Allosteric Properties of Nucleoside Diphosphatase and Its Identity with Thiamine Pyrophosphatase*

(Received for publication, January 3, 1968)

MITSUO YAMAZAKI and OSAMU HAYAISHI

From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan

SUMMARY

A procedure has been described for the purification of nucleoside diphosphatase from bovine liver microsomes. The purified enzyme is shown, by analysis in the ultracentrifuge and by polyacrylamide disc gel electrophoresis, to be nearly homogeneous. The enzyme has an $s_{20,w}$ of 4.9 S.

Thiamine pyrophosphatase was purified together with nucleoside diphosphatase. Evidence has been obtained which indicates that the nucleoside diphosphates and thiamine pyrophosphate are hydrolyzed by a single enzyme, although the pH activity curves were not identical with the two types of substrates.

Both enzyme activities were markedly enhanced by the presence of adenosine triphosphate. The effect of ATP was more pronounced at low substrate concentrations and disappeared as the substrate level increased. ATP was not consumed during the reactions. The effect of ATP was exerted without a measurable lag when it was added during the course of the reaction, and the stimulatory effect was lost immediately when ATP was removed from the reaction mixture. ATP protected the enzyme against heat inactivation, and gel filtration experiments showed ATP to be bound to the enzyme protein. Inosine triphosphate, guanosine triphosphate, and deoxyadenosine triphosphate showed the same degree of stimulatory effect as ATP.

Nucleoside diphosphatase, a microsomal enzyme, catalyzes the hydrolysis of the terminal phosphate of inosine diphosphate, guanosine diphosphate, uridine diphosphate, and D-ribose 5'-pyrophosphate (1-5). Gregory (6) reported that a nucleoside diphosphatase preparation from lamb liver also possessed thiamine pyrophosphatase activity, which hydrolyzed the terminal phosphate of thiamine pyrophosphate. However, Novikoff and Heus (8) concluded that these two activities are due to two distinct enzymes, because of the failure to detect the thiamine pyrophosphatase activity in the nucleoside diphosphatase preparation from rat liver and the different intracellular localization of the two enzymes by histochemical methods.

Recently we have found that thiamine pyrophosphatase is present in a partially purified preparation of nucleoside diphosphatase from rat liver, and that both enzyme activities were enhanced in the presence of adenosine triphosphate in the reaction mixture (7, 9). The present investigation was undertaken in order to clarify the relationship of nucleoside diphosphatase to thiamine pyrophosphatase and to elucidate the mechanism by which ATP exerts the stimulatory effect.

This paper describes a procedure for the purification of nucleoside diphosphatase from bovine liver microsomes, which yields this enzyme in nearly homogeneous form. Available evidence indicates that both nucleoside diphosphatase and thiamine pyrophosphatase activities are exhibited by a single enzyme. The role of ATP in the activation of the reaction is described, and a possible physiological significance of this activation is discussed.

EXPERIMENTAL PROCEDURE

Materials—IDP was prepared from ADP by deamination with nitrous acid according to the method of Kleinzeiler (10) and was purified by column chromatography on Dowex 1-formate with the use of ammonium formate as the eluting system (11). ADP was a gift of the Takeda Research Laboratories (Osaka, Japan). Other nonradioactive nucleotides were purchased from Sigma. ATP-S-14C and AMP-S-14C were obtained from Schwarz BioResearch. ATP-γ-32P was prepared by photophosphorylation of ADP with spinach chloroplasts according to the method of Jagendorf and Avron (12). Thiamine pyrophosphatase was obtained from Tokyo Kasei Company (Tokyo, Japan) and purified by chromatography on a column of Amberlite IRC-50 (H⁺) (13). Thiamine triphosphate, thiamine monophosphate, and S-benzoylthiamine pyrophosphate diphosphatase preparation from lamb liver also possessed thiamine pyrophosphatase activity, which hydrolyzed the terminal phosphate of thiamine pyrophosphate. However, Novikoff and Heus (8) concluded that these two activities are due to two distinct enzymes, because of the failure to detect the thiamine pyrophosphatase activity in the nucleoside diphosphatase preparation from rat liver and the different intracellular localization of the two enzymes by histochemical methods.

Recently we have found that thiamine pyrophosphatase is present in a partially purified preparation of nucleoside diphosphatase from rat liver, and that both enzyme activities were enhanced in the presence of adenosine triphosphate in the reaction mixture (7, 9). The present investigation was undertaken in order to clarify the relationship of nucleoside diphosphatase to thiamine pyrophosphatase and to elucidate the mechanism by which ATP exerts the stimulatory effect.

This paper describes a procedure for the purification of nucleoside diphosphatase from bovine liver microsomes, which yields this enzyme in nearly homogeneous form. Available evidence indicates that both nucleoside diphosphatase and thiamine pyrophosphatase activities are exhibited by a single enzyme. The role of ATP in the activation of the reaction is described, and a possible physiological significance of this activation is discussed.

Nucleoside diphosphatase, a microsomal enzyme, catalyzes the hydrolysis of the terminal phosphate of inosine diphosphate, guanosine diphosphate, uridine diphosphate, and D-ribose 5'-pyrophosphate (1-5). Gregory (6) reported that a nucleoside

* This investigation was supported in part by Public Health Service Research Grants CA-01222 from the National Cancer Institute and AM-10333 from the National Institute of Arthritis and Metabolic Diseases, and by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Squibb Institute for Medical Research, and the Scientific Research Fund of the Ministry of Education of Japan.

† Visiting scientist from the Central Research Laboratories, Sankyo Company, Tokyo, Japan.

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* In the previous paper (7), the name thiamine pyrophosphate phosphohydrolase was used.
were gifts of Sankyo Company (Tokyo, Japan). Crystalline hexokinase was obtained from Boehringer and Sohne. DEAE-Sephadex A-50 was a product of Pharmacia. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (14). Hydroxyapatite was prepared by the method of Tiselius, Hjertén, and Levin (15). Other chemicals were of analytical grade.

**Nucleotide Diphosphatase Assay**—Nucleotide diphosphatase activity was routinely assayed with IDP as substrate. The standard assay system contained 20 μmoles of Tris-HCl, pH 8.5, 2 μmoles of MgCl₂, 1 μmole of IDP, 0.1 μmole of ATP where indicated, and enzyme, in a total volume of 0.5 ml. The reaction mixture was incubated in the absence of enzyme at 37°C for 5 min, and the reaction was then started by the addition of enzyme. After further incubation for 7 min at 37°C, the reaction was stopped by the addition of 0.1 ml of 10 N H₂SO₄. The formation of Pi was measured as described above.

**Thiamine Pyrophosphatase Assay**—The standard reaction mixture was the same as that with nucleoside diphosphate except that IDP was replaced by thiamine pyrophosphate. The activity was followed fluorometrically by the formation of thiamine monophosphate, at 355 nm, and Pi, at 250 nm. The fluorescence due to thiochrome monophosphate was measured by the method of Ornstein and Davis (19). Ultrasound was used to stop the reaction. The reaction mixture was incubated in the absence of enzyme at 37°C for 5 min, and the reaction was then started by the addition of enzyme. After further incubation for 7 min at 37°C, the reaction was stopped by the addition of 0.1 ml of 10 N H₂SO₄. The formation of Pi was measured by the method of Fiske and SubbaRow (16). The fluorescence of the reaction mixture was measured as described above.

**RESULTS**

**Purification of Enzyme**

All procedures were carried out at 0-4°C.

**Preparation of Microsome Fraction**—Fresh bovine liver (1.9 kg) was homogenized for 1 min in a Waring Blender with 5 liters of cold 0.25 M sucrose. The mixture was centrifuged at 13,300 × g for 10 min. The supernatant fluid was then centrifuged for 60 min at 44,300 × g in a Spinco model L ultracentrifuge (No. 21 rotor, 21,000 rpm). The sedimented microsomal fraction was suspended in 2 liters of 0.25 M sucrose (microsome fraction, 2,500 ml).

**Solubilization from Microsome—Solubilization of the enzyme was accomplished by the treatment of microsomes with NH₄OH. The microsome suspension was adjusted to pH 10.8 by the addition, with vigorous stirring, of 30 ml of 28% NH₄OH and was then immediately brought back to pH 6.5 with 30 ml of glacial acetic acid. Prolonged treatment of the microsome suspension at the alkaline pH resulted in a pronounced loss of the enzyme activity. The suspension was then centrifuged for 60 min at 44,300 × g and the residue was discarded.**

As shown in Fig. 1, significant amounts of the enzyme activity and protein were released into the supernatant solution from microsomes by the alkali treatment and pH 10.8 was optimal for solubilization of the enzyme. The total activity of the suspension of microsomes was increased about 2.5-fold after treatment with NH₄OH, and 52% of this activity was recovered in the supernatant solution. The pH of the microsome suspension (0.6 mg of protein per ml) in 0.25 M sucrose was adjusted to the values shown on the abscissa by the addition of NH₄OH and then was brought to pH 6.5 with acetic acid. After centrifugation at 105,000 × g for 60 min, the supernatant solutions were assayed for the enzyme activity and protein concentration.

**Fig. 1.** Effect of pH on the solubilization of nucleoside diphosphatase from microsomes. The pH of the microsome suspension (0.6 mg of protein per ml) in 0.25 M sucrose was adjusted to the values shown on the abscissa by the addition of NH₄OH and then was brought to pH 6.5 with acetic acid. After centrifugation at 105,000 × g for 60 min, the supernatant solutions were assayed for the enzyme activity and protein concentration.
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Fig. 2. Chromatography steps in the purification of nucleoside diphosphatase and thiamine pyrophosphatase. Chromatographic procedures are described in the text. A, DEAE-Sephadex chromatography. Nucleoside diphosphatase activity was assayed in the absence of ATP and thiamine pyrophosphatase (TPP) activity was assayed in the presence of ATP. B, hydroxylapatite chromatography of the DEAE-Sephadex peak. Both activities were determined in the presence of ATP.

Ammonium Sulfate Fractionation—To the microsome extract, protein solution was then removed by dialysis against various buffers described below. These concentrated preparations were used in electrophoresis and ultracentrifuge analyses.

By the above procedures, nucleoside diphosphatase was purified about 80-fold from the extract of microsomes with an overall yield of 8% (Table I). Thiamine pyrophosphatase was purified together with nucleoside diphosphatase, and the ratio of the two activities remained essentially constant throughout the various steps of the purification. Both enzyme activities were enhanced by the addition of ATP to the reaction mixture. This activation by ATP was observed in each step of the purification procedures. In the presence of 0.2 mM ATP, the specific activities of nucleoside diphosphatase and thiamine pyrophosphatase in the hydroxylapatite fraction were 378 and 74.2, respectively.

The enzyme was quite stable throughout all purification steps at -15°C and could be stored for several months without an appreciable loss of activity. When the purified enzyme was stored...
stored at 4° in 0.02 M Tris-HCl, pH 7.4, containing 0.2 M NaCl, about 60% of the original activity remained after 6 days.

Analytical in Ultracentrifuge and Electrophoresis—The protein in the hydroxylapatite fraction appeared nearly homogeneous in a Spinco model E analytical ultracentrifuge, with a sedimentation constant of $s_{20,w} = 4.9$ S (Fig. 3). The slight asymmetry of the peak might reflect the presence of a small amount of a slower moving protein. ATP at a concentration of 0.2 mM did not alter the sedimentation constant of the enzyme.

Polyacrylamide disc gel electrophoresis at pH 8.3 of the hydroxylapatite fraction resulted in one principal band and a second very faint band of slower mobility.

Catalytic Properties of Purified Enzyme Preparation

Substrate Specificity—The purified enzyme preparation catalyzed rapid hydrolysis of IDP, GDP, UDP, and dUDP, but had little activity with CDP and showed no activity toward ADP or dTDP, either in the presence or absence of ATP (Table II). Thiamine pyrophosphate was hydrolyzed at a considerably more rapid rate in the presence of ATP. The following compounds did not serve as substrate when tested at 2 μM: ATP, ITP, 5’-AMP, 5’-IMP, thiamine triphosphate, thiamine monophosphate, S-benzoylthiamine pyrophosphate, and PP.

The reaction products were identified as 5’-IMP by paper chromatography with isobutyric acid-2 M ammonia (66:20:15) as the solvent (28), and as thiamine monophosphate with 7-t-butyl alcohol-water-1 M sodium formate buffer, pH 5.0, respectively. Fig. 4 shows the paper chromatogram of the reaction mixture with thiamine monophosphate, S-benzoylthiamine pyrophosphate, and PP.

Effect of pH on Activity and Stability—The optimum pH for the hydrolysis of IDP was found to be between pH 6.5 and 7.4 in the presence and absence of ATP (Fig. 5A). With thiamine pyrophosphate as substrate, the pH optimum was about 8.8, and there was very little activity below pH 7.4 in the absence of ATP. However, on addition of ATP, an appreciable activity was obtained in the neutral pH range (Fig. 5B).

Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Nucleoside diphosphatase A</th>
<th>Thiamine pyrophosphatase B</th>
<th>Ratio, A:B</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsome extract</td>
<td>13,800</td>
<td>2.6</td>
<td>0.30</td>
<td>6.7</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>3,600</td>
<td>4.9</td>
<td>0.62</td>
<td>7.9</td>
<td>51</td>
</tr>
<tr>
<td>Ethanol fraction</td>
<td>1,050</td>
<td>10.5</td>
<td>1.18</td>
<td>8.9</td>
<td>31</td>
</tr>
<tr>
<td>Calcium phosphate gel fraction</td>
<td>220</td>
<td>24.6</td>
<td>2.98</td>
<td>8.3</td>
<td>15</td>
</tr>
<tr>
<td>DEAE-Sephadex fraction</td>
<td>25</td>
<td>187</td>
<td>20.5</td>
<td>9.1</td>
<td>13</td>
</tr>
<tr>
<td>Hydroxylapatite fraction</td>
<td>15</td>
<td>199</td>
<td>21.2</td>
<td>9.4</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* Enzyme activity was assayed in the standard assay system in the absence of ATP.

** Yield of nucleoside diphosphatase.

The compound were tested at a concentration of 2 μM in the presence and absence of 0.2 mM ATP in the standard assay system with 0.08 μg of the enzyme.

Table II

Substrate specificity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>With ATP</th>
<th>Without ATP</th>
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<tbody>
<tr>
<td>IDP</td>
<td>31.3</td>
<td>16.0</td>
</tr>
<tr>
<td>UDP</td>
<td>21.8</td>
<td>13.5</td>
</tr>
<tr>
<td>GDP</td>
<td>24.8</td>
<td>18.5</td>
</tr>
<tr>
<td>dUDP</td>
<td>16.8</td>
<td>9.0</td>
</tr>
<tr>
<td>CDP</td>
<td>3.5</td>
<td>1.2</td>
</tr>
<tr>
<td>ADP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dTDP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>12.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>

The enzyme exhibited a relatively broad range of stability at 4° between pH 6.0 and 8.0 and was rapidly inactivated at pH 5.0. The pH stability curves were identical when the enzyme was tested with IDP or thiamine pyrophosphate as the substrate.

Effect of Substrate Concentration—The stimulatory effect of ATP on the reaction varied with the concentration of substrate. The degree of stimulation was more pronounced at low substrate concentrations, and the effect disappeared as the substrate level increased. Fig. 6A shows the substrate concentration versus velocity curve with IDP as substrate. The Lineweaver-Burk plot gave a slightly concave curve in the absence of ATP, but was linear in its presence. The value of $V_{max}$ was essentially unchanged by the addition of ATP. Similar curves were obtained when GDP or UDP was used in place of IDP. Fig. 6B shows the effect of ATP on the reaction with thiamine pyrophosphate as substrate. The effect of ATP was more pronounced at low concentrations of thiamine pyrophosphate. The value of $V_{max}$ was essentially unchanged.

The $K_m$ values calculated from the double reciprocal plots with higher concentrations of the substrate are summarized in Table III. ATP decreased the $K_m$ values by about one-third and one-sixth with nucleoside diphosphates and thiamine pyrophosphate as substrate, respectively. The $K_m$ values for thiamine pyrophosphate were much larger than those for the nucleoside diphosphates, both in the presence and absence of ATP. On the other hand, the values of $V_{max}$ did not differ

* The concave nature of this curve has also been reported recently by Schramm and Morrison (25).
significant when IDP and thiamine pyrophosphate acted as substrate (540 and 354 units per mg of protein, respectively).

Effect of ATP Concentration—Maximal activity was obtained with 0.2 mM ATP, either with IDP or with thiamine pyrophosphate as substrate (Fig. 7). The $K_m$ values for ATP were calculated to be 0.040 and 0.046 mM with IDP and thiamine pyrophosphate as substrate, respectively. ATP at concentrations higher than 2 mM was inhibitory in the thiamine pyrophosphatase reaction.

Nucleotide Specificity for Activation—Nucleoside triphosphates other than ATP also produced similar stimulatory effects on nucleoside diphosphatase and thiamine pyrophosphatase activities. In both reactions, ITP, GTP, and dATP were as effective as ATP, and UTP and CTP were 30 to 40% effective as ATP when these compounds were tested at a concentration of 0.1 mM (Table IV). The following compounds were ineffective at a concentration of 0.1 mM: thiamine triphosphate, ADP, 5'-AMP, 3'-AMP, 3',5'-cyclic-AMP, and NAD.

Nature of ATP Activation—The conversion of ATP to ADP or AMP could not be detected during the reaction, and there was no evidence that ATP was consumed. In these experi-

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**Fig. 4.** Paper chromatogram of the reaction mixture of thiamine pyrophosphatase. Reactions were performed under the standard assay conditions in the presence (A) and absence (B) of ATP, and in the absence of enzyme (C). The reaction mixture was chromatographed on Toyo Roshi No. 51A paper with n-propyl alcohol-water-1 m sodium formate buffer, pH 5.0 (65:20:15), as the solvent (24). The chromatograms were sprayed with a mixture of 2 parts of ethanol, 1 part of 10% NaOH, and 0.05 part of 2.5% K$_3$Fe(CN)$_6$ (13) and were studied under an ultraviolet lamp for the presence of fluorescent spots. D, pure reference compounds. TMP, thiamine monophosphate; TPP, thiamine pyrophosphate.

**Fig. 5.** Effect of pH on the rate of reaction in the presence and absence of ATP. A, nucleoside diphosphatase activity; B, thiamine pyrophosphatase activity. The enzyme activities were determined with 0.1 µg (A) or 0.4 µg (B) of the enzyme under the standard assay conditions in the presence (●) and absence (○) of ATP, except that the following buffers were used; 0.04 M sodium acetate (pH 5.5 to 6.0), Tris acetate (pH 6.5 to 7.2), and Tris-Cl (pH 7.4 to 8.3). TMP, thiamine monophosphate.

**Fig. 6.** The rate of the reaction as a function of substrate concentration in the presence and absence of ATP. A, nucleoside diphosphatase activity. The reaction mixture (2.0 ml) contained 80 µmoles of Tris-HCl, pH 8.5, 8 µmoles of MgCl$_2$, 0.1 µg of enzyme, 0.4 µmoles of ATP where indicated, and varying amounts of IDP. B, thiamine pyrophosphatase activity. The reaction mixture (0.5 ml) contained 20 µmoles of Tris-HCl (pH 8.5), 2 µmoles of MgCl$_2$, 0.4 µg of enzyme, 0.1 µmoles of ATP where indicated, and varying amounts of thiamine pyrophosphate. ●, activity in the presence of ATP; ○, activity in the absence of ATP. TMP, thiamine monophosphate; TPP, thiamine pyrophosphate.

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>With ATP $K_m$ (mM)</th>
<th>Without ATP $K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDP</td>
<td>0.94</td>
<td>3.5</td>
</tr>
<tr>
<td>GDP</td>
<td>1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>UDP</td>
<td>0.63</td>
<td>2.1</td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>3.5</td>
<td>20.7</td>
</tr>
</tbody>
</table>

The assay conditions were the same as in Fig. 6A for nucleoside diphosphates and as in Fig. 6B for thiamine pyrophosphate as substrate.
ments the standard incubation mixture contained 40 mmoles of ATP-14C (1200 cpm per mpmole) and 1.0 pmole of unlabeled IDP. With the enzyme preparation used, 105 mmoles of P_i were formed after incubation at 37°C for 6 min. The reaction was stopped by heating and the reaction mixture was chromatographed on a small column of Dowex 1-formate to separate ATP, ADP, and AMP (11). A zero time control was also treated in the same manner. No metabolites of ATP were found under these conditions, and essentially all of the radioactivity was recovered in the ATP fraction. Furthermore, in the presence of IDP and ATP-14C, the P_i formed did not contain radioactivity and therefore must have been derived from IDP only. These experiments were performed in the presence of 40 mmoles of ATP-14C (2000 cpm per mpmole), and radioactive P_i was determined as described under "Experimental Procedure."

The stimulatory effect of ATP was observed to occur without a measurable lag when ATP was added to an otherwise complete reaction mixture during the course of reaction (Fig. 8). The stimulatory effect disappeared immediately when ATP was removed by the addition of excess amounts of hexokinase and glucose.

**Binding Studies**—Gel filtration experiments with Sephadex G-50 showed that ATP (8-14C and 8-32P) was bound to the enzyme protein (Fig. 9A). A significant amount of radioactivity (0.4% of total) was recovered in the tubes containing the enzyme activity, although the bulk of the radioactivity was retained by the column; the ratio (1.37) of 14C:32P in the fractions containing the enzyme activity was essentially the same as the ratio (1.25) in the original mixture. The binding ratio of ATP to enzyme in these fractions was estimated to be about 0.6 eq per mole of protein, assuming the molecular weight of the enzyme to be 100,000 from the values of sed, 4 = 4.9 S. The low value for the number of bound ATP molecules might be due to the partial dissociation of ATP from the enzyme molecule during the passage through the column.

Control experiments, in which ATP was replaced by the same amounts of AMP-8-14C or in which the enzyme was replaced by bovine serum albumin (1 mg), showed no radioactivity in fractions containing the enzyme or albumin (Fig. 9B). It should be noted in Fig. 9, A and B, that ATP not bound to the enzyme was eluted from the column 4 tubes ahead of AMP under the same conditions.

**Heat Inactivation**—Nucleoside diphosphatase is a very heat-labile enzyme (2, 3). At 45°C in 0.02 m Tris-acetate, pH 7.4, about 85% of the enzyme activity was destroyed in 15 min. This inactivation was decreased by 4 mM ATP. Fig. 10 shows that the rate of inactivation by heat was the same for both enzyme activities. In the presence of 0.2 mM ATP, there was no protection of either activity against heat inactivation.

**Competition between IDP and Thiamine Pyrophosphate**—The hydrolysis of thiamine pyrophosphate was inhibited by IDP. The hydrolysis of thiamine pyrophosphate in the presence of IDP was determined by measurement of the formation of thiamine monophosphate. This inhibition was observed either

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** Effect of ATP concentration on the activities of nucleoside diphosphatase and thiamine pyrophosphatase. The reaction systems were the same as in Fig. 6, A or B, except that 0.25 mM IDP or 2 mM thiamine pyrophosphate was used as substrate, and the concentration of ATP was varied as indicated. [○—○], nucleoside diphosphatase activity; [●—●], thiamine pyrophosphatase activity. TMP, thiamine monophosphate.

![Fig. 8](http://www.jbc.org/)

**Fig. 8.** Effect of addition or removal of ATP during the course of reaction. The reactions were performed in the presence (●—●) and absence (○—○) of ATP under the conditions described in Fig. 6A, except that 0.25 mM IDP was used as substrate. At the time indicated by the arrow, 0.4 μmole of ATP (X—X) or sufficient amounts of hexokinase and glucose (△—△) were added.
Fig. 9. Elution of the enzyme and ATP from a column of Sephadex G-50. A, chromatography of a solution containing the enzyme and doubly labeled ATP (8-14C and y-32P). The enzyme (0.3 mg) were mixed with 80 mmoles of ATP (specific activity, 3.6 ,uCi per pmole for 14C and 3.0 pCi per pmole for 32P) and 30 mmoles of Tris-HCl, pH 7.2, in a total volume of 0.2 ml. After incubation at 20° for 5 min, the mixture was placed on a column of Sephadex G-50 (0.8 X 8 cm) equilibrated with 0.04 M Tris-acetate, pH 7.2, and the protein was eluted with the same buffer. Four drops (about 0.15 ml) were collected in each fraction. Aliquots were assayed for the enzyme activity with ATP as substrate and for radioactivity. B, chromatography of a solution containing the enzyme and AMP-8-32P. The same procedures were performed with 80 mmoles of AMP-14C (specific activity, 3.6 ,uCi per pmole).

Fig. 10. Effect of ATP on heat inactivation of nucleoside diphosphatase and thiamine pyrophosphatase. The enzyme, 30 ng, was incubated at 45° in 1.0 ml of 0.02 M Tris-acetate, pH 7.4, with (- -) or without (— —) 4 mM ATP. At indicated times aliquots were withdrawn and assayed for the activities of nucleoside diphosphatase (○) and thiamine pyrophosphatase (■) under the standard assay conditions in the presence of ATP.

Table V

<table>
<thead>
<tr>
<th>Metal Substrate</th>
<th>IDP</th>
<th>Thiamine pyrophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With ATP</td>
<td>Without ATP</td>
</tr>
<tr>
<td>MgCl₂ (4 mM)</td>
<td>375</td>
<td>195</td>
</tr>
<tr>
<td>MnCl₂ (2 mM)</td>
<td>322</td>
<td>288</td>
</tr>
<tr>
<td>CaCl₂ (4 mM)</td>
<td>257</td>
<td>221</td>
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<tr>
<td>CoCl₂ (4 mM)</td>
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<td>57</td>
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<td>ZnCl₂ (4 mM)</td>
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<td>NiCl₂ (4 mM)</td>
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<tr>
<td>None</td>
<td>12</td>
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</tr>
</tbody>
</table>

Effect of Metal Ions—The enzyme exhibited an absolute requirement for a divalent metal ion. As shown in Table V, Mg++, Mn++, and Ca++ were effective in nucleoside diphosphatase and thiamine pyrophosphatase.

whether thiamine pyrophosphatase was, in turn, a competitive inhibitor of the hydrolysis of IDP. The hydrolysis of IDP was inhibited competitively by thiamine pyrophosphatase (Fig. 11B). The hydrolysis of IDP in the presence of thiamine pyrophosphatase was estimated by the difference between the total amount of P_i liberated and the amount of thiamine monophosphate formed. From the above experiments, the apparent inhibition constant (K_i) was calculated to be 111 and 333 mM for IDP and thiamine pyrophosphatase, respectively. K_i values were calculated from the double reciprocal plots with higher concentrations of the substrate. These K_i values are very close to the Michaelis constants of 0.94 and 3.5 mM for IDP and thiamine pyrophosphatase, respectively.
Standard assay conditions were used with 2 mM IDP as substrate in the presence and absence of ATP.

**Table VI**  

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>IDP</th>
<th>Thiamine pyrophosphate</th>
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<tbody>
<tr>
<td></td>
<td>With ATP</td>
<td>Without ATP</td>
<td>With ATP</td>
</tr>
<tr>
<td>None</td>
<td>222 (100)</td>
<td>231 (100)</td>
<td>38 (100)</td>
</tr>
<tr>
<td>KF, 0.1 M</td>
<td>118 (50)</td>
<td>80 (35)</td>
<td>24 (28)</td>
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<tr>
<td>PP, 0.4 mM</td>
<td>181 (46)</td>
<td>125 (54)</td>
<td>30 (45)</td>
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<td>p-Mercuribenzoate, 0.2 mM</td>
<td>222 (57)</td>
<td>104 (45)</td>
<td>54 (63)</td>
</tr>
</tbody>
</table>

*The values in parentheses are the relative rates given as percentages.*

Phosphatase and thiamine pyrophosphatase reactions. On the other hand, Co²⁺ and Zn²⁺ were less active, and Ni²⁺ was inactive. In both reactions, the stimulatory effect of ATP was more pronounced with Mg²⁺ than with Mn²⁺ or Ca²⁺. The maximal activity was obtained at a Mg²⁺ concentration of 4 mM with 2 mM IDP as substrate (Fig. 12). The stimulatory effect of ATP was observed over the entire range of Mg²⁺ concentrations tested, and the Kₘ value for Mg²⁺, which was not varied by the addition of ATP, was estimated to be 1.8 mM.

**Inhibitors**—Potassium fluoride (0.1 M), PP₁ (0.4 mM), and p-mercuribenzoate (0.2 mM) inhibited the enzyme reaction. About the same degree of inhibition was produced by these compounds with IDP or thiamine pyrophosphatase as substrate, in the presence and absence of ATP (Table VI).

**Discussion**

Partially purified preparations of nucleoside diphosphatase have been obtained from acetone powder extracts of liver and kidney by Flaut (1), Gibson, Ayengar, and Sanadi (2), Heppel, Strominger, and Maxwell (3), and Novikoff and Heus (8). In the present experiments, the nucleoside diphosphatase was purified after solubilization from microsomes. Although Ernster and Jones solubilized the nucleoside diphosphatase from rat liver microsomes by deoxycholate (4), only a small amount of nucleoside diphosphatase was solubilized from bovine liver microsomes by deoxycholate (0.1%), and higher concentrations (0.2 and 0.4%) of the substance inactivated the enzyme. The treatment with NH₂OH released the soluble enzyme in good yield and, at the same time, resulted in a substantial increase in total activity. Stetten and Burnett (26) have reported that the activity of glucose 6-phosphatase in microsome suspensions was also increased by treatment with NH₂OH at pH 9.5 to 9.8.

A high degree of purity of the final hydroxyapatite fraction was indicated by (a) the monodisperse peak in the sedimentation velocity analysis, (b) the nearly homogeneous band upon polyacrylamide gel disc electrophoresis, (c) the constant specific activity in the several fractions of the hydroxyapatite peak, and (d) a specific activity as high as 370 units per mg of protein, with IDP as substrate, in the presence of ATP.

Thiamine pyrophosphatase activity during purification paralleled that of nucleoside diphosphatase. Although the thiamine pyrophosphatase activity has been detected in homogenates of animal tissues, purified preparations of this activity have not been so far obtained (27, 28). Thus, the following evidence is consistent with the conclusion that both enzyme activities are the properties of a single enzyme: (a) the ratio of activities with the two types of substrate remained essentially constant throughout the purification procedure, including DEAE Sephadex and hydroxyapatite chromatography; (b) partial heat denaturation produced the same degree of inactivation in both enzyme activities, and this inactivation was similarly prevented by ATP; (c) each substrate behaved as a competitive inhibitor of the hydrolysis of the other, and the Kₘ and Kₜ values were in agreement; (d) both activities were increased by ATP and other nucleoside triphosphates, (e) both activities were activated by Mg²⁺, Mn²⁺, or Ca²⁺, and inhibited by fluoride, PP₁, or p-mercuribenzoate; (f) the pH stability curves were apparently the same for both activities.

Only the pH activity curves differed for the two classes of substrate. Thiamine pyrophosphatase gave very little activity below pH 8.0 in the absence of ATP. Although Novikoff and Heus (8) failed to detect thiamine pyrophosphatase activity in a nucleoside diphosphatase preparation at pH 7.4, the thiamine pyrophosphatase activity might have been detected if it had been measured at about pH 9, particularly in the presence of ATP.

The activities of nucleoside diphosphatase and thiamine pyrophosphatase were markedly enhanced by the presence of ATP. ATP was active at low concentrations (viz. 10⁻⁵ M) and was not consumed during the reaction. ATP appeared to be bound noncovalently to the enzyme rather than to direct participation of ATP in the reaction. ATP therefore appears to act as allosteric effectors of nucleoside diphosphatase and thiamine pyrophosphatase. A similar type of activation by ATP has been described for nucleotide reductase of Escherichia coli (31) and Lactobacillus leichmannii (32, 33) and for adenylate deaminase of brain (34). In this type of activation, ATP is bound to the enzyme at a site different from the substrate site, thereby stabilizing certain conformations or states of the protein molecule. The hypothesis that ATP causes a conformational change in the nucleoside diphosphatase and thiamine pyro-
phosphatase structure is supported by the studies in which ATP protected the enzyme against heat denaturation, although the concentration of ATP required for stabilization of the enzyme was relatively higher than the corresponding value of activation of the reaction. However, this difference might be attributable to variations in experimental conditions, such as, for example, changes in temperature of incubation. ATP did not alter the sedimentation coefficient of the enzyme under the experimental conditions used in our studies. Thus, the possibility that the activation of the enzyme by ATP was accompanied by the association or dissociation of subunits appears to be improbable.

Nucleoside diphosphatase is specific for the nucleoside diphosphate linkage, but neither ADP nor CDP serves as substrate. These compounds bear an amino group in the pyrimidine portion of the ring. It is of interest that only nucleoside triphosphates showed the stimulatory effect on the reaction and the most potent activators were purine ribonucleoside or deoxyribonucleoside triphosphates.

It is reasonable to assume that the physiological significance of this finding is related to a homeostatic mechanism. A role of nucleoside diphosphatase in shifting the equilibrium of the phosphoenolpyruvate carboxykinase reaction and the synthetic reaction of succinyl-CoA was suggested by Plant (1) and by Gibson et al. (2), respectively. From the present finding that ATP and other nucleoside triphosphates stimulate the nucleoside diphosphatase reaction, we proposed that this effect would favor glycogenogenesis by leading to acceleration of the phosphoenolpyruvate carboxykinase reaction when the levels of ATP and other purine nucleoside triphosphates are high (9).

Hydrolysis of UDP should also favor glycogen synthesis, since UDP appears to be an inhibitor of glycogen synthetase (35). It should be noted that liver and kidney are thus far the only tissues which have been shown to contain both nucleoside diphosphatase (36) and phosphoenolpyruvate carboxykinase (37), and both tissues are known to synthesize glycogen from pyruvate or lactate via the phosphoenolpyruvate carboxykinase-dependent pathway (38).

A possible physiological implication of the metabolic regulation of thiamine pyrophosphatase activity has already been discussed as the control mechanism of ATP generation through the citric acid cycle (7).

Acknowledgments—The senior author wishes to thank Dr. G. Sunagawa, Director of the Central Research Laboratories, Sankyo Company (Tokyo, Japan), for his encouragement throughout this work, and to Drs. M. Tokushige, A. Nakazawa, and B. Maruo for their generous advice and helpful discussions. We are indebted to Miss E. Komabayashi for her skillful and devoted assistance during this investigation and to Dr. B. L. Horecker for his stimulating discussions and kind help during the preparation of this paper.

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