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A Pyrimidine Nucleoside Monophosphate Kinase from Rat Liver†

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ABSTRACT: A pyrimidine nucleoside monophosphate kinase has been purified 2100-fold from rat liver. With ATP and dATP as phosphate donors the kinase uses CMP, dCMP, and UMP as phosphate acceptors. Ara-CMP is also phosphorylated by the kinase. In contrast to dCMP and UMP, CMP can be phosphorylated by dCTP. CTP and ara-CTP cannot substitute for dCTP. The stringent specificity of the phosphate donor site for ATP and dATP is lost when CMP serves as acceptor. All nucleoside triphosphates act as donors to a significant extent. No evidence has been found to suggest more than one enzyme. All activities, to different degrees, are strictly dependent upon preincubation at 37° with a sulfhydryl reducing agent. Various reagents (85 m*M*) are ranked in order of increasing effectiveness of reactivation as follows: dithiothreitol > glutathione ≥ 2-mercaptoethanol > L-cysteine > DL-α-lipoic acid. A $NADP^+$-dependent thioredoxin (17 μ*M*)-thioredoxin reductase system from Novikoff ascites rat tumor was found to be the most powerful reducing agent tested. CTP, dCTP, UTP, and dTTP (1 m*M*) do not affect the kinase activity regardless of the phosphate acceptor.

Maley and Ochoa (1958) partially purified a nucleoside monophosphate kinase from *Azotobacter vinelandii* which phosphorylated CMP and dCMP. UMP was not tested as substrate. In *Escherichia coli* UMP kinase was found to be separable from CMP kinase. The latter enzyme could also phosphorylate dCMP (Hiraga and Sugino, 1966). However, in preparations from calf liver close association of CMP with UMP kinase activities occurred. dCMP was not tested (Strominger et al., 1959). Later, Sugino and coworkers reported a preparation purified from calf thymus which catalyzed phosphorylation of UMP, CMP, and dCMP (Sugino et al., 1966). In spite of the fact that dCMP was

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the poorest substrate, the kinase was classified as ATP: deoxycytidine 5′-monophosphate phosphotransferase (EC 2.7.4.14).

The enzyme occupies a strategic position in biosynthesis of pyrimidine nucleotides, since its phosphate acceptor substrates are products of both the de novo and the salvage pathways. The phosphate acceptor specificity is peculiar since the enzyme catalyzes the phosphorylation of CMP, dCMP, and UMP, but not dUMP, which differs from UMP by only one atom of oxygen, just as dCMP differs from CMP. All three activities are strictly dependent upon the presence of a sulfhydryl reducing agent for activity. The dCMP kinase activity is the most unstable of all three activities, as it is completely lost after a short storage at low concentrations of 2-mercaptoethanol (5 m*M*). The activity can be restored, however, after 1 hr of preincubation at 37° in the presence of higher concentrations of 2-mercaptoethanol (50 m*M*) (Sugino et al., 1966). The instability of the kinase has hampered further study.

We report a reproducible protocol which yields a stable, 2100-fold purification of the kinase from rat liver homogenate. In studying the substrate specificity we found that the kinase is able to recognize deoxycytidine 5′-triphosphate (dCTP) in addition to the known phosphate donors ATP and dATP. This property is evident only when CMP serves as phosphate acceptor. Both the activity and substrate specificity of the liver kinase depend reversibly upon the presence of a sulfhydryl reducing agent.

Materials and Methods

The ^{14}C-labeled nucleotides were purchased from Schwarz BioResearch, Inc. [γ-^{32}P]ATP was prepared according to Glynn and Chappell (1964). Streptomycin sulfate was obtained from Charles Pfizer Co., Inc. Calcium phosphate gel, phenylmethylsulfonyl fluoride, sodium phosphoenolpyruvate, and pyruvate kinase (rabbit muscle) were purchased from Calbiochem. Lactate dehydrogenase (chicken heart) and all other nucleotides were reagent grade products obtained from P-L Biochemicals. DEAE-cellulose DE-52 and cellulose phosphate P-11 were Whatman products. Cytosine β-D-arabinofuranoside-5′-monophosphoric acid (ara-CMP), cytosine β-D-arabinofuranoside-5′-triphosphate (ara-CTP), and DL-α-lipoic acid, reduced form, were purchased from Sigma Chemical Co. Thioredoxin (Herrmann and Moore, 1973) and thioredoxin reductase (Moore, 1973) were prepared from Novikoff ascites rat tumor cells.

Assay of the Enzyme. Two methods of assay are used. The radiochemical assay measures the conversion of nucleoside monophosphate to nucleoside diphosphate, or the transfer of γ-phosphate from ATP to the nucleoside monophosphate by separation of reactants and products on paper electrophoresis. The optical assay measures the oxidation of NADH. In the latter assay, pyruvate kinase is coupled with lactate dehydrogenase in order to measure ADP formation. The disappearance of NADH, measured by the decrease in absorbance at 340 nm, is a measure of the kinase activity. With dCMP kinase as substrate, NADH oxidation is stoichiometric with dCDP formation. However, when CMP or UMP are used as substrates, higher values are obtained, since ADP, and to a lesser degree UDP and CDP, are substrates of pyruvate kinase (Plowman and Krall, 1965).

The standard conditions of the radiochemical assay are as follows: 40 m*M* Tris-Cl (pH 7.5), 6.00 m*M* ATP, 22.20 m*M* $MgCl_2$, 2.77 m*M* [^{14}C]CMP (1.3 μCi/μmol), enzyme, and water, to a final volume of 50 μl. The enzyme was preincubated for 60 min at 37° in 50 m*M* dithiothreitol. The reaction was carried out at 37° for a length of time which converts less than 10% of substrate to product. The reaction was stopped by freezing in an acetone–Dry Ice bath following the addition of 10 μl of a solution which contained CMP, CDP, and CTP, each at a concentration of 10 m*M*.

The separation of the mono-, di-, and triphosphates was accomplished by spotting 50 μl of the sample on a sheet of Whatman No. 3MM paper (57 × 20.5 cm) which had been dampened with 50 m*M* citric acid–citrate buffer (pH 3.5). The electrophoresis was run at 4000 V, 50 mA for 2 hr at 10°. The nucleotide spots were viewed with an ultraviolet (uv) light, cut out, and placed in vials containing 20 ml of Packard Permafluor liquid scintillation fluid diluted 25-fold with toluene. Samples were counted in a Nuclear-Chicago Model 6848 liquid scintillation system.

The standard conditions of the spectrophotometric assay are as follows: 70 m*M* Tris-Cl (pH 7.6), 9.00 m*M* ATP, 19.80 m*M* $MgCl_2$, 3.00 m*M* sodium phosphoenolpyruvate, 20.00 m*M* KCl, 0.131 m*M* NADH, 7 μg of pyruvate kinase (1930 units/mg), 10 μg of lactate dehydrogenase (300 units/mg), 8.00 m*M* dCMP, 50 m*M* dithiothreitol, enzyme, and water to a final volume of 1.00 ml. Particular care was taken in using ATP and sodium phosphoenolpyruvate with minimal amounts of ADP and pyruvate. Enzyme activity was measured at 37° on a Cary 15 recording spectrophotometer. A small rate obtained with the enzyme in the absence of nucleoside monophosphate was subtracted.

Steady-State Kinetics and Treatment of Data. Initial velocities were measured. In all cases the conversion of substrate to product was maintained close to 10% by varying time of incubation. The K_m values were obtained by applying a weighted least-squares analysis of the data (Wilkinson, 1961).

The concentrations of nucleotides were determined spectrophotometrically, using published molar extinction coefficients. Protein was measured with Folin phenol reagent (Lowry et al., 1951), after precipitation with 10% trichloroacetic acid and washing of the precipitate with 5% Cl_3CCOOH.

Results

Purification of the Enzyme. SOURCE OF THE ENZYME. Thirty-day-old, female, Sprague-Dawley rats (albino), which had been made pseudopregnant (Albert, 1961) for other studies, were kindly donated by Dr. Darrell N. Ward. We have used this liver to defray expenses which we would have incurred otherwise. The rats were etherized, and the livers dissected out, frozen on a block of Dry Ice, and stored until use.

PREPARATION OF LIVER EXTRACT. Purification of the enzyme was carried out at 4°. Frozen rat liver (150 g) was minced and homogenized under nitrogen in a Tec-Mar homogenizer for approximately 2 min in 25 m*M* potassium phosphate (pH 7.5)–250 m*M* sucrose–30 m*M* KCl–10 m*M* dithiothreitol (250 ml). Phenylmethylsulfonyl fluoride (1 *M*, 1.5 ml) dissolved in isopropyl alcohol was added during homogenization. The homogenate (fraction I) was centrifuged at 8000*g* for 30 min. The supernatant was then centrifuged at 123,000*g* for 2 hr. This supernatant yielded fraction II.

STREPTOMYCIN TREATMENT. Streptomycin sulfate (5%, 0.25 vol of fraction II) was added to fraction II with

Table I: Purification of CMP Kinase from Rat Liver.[a]

Fractions and Steps	Act. (μmol hr^{-1} ml^{-1})	Total Act. (μmol hr^{-1})	Protein (mg/ml)	Sp Act. (μmol hr^{-1} mg^{-1})
(I) Homogenate	151	64,175	176	0.86
(II) Extract	146	51,100	23.9	6.1
(III) Streptomycin	130	55,250	18.6	7.0
(IV) pH 5	134	56,950	17.4	7.7
(V) 70–85% ammonium sulfate	43	35,192	2.51	16.9
(VI) Calcium phosphate gel	315	29,008	3.31	95.3
(VII) DEAE-cellulose chromatography	429	14,157	1.44	298
(VIII) Cellulose phosphate chromatography	47	9,298	0.026	1810

[a] The radiochemical assay was used to measure the enzymic activity. Proteins were determined by the procedure of Lowry et al. (1951) after precipitation with 10% Cl_3CCOOH and washing with 5% Cl_3CCOOH.

continuous stirring under nitrogen. After 30 min of standing, the suspension was centrifuged at 8000*g* for 30 min. The supernatant yielded fraction III.

pH TREATMENT. Acetic acid (7.5%) was added dropwise to fraction III with continuous stirring until the pH of the suspension reached 5.0. After standing for 10 min, the suspension was centrifuged at 8000*g* for 15 min. The supernatant was brought to pH 7.5 with ammonium hydroxide yielding fraction IV.

AMMONIUM SULFATE TREATMENT. Fraction IV was brought to 50% saturation by the slow addition of solid ammonium sulfate with continuous stirring under nitrogen. After 1 hr of standing, the suspension was centrifuged at 8000*g* for 30 min. The supernatant was brought to 70% saturation, stored for 1 hr, and centrifuged at 8000*g* for 30 min. This supernatant was brought to 85% saturation, stored for 1 hr, and centrifuged at 8000*g* for 30 min. The pellet was dissolved in 25 m*M* potassium phosphate (pH 7.5)–10 m*M* dithiothreitol. Phenylmethylsulfonyl fluoride (1 *M*, 0.12 ml) in isopropyl alcohol was added for every 25 ml of redissolved pellet.

SEPHADEX G-25 DIALYSIS I. The redissolved 70–85% ammonium sulfate fraction was applied to a Sephadex G-25 column (2.5 × 73 cm) previously equilibrated with 1 m*M* potassium phosphate (pH 7.5)–5 m*M* 2-mercaptoethanol. Fractions of 5 ml were collected and assayed by the optical method. The enzymically active fractions which appeared in the void volume were pooled and diluted in 1 m*M* potassium phosphate (pH 7.5)–5 m*M* 2-mercaptoethanol to a protein concentration of 3.0 mg/ml, yielding fraction V.

CALCIUM PHOSPHATE GEL TREATMENT. For every 100 ml of fraction V, 30 ml of calcium phosphate gel (32 mg/ml) was added with continuous stirring. The suspension was stirred for 15 min, allowed to stand for 15 min, and then centrifuged at 8000*g* for 10 min. The pellet was suspended in a volume of 1 m*M* potassium phosphate (pH 7.5)–150 m*M* NaCl–5 m*M* 2-mercaptoethanol equal to the volume of fraction V. This suspension was allowed to stir for 45 min and then centrifuged at 8000*g* for 10 min. The pellet was suspended in 5 m*M* potassium phosphate (pH 7.5)–8% ammonium sulfate–10 m*M* dithiothreitol (21 ml for every 100 ml of fraction V). This suspension was stirred for 30 min and then centrifuged at 8000*g* for 10 min. The supernatant was concentrated to 30 ml in an Amicon Diaflo ultrafiltration apparatus using an Amicon PM 10 membrane at 40 psi of nitrogen.

SEPHADEX G-25 DIALYSIS II. Concentrated enzyme was applied to a Sephadex G-25 column (2.5 × 73 cm) equilibrated with 5 m*M* potassium phosphate (pH 7.5)–5 m*M* 2-mercaptoethanol. Fractions of 5 ml were collected and assayed by the optical method. The enzymically active fractions, which appeared in the void volume, were pooled, yielding fraction VI.

DEAE-CELLULOSE CHROMATOGRAPHY. DEAE-cellulose DE-52 was washed with distilled water and then with 50 m*M* Tris-acetate (pH 7.5). A column (2.5 × 29 cm) was packed with the resin and washed with 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol until the effluent was of constant pH (7.5) and conductance (2.4×10^{-3} mho). Fraction VI, containing 300 mg of protein, was applied to the column at a rate of 0.18 ml/min. Fractions of 3.3 ml were collected, monitored at 280 nm to follow the protein elution, and assayed by the optical method. The column was washed with 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol (300 ml). A 700-ml linear gradient of 0–0.25 *M* KCl in 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol was used to elute the enzyme. The enzyme eluted between 0.14 and 0.18 *M* KCl. The enzymically active fractions were pooled, yielding fraction VII.

CELLULOSE PHOSPHATE CHROMATOGRAPHY. Cellulose phosphate was washed with 0.5 *M* KOH, water, 0.5 *M* HCl, and again with water. These washes were followed with disodium (ethylenedinitrilo)tetraacetate (5 m*M*) and 5 m*M* Tris-acetate (pH 7.5). A cellulose phosphate column (1 × 12 cm) was packed with the resin and equilibrated with 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol until the effluent was of constant pH (7.5) and conductance (2.4×10^{-3} mho). Fraction VII (50 mg of protein) was diluted into 3 vol of 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol, and applied to the column at a rate of 0.34 ml/min. The column was washed with 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol (160–200 ml) until protein no longer eluted. Activity was measured by the optical assay. A small amount of activity appeared in the washing. A 300-ml linear gradient of 0–0.5 *M* KCl in 5 m*M* Tris-acetate (pH 7.5)–5 m*M* 2-mercaptoethanol was used to elute the enzyme (0.2 *M* KCl). The active fractions were pooled, yielding fraction VIII, and were concentrated to 30 ml in an Amicon Diaflo ultrafiltration apparatus using a Pellicon type PS membrane at 40 psi of nitrogen.

When 2-mercaptoethanol removal was necessary, concentrated fraction VIII was dialyzed for 16 hr against 6 l. of 5 m*M* Tris-acetate (pH 7.5), yielding fraction VIIId. The results of a typical purification are presented in Table I.

Phosphate Acceptor and Donor Specificity. CMP, dCMP, and UMP are the only nucleotides capable of functioning as substrate. The radiochemical assay (ATP, 1.38 m*M;* nucleoside monophosphate, 0.20 m*M*) was used to measure production of nucleoside diphosphate from [^{14}C]CMP (32 μmol hr^{-1} mg^{-1}), [^{14}C]dCMP (6 μmol hr^{-1} mg^{-1}), and [^{14}C]UMP (24 μmol hr^{-1} mg^{-1}). [γ-^{32}P]ATP was used in testing dUMP (6.6 m*M*), dTMP (6.0 m*M*), GMP (2.6 m*M*), dGMP (3.6 m*M*), and ara-CMP (0.9 m*M*). Of these, only ara-CMP serves as phosphate acceptor (6 μmol hr^{-1} mg^{-1}).

Phosphate donor specificity was examined for CMP, UMP, and dCMP kinase activities. ATP and dATP are

Table II: Phosphate Donor Specificity (Relative to ATP Rate, 100%).[a]

Phosphate Donor (6.86 m*M*)	Phosphate Acceptor (1 m*M*)		
	CMP	UMP	dCMP
ATP	100	100	100
dATP	95	82	105
dTTP	24	0	1
UTP	36	4	2
dUTP	35	7	5
GTP	29	2	5
dGTP	26	9	0
CTP	36	9	0
dCTP	95	18	1
XTP	15	4	0
ITP	36	32	5
AMP	2	0	0
ADP	1	9	0
Ara-CTP	0	0	0

[a] The radiochemical assay was used to measure enzymic activity of fraction VIII.

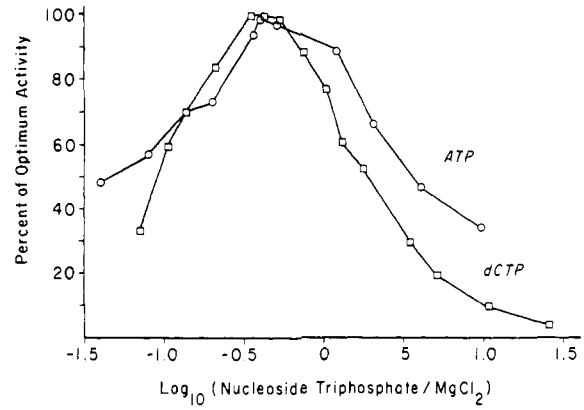


FIGURE 1: The radiochemical assay was used to measure activity of fraction VIII. For the dCTP curve, concentrations used were: CMP, 9.34 m*M*; dCTP, 8.01 m*M*; $MgCl_2$, 0.3–112.50 m*M*. For the ATP curve, concentrations used were CMP, 2.27 m*M*; ATP 3.07 m*M*; $MgCl_2$, 0.3–75.00 m*M*. The optimum ratio of concentrations of nucleoside triphosphate to $MgCl_2$ was approximately 2.45 for both cases.

phosphate donors for each of the three kinase activities (Table II). When CMP serves as phosphate acceptor, many nucleoside triphosphates act as donor to a significant extent, particularly dCTP. However, CTP cannot donate phosphate as well, and ara-CTP cannot donate at all. When UMP acts as phosphate acceptor only ATP and dATP serve as efficient phosphate donors. dCTP acts as donor to a slight, but significant degree. ITP can also phosphorylate UMP. When dCMP serves as phosphate acceptor, only ATP and dATP are effective substrates. None of the other nucleotides act as donor to a significant extent.

Mg^{2+} was found to be necessary for the phosphorylation of CMP, UMP, and dCMP by either ATP or dCTP. With CMP (2.66 m*M*) as phosphate acceptor and ATP (3.00 m*M*) as phosphate donor, Mn^{2+}, Ni^{2+}, and Ca^{2+} are able to substitute for Mg^{2+} but are less effective. The relative rates obtained are Mg^{2+} (100%), Mn^{2+} (42%), Ni^{2+} (16%), and Ca^{2+} (13%). No activity could be observed with Zn^{2+}, Co^{2+}, Ba^{2+}, or Cu^{2+}, or with the following monovalent cations: NH_4^+, Li^+, Na^+, or K^+. With CMP (9.34 m*M*) as phosphate acceptor and dCTP (8.67 m*M*) as phosphate donor, Mn^{2+} (100%) and Mg^{2+} (94%) are almost equally effective, followed by Co^{2+} (20%) and Ni^{2+} (17%). No activity was observed with Zn^{2+}, Ca^{2+}, Ba^{2+}, or Cu^{2+}. All cations were used at a concentration of 7.14 m*M* and were chloride salts.

The optimum ratios of Mg^{2+} to ATP and to dCTP (Figure 1) were found to be very close, although the profiles of the curves were somewhat different.

Steady-State Kinetics. Kinetic parameters for CMP, UMP, and dCMP with $MgATP^{2-}$, $MgdATP^{2-}$, or $MgdCTP^{2-}$ as phosphate donors were determined from initial velocity measurements using the radiochemical assay. The data were plotted according to a linear transformation of the Michaelis–Menten equation (Lineweaver and Burk, 1934). With ATP (1.47 m*M*) as phosphate donor, K_m values are for CMP 0.030 m*M* ± 0.007, for dCMP 2.77 m*M* ± 0.39, and for UMP 0.040 m*M* ± 0.009. With dATP (1.47 m*M*) K_m values are for CMP 0.027 m*M* ± 0.004, for dCMP 1.10 m*M* ± 0.18, and for UMP 0.053 m*M* ± 0.005. With dCTP (5.60 m*M*) the K_m value for CMP is 0.98 m*M* ± 0.07. Fraction VIII was used as source of the enzyme with the range of concentrations of the phosphate acceptor as follows: CMP (ATP or dATP), 0.004–0.048 m*M*; CMP (dCTP), 0.012–0.598 m*M*; UMP, 0.017–0.180 m*M*; dCMP, 0.165–1.725 m*M*. With CMP (0.13 m*M*) as phosphate acceptor, K_m values are for ATP 0.32 m*M* ± 0.13, for dATP 0.074 m*M* ± 0.004, and for dCTP 0.82 m*M* ± 0.07. With dCMP (1.17 m*M*), K_m values are for ATP 0.68 m*M* ± 0.09 and for dATP 0.61 m*M* ± 0.06. With UMP (0.58 m*M*), K_m values are for ATP 0.067 m*M* ± 0.022 and for dATP 0.42 m*M* ± 0.04. Fraction VIII was used as source of the enzyme with the range of concentrations of the phosphate donors as follows: ATP, 0.022–0.256 m*M*; dATP, 0.014–0.586 m*M*; dCTP, 0.330–3.080 m*M*.

When ATP or dATP serve as phosphate donor, substrate inhibition occurs at concentrations of CMP greater than 0.13 m*M*. No substrate inhibition is observed with dCMP (3 m*M*) or UMP (1 m*M*). With dCTP as phosphate donor, no substrate inhibition is observed with CMP (0.6 m*M*).

The UMP:CMP ratio remains nearly constant throughout the purification: fraction II, 3.12; III, 3.26; IV, 2.80; V, 2.84; VI, 2.20; VII, 1.86; VIII, 2.74 (Table I).

Although the ratios of the kinase activities remained more or less constant during the purification, competition experiments were performed in order to investigate whether a single enzyme was responsible for all three activities. By adding labeled dCMP along with increasing amounts of unlabeled CMP or UMP, the amount of dCDP formed decreased. However, the production of ADP did not decrease (Table III). When dCMP was used alone, the amount of [^{14}C]dCDP formed was equivalent to the amount of ADP, indicating that the enzyme was free of contaminating enzymic activities which would interfere in the stoichiometry of the reaction. By adding CMP (0.27 m*M*) the production of [^{14}C]dCDP was reduced by 50%. In contrast, the ADP produced in the presence of dCMP and CMP was slightly higher than the amount formed when CMP was used alone. These data are consistent with the interpretation that CMP, due to its lower K_m for the phosphate acceptor site, is phosphorylated preferentially by a single enzyme. Similar results are obtained with UMP.

Competition experiments were also performed using labeled UMP and increasing amounts of unlabeled CMP. [^{14}C]UDP formed in the absence of CMP was 550 μmol hr^{-1} mg^{-1}; in the presence of 0.50 m*M* CMP, 424 μmol

Table III: Formation of [^{14}C]dCDP and ADP in Presence of [^{12}C]CMP or [^{12}C]UMP.[a]

Addition	μmol hr^{-1} mg^{-1} [^{14}C]dCDP	ADP	Addition	μmol hr^{-1} mg^{-1} [^{14}C]dCDP	ADP
dCMP (8.0 m*M*)	56	57	dCMP (8.0 m*M*)	56	57
dCMP (8.0 m*M*) + CMP (0.09 m*M*)	40		dCMP (8.0 m*M*) + UMP (1.03 m*M*)	20	
dCMP (8.0 m*M*) + CMP (0.18 m*M*)	34		dCMP (8.0 m*M*) + UMP (2.06 m*M*)	13	
dCMP (8.0 m*M*) + CMP (0.27 m*M*)	25		dCMP (8.0 m*M*) + UMP (3.09 m*M*)	0	
dCMP (8.0 m*M*) + CMP 0.36 (m*M*)		129	dCMP (8.0 m*M*) + UMP (2.16 m*M*)		184
CMP (0.36 m*M*)		118	UMP (2.16 m*M*)		185

[a] [^{14}C]dCDP was measured by the radiochemical assay as described in the Materials and Methods section with the following modifications: 9.0 m*M* ATP, 8.0 m*M* [^{14}C]dCMP, varying concentrations of CMP and UMP, and fraction VIII (1 μg). ADP was measured by the spectrophotometric assay exactly as described in the Materials and Methods section using fraction VIII (2.2 μg) and 0.36 m*M* CMP or 2.16 m*M* UMP where indicated.

Table IV: Activation by Sulfhydryl Reducing Agents (Relative to Dithiothreitol Rate, 100%).[a]

SH Reducing Agents	CMP (ATP)	CMP (dCTP)	dCMP (ATP)	UMP (ATP)
None	0	0	0	0
Dithiothreitol (85 m*M*)	100	100	100	100
2-Mercaptoethanol (85 m*M*)	67	22	46	95
Glutathione (reduced) (85 m*M*)	77	41	46	82
L-Cysteine (85 m*M*)	59	10	40	79
DL-α-Lipoic acid (reduced) (85 m*M*)	55	28	29	57
Thioredoxin (reduced) (17 μ*M*)	160	100	270	340

[a] The radiochemical assay was used as described in the Materials and Methods section. Fraction VIIId (0.1 μg) was preincubated at the cited concentration of reducing agent for 30 min at 37° in a volume of 20 μl. Although freshly prepared solutions were used, no attempt was made to measure the concentration of free sulfhydryl groups. Thioredoxin reductase (3.9 μg), NADPH (60 μg), and fraction VIIId (0.1 μg) were preincubated for 30 min at 37° in a volume of 20 μl. The purity of the thioredoxin preparation was judged to be 50% on the basis of polyacrylamide gel electrophoresis.

hr^{-1} mg^{-1}; in the presence of 1.00 m*M* CMP, 269 μmol hr^{-1} mg^{-1}; in the presence of 150 m*M* CMP, 225 μmol hr^{-1} mg^{-1}.

Competition experiments were also performed using [^{14}C]CMP (1.0 m*M*) and unlabeled ATP and dCTP. [^{14}C]CDP formed in the presence of ATP (3.43 m*M*) was 90 μmol hr^{-1} mg^{-1}; in the presence of dCTP (6.86 m*M*), 64 μmol hr^{-1} mg^{-1}; in the presence of ATP (3.43 m*M*) and dCTP (6.86 m*M*), 73 μmol hr^{-1} mg^{-1}. These data show that the rate obtained with ATP alone does not increase when ATP and dCTP are present together, as would be expected if separate enzymes were responsible.

Activation by Sulfhydryl Reducing Agents. All four enzymic activities depend strictly upon sulfhydryl reducing agents. Reduced thioredoxin is by far the most effective. The naturally occurring thiols reduced glutathione, reduced DL-α-lipoic acid, and L-cysteine can substitute for thioredoxin. Dithiothreitol and 2-mercaptoethanol are also effective. All the rates relative to dithiothreitol (100%) are presented in Table IV.

The enzyme does not appear to be subject to inhibition by CTP, dCTP, UTP, or dTTP (1.0 m*M*), regardless of the phosphate acceptor. It is possible that the activity of the enzyme in vivo is regulated by the concentration of thiols.

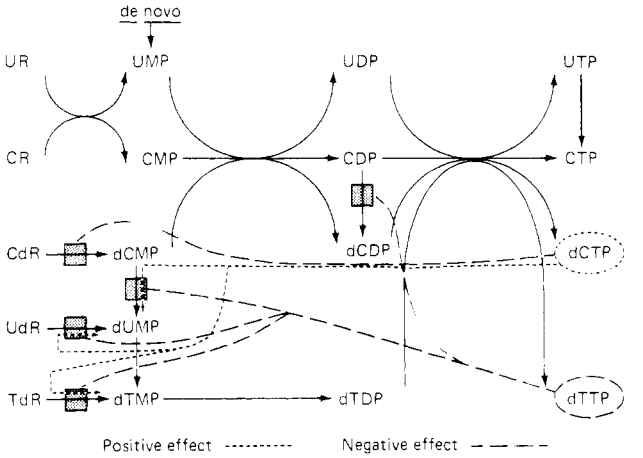
Discussion

From competition experiments presented in Table III, it appears that only one enzyme is catalyzing phosphorylation of UMP, CMP, and dCMP. Ara-CMP is also phosphorylated by the kinase and therefore is responsible for the synthesis of ara-CTP which appears to be the active metabolite in chemotherapy of leukemia (Furth and Cohen, 1968). Although the enzyme is labeled as a dCMP:ATP phosphotransferase, it would be more appropriately termed a UMP-CMP:ATP phosphotransferase. The possibility still exists that particular conditions in the cell may potentiate the dCMP kinase activity.

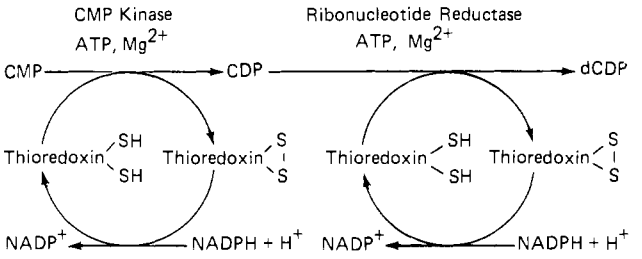
In studying the phosphate donor specificity, it was found that dCTP can substitute for ATP and dATP only when CMP acts as phosphate acceptor. CTP is a relatively poor phosphate donor and ara-CTP will not serve as phosphate donor. This specificity illustrates the importance of the 2′- and 3′-hydroxyl groups in the binding of nucleotide substrates. It is difficult to assign a physiological meaning to this in vitro finding since the ratio of dCTP to ATP in the cell is very low. Thus, one must conclude that the donor of choice is ATP. However, local intracellular concentrations of nucleotides may be different from the average found in cellular extracts.

The kinase may play a role in the regulation of DNA synthesis, inasmuch as it furnishes CDP to the ribonucleotide reductase and competes with dCMP deaminase for dCMP (Scheme I). Recent reports have pointed out that

Scheme I: Regulation of Pyrimidine Nucleotide Biosynthesis.



Scheme II: Action of Thioredoxin in the Phosphorylation of CMP and the Reduction of CDP.



the sizes of the dCTP and dTTP pools are critical for the onset of DNA synthesis (Nordenskjold et al., 1970; Bjursell et al., 1972; Skoog et al., 1973; Bjursell and Reichard, 1973). The dCMP kinase may bypass the regulation of ribonucleotide reductase, especially under conditions of high concentration of dTTP, since this effector can simultaneously inhibit the conversion of CDP to dCDP (Moore and Hurlbert, 1966) and the deamination of dCMP (Maley and Maley, 1964, 1965; Geraci et al., 1967) leaving the dCMP kinase free to operate. The dCMP kinase activity would be diminished under conditions of high concentration of dCTP, an effector which inhibits deoxycytidine kinase activity (Maley and Maley, 1962; Durham and Ives, 1967), and activates dCMP deaminase activity (Maley and Maley, 1964, 1965; Geraci et al., 1967). The inability of dCMP kinase to phosphorylate dUMP avoids competition with thymidylate synthetase for its substrate dUMP (Sugino, 1965). The phosphorylation of dUMP would interfere with the pool size of dTTP.

All three activities are strictly dependent upon the presence of sulfhydryl reducing agents. Sulfhydryl concentration has been shown to be related to the rate of cell proliferation (Needham, 1942). Recently, a low molecular weight protein, thioredoxin, was discovered, which couples the reductive potential of NADPH to the reduction of ribonucleotides (Laurent et al., 1964). Thioredoxin has been purified from normal rat liver (Larson and Larsson, 1972) and rat Novikoff ascites hepatoma (Herrmann and Moore, 1973). The strict dependence of the dCMP kinase on very low concentrations of thioredoxin furnishes another function for thioredoxin. The possibility that thioredoxin may be responsible for both the phosphorylation of the pyrimidine nucleoside monophosphate and the reduction of the nucleoside diphosphate (Scheme II) warrants further investigation.

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