THE MONOESTER PHOSPHATE GROUPING OF COENZYME A*

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Coenzyme A (CoA) has been found to contain three phosphate groupings per molecule (4, 5). One of these has been found in the monoester form, since 1 mole of phosphate is liberated from 1 mole of CoA by the prostatic monoesterase (4, 5). This monoester phosphate has been ascertained to be on the adenylic acid moiety of the coenzyme (6). Through the use of a specific 3'-nucleotidase (1), preliminary evidence has been obtained indicating that CoA is a 3' derivative (2). In contrast, triphosphopyridine nucleotide (TPN) contains a monoester phosphate in the 2' position (7). The present paper presents detailed information showing the differences in position of the monoester linkage of CoA and TPN.

Materials

Two samples of CoA were used in this study. One had an activity of 390 units per mg. and was kindly supplied by Dr. F. Lipmann and Dr. G. D. Novelli. The second was a preparation obtained from the Pabst Laboratories and assayed 270 units per mg. TPN was obtained from the Sigma Chemical Company and was a preparation of 75 per cent purity.

The specific 3'-nucleotidase was obtained from barley and rye grass by the methods outlined by Shuster and Kaplan (1). Prostatic phosphatase was prepared by Markham and Smith's procedure (8) and phosphotransacetylase by the method of Stadtman (9). The specific 5'-adenylic acid deaminase was purified by the method of Nikiforuk and Colowick. The taka-diastase deaminase used was the purified preparation of Kaplan, Colowick, and Ciotti (10). 5'-Nucleotidase from potato was purified according

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to Kornberg and Pricer (7). Nucleotide pyrophosphatase was obtained from snake venom powder (Crotalus adamanteus) by dissolving 5 mg. of powder in 1 ml. of H$_2$O and removing the insoluble material. Intestinal adenosine deaminase was prepared from the Armour intestinal phosphatase preparation according to the procedure of Kornberg and Pricer (11).

**Results**

**Action of 3'-Nucleotidase on CoA**—Gregory et al. (4) have shown that the prostatic monoesterase acts on CoA and removes one phosphate group. This product is inactive in the phosphotransacetylase reaction (12). The compound which is formed from the action of monoesterase has been termed dephosphorylated coenzyme A (DPCoA) (13). The specific 3'-nucleotidase from barley, which attacks monoester groupings only in the 3'- and not 2'- or 5'-nucleotides (1), liberates 1 mole of phosphate per mole of coenzyme A (Table I). As indicated in Table I, the barley enzyme also inactivates the coenzyme for the phosphotransacetylase reaction. Treatment with the 3'-nucleotidase does not inactivate the coenzyme for the acetylation of sulfanilamide system of Kaplan and Lipmann (15); this is also the case with the action of the prostatic enzyme on CoA.

The monoester phosphate grouping in TPN is not attacked by the 3'-nucleotidase (1). This confirms Kornberg and Pricer's finding that the TPN is a 2' derivative and would also indicate that the monoester linkages in coenzyme A and TPN are located in different positions of the adenosine ribose moiety.

Although the specific 3'-nucleotidase does not attack TPN, the unspecific prostatic enzyme liberates the monoester phosphate of TPN to form di-phosphopyridine nucleotide (DPN) (10). This is indicated in Table II, where the activities of the prostatic enzyme on 2'-adenylic acid and TPN are compared. As can be seen from Table II, the enzyme acts on the mononucleotide 900 times as rapidly as on TPN.

**Table I**

*Splitting of CoA by 3'-Nucleotidases*

<table>
<thead>
<tr>
<th></th>
<th>Inorganic $P$</th>
<th>Arsenobis of acetyl $PO_4$</th>
<th>Acetylation of sulfanilamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA (0.05 $\mu$M)</td>
<td>0</td>
<td>14.8</td>
<td>14.8</td>
</tr>
<tr>
<td>‒ (0.05 &quot;&quot;) + 3'-nucleotidase</td>
<td>0.052</td>
<td>0</td>
<td>13.9</td>
</tr>
</tbody>
</table>

* Carried out by the procedure of Stadtman, Novelli, and Lipmann (14).
† Method of Kaplan and Lipmann (15).
Table II also compares the action of the prostatic enzyme on CoA and 3'-adenylic acid. The data indicate that it takes approximately 400 times more prostate enzyme to split 1 \( \mu M \) of phosphate from CoA than from the 3'-adenylate. In contrast, CoA and 3'-adenylic acid are attacked by the specific 3'-nucleotidase at about equal rates (1). The great differences in the action of prostatic enzyme on the monoester groupings of the coenzymes and on the mononucleotides certainly suggest that the specificity of the enzyme is much greater than was previously indicated. However, the 3'-nucleotidase, although specific in its action with respect to the position of the phosphate, seems to handle more complex compounds almost as well as mononucleotides. This is illustrated by the action of the 3' enzyme on CoA.

### Table II

**Action of Prostatic Phosphatase on Adenylic Acids, TPN, and CoA**

The incubation mixture consisted of 3 \( \mu M \) of substrates and 0.1 M acetate buffer, pH 5.1, in a volume of 2.0 ml. In the case of the mononucleotides, a smaller amount of enzyme was used. All values are corrected for controls and calculated to the same basis.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( \mu M ) P per hr. per 0.2 ml. enzyme</th>
<th>Rate compared to TPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPN</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>CoA</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>5'-Adenylic acid</td>
<td>376.0</td>
<td>418</td>
</tr>
<tr>
<td>2'-Adenylic acid</td>
<td>820.0</td>
<td>910</td>
</tr>
<tr>
<td>3'-Adenylic acid</td>
<td>1032.0</td>
<td>1182</td>
</tr>
</tbody>
</table>

**Comparison of Diphosphoadenosine Fragments from CoA and TPN**—Since both CoA and TPN contain a diphosphoadenosine component in their structure, a comparison between the diphosphoadenosine fragment from CoA with that from TPN would give an idea as to the nature of the monoester phosphate in CoA. If the diphosphate from CoA is identical with that from TPN, the monophosphate group in CoA must be in the 2' position. If the diphosphates are different, then the CoA derivative must be 3',5'-diphosphoadenosine, in contrast to the 2',5'-diphosphate from TPN.

200 \( \gamma \) of CoA (370 units per mg.) and 200 \( \gamma \) of TPN (80 per cent pure) were incubated separately with 0.1 ml. of snake venom extract, 30 \( \mu M \) of MgCl\(_2\), and 250 \( \mu M \) of Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, in a total volume of 0.38 ml. After 60 minutes of incubation at 37\(^\circ\), the reaction was stopped by placing the mixture in a boiling water bath for 2 minutes. The mixture was then placed on Whatman No. 3 filter paper and developed with a Carter solvent system (16) of 5 per cent Na\(_2\)HPO\(_4\).
and isoamyl alcohol. The spots on the filter paper were located with a Mineralight lamp. The $R_p$ values for the diphosphoadenosine fragments from CoA and from TPN are 0.83 and 0.85, respectively. Although the difference in the rate of migration of these two compounds is rather small, yet their relative positions on a paper chromatogram were constant in several trials. The small difference is actually expected, since with a phosphate group in the 5' position common to both compounds the difference in the rate of migration of these two compounds should be smaller than that between 2'- and 3'-adenylic acid. It thus appears from the chromatographic data that the fragments from the two coenzymes are different.

Fig. 1. Formation of 5-adenylic acid from CoA but not from TPN split-products. Split-products of CoA and of TPN, eluted from the paper chromatogram (see the text), were incubated with 3'-nucleotidase and then tested with 0.02 ml. of 1:10 5'-adenylic acid deaminase in citrate buffer, pH 6.5.

5-Adenylic Acid from Diphosphoadenosine Fragment of CoA—The diphosphoadenosine fragments of both CoA and TPN were cut from the filter paper as described above and eluted with water. To 0.2 ml. of the eluates were added 0.02 ml. of the purified 3'-nucleotidase and 10 μM of Tris (pH 7.5); the mixture was incubated for 30 minutes at 37°. After the incubation period, the mixtures were heated and then diluted with 0.1 M citrate buffer (pH 6.5) to a volume of 3 ml. The specific 5'-adenylic acid deaminase from muscle was added and the deamination followed at 265 μ. As can be seen from the data in Fig. 1, deamination occurs only with the fragment from CoA. This indicates that only the CoA fragment yields 5'-adenylic acid on treatment with the 3'-nucleotidase. The diphosphoadenosine fragment from TPN is 2',5'-diphosphoadenosine; this compound is not attacked by the 3'-nucleotidase, and hence no 5'-adenylic acid is
formed by the action of this enzyme. The data presented in Fig. 1 clearly illustrate the differences in positions of the monoester linkages of CoA and TPN.

Similar results can be obtained by incubating CoA with the snake venom extract and then treating with the 3'-nucleotidase without chromatographic separation. In order to obtain 5'-adenylic acid under these conditions, it is essential to inactivate the snake venom system by heating before incubating with the 3' enzyme. Otherwise, adenosine would be the end-product, since the snake venom contains a potent 5'-nucleotidase. The 5'-nucleotidase from snake venom does not attack the diphosphoadenosine fragments from either CoA or TPN.

The 5'-adenylic acid liberated from the diphosphoadenosine fragment of CoA, after treatment with the 3'-nucleotidase, was further established by paper chromatography. In a 5 per cent Na₂HPO₄-isoamyl alcohol solvent mixture, the product moves at the same rate as the authentic 5'-adenylate.

Adenosine from Coenzyme A, But Not from TPN—If CoA is incubated together with the 3'-nucleotidase and the crude snake venom extract, the resulting product is adenosine. This occurs through the action of the snake venom pyrophosphatase and the 3'-nucleotidase to form 5'-adenylic acid, which is then dephosphorylated by the specific 5'-nucleotidase of the snake venom to yield adenosine. The adenosine liberated can be determined with the adenosine deaminase from intestine. This is illustrated in Fig. 2. TPN treated under identical conditions does not yield adenosine. The above results again indicate a difference in the monoester groupings between CoA and TPN.

Isolation of 2',5'-Diphosphoadenosine and 3',5'-Diphosphoadenosine—50 mg. of TPN were incubated with 2 ml. of snake venom extract, 20 µM of MgCl₂, and 300 µM of Tris (pH 9.5) in a total volume of 3 ml. and incubated at 37°. The splitting of the TPN was followed by using the TPN-specific isocitric dehydrogenase from pig heart (17). The formation of nicotinamide mononucleotide and diphosphoadenosine was complete in 24 hours. The reaction mixture was then placed on a Dowex 1 formate column. Nicotinamide mononucleotide is not adsorbed on this column, whereas the 2',5'-diphosphoadenosine is removed. After washing, the diphosphoadenosine was eluted with 0.02 N HNO₃ + 0.02 N NaNO₃ mixture; the compound could be followed by the absorption at 260 mμ. The eluates containing the compound were combined (27 ml.) and 1 ml. of 20 per cent mercuric acetate (in 0.1 N HAC) was added. The resulting precipitate was then suspended in a small volume of H₂O and treated with H₂S. After removal of the sulfide, the solution was aerated and assayed for adenine

\^Approximately 50 ml. of 0.02 N HNO₃ + 0.02 N NaNO₃ mixture were required before the diphosphoadenosine fragments appeared in the eluates.
and total organic phosphate. The ratio of adenine to total P was found to be close to 1:2. Approximately 40 μM of compound were recovered in this solution.

50 mg. of CoA were treated with snake venom in the same way as with the TPN. The splitting was followed by the phosphotransacetylase assay. The reaction was somewhat slower than the TPN splitting, and complete

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

**Fig. 2.** Formation of adenosine from CoA, but not from TPN. Curve 1, 50 γ of TPN were incubated with 0.2 ml. of snake venom extract, 0.05 ml. of 3'-nucleotidase, 4 μM of MgCl₂, and 50 μM of Tris buffer, pH 7.5, in a total volume of 0.44 ml. at 37° for 60 minutes. Then the mixture was tested with 0.01 ml. of 1:10 intestinal adenosine deaminase in phosphate buffer, pH 7.5. Curve 2, same as Curve 1, but without 3'-nucleotidase; Curve 3, same as Curve 2, but with CoA (370 units per mg.) instead of TPN; Curve 4, same as Curve 1, but with CoA (370 units per mg.) instead of TPN.

hydrolysis of the CoA occurred after about 5 hours. After placing the reaction mixture on a Dowex 1 formate column, the 3′,5′-diphosphoadenosine was eluted with the 0.02 N HNO₃ + 0.02 N NaN₂ mixture. The CoA product is eluted from the column with approximately the same volume as is the 2′,5′-diphosphoadenosine. In contrast to the 2′,5′-diphosphoadenosine, the 3′,5′-diphosphoadenosine is not precipitated by the mercuric acetate. It was found necessary to add lead acetate (0.5 ml. of a 25 per cent solution) to precipitate the 3′,5′ compound. The lead salt was decomposed with H₂S and the solution aerated. 22 μM of the compound were obtained by this method. A ratio of adenine to total P of close to
1:2 was found. The CoA product was attacked by 3'-nucleotidase, liberating 1 mole of P, and resulted in the formation of 5'-adenylate. No inorganic P was obtained by the action of the 3' enzyme on the TPN product.

**Deamination of Diphosphoadenosine by Taka-diastase Deaminase**—It has been reported previously (10) that the deaminase from taka-diastase attacks 3'-adenylic acid, but not 2'-adenylic acid. Therefore, it was of interest to test the action of the enzyme on the diphosphoadenosine compounds. As can be seen from the data in Table III, the taka-diastase deaminase catalyzes the deamination of the diphosphoadenosine fragment from CoA, but does not attack the TPN fragment. This further indicates a difference in the position of the monoester grouping of CoA and TPN.

**Action of 5'-Nucleotidase from Potato on Diphosphoadenosine**—The 5'-nucleotidases from snake venom and bovine seminal fluid do not liberate the

### Table III

**Deamination of Diphosphoadenosines from TPN and CoA by Taka-diastase Deaminase**

The final concentration of nucleotide in all cases was $8 \times 10^{-3}$ M. The reaction was carried out in 0.1 M phosphate (pH 6.8) and started with 35 units of purified deaminase. Results in optical density units.

<table>
<thead>
<tr>
<th>Decrease in E_{420}</th>
<th>2'-Adenylate</th>
<th>3'-Adenylate</th>
<th>TPN fragment</th>
<th>CoA fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.000</td>
<td>0.040</td>
<td>0</td>
<td>0.010</td>
</tr>
<tr>
<td>2</td>
<td>0.002</td>
<td>0.082</td>
<td>0</td>
<td>0.018</td>
</tr>
<tr>
<td>5</td>
<td>0.004</td>
<td>0.132</td>
<td>0.002</td>
<td>0.083</td>
</tr>
<tr>
<td>10</td>
<td>0.004</td>
<td>0.190</td>
<td>0.004</td>
<td>0.049</td>
</tr>
</tbody>
</table>

5' grouping from the diphosphoadenosine fragment of TPN (7). Kornberg and Pricer (7), however, have found that the potato 5'-nucleotidase will attack the 5' grouping of the TPN fragment and yield 2'-adenylic acid. This finding was used by these authors as a basis for establishing that the TPN molecule contained a monoester grouping in the 2' position. It was, therefore, thought that by the use of the potato enzyme the 3'-adenylate could be obtained from the diphosphoadenosine fragment of CoA. However, all of our potato fractions would attack 3'-adenylic acid as well as the 5'-nucleotide, even at pH 9.5. The ratio of 5:3 activity was approximately 6:1 at pH 9.5. There was no splitting of 2'-adenylic acid at this pH. Hence, any attempts to obtain 3'-adenylate from the CoA product with the potato enzyme resulted in the liberation of more than 1 mole of phosphate per mole of compound, and usually close to 2 moles of inorganic P were obtained. This is illustrated in Table IV. The TPN derivative yielded close to 1 mole of phosphate per mole of compound (Table IV), in confirmation of the experiments of Kornberg and Pricer. Because of the
association of 3'-nucleotidase activity with the 5'-nucleotidase preparation, the isolation of 3'-adenylic acid from the CoA fragment could not be achieved. The data in Table IV, however, further indicate a difference in the position of the phosphate grouping in the two diphosphoadenosines, since the CoA fragment yields close to 2 moles of phosphate per mole, whereas the TPN product gives only 1 mole of phosphate under identical conditions.

Preparation of TPN with Monoester Grouping in 3' Position—Much of the evidence which has been presented for a difference in the positions of the monoester grouping of CoA and TPN has been based on the action of the specific 3'-nucleotidase. If TPN contained a phosphate in the 3' position, it was our view that the 3' enzyme would attack the monoester grouping. Therefore, we prepared TPN with a monoester grouping in the

<table>
<thead>
<tr>
<th>Compound</th>
<th>Phosphate liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td></td>
<td>μM</td>
</tr>
<tr>
<td>Diphosphoadenosine from TPN</td>
<td>0.21</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* Incubated 60 minutes at 37°.
† Incubated 30 minutes at 37°.

3’ position to see whether the 3’ enzyme would liberate phosphate from such a compound.

Brown and Todd (18) have found that acid causes a migration of phosphate between positions 2' and 3' and, with 2'- and 3'-nucleotides, yields an equilibrium mixture under these conditions. We have been able to isomerize the natural TPN and obtain a mixture of two isomers. One is the natural TPN containing the monoester in the 2' position; the second is a 3' derivative. The results of such isomerization experiments are summarized in Table V.

As can be seen from the data, no phosphate is liberated from the control TPN by the 3' enzyme; however, over 40 per cent of the monoester phosphate is liberated from the isomerized material. This indicates an equilibrium mixture of the two forms of the coenzyme after the acid treatment. There is no DPN present, in either the control or isomerized preparations. Treatment, however, with the 3'-nucleotidase results in the formation of DPN from the isomerized material. The amount of DPN formed is al-
most stoichiometric with the amount of inorganic phosphate liberated. No DPN is obtained from the normal TPN. The fact that phosphate is liberated from the isomeric material and that DPN results from this reaction indicates the presence of TPN with a monoester grouping in the 3′ position. The finding that isomerization of TPN does result in material which

<table>
<thead>
<tr>
<th>Table V</th>
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</thead>
<tbody>
<tr>
<td><strong>Isomerization of TPN</strong></td>
</tr>
<tr>
<td>All values in micromoles. 20 mg. of Sigma No. 85 TPN were allowed to stand in 1.0 ml. of 0.5 n HCl for 24 hours at room temperature. 5 volumes of cold acetone were then added. The resulting precipitate was spun down, washed with 5 volumes of acetone, and dissolved in 2.0 ml. of H2O. The amount of compound was assayed by the cyanide procedure (19). DPN was determined before and after treatment with 3′-nucleotidase by the use of crystalline yeast alcohol dehydrogenase (20).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table VI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analysis of Dephosphorylated CoA</strong></td>
</tr>
<tr>
<td>μM per mg.</td>
</tr>
<tr>
<td>0.78</td>
</tr>
<tr>
<td>1.04</td>
</tr>
</tbody>
</table>

* Estimated by ultraviolet absorption at 260 μm, based on a millimolar absorption coefficient of 16,000.
† Determined by the orcinol method with adenylic acid as the standard (21).
‡ Determined by the procedure of Novelli, Kaplan, and Lipmann (22). We wish to thank Dr. Robert Van Reen and Miss Grace Cunningham for these assays.

is susceptible to the action of the 3′ enzyme further strengthens our view of the nature of the monoester phosphate in CoA.

Preliminary experiments have indicated that CoA can also be isomerized. The biological activities of the 3′-TPN and the 2′-CoA will be described elsewhere.

Preparation of DPCoA—40 mg. of CoA (270 units per mg.), dissolved in 70 mg. of water (pH about 3.5), were neutralized with solid KHCO3 to pH 7.5. With occasional stirring or shaking, the solution was kept at room temperature until the characteristic odor of —SH compounds dis-
appeared. 5 ml. of 3'-nucleotidase were added to the solution, which was then incubated at 37°. Small samples (corresponding to 10 units of CoA) were taken after 2 hours incubation for the arsenolysis test. As soon as all the CoA was dephosphorylated (indicated by a negative arsenolysis test), the solution was evaporated under reduced pressure to a small volume (about 8 ml.). Some precipitate formed at this stage was removed by centrifugation. The clear solution was then acidified to about pH 4 with 4 N HNO₃, and 6 volumes of cold acetone (−15°) were added to the acidified mixture. After standing in a deep freeze (−15°) overnight, the precipitate was collected by centrifugation. It was then redissolved in a small volume of water (5 ml. of water) and reprecipitated with acetone as before. Any insoluble materials were removed by centrifugation or filtration before the acetone was added. 40 mg. of DPCoA of 50 per cent purity were obtained. Analyses were made for its adenine, ribose, pantothenate, and phosphate content; the findings are summarized in Table VI.

**SUMMARY**

The specific 3'-nucleotidase from barley or rye grass has been found to hydrolyze the monoester phosphate grouping of CoA to form dephosphorylated CoA. The 3' enzyme does not attack the monoester grouping of TPN.

The unspecific monoesterase from prostate attacks the primary monoester grouping of CoA at approximately 1/400th that of 3'-adenylic acid, and the monoester phosphate of TPN at a rate approximately 1/900th that of 2'-adenylic acid.

Through the use of the snake venom pyrophosphatase, the diphosphoadenosine fragments of CoA and TPN have been obtained. The fragments from the two coenzymes have been found to be different by paper chromatographic techniques. The diphosphoadenosine from CoA yields 5'-adenylic acid on treatment with the 3'-nucleotidase and yields adenosine when it is treated with the 3' enzyme and the 5'-nucleotidase from snake venom together. 5'-Adenylic acid or adenosine is not formed from the diphosphoadenosine fragment of TPN under identical conditions. The taka-diastase deaminase attacks the diphosphoadenosine fragment from CoA, but not from TPN.

Consideration of the above data places the monoester grouping of CoA at the 3' position on the adenosine moiety of the molecule, and confirms Kornberg and Pricer's finding that TPN is a 2'-adenosine derivative. Further, CoA in the −SH form is not split by the 3'-nucleotidase. This is due to the inhibition by the SH group, as it has been shown that cysteine and reduced glutathione are strong inhibitors of the enzyme (1).
ther evidence is also given for the difference in positions of the monoester groupings of the two coenzymes.

Isomerization of TPN by acid yields two forms of TPN. One contains a monoester grouping in the natural 2' position; the second has the monoester linkage as a 3' grouping. The 3'-nucleotidase liberates phosphate from the 3' isomer to form DPN.

The isolation of dephosphorylated CoA from the action of the 3'-nucleotidase on CoA is described.

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