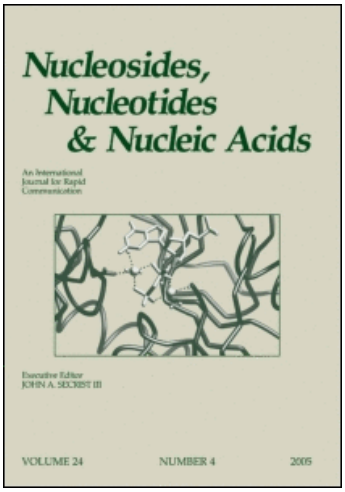

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Cytidine Kinase: A Novel Pyrimidine Ribonucleoside Phosphorylating Enzyme in *Escherzchza Coli*

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CYTIDINE KINASE: A NOVEL PYRIMIDINE RIBONUCLEOSIDE PHOSPHORYLATING ENZYME IN *ESCHERICHIA COLI*

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ABSTRACT: A previously undescribed pyrimidine ribonucleoside kinase that preferentially phosphorylates cytidine to yield its 5'-monophosphate form has been purified to near homogeneity from *Escherichia coli*. The enzyme was distinct from the known pyrimidine nucleoside kinase in the chromatographic profile, substrate specificity and molecular weight.

INTRODUCTION

There are two basic pathways for the biosynthesis of nucleoside triphosphates: (a) *de novo* synthesis from small precursor molecules, and (b) formation via salvage pathway. The latter involves the reutilization of preformed pyrimidine bases which occur in the cell as a result of nucleic acid degradation or uptake from outside of the cell. Since the pioneering work of Valentin-Hansen[1], only one enzyme named pyrimidine nucleoside kinase (EC 2.7.1.48) has been believed to participate in the conversion of pyrimidine ribonucleosides into their monophosphate forms; it catalyzes the phosphorylation of uridine and cytidine with similar efficiencies[2-4]. Through attempts to isolate the pyrimidine nucleoside kinase under various conditions, we came across a novel enzymatic activity which phosphorylates predominantly cytidine.

Here we describe a method for isolating this new enzyme and its biochemical properties.

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T.K. submitted this work as partial fulfillment of the requirement for Ph.D. at Kanazawa University.

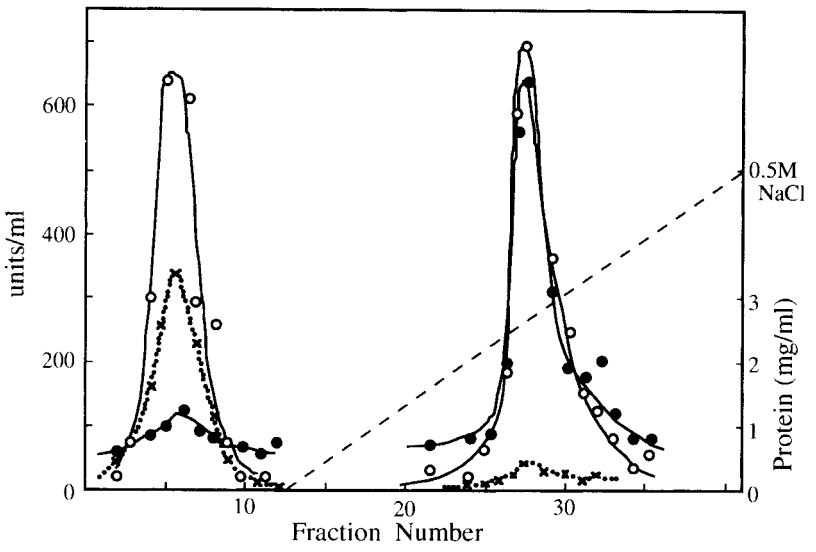

FIG. 1. Chromatography of pyrimidine ribonucleoside kinase activity on phospho-cellulose. The column volume was 50 ml. Equilibrating buffer, TCE-5G. Load, 10 ml of crude extract. Flow rate, 30 ml/h. Fraction volume, 3 ml. All other details are given in the text under Experimental Section and under Results. o—o, CMP forming activity. ●—●, UMP forming activity. x---x, protein concentration. ---, NaCl concentration.

RESULTS

Enzyme Purification. All manipulations were carried out at 4 °C.

Phospho-cellulose Chromatography. The crude extract from *E. coli* Y70-272 (*cdd*⁻) cells was first subjected to phospho-cellulose column chromatography. The column (5.0 x 25 cm) was preequilibrated with TCE-5G buffer onto which the sample was layered, then run with the same buffer and monitored for absorption at 280 nm. After the unbound proteins flowed through, the column was washed until the eluate no longer absorbed 280-nm ultraviolet light. A linear gradient of 0-0.5 M NaCl in TCE-5G was then administered. Eluates were fractionated and assayed for cytidine kinase activity. As shown in FIG. 1, clear peaks could be observed: one in the flow-through, unbound fractions, and the other in fractions bound in TCE-5G but eluted by the NaCl gradient at around 0.3 M.

These two peaks were consistently observed in several independent experiments. The average ratio of cytidine kinase activity in unadsorbed and NaCl-eluted fractions was 36% : 64%. We then examined the properties of the enzymatic activities. For this, fractions with high specific activity were combined for each peak. First, the heat stability was

measured. The cytidine kinase activity in the eluted fractions was 60% inactivated after the incubation at 50°C for 2 min. However, the unadsorbed fractions were only 15% inactivated under the same conditions. The two peaks also differed in substrate preference. The enzyme in the NaCl eluate utilized uridine and cytidine equally well, thus being comparable with the pyrimidine nucleoside kinase previously reported[1]. On the other hand, the unadsorbed fractions phosphorylated cytidine as efficiently as the eluted fractions, but could utilize uridine only to a much lesser extent. Therefore, we investigated the nature of the activity unadsorbed by the phospho-cellulose column.

DEAE-cellulose Chromatography. A pool of the unadsorbed fractions was applied to a DEAE-32-cellulose column (4.3 x 23 cm) which had been equilibrated with TCE-5G. The column was first washed with about 150 ml of TCE-5G until the eluate no longer absorbed 280-nm light. Bound materials were then eluted with a linear gradient of potassium phosphate ranging from 0 M to 0.7 M. The cytidine kinase activity was recovered as a single peak at 0.35 M. Fractions with the highest activity were pooled and underwent subsequent purification procedures.

Hydroxyapatite Column Chromatography. The combined fractions were dialyzed twice against 10 mM potassium phosphate buffer, pH 7.0, and applied to a hydroxyapatite column (2.1 x 25 cm). The chromatography was performed with a potassium phosphate buffer with linearly increasing ionic strength. The enzymatic activity was eluted as a single peak at 0.17 M. The active fractions were then pooled.

Affinity Chromatography. Pooled fractions were desalted on a Sephadex G-25 column (1.0 x 7 cm) equilibrated with TCE-5G. The desalted materials were applied to a CMP-agarose equilibrated with TCE-5G. Proteins were eluted by stepwise addition of TCE-5G containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 1.0 M NaCl. The most of the enzyme activity was recovered in 0.3-M NaCl-eluted fractions (FIG. 2).

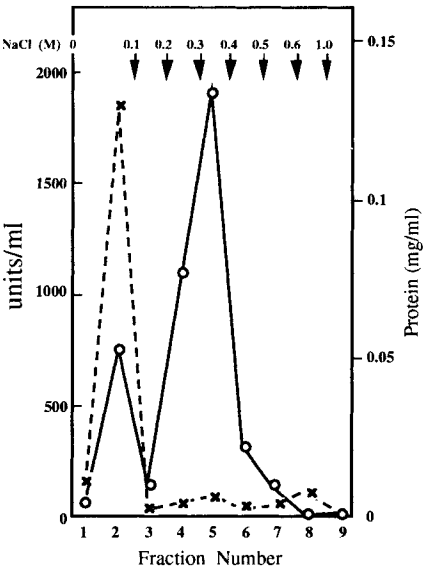

FIG. 2. Chromatography of cytidine kinase activity on CMP-agarose. The column volume was 7 ml. Equilibrating buffer, TCE-5G. The column was eluted with TCE-5G buffer containing indicated concentrations of NaCl. Fraction volume, 5 ml. o—o, CMP forming activity. x---x, protein concentration.

The stages of purification are summarized in TABLE 1. The materials obtained at each purification step were analyzed by SDS-PAGE. As shown in FIG. 3, the number of bands stained with Coomassie brilliant blue decreased as the purification procedures proceeded, and only a single band was marked after the CMP-agarose affinity chromatography. The purified materials were found to migrate to the position of *ca* 43 kDa under the reducing conditions.

Molecular Weight of the Native Enzyme. The molecular weight of the native enzyme was estimated by molecular-exclusion chromatography using a Sephacryl S-200 column. The column was calibrated with markers of known molecular weights including myoglobin, horseradish peroxidase, bovine serum albumin and phosphorylase b. The affinity-purified enzyme was applied to the column, and its molecular weight was estimated from plots of the elution volume versus log molecular weight (FIG. 4). The average value in separate determinations was *ca* 80,000.

Optimum pH. The activity profile of the purified materials was determined as a function of pH. Optimal activity was noted when the pH was between 7.0 and 8.0 in either Tris-HCl or MOPS buffers.

Divalent Cation Requirement. With cytidine as a substrate, Mg^{2+} was required for kination. Replacement of Mg^{2+} by Mn^{2+} decreased the enzyme activity to 15% and that by Co^{2+} to 10%. Only marginal activity was detected with Ca^{2+} or Cu^{2+}. Thus, the rank order of divalent cations in promoting cytidine phosphorylation was $Mg^{2+} >> Mn^{2+} > Co^{2+} >> Ca^{2+}/Cu^{2+}$.

Phosphate Acceptor. TABLE 2 lists the nucleosides tested for their acceptor activities in the kination reaction containing pyrimidine or purine as the base, with ribose or deoxyribose as the sugar moiety, and with various substitutions on their pyrimidine rings. Cytidine was the most preferred substrate (TABLE 2). Uridine exhibited an acceptor activity one order of magnitude lower than cytidine.

Km and Vmax Values. The dependence of the reaction rate on the concentrations of cytidine and uridine was determined. The Km value for cytidine was 0.15 mM. Uridine required much higher concentrations, its Km being 1.2 mM. The value for GTP was 0.17 mM. The Vmax values for cytidine, uridine and GTP were 57, 41 and 53 μmoles/min/mg protein, respectively.

Phosphate Donors and Feedback Inhibition. A variety of nucleoside triphosphates were tested for their ability to donate phosphate to cytidine. GTP was found to be the most effective among the nucleoside triphosphates tested; dGTP, ATP and dATP followed in this order (TABLE 3). UTP and CTP are the end products of this enzyme-driven reaction. Even in the presence of GTP as a phosphate donor, these pyrimidine nucleoside triphosphates were found to inhibit the kination reaction. This suggests that there could be feedback inhibitions of this enzyme. We thus determined the Ki values of CTP and UTP by

TABLE 1. Purification of cytidine kinase from *E. coli*

Step	Total activity (units[1])	Total protein (mg)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extract	3.5×10^5	3680	95	–	100
Phospho-cellulose[2]	1.5×10^5	2980	50	1	43
DEAE-cellulose	1.1×10^5	195	564	11	31
Hydroxyapatite	7.6×10^4	8.5	8,940	177	22
CMP-agarose	4.6×10^4	0.5	92,000	1864	13

[1]Unit definition is according to the Commission of Enzymes of the International Union of Biochemistry[5].
[2]Flowthrough fractions unbound to phospho-cellulose.

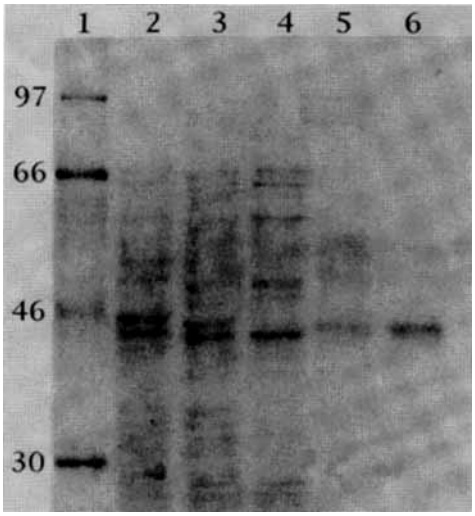

FIG. 3. SDS-polyacrylamide electrophoresis of enzyme preparations after different stages of purification. Samples were treated with SDS and β–mercaptoethanol before loading on Laemmli gel. Molecular weight markers (lane 1) contained phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (46,000) and carbonic anhydrase (30,000). Lane 2, crude extract; lane 3, unadsorbed fractions in phospho-cellulose column chromatography; lane 4, 0.35-M potassium phosphate-eluted fractions in DEAE column chromatography; lane 5, 0.17-M potassium phosphate-eluted fractions in hydroxyapatite column chromatography; lane 6, CMP-agarose affinity purified materials. After electrophoresis, the gel was stained with 0.05% Coomassie brilliant blue and photographed.

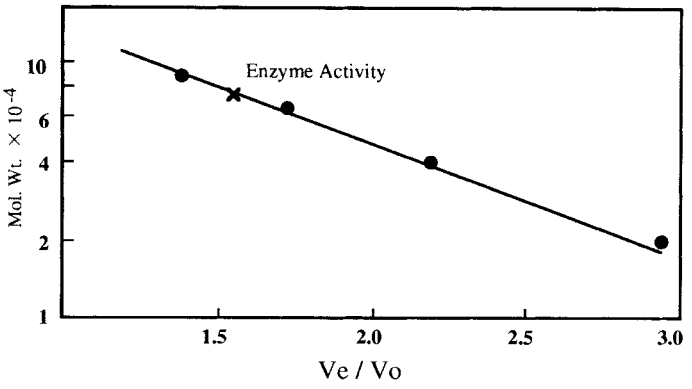

FIG. 4. Estimation of the molecular weight of the native enzyme by gel filtration. Purified proteins were loaded on the top of a Sephacryl S-200 column (0.6 x 110 cm), which had been equilibrated with TCE-5G buffer, and eluted with the same buffer at a flow rate of 5 ml/h. The column was calibrated with phosphorylase b, bovine serum albumin, horseradish peroxidase and myoglobin. *Ordinate*, logarithm of molecular weights; *abscissa*, ratio of elution volume *(Ve)* divided by void volume *(Vo)*.

TABLE 2. List of nucleosides assayed for phosphate acceptor

Acceptor molecule	Relative activity(%)
Cyd	100
Urd	9.6
Ado	8.4
Guo	13.4
dCyd	4.4
dUrd	20
dThd	35
dAdo	18
dGuo	21
FUrd	25
BrUrd	36
IUrd	25
FCyd	18
AraC	34

The nucleosides were assayed as potential phosphate acceptors at 1mM each in place of cytidine by coupled spectrophotometric assay using the purified enzyme. Values are related to the amount of GDP formed with cytidine and expressed as %. Cyd, cytidine; Urd, uridine; Ado, adenosine; Guo, guanosine; dThd, thymidine; FUrd, 5-fluoro substituted uridine; BrUrd, 5-bromo substituted uridine; IUrd, 5-iodo substituted uridine; FCyd, 5-fluoro substituted cytidine; AraC, arabinosyl cytosine.

TABLE 3. Phosphate donors

Donor	Relative activity (%)
GTP	100
ATP	11
CTP	0
UTP	0
dGTP	80
dATP	5
dCTP	0
dUTP	0

The nucleoside triphosphates were assayed as potential phosphate donors at 2 mM each in place of GTP. Values are related to the amount of CMP formed with GTP and expressed as %.

double-reciprocal plotting (FIG. 5 and 6). The Ki value for CTP was 0.03 mM and that for UTP was 1.13 mM. The double-reciprocal plots revealed the mode of the inhibitions as mixed-type.

DISCUSSION

In the present study, we have demonstrated the presence of a previously undescribed enzymatic activity that converts cytidine to its ribonucleotide form in *E. coli*. It was purified to near homogeneity through several chromatographic procedures, including phosphocellulose, DEAE-cellulose, hydroxyapatite and CMP-agarose column chromatography. The estimated molecular weight of the new cytidine kinase was *ca* 80,000 on gel permeation (FIG. 4). In reducing SDS-PAGE, however, it migrated at *ca* 43,000 (FIG. 3). This suggests that the native enzyme would exist and act as a homodimer form of the 43-kDa subunit. The enzyme was separated from the known pyrimidine ribonucleoside kinase on phospho-cellulose where the latter activity was recovered in adsorbed, salt-eluted fraction as described[4] but the former in flow-through, unadsorbed fractions. The enzyme isolated had properties distinct from the EC 2.7.1.48 pyrimidine ribonucleoside kinase as follows. First, it phosphorylates preferentially cytidine, whereas the EC 2.7.1.48 enzyme phosphorylates both uridine and cytidine and so has been named uridine-cytidine kinase or even uridine kinase[1]. The enzyme described herein also was capable of catalyzing the uracil nucleoside-nucleotide conversion, but the Km value for uridine (1.2 mM) was about one order of magnitude higher than that for cytidine (0.15 mM). We thus propose naming the novel enzyme cytidine kinase. Second, this kinase is relatively heat-resistant. Third, it was

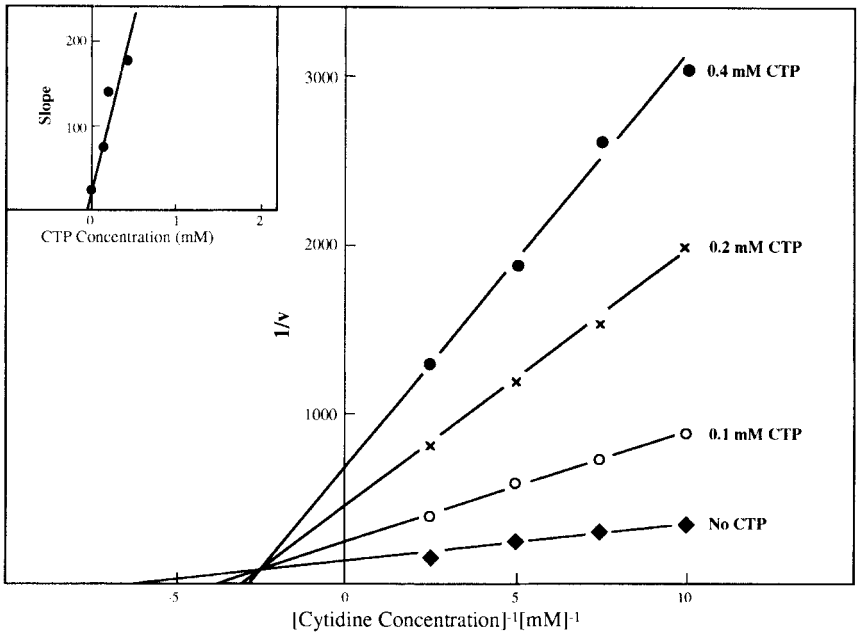

FIG. 5. Lineweaver-Burk plots showing inhibition of cytidine phosphorylation by CTP. Phosphorylation of cytidine was measured, without CTP (◆) and in the presence of increasing amounts of CTP (○: 0.1 mM; **x** : 0.2 mM; ● : 0.4 mM). The inset shows the linear relationship between the slope of the lines from Lineweaver-Burk representation and the inhibitor concentration.

about 10 kDa smaller than the uridine-cytidine kinase[1]; the size of the subunit was also distinct from that reported for uridine-cytidine kinase[6]. Fourth, the reaction catalyzed by the cytidine kinase could be susceptible to feedback inhibition, but, contrasting with the case of the uridine-cytidine kinase[1], the Ki value for UTP was much higher than CTP (FIG. 5 and 6). In parallel assays, the enzyme activity that bound to phospho-cellulose, thereby being separated from the cytidine kinase (FIG. 1), met the criteria documented[1] for the EC 2.7.1.48 pyrimidine ribonucleoside kinase (data not shown). Features common to the two types of nucleoside kinase were also noted. Both exhibited a similar specificity with regard to phosphate donors. It was GTP that could most effectively donate phosphate to cytidine (TABLE 3). dGTP could replace GTP, but ATP and dATP were much less effective in the *E. coli* enzymes. For phosphate-accepting substrates, the ribofuranosyl moiety is required in both types of kinases. 2'-Deoxyribonucleosides were less phosphorylated even when their bases were cytosine (TABLE 2).

The presence of cytidine kinase would seem to be of significance in several aspects. First, it could provide an alternative route for synthesizing cytosine nucleotides. In the

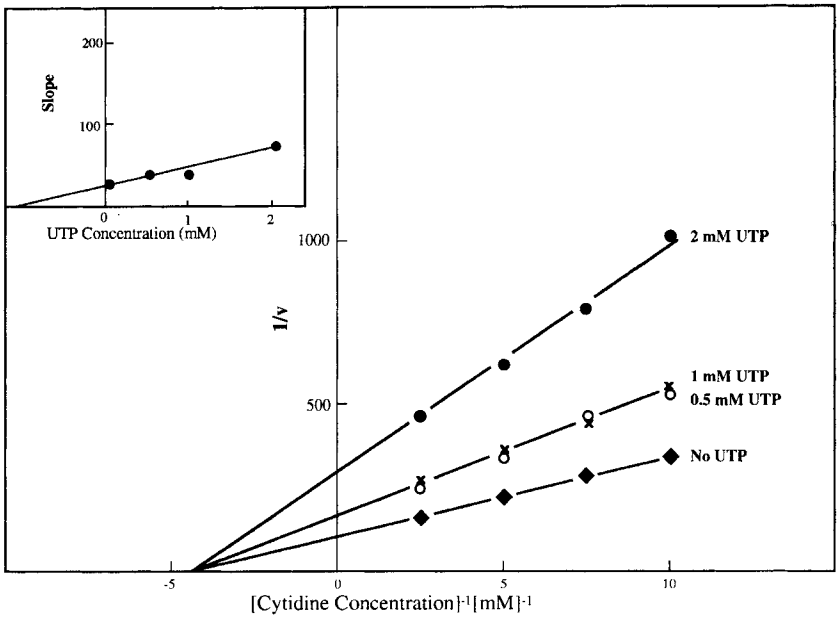

FIG. 6. Lineweaver-Burk plots showing inhibition of cytidine phosphorylation by UTP. Phosphorylation of cytidine was measured, without UTP (◆) and in the presence of increasing amounts of UTP (○: 0.5 mM; x : 1 mM; ●: 2 mM). The inset shows the linear relationship between the slope of the lines from Lineweaver-Burk representation and the inhibitor concentration.

salvage pathway, cytosine and cytidine are rapidly deaminated to yield their uracil counterparts. The *de novo* pathway of pyrimidine synthesis enters at uridylate to which flows from the deamination products as well as from salvaged uracil and uridine converge. Further, it is not before the step of UTP amination that conversion of uracil into cytosine takes place. Thus, cytidine kinase would supplement the enzyme network for pyrimidine triphosphate synthesis, which looks rather stressed on the uracil side, ensuring balanced levels of building blocks for RNA synthesis. Second, the cytidine kinase could also contribute to the maintenance of balanced levels of pyrimidine deoxyribonucleotides, the precursors for DNA synthesis. For dCTP production, the *de novo* pathway takes a circuitous route via UTP→CTP→CDP, CDP then being converted by ribonucleotide reductase to dCDP. Cytidine kinase-mediated salvage could thus help provide promptly the substrate for nucleotide reduction. Third, cytidine kinase could offer an additional point for regulation of nucleotide synthesis. CTP inhibition and the GTP requirement of this enzyme would seem to be important from this aspect. The former would serve as a device of feedback regula-

tion to prevent an oversupply, and the latter may be implicated as one of numerous lines linking the purine- and the pyrimidine-nucleotide synthetic systems.

The possibility that there should be in cells a kinase that directly and preferentially converts cytidine to its very nucleotide form has been inferred[7]. However, previous attempts to identify it, including those to isolate the corresponding mutants, have failed. This may be ascribed in part to the isolation procedures employed. We found that the chromatographic separation on phospho-cellulose is essential for discriminating the cytidine kinase activity from the known pyrimidine ribonucleoside kinase. The fact that these two kinases share some common characteristics, exemplified by the usage of cytidine as a substrate, would seem to have masked the presence of the cytidine kinase. The nature of this enzyme remains to be clarified. For this, it would be necessary to determine the primary structure of the enzyme protein and to establish whether the two kinases are encoded by different genes or are derived from one and the same gene. In addition, the presence of such cytidine kinase activity should also be explored in other species. In our preliminary experiment, an enzymatic activity similar to *E. coli* cytidine kinase was not detected in rat liver extract[8]. However, to conclude that it is absent in eukaryotes we feel is premature, because it is known that, whereas *de novo* routes of purine and pyrimidine nucleotide synthesis are virtually the same in all cells, the salvage routes are far more diverse in nature and distribution, and because kinases like those specific to pyrimidine nucleosides are known to be abundant in some cells but not in others[9].

Nevertheless, even if the enzyme described in this paper might be an alternatively processed or modified form of the known pyrimidine ribonucleoside kinase, its presence, its relative abundance and its peculiar characteristics would seem to aid efficient salvage of the available precursors and to contribute to the intricate regulation of nucleotide synthesis.

EXPERIMENTAL SECTION

Materials. [2-^{14}C]Uridine (55 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). [u-^{14}C]Cytidine (400 mCi/mmol) was from C.E.A. (Saclay, France). Hydroxyapatite HTP and molecular weight markers were from Bio-Rad Laboratories (Richmond, CA). DEAE-cellulose filters (DE 81), DEAE-cellulose (DE 32) and phospho-cellulose (P11) were from Whatman, and CMP-agarose from Sigma. Other reagents and solvents were of the purest grade available.

Cells. *E. coli* strain Y70-272 (*cdd*$^-$) was donated by Dr. R. Hosono, Kanazawa University School of Medicine. This strain totally lacks cytidine deaminase, and was used to exclude the possibility that the apparent substrate specificity preference might be due to contaminating the deaminase activity. Cells were grown in a nutrient broth at 37 °C.

Preparation of the Enzyme Extracts. The enzyme was extracted from *E. coli* Y70-272. Harvested bacteria were suspended in a buffer containing 50 mM Tris-Base, 0.01 mM EDTA and 5% (v/v) glycerol which had been titrated to pH 7.5 with 1 M citric acid (TCE-5G buffer)[4], and disrupted by sonication (60 s, 4 °C). The lysates were centrifuged (10, 000 *g*, 4 °C, 60 min), and the resultant supernatant was used as the crude extract.

Protein Determination. Protein concentrations were determined by the method of Bradford[10].

Enzyme Assays. Two types of assays were used. One was the radiochemical assay, which measures the conversion of nucleosides to their corresponding nucleoside monophosphates. The incorporation of radioactivity into nucleotides was determined by the filter-disc method of Ahmed[11] with minor modifications. The assay mixture contained 0.1 μCi [^{14}C]cytidine or [^{14}C]uridine adjusted to 1.0 mM with the unlabeled nucleoside, 2.0 mM GTP, 3.0 mM $MgCl_2$, 100 mM MOPS (pH 7.5), 1 mg/ml bovine serum albumin and about 50 μg of the enzyme preparation in a final volume of 0.1 ml. The tubes were kept at 37 °C for 30 min, after which the reaction was stopped by heating the tubes in a water bath (100 °C) for 3 min, followed by chilling on ice. After denatured proteins were removed by centrifugation, the supernatant fluid was spotted on DE 81 discs. Radiolabeled nucleotides were then eluted with 0.1 N HCl in 0.5 M NaCl and measured in a Beckman Model LS-6500 liquid scintillation counter. When the enzymatic formation of uridine nucleotides was monitored as functions of the enzyme concentration and time, the reaction proceeded linearly with up to 100 μg of enzyme protein and during time periods of up to 60 min. Accordingly, most of the assays were carried out with 40 to 60 μg of enzyme protein for 30 min. The reaction product was also assayed on thin-layer sheet of polyethylene iminocellulose, developed with 0.05 M sodium citrate (pH 3.5) and was confirmed to be CMP.

The other assay[1] was coupled spectrophotometry. GDP production was measured by the change in absorbance at 340 nm, which was caused by the oxidation of NADH in a coupled enzyme system using the nucleosides and GTP as substrates and phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase as coupling factors.

SDS-Polyacrylamide Gel Electrophoresis. Slab gels containing 12% acrylamide and 0.32% N, N'-methylene-*bis*-acrylamide were cast and run at room temperature essentially according to the procedure of Laemmli[12]. Electrophoresis was initiated at 7.5 mA per gel until samples had completely entered the resolving gel, and then conducted at 15 mA.

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Abbreviation used: cdd, cytidine deaminase (EC.3.5.4.5).

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