Metabolism of Deoxyribonucleotides

I. PURIFICATION AND PROPERTIES OF DEOXYCYTIDINE MONOPHOSPHOKINASE OF CALF THYMUS*

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SUMMARY

A procedure for the partial fractionation and purification of dAMP, dGMP, dCMP, and dTMP kinases from calf thymus is described. The results obtained indicate that there are at least four distinct nucleoside monophosphokinases, specific for adenine, guanine, cytosine and uracil, and thymine.

The kinase specific toward dCMP was further purified and it was shown that the stability of this enzyme was absolutely dependent on the presence of thiol.

The purified dCMP kinase preparation also catalyzed the phosphorylation of CMP and UMP. These three pyrimidine nucleotides were found to be competitive inhibitors for each other. However, under appropriate conditions in the absence of thiol, dCMP kinase activity was lost without appreciable change in the other two kinase activities. The dCMP kinase activity that had been lost was easily restored by incubating with thiol compounds.

Several other properties of the purified enzyme are also described.

The biosynthesis of deoxyribonucleic acid is presently thought of as a polymerization reaction of deoxyribonucleoside monophosphate units which in turn originate from deoxyribonucleoside triphosphates, i.e. dATP, dGTP, dCTP, and dTTP (5-8). The cellular activities required to supply these triphosphates may thus be among the factors which participate in the control of DNA synthesis (9). From this point of view, it will be of considerable interest to study the fundamental steps in the synthesis of deoxyribonucleoside triphosphates from the corresponding mono- and diphosphates in rapidly proliferating tissues.

Although there have been many papers dealing with deoxyribonucleotide kinases which catalyze phosphorylation by ATP of various deoxyribonucleotides (8, 10-38), only a very limited number of investigations have been done with purified enzyme preparations to elucidate their fundamental enzymatic properties (39-42).

This paper and papers to follow will be devoted to the description of the purification and properties of various enzymes which catalyze the phosphorylation of deoxyribonucleosides and their mono- and diphosphates. In this paper, procedures for the fractionation and purification from calf thymus of deoxycytidine monophosphokinase and its several properties are reported.

EXPERIMENTAL PROCEDURE

32P-Ribo- and Deoxyribonucleoside Monophosphates—Escherichia coli B was grown in the presence of 32P-orthophosphate and the cells were harvested as described by Lehman et al. (5). A mixture of 32P-DNA and 32P-RNA was extracted from the washed cells according to Marmur (43) and this was then subjected to hydrolysis with a bacterial nuclease-phosphodiesterase system to yield a mixture of 5'-ribo- and 5'-deoxyribonucleoside monophosphates (44,45). The hydrolyzed mixture was chromatographed on a column of Dowex 1-formate (X-8, 200 to 400 mesh) by a gradient elution technique (46) to separate the four nucleotide pairs, i.e. CMP plus dCMP, AMP plus dAMP, GMP plus dGMP, and UMP plus dTMP (5-8). The cellular activities required to supply these triphosphates may thus be among the factors which participate in the control of DNA synthesis (9). From this point of view, it will be of considerable interest to study the fundamental steps in the synthesis of deoxyribonucleoside triphosphates from the corresponding mono- and diphosphates in rapidly proliferating tissues.

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These purified 32P-ribo- and 32P-deoxyribonucleoside monophosphates were found to be chromatographically homogeneous in the above mentioned systems, and more than 99% of 32P-radioactivity of each nucleotide was converted to inorganic ortho-
phosphate by treatment either with bull semen 5'-nucleotidase or with prostate phosphomonoesterase as checked by a specific P1 precipitation procedure (49).

Nonradioactive Chemicals—All the nucleosides and nucleoside mono-, di-, and triphosphates were the products of Sigma. Diethylaminoethyl cellulose was the product of Serva Company, West Germany, and prepared for use as described by Peterson and Sober (50). Preconditioning of the columns of the ion exchange agent for chromatography was carried out as described in each section in "Results." Sephadex G-25 was obtained from Pharmacia. Alumina Cy gel was prepared according to Willstätter and Kraut (see Reference 51). Other chemicals of reagent grade were obtained from commercial sources.

Enzymes—Phosphomonoesterase was prepared from human prostate gland as described by Schmidt (52). Bull semen 5'-nucleotidase, prepared according to the method of Heppel and Hilmoe (53), was obtained through the courtesy of Dr. E. Ohmura, Research Laboratories, Takeda Chemicals Industry, Ltd., Japan.

Assay of Nucleoside Monophosphokinase—The principle of the deoxyribonucleoside monophosphokinase assay was the same as that described by Lehman et al. (5). The assay depends on the conversion of a substrate sensitive to phosphomonoesterase (nucleoside monophosphate) to a product or products insensitive to phosphomonoesterase (nucleoside di- or triphosphate or both). In their original procedure, the reaction mixture containing residual 32P-deoxyribonucleoside monophosphate was subjected to hydrolysis by phosphomonoesterase, to yield the deoxynucleoside and 32Pi. The mixture was then treated with charcoal, which adsorbs 32P-deoxyribonucleoside polyphosphates, and the charcoal was washed several times with water and the radioactivity counted. The procedure involving charcoal treatment was tedious and time consuming.

In all the kinase assays reported in this paper, 32P1 released by treatment with phosphomonoesterase was removed by precipitation with triethylammonium molybdate (49). The modified assay procedure was as follows.

A reaction mixture which contained, in a volume of 0.25 ml, 10 μmoles of Tris (pH 7.5), 1.0 μmole of ATP, 2.0 μmoles of MgCl2, 0.1 μmole of 32P-deoxyribo- or 32P-ribonucleoside monophosphate (5,000 to 10,000 cpm), and 0.02 to 0.15 unit (0.05 μl) of enzyme preparation, was incubated at 37° for a given period of time. The incubation time employed was usually 10 to 20 min for AMP, dAMP, GMP, dGMP, CMP, dCMP, and UMP kinases, and 60 min for dTMP and dUMP kinases (54). After incubation, 0.5 ml of water was added and the reaction was terminated by heat treatment (100° for 5 min). Then 0.1 ml of a phosphomonoesterase solution (previously titrated), 0.1 ml of 1 M acetate buffer (pH 5.0), and 0.05 ml of 0.2 M MgCl2 were added to the reaction mixture and incubated for 60 min at 37°.

As purification proceeded, the kinase activities became unstable on dilution. For diluting such enzyme preparations to yield an appropriate range of activity for assay, 0.005 M potassium phosphate solution (pH 7.5) containing bovine serum albumin at a concentration of 1.0 mg per ml was used in place of Buffer I or Buffer II.

As the extent of purification increased, some of the kinases became less susceptible to the heat treatment at pH 7.5. In such cases, reaction was terminated by adding 0.1 ml of 1 M acetate buffer (pH 5.0) followed by heat treatment (100°, 3 min). After cooling to room temperature, 0.1 ml of the phosphomonoesterase solution was added.

After cooling to room temperature, 0.05 ml of 0.01 M KH2PO4 (carrier) and 0.35 ml of a P1 precipitation mixture (49) were added, mixed well, and allowed to stand for several minutes. The precipitate of P1 was removed by centrifugation at 1500 × g for 3 min. An aliquot (0.5 to 1.0 ml) taken from the supernatant fluid was directly plated on an aluminum planchet, dried, and counted. No neutralization of the sample prior to plating was necessary when aluminum planchets were used (see Footnote 4 of Reference 49).

For the control run, the enzyme preparation was replaced by water or by a heat-killed enzyme preparation. The rest of the procedure was the same as described above. For the assay of dCMP kinase, each enzyme preparation was preincubated with 0.05 M β-mercaptoethanol for 60 min at 37° to ensure its full activation (see "Results" and Fig. 5).

A unit of enzyme is that amount catalyzing the formation of 1 μmole of nucleoside polyphosphate or polyphosphates per hour. Specific activity is expressed as units per mg of protein.

Identification of Reaction Products of dCMP Kinase—An aliquot (0.02 ml, 10,000 to 20,000 cpm) was taken from the reaction mixture, applied on a strip of Whatman No. 3 paper, and developed with an isobutyric acid-ammonia system (48). After drying, the strip was cut into small pieces 5 mm long in the direction of the development, and each piece was counted for 32P. By this procedure, 32P radioactivity was detected only in three spots corresponding to authentic dCMP, dCDP, and dCTP. The radioactive pieces which constitute each spot were pooled and eluted with 0.01 M ethylenediaminetetraacetate (pH 7.5). Aliquots of the eluates from dCMP and dCTP spots were plated, dried, and counted. In the paper chromatography mentioned above, Pi migrated at the same rate as dCTP. Therefore, another aliquot of the eluate from the dCTP spot was treated with triethylammonium molybdate (49) to remove any contaminating 32Pi, which might be liberated by the action of contaminating phosphatase, and the resulting supernatant solution was plated, dried, and counted.

As the purification of the enzyme proceeded, the value obtained after the removal of 32Pi by the triethylammonium molybdate treatment, which represented the net amount of dCTP formed, approached the value obtained without the prior treatment for removing Pi. This indicated that the contaminating phosphatase was removed by the purification.

The sum of the counts in dCDP and dCTP spots coincided well with the value calculated from the result of the enzyme assay which measured the amount of 32P-nucleotides insensitive to prostate phosphomonoesterase.

Determination of Stoichiometry of Reactions—Substrates and products of each reaction mixture indicated in Table VIII were separated by ion exchange chromatography on a Dowex-1 (X-8) chloride column. The reaction mixture was diluted to 50 ml with water and applied to a column, 1 × 5 cm. The separation procedure was as follows: in the case of the dCMP or CMP kinase reaction, dCMP and dCDP or CMP and CDP were separately eluted by a 0 to 0.06 M LiCl linear gradient, and then ADP and ATP were eluted by a 0.06 to 0.4 M LiCl linear gradient. In the case of the UMP kinase reaction, a 0 to 0.4 M linear gradient was employed. The third peak (UDP) and the fourth peak (ATP) were partially overlapping. The amounts of these nucleotides in the overlapping fraction were determined by optical density measurement at two different wave lengths, 260 μμ and 280 μμ.
Partial fractionation of four deoxynucleoside monophosphokinases

Activity was measured as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>dAMP kinase</th>
<th>dGMP kinase</th>
<th>dCMP kinase</th>
<th>dTMP kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td>A (crude extract)</td>
<td>100</td>
<td>0.31</td>
<td>116</td>
<td>0.36</td>
</tr>
<tr>
<td>B (pH 5 supernatant)</td>
<td>96</td>
<td>1.0</td>
<td>143</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>0</td>
<td>27</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>CII (35 to 50%)</td>
<td>88</td>
<td>2.8</td>
<td>63</td>
<td>2.0</td>
</tr>
<tr>
<td>CIII (60 to 75%)</td>
<td>3</td>
<td>0.4</td>
<td>45</td>
<td>5.7</td>
</tr>
<tr>
<td>Alumina C4 gel fraction</td>
<td>3</td>
<td>0.8</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>IIIa</td>
<td>4</td>
<td>0.6</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>IIIb</td>
<td>10</td>
<td>2.4</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>IIIc</td>
<td>12</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IIId</td>
<td>15</td>
<td>8.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IIIe</td>
<td>6</td>
<td>8.9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* The data for total activity were normalized taking the value for dAMP kinase in Fraction A (crude extract) as 100.

† In the experiment presented in this table, dCMP kinase activity was determined without preincubation with 0.05 M β-mercaptoethanol.

RESULTS

Separation of Deoxycytidine Monophosphokinase from Other Deoxynucleoside Monophosphokinases—A summary of procedures used to achieve separation and partial purification of deoxycytidine monophosphokinase is presented in Tables I and II.

Preparation of Crude Extract—All the procedures described below were done in a cold room at 4°C. Frozen calf thymus, 100 g, was homogenized in a Waring Blendor with 400 ml of Buffer I. The homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant fluid was collected.

**pH 5 Treatment**—Fraction A was adjusted to pH 5.0 by the addition of 5 ml of 2 N acetic acid. After 10 min of additional stirring the suspension was centrifuged at 10,000 × g for 30 min. The precipitate (pH 5 precipitate) was discarded and the clear supernatant fluid was brought to pH 7.5 with 5 ml of 2 N aqueous ammonia (Fraction B).

Ammonium Sulfate Fractionation—To 350 ml of Fraction B were added 73 g of ammonium sulfate with constant mixing over a 30-min period. After 20 min the mixture was centrifuged at 10,000 × g for 15 min and the precipitate (Fraction CII) was discarded. To the supernatant (370 ml) was referred to as Fraction A (crude extract).

Ammonium sulfate fractionation—To 350 ml of Fraction B were added 73 g of ammonium sulfate with constant mixing over a 30-min period. After 20 min the precipitate was collected by centrifugation and dissolved in 35 ml of Buffer II* (Fraction CIII).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Yield</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (crude extract)</td>
<td>37,000</td>
<td>33,000</td>
<td>500,000</td>
<td>100</td>
<td>0.066</td>
</tr>
<tr>
<td>B (pH 5 supernatant)</td>
<td>35,000</td>
<td>29,500</td>
<td>301,000</td>
<td>89</td>
<td>0.098</td>
</tr>
<tr>
<td>CII (ammonium sulfate fraction)</td>
<td>3,500</td>
<td>13,000</td>
<td>39,000</td>
<td>40</td>
<td>0.33</td>
</tr>
<tr>
<td>IIIb (Cy eluate)*</td>
<td>6,000</td>
<td>8,150</td>
<td>9,150</td>
<td>25</td>
<td>0.8</td>
</tr>
<tr>
<td>E (DEAE-cellulose)*</td>
<td>1,450</td>
<td>2,160</td>
<td>682</td>
<td>6.6</td>
<td>3.15</td>
</tr>
<tr>
<td>F (DEAE-Sephadex)*</td>
<td>233</td>
<td>2,040</td>
<td>210</td>
<td>0.2</td>
<td>9.7</td>
</tr>
</tbody>
</table>

* At each step after IIIb, only a portion of the pooled fraction was used for the succeeding purification step (see text). Values in the latter three fractions were corrected for the total amount of the starting material.

As seen in Table I, dTMP kinase was recovered in CII and dCMP and dAMP kinases in CIII. Approximately two-thirds of dGMP kinase was recovered in CIII and one-third in CII.

Fractionation of Fraction CIII by Amidonium C4 Gel Adsorption and Elution—Fraction CIII which contained dAMP, dGMP, and dCMP kinases was further fractionated by amidonium C4 gel adsorption and elution. The fractionation procedure was as follows: Fraction CIII (35 ml) was dialyzed for 20 hours against three changes of 350 ml of Buffer II. An aliquot (35 ml) of the
dialyzed solution which contained 11 mg of protein per ml was mixed with 23 ml of alumina C\textsubscript{3} gel suspension (15 mg per ml), stirred for 15 min, and centrifuged. The gel was washed with 10 ml of Buffer II and again centrifuged. The supernatant fluid and the washings were combined and referred to as III-S (65 ml). The packed gel was eluted successively with 20-ml and 10-ml portions of each of the following solutions which contained 0.005 M \( \beta \)-mercaptoethanol and were adjusted to pH 7.5: (a) 0.01 M potassium phosphate; (b) 0.025 M potassium phosphate; (c) 0.025 M potassium phosphate plus 0.04 M potassium chloride; (d) 0.025 M potassium phosphate plus 0.1 M potassium chloride; (e) 0.025 M potassium phosphate plus 0.2 M potassium chloride; (f) 0.025 M potassium phosphate plus 0.5 M potassium chloride. Eluates thus obtained were designated as IIIa, IIIb, IIIc, IIId, IIIe, and IIIf according to the numbering of the clinic used.

dGMP kinase was recovered in III-S, dCMP kinase in IIIf, and dAMP kinase in IIIc, d, e, and f (Table I).

For the further purification of these enzymes, several large scale preparations, starting from 1 to 2 kg of thymus, were performed and the partially purified individual kinases were pooled.

**Further Purification of Deoxycytidine Monophosphokinase**

**Chromatography on Diethylaminoethyl Cellulose**—Samples of Fraction IIIb were pooled (5000 ml) and applied to a column of diethylaminoethyl cellulose (5.2 \( \times \) 30 cm, previously equilibrated with Buffer II). A linear gradient was established with 4000 ml of Buffer II in the mixing vessel and 4000 ml of 0.5 M KCl in Buffer II in the reservoir. Elution proceeded at a flow rate of 3.7 ml per min, and 10-ml fractions were collected. The elution profile is shown in Fig. 1. As seen in Fig. 1, CMP kinase activity was eluted in the fractions where dCMP kinase was eluted. Tubes 24 to 34 (see Fig. 1) were pooled and constitute Fraction E (DEAE-cellulose eluate, 1450 ml).

**Chromatography on Diethylaminoethyl Sephadex**—A portion, 500 ml, of Fraction E was diluted with 2000 ml of Buffer II to lower the salt concentration and was applied to a column of diethylaminoethyl Sephadex (2 \( \times \) 25 cm, previously equilibrated with Buffer II). A linear gradient was established with 360 ml of Buffer II in the mixer and 360 ml of 0.5 M KCl in Buffer II in the reservoir. Elution proceeded at a flow rate of 1.3 ml per min, and 20-ml fractions were collected. The elution profile is shown in Fig. 2. The elution pattern of CMP and UMP kinases coincided almost completely with that of dCMP kinase. Tubes 13 to 16 were pooled and constitute Fraction F (DEAE-Sephadex eluate, 80 ml). The extent of purification of dCMP kinase in Fraction F was about 150-fold over the crude extract (Table II).

**Properties of Deoxycytidine Monophosphokinase**

**Stability**—The activity of dCMP kinase in Fraction A, Fraction B, or Fraction CIIIB dissolved in Buffer I was lost very rapidly at 4\textdegree or at -10\textdegree (Fig. 3). Fraction CIIIB dissolved in Buffer II and dialyzed against the same solution for 16 hours was stable at 4\textdegree for several days. Dialysis against Buffer II for 16 hours followed by storage at 4\textdegree for 24 to 48 hours could restore the activity that had been lost in Buffer I. Inactivation in Buffer I and reactivation in Buffer II could be repeated as shown in Fig. 4. The difference in composition between Buffer I and Buffer II suggested that the concentration of salts might have an important role in the reversible inactivation of dCMP kinase. In addition, it was found that the stability of dCMP kinase was absolutely
dependent on the presence of such thiol compounds as β-mercaptoethanol, cysteine, and glutathione. Furthermore, the activity once lost in Buffer I was completely restored by incubating the kinase preparation with 0.05 M β-mercaptoethanol for 1 to 2 hours at 37°C (Fig. 5). Glutathione and cysteine similarly reactivated the kinase, whereas no restoration of activity was achieved with ascorbic acid (Table III). Thus, except where otherwise mentioned, each enzyme preparation was incubated with 0.05 M β-mercaptoethanol for 1 hour prior to the assay to ensure the full restoration of the activity. In some cases, the preincubation was omitted to check the extent of inactivation.

Effect of pH—The effect of pH on the reaction was measured over the pH range of 6.5 to 11.0, with 0.02 M imidazole-acetate, Tris-HCl, and glycine-NaOH buffers, and Fraction F as the enzyme source. The plot of enzyme activity against pH (Fig. 6) shows that dCMP kinase has a pH optimum at 9.0.

Requirement for Cations—The requirement for cations was tested with Fraction F as the enzyme source. As shown in Table IV, the kinase activity was found to be absolutely dependent on the presence of Mg²⁺ or Mn²⁺. Co²⁺ and Ni²⁺, tested at the same concentration, could partially substitute for Mg²⁺ or Mn²⁺. Ca²⁺ and Fe²⁺ were ineffective.

Specificity for Phosphate Donors—The specificity of the dCMP kinase toward the nucleoside triphosphates as phosphate donors was tested with Fraction F as the enzyme source. Of the nine ribo- and deoxyribonucleoside triphosphates tested only ATP and dATP were found to be active as the phosphate donors (Table V). dATP was about 80% as active as ATP under the routine assay conditions.
TABLE V

Specificity for nucleoside triphosphates as phosphate donors

Activity was measured as described in "Experimental Procedure." ATP was replaced by the same concentration of various nucleoside triphosphates, as indicated. Each nucleoside triphosphate was purified by ion exchange chromatography just before use in order to avoid contamination with diphosphate and monophosphate. Fraction F was used as the enzyme source.

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>&lt;2</td>
</tr>
<tr>
<td>CTP</td>
<td>0</td>
</tr>
<tr>
<td>UTP</td>
<td>0</td>
</tr>
<tr>
<td>ITP</td>
<td>&lt;2</td>
</tr>
<tr>
<td>dATP</td>
<td>81</td>
</tr>
<tr>
<td>dGTP</td>
<td>0</td>
</tr>
<tr>
<td>dCTP</td>
<td>0</td>
</tr>
<tr>
<td>dTTP</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE VI

Specificity for nucleoside monophosphates as phosphate acceptors

Activity was measured as described in "Experimental Procedure." dCMP was replaced by the same concentration of various nucleoside monophosphates, as indicated. Fraction F was used as the enzyme source.

<table>
<thead>
<tr>
<th>Nucleoside monophosphate</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>&lt;5</td>
</tr>
<tr>
<td>GMP</td>
<td>&lt;3</td>
</tr>
<tr>
<td>CMP</td>
<td>400</td>
</tr>
<tr>
<td>UMP</td>
<td>80</td>
</tr>
<tr>
<td>dAMP</td>
<td>&lt;1</td>
</tr>
<tr>
<td>dGMP</td>
<td>&lt;1</td>
</tr>
<tr>
<td>dCMP</td>
<td>100</td>
</tr>
<tr>
<td>dTMP</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>dUMP</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE VII

Relative activities toward dCMP, CMP, and UMP in various fractions

Activity was measured as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Relative purity</th>
<th>CMP: dCMP</th>
<th>UMP: dCMP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (crude extract)</td>
<td>1.0</td>
<td>5.4</td>
<td>5.1</td>
</tr>
<tr>
<td>B (pH 5 supernatant)</td>
<td>1.5</td>
<td>6.4</td>
<td>5.4</td>
</tr>
<tr>
<td>CIII (ammonium sulfate)</td>
<td>5.0</td>
<td>4.8</td>
<td>5.3</td>
</tr>
<tr>
<td>IIIb (alumina Cy eluate)</td>
<td>14.0</td>
<td>6.9</td>
<td>5.2</td>
</tr>
<tr>
<td>E (DEAE-cellulose)</td>
<td>46.0</td>
<td>4.5</td>
<td>4.0</td>
</tr>
<tr>
<td>F (DEAE-Sephadex)</td>
<td>147.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A separate purification was carried out to determine the ratio UMP:dCMP. The ratios obtained in this experiment are different from that calculated from values shown in Table VI which gives a value of 0.8 for Fraction F (see Fig. 2 and Table VI). The reason for this discrepancy is not clear.

Specificity for Phosphate Acceptors—Fraction F was also tested for its specificity toward various nucleoside monophosphates with ATP as the phosphate donor (Table VI). Of the nine ribo- and deoxyribonucleoside monophosphates tested, dCMP and CMP were active as acceptors. In addition, quite surprisingly, UMP but not dUMP was found to be active as a phosphate acceptor. The ratio of the rate of the phosphorylation of CMP or UMP to that of dCMP with enzyme preparations at various stages of purification did not vary widely (Table VII) except for Fraction F, suggesting the possible identity of dCMP kinase with CMP kinase and with UMP kinase (see also Fig. 2).

K_m values for dCMP, CMP, and UMP were determined with Fraction E as the enzyme source. Values of $1.4 \times 10^{-3}$ M, $8.5 \times 10^{-8}$ M, and $1.7 \times 10^{-4}$ M were obtained for dCMP, CMP, and UMP, respectively (Figs. 7 to 9).

Reaction Products—The reaction products were identified as dCDP and dCTP as described in "Experimental Procedure."

![Fig. 7](http://www.jbc.org/)  
**Fig. 7.** Competitive inhibition of dCMP kinase activity by CMP and UMP. Activity was measured as described in "Experimental Procedure" except for the addition of 500 mmoles of CMP or UMP where indicated. Fraction E was used as the enzyme source. •, dCMP^2P; ○, dCMP^2P + CMP; □, dCMP^3P + UMP.

![Fig. 8](http://www.jbc.org/)  
**Fig. 8.** Competitive inhibition of CMP kinase activity by dCMP and UMP. Activity was measured as described in "Experimental Procedure" except for the addition of 500 mmoles of dCMP or 100 mmoles of UMP where indicated. Fraction E was used as the enzyme source. •, CM^2P; ○, CM^3P + dCMP; □, CM^3P + UMP.
Fig. 9. Competitive inhibition of UMP kinase activity by dCMP and CMP. Activity was measured as described in "Experimental Procedure" except for the addition of 500 mmol of dCMP or 50 mmol of CMP where indicated. Fraction E was used as the enzyme source.

\[ \text{dCMP} + \text{ATP} \rightarrow \text{dCDP} + \text{ADP} \quad (1) \\
\text{CMP} + \text{ATP} \rightarrow \text{CDP} + \text{ADP} \quad (2) \\
\text{UMP} + \text{ATP} \rightarrow \text{UDP} + \text{ADP} \quad (3) 

Inhibition by Pyrimidine Nucleoside Monophosphates—Various nucleoside monophosphates were tested for their inhibitory activity against dCMP kinase. Table IX shows that among the 10 nucleoside monophosphates tested, those which acted as phosphate acceptors (Table VI) and \( \psi \) UMP exerted significant inhibition on the phosphorylation of dCMP. dUMP, which was inactive as a phosphate acceptor, slightly inhibited the reaction. The type of inhibition exerted by CMP and UMP was a competitive one (Fig. 7). When CMP or UMP was used as a phosphate acceptor, dCMP and UMP, or dCMP and CMP acted as competitive inhibitors, respectively (Figs. 8 and 9).

### DISCUSSION

Fractionation of calf thymus extracts for the study of deoxyribonucleoside monophosphokinase activity revealed the presence of at least four distinct enzymes, specific toward the base components, adenine, guanine, cytosine, and thymine.

Among these four kinases, deoxycytidine monophosphokinase was found of particular interest because of its extreme instability. Our results indicate that the stability of this kinase is absolutely dependent on the presence of thiol compounds such as \( \beta \)-mercaptoethanol, glutathione, and cysteine. Furthermore, the activity once lost in the absence of thiol was completely restored by incubating the inactive preparation with \( \beta \)-mercaptoethanol or glutathione. These facts indicate the presence in the kinase protein of one or more reactive thiol groups which are essential to maintain the enzyme in its active form.

During the purification described in this paper, kinase activities toward dCMP, CMP, and UMP were inseparable, and the ratios of activities between these kinases at various stages of purification remained essentially constant. Furthermore, these three pyrimidine nucleotides were competitive inhibitors for each other.

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6 Fraction E was contaminated with a very minute amount of ATPase which was inactivated by heat treatment at 100° for 3 min. This heat treatment caused only 50% loss of the kinase activity (see Footnote 5). The heat-treated enzyme was used for determining stoichiometry.
other. These facts suggest that the phosphorylation of dCMP, CMP, and UMP may be catalyzed by a single enzyme or by the same catalytic site. In other words, the enzyme preparation purified as dCMP kinase does not seem to exhibit absolute specificity for either cytosine or uracil, or for either ribose or deoxyribose. However, the same enzyme preparation did not catalyze the phosphorylation of dUMP. dUMP kinase activity was recovered in another fraction which was clearly distinct from dCMP kinase (54). Thus, no simple mechanistic prediction as to the acceptor specificity can be made on the basis of the structures of the components of the nucleotide. As phosphate donors only ATP and dATP were found to be active.

In striking contrast to the findings mentioned above, only dCMP kinase activity, of the three kinase activities associated with the purified enzyme preparation, was lost under appropriate incubation conditions in the absence of added thiol. Similar selective and reversible inactivation of dCMP kinase activity was observed in rat liver and hepatoma extracts (55). This selective and reversible inactivation of dCMP kinase activity, of the three kinase activities associated with the purified preparation are needed. These are now under way.

In E. coli extracts, dCMP kinase activity is stable in the absence of added thiol and, furthermore, UMP kinase is easily separable by DEAE-cellulose chromatography from dCMP kinase, the latter being inseparable from CMP kinase (56). The dCMP kinase purified from Azotobacter vinelandii also catalyzes the phosphorylation of CMP, but UMP has not been tested as a phosphate acceptor (40). A close association of UMP kinase with CMP kinase has been reported for partially purified enzyme preparations from calf liver (57). The evidence, however, is not conclusive, and no investigation has been carried out with dCMP as a phosphate acceptor.

In order to clarify the substrate specificity and the mechanism of the selective and reversible inactivation of dCMP kinase, further purification by a procedure which may involve subunit dissociation techniques as well as a more extensive investigation with the purified preparation are needed. These are now under way.

REFERENCES

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