency were Rib-1-P and potassium phosphate, which under these conditions gave the same degree of protection. However, when these two stabilizers were compared at a much lower enzyme concentration (1.1 μg/mL), the concentration of phosphate necessary for 50% protection at 53 °C for 10 min was greater than that for Rib-1-P (50 and 3 mM, respectively). Uracil protected appreciably, whereas thymine destabilized the enzyme. Analogously, uridine was a better stabilizer than was thymidine.

Scheme I: Reactions Catalyzed by Nucleoside Phosphorylases in Pentosyl Transfer from a Pyrimidine Nucleoside to a Purine Base



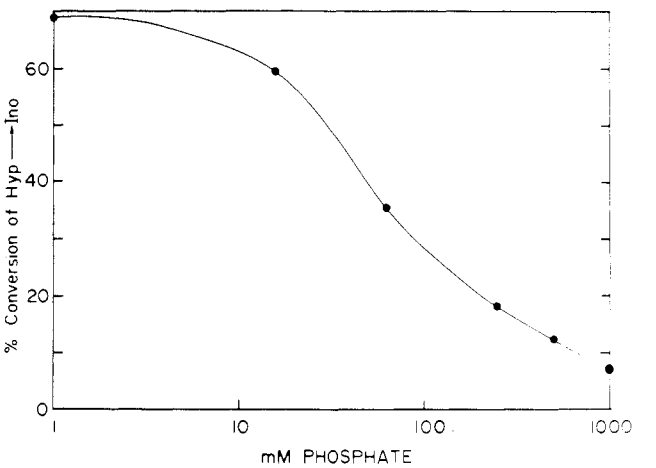


FIGURE 4: Effect of phosphate concentration on inosine synthesis with uridine as the pentosyl donor. Reaction mixtures were incubated at 37 °C and contained, in a final volume of 0.125 mL, 1.38 mM [8-^{14}C]Hyp, 1.38 mM Urd, potassium phosphate buffer, pH 7.0, at the specified concentrations, 0.0037 unit of PN Pase, and 0.0133 unit of Urd Pase. Quantitation was as described under Experimental Procedures. Assurance that equilibrium had been reached was obtained by adding more enzyme to each reaction after 18 h. No change was observed 4 h after this addition.

Synthetic Reactions. Pentosyl transfer catalyzed by nucleoside phosphorylases has been shown to involve the formation and utilization of pentose 1-phosphate (Krenitsky, 1968). Accordingly, the method for the enzymatic synthesis of nucleosides of purines and their analogues which is described below involves a pair of coupled reactions, each catalyzed by a separate enzyme. The first reaction (A in Scheme I) is the phosphorolysis of a pyrimidine nucleoside. The purpose of this reaction is the formation of pentose 1-phosphate to be utilized in the second reaction. Either dThd Pase or Urd Pase catalyzes the phosphorolysis, depending on the structure of the pyrimidine nucleoside. The second reaction (B in Scheme I) is the synthesis of the product nucleoside from the pentose 1-phosphate ester generated in the first reaction and a purine or purine analogue base. This reaction is catalyzed by PN Pase. The net effect of reactions A and B in Scheme I is the transfer of a pentosyl moiety from a pyrimidine nucleoside to a purine or purine analogue base (C in Scheme I). Both donor and product nucleosides are of the β configuration. A double inversion occurs with α-pentose 1-phosphate ester as the intermediate.

(*1*) *Phosphate Concentration.* The amount of hypoxanthine converted to inosine with uridine as the ribosyl donor was a function of the phosphate concentration (Figure 4). The

Table VI: A Comparison of the Efficiency of Various Pentosyl Donors[a]

pentosyl donor	% [^{14}C]nucleoside formed[b]
Rib-1-P	55
uridine[c]	58
inosine	42
adenosine	33
dRib-1-P	55
thymidine[d]	49
2′-deoxyinosine	41
2′-deoxyadenosine	32

[a] Reaction mixtures were incubated at 37 °C and pH 7.0 and contained the following in a final volume of 0.234 mL: 2.3 mM [8-^{14}C]Hyp; 2.3 mM donor; 20 mM PO_4(K); 40 mM Pipes·KOH, 0.015 unit of PN Pase. Additional enzyme was added after 18 h to ensure that equilibrium had been reached. No change in nucleoside formation was noted over the next 2 h. Quantitation was as described under Experimental Procedures. [b] Values did not change significantly from 1.3 to 18 h. [c] Urd Pase (0.027 unit) was added. [d] dThd Pase (0.015 unit) was added.

concentration of inosine at equilibrium decreased with increasing phosphate concentration. By itself, this would suggest that very low phosphate concentrations would give optimal product yields. However, at concentrations of phosphate below 1 mM the time required to reach equilibrium was significantly increased (data not shown). This is consistent with the kinetic constants reported for phosphate and Rib-1-P with the enzymes involved (Krenitsky, 1976; Jensen, 1976).

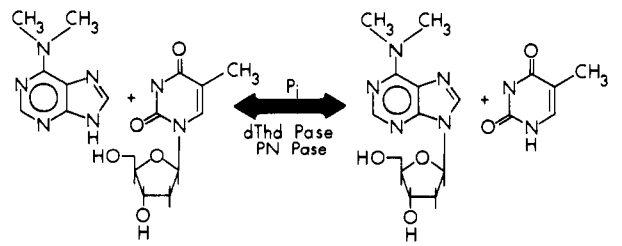
(*2*) *Pentosyl Donors.* Uridine and Rib-1-P were more effective as ribosyl donors than were inosine and adenosine (Table VI). Similarly, thymidine and dRib-1-P were more effective donors than were 2′-deoxyinosine or 2′-deoxyadenosine.

(*3*) *Desalting Enzymes.* As indicated above, all three purified nucleoside phosphorylases were very stable when stored as ammonium sulfate suspensions. Since high concentrations of this salt inhibited the catalytic activity of these enzymes, the suspensions were centrifuged and the pellets were dissolved in water before addition to reaction mixtures. When a high concentration of enzyme was used, it was desirable to further desalt this solution by dialysis or by gel filtration before adding the enzyme to the reaction mixture.

(*4*) *Microbial Growth Inhibition.* In some instances, microbial growth was observed in reaction mixtures. This was especially prevalent with lengthy incubations where the pentosyl donor was uridine. In most cases, this problem was prevented by the addition of potassium azide (0.2%) to the reaction mixtures.

Examples of the Method

(*I*) *6-(Dimethylamino)-9-(β-D-2′-deoxyribofuranosyl)purine* (*Iwamoto et al., 1962*). A suspension of 1 g (5.8 mmol) of



6-(dimethylamino)purine (Elion et al., 1952) and 3.25 g (13.4 mmol) of thymidine in 150 mL of 10 mM potassium phosphate was adjusted to pH 7.4. To this suspension, 18 units of purified PN Pase (Table III) and 5 units of purified dThd Pase (Table II) were added. After 26 h at 36 °C, the reaction mixture was made alkaline by the addtion of 4 mL of 15 M NH_4OH and applied to a Dowex 1-X8 (formate) column, 2.5 cm in diameter and 10 cm in height. The column was then washed with 50 mL of H_2O. The effluent and water wash were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 25 mL of 30% 1-propanol and the solution was applied to a polyacrylamide gel (P-2) column, 5 cm in diameter and 90 cm in height, equilibrated with 30% 1-propanol. The column was washed with this solvent and the eluate was monitored for product by thin-layer chromatography on cellulose with H_2O. Compounds were visualized under ultraviolet light. Fractions containing only the product spot were combined. The bulk of the 1-propanol was evaporated under reduced pressure and the remainder of the fluid was lyophilized, yielding 1.35 g (4.68 mmol) (81%) of a white powder: UV (0.1 N HCl) λ_{max} 268, λ_{min} 232; UV (0.1 N NaOH) λ_{max} 276, λ_{min} 235. [The spectra were in agreement with those reported (Iwamoto et al., 1962).] Anal. Calcd for $C_{12}H_{17}N_5O_3 \cdot 0.5H_2O$: C, 49.99; H, 6.29; N, 24.29. Found: C, 50.12; H, 6.27; N, 24.35.

(*II*) *2-Amino-6-chloro-9-β-D-ribofuranosylpurine* (*Gerster et al., 1963*). A suspension of 1.44 g (8.5 mmol) of 2-amino-6-chloropurine (Hitchings & Elion, 1957) and 4.2 g (17 mmol) of uridine in 200 mL of 10 mM potassium phosphate was adjusted to pH 7.4. To this suspension, 18 units of purified PN Pase (Table III) and 1 unit of purified Urd Pase (Table I) were added. After 7 days at 36 °C, the reaction mixture was filtered. The filtrate was made alkaline by the addition of 4 mL of 15 M NH_4OH and applied to a Dowex 1-X8 (formate) column, 2.5 cm in diameter and 10 cm in height. The column was then washed with water. Fractions were monitored for the presence of product by thin-layer chromatography on cellulose in H_2O. Fractions containing the bulk of the product were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 25 mL of 30% 1-propanol and applied to a polyacrylamide gel (P-2) column, 5 cm in diameter and 90 cm in height, equilibrated with 30% 1-propanol. The column was washed with this solvent and fractions rich in product were evaporated to dryness under reduced pressure. The residue was dissolved in 25 mL of 30% 1-propanol and the above-described P-2 chromatographic step was repeated. Fractions containing only the product spot by thin-layer chromatography were combined, the 1-propanol was evaporated under reduced pressure, and the remaining fluid was lyophilized, yielding 1.95 g (6.5 mmol) (76%) of product: UV (0.1 N HCl) λ_{max} 311, 247, λ_{min} 266, 236; UV (0.1 N NaOH) λ_{max} 388, λ_{min} 265, λ_{sh} 244. [The spectra were in agreement with those reported (Gerster et al., 1963).] Anal. Calcd for $C_{10}H_{12}N_5O_4Cl$: C, 39.81; H, 4.01; N, 23.21. Found: C, 39.60; H, 4.09; N, 23.16.

Discussion

It was shown that *E. coli* is a rich source of readily isolated and stable forms of three separate nucleoside phosphorylases: Urd Pase, dThd Pase, and PN Pase (Tables I–III). Efficient use of these enzymes as synthetic catalysts requires knowledge of factors that influence both their activity and their stability. Kinetic parameters for each enzyme have been reported (Schwartz, 1971; Jensen & Nygaard, 1975; Jensen, 1976; Krenitsky, 1976). The results of the present study reveal several important factors that influence the stability of the enzyme catalysts. Clearly, extremes of pH are to be avoided (Figure 1). Consequently, it is advisable to adjust the pH of the reaction mixture components prior to the addition of enzyme. A small aliquot withdrawn before enzyme addition is

also useful as a control to be used in monitoring the course of the reaction by thin-layer chromatography.

The stability of dThd Pase, PN Pase, and to a lesser extent Urd Pase was greater at higher enzyme concentrations (Figure 3). For this reason, reaction mixture volumes were kept at a minimum. However, in some cases, limitations in the solubility of the pentosyl acceptor necessitated large reaction mixture volumes. Studies on immobilization of these enzymes are currently under way in this laboratory. It is hoped that this approach will overcome the need to compromise on reaction volumes.

Stabilization of the enzymes by substrates (Tables IV and V) is clearly an important factor under reaction mixture conditions. Catalytic efficiency might be a reflection not only on the ability of analogues to serve as substrates but also of their ability to stabilize the catalysts.

The appreciable stabilization of PN Pase from *E. coli* by phosphate, pentose 1-phosphates, and nucleosides, but not by free purine bases (Table IV), is consistent with the mechanism proposed for this enzyme, where all substrates except free bases form binary complexes with the free enzyme (Jensen, 1976). Further, the enhanced stabilization observed with the combination of phosphate and free base (Table IV) supports the proposal that enzyme, phosphate, and purine base form a "dead-end" complex (Jensen, 1976).

The somewhat greater efficiency of uridine as a pentosyl donor compared with inosine (Table VI) is in agreement with the published equilibrium constants for the reactions involved. The reported K_{eq} values for the phosphorolysis of inosine, at pH values near neutrality, range from 0.018 to 0.048, with an average value of 0.03 (Kalckar, 1947; Heppel & Hilmoe, 1952; Tarr, 1958; De Verdier & Gould, 1963; Jensen & Nygaard, 1975; Murakami & Tsushima, 1975). The reported K_{eq} values for the phosphorolysis of uridine range from 0.031 to 0.44 with an average value of 0.2 (Paege & Schlenk, 1952; Pontis et al., 1961; Sakai et al., 1967; Bose & Yamada, 1974). The larger equilibrium constant for the phosphorolysis of uridine as compared with that for inosine is consistent with its greater efficiency as a pentosyl donor. Similarly, the reported K_{eq} for the phosphorolysis of thymidine (0.102) (Bose & Yamada, 1974) is higher than that for the phosphorolysis of 2′-deoxyinosine (0.031) (Tarr, 1958). Perhaps more important than this thermodynamic advantage is the generally greater ease of product isolation that results from the use of pyrimidine rather than purine nucleosides as donors.

Although phosphate stabilized PN Pase and dThd Pase (Tables IV and V), high concentrations in reaction mixtures are generally to be avoided because of its undesirable effect on the yield of the reaction (Figure 4). The possibility also exists that high levels of phosphate might result in the formation of significant amounts of the dead-end complex of PN Pase, phosphate, and purine or purine analogue base as discussed above. On the other hand, very low phosphate concentrations diminish the rate of reaction A in Scheme I (Schwartz, 1971; Krenitsky, 1976) and could result in levels of pentose 1-phosphate esters in the reaction mixture below the K_m value for PN Pase (Jensen, 1976) and too low to stabilize PN Pase and dThd Pase (Tables IV and V). In general, reaction mixture concentrations of phosphate in the range of 1–20 mM were found satisfactory.

Although other parameters such as acceptor base solubility are important, the versatility of this method of nucleoside synthesis is largely a function of the substrate specificity of the enzyme catalysts, especially of PN Pase. The specificity of this enzyme from *E. coli* has been studied in detail (Doskocil & Holy, 1977), and the results suggest that it can be used to synthesize a wide variety of analogues of purine nucleosides. One important difference in the substrate specificity of PN Pase from *E. coli* as compared with its mammalian counterpart is that adenine and some of its congeners are good substrates for the former but not the latter enzyme. This makes possible the enzymatic synthesis of nucleosides of adenine analogues which might not be rapidly cleaved to the free base in mammals. On the other hand, it appears that mammalian PN Pase is more efficient in the synthesis of nucleosides of 2-oxo-6-substituted purines and their analogues than is the *E. coli* enzyme (Doskocil & Holy, 1977; Krenitsky, 1967; Krenitsky et al., 1967, 1968). The wide distribution of the nucleoside phosphorylases and their variation in substrate specificity suggest that appropriate catalysts for the synthesis of a very wide variety of nucleosides might be found in nature.

One of the most important practical features of the enzymatic method for nucleoside synthesis presented here is its adaptability to large-scale work. It appears that enzyme catalysts might play an important role in future developments in nucleoside chemistry.

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Characterization of the Respiratory NADH Dehydrogenase of *Escherichia coli* and Reconstitution of NADH Oxidase in *ndh* Mutant Membrane Vesicles†

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ABSTRACT: Highly purified preparations of the cholate-solubilized respiratory NADH dehydrogenase, isolated from genetically amplified *Escherichia coli* strains [Jaworowski, A., Campbell, H. D., Poulis, M. I., & Young, I. G. (1981) *Biochemistry 20*, 2041–2047], have been characterized. Enzyme preparations were shown to contain 70% (w/w) lipid, predominantly phosphatidylethanolamine. One mol of noncovalently bound FAD and ~1 mol of ubiquinone/mol of enzyme subunit were detected. The purified enzyme was shown to contain only low levels of Fe and acid-labile S, indicating the absence of iron–sulfur clusters. No Cu, Mo, W, or covalently bound P was detected, and no evidence for other chromophores was obtained from visible and ultraviolet absorption spectra of the purified enzyme or of the delipidated polypeptide prepared by gel filtration in sodium dodecyl sulfate. Protein chemical studies verified that the enzyme consists of a single polypeptide species of M_r 47 000, and the N- and C-terminal cyanogen bromide peptides were identified. The pure enzyme was shown to reconstitute membrane-bound, cyanide-sensitive NADH oxidase activity in membrane vesicles prepared from *ndh* mutant strains.

The oxidation of NADH via the respiratory chain with concomitant generation of energy is one of the primary features of aerobic respiration. In *Escherichia coli*, the enzyme catalyzing the first step in this pathway, the respiratory NADH dehydrogenase, is present in the cytoplasmic membrane together with the other components of the electron transport chain.

The low levels of this enzyme in wild-type cells, and the presence of high levels of contaminating NADH dehydrogenase activities, have hampered its isolation and characterization (Dancey et al., 1976; Gutman et al., 1968; Bragg & Hou, 1967a,b). The recent cloning of the *E. coli* respiratory NADH dehydrogenase structural gene (Young et al., 1978), however, has allowed several new approaches to be used to study this enzyme. Amplification of enzyme levels in vivo to 100 times those found in the wild type has enabled it to be purified for the first time with high catalytic activity toward ubiquinone as electron acceptor and in quantities permitting detailed characterization (Jaworowski et al., 1981). The purified enzyme shows a single band of apparent molecular weight 45 000 on $NaDodSO_4$[1] gel electrophoresis. DNA sequencing (Young et al., 1981) has revealed the complete primary structure of the enzyme, which consists of 433 amino acid residues, with a molecular weight of 47 200. By use of the cloned DNA as template, a catalytically active, membrane-bound form of the enzyme has been produced in vitro in a cell-free, coupled transcription–translation system (Poulis et al., 1981).

We describe here the characterization of the purified cholate-solubilized enzyme preparation and show that the enzyme retains the ability to reconstitute functional NADH oxidase activity in membrane particles prepared from *ndh* mutant strains.

Experimental Procedures

Bacterial Strains and Plasmids. The *ndh* mutant strain IY12, a derivative of *E. coli* K12, has been described previously (Young et al., 1978). Plasmid pIY1 (Young et al., 1978) possesses the 2500 base pair *ndh* fragment cloned into the *Eco*RI site of pSF2124 (So et al., 1975). Plasmid pLJ3 (Johnsrud, 1978) carries a double *lac* promoter fragment at the *Eco*RI site of pMB9 (Rodriguez et al., 1976). Plasmid pIY10 is a derivative of pMB9, carrying both the *ndh* and

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[1] Abbreviations used: *ndh*, structural gene for NADH dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; ubiquinone-*n*, ubiquinone isoprenologue containing *n* isoprene units in the side chain; fluorescamine, 4-phenylspiro[furan-2(3*H*)-1′-phthalan]-3,3′-dione; EDTA, ethylenediaminetetraacetic acid disodium salt; TPCK, L-1-(*p*-toluenesulfonyl)-amido-2-phenylethyl chloromethyl ketone.

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