We have studied the effects of adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase on the phosphorylative and functional modification of bovine adrenal tyrosine hydroxylase. Incubation of partially purified tyrosine hydroxylase with cAMP-dependent protein kinase in the presence of [γ-32P]ATP and 5 μM cAMP led to a 3- to 5-fold activation of tyrosine hydroxylase and to incorporation of [32P]phosphate into protein. When tyrosine hydroxylase preparations activated by exposure to enzymatic phosphorylating conditions were analyzed by sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, and gel electrofocusing, the radioactivity of 32P was coincident with the activity of tyrosine hydroxylase, suggesting incorporation of 32P from [γ-32P]ATP into tyrosine hydroxylase. Polyacrylamide gel electrophoresis of the phosphorylated tyrosine hydroxylase preparation in the presence of 0.1% sodium dodecyl sulfate revealed that the 60,000-dalton polypeptide subunit of tyrosine hydroxylase served as the phosphate acceptor.

Tyrosine hydroxylase (EC 1.14.16.2) catalyzes the conversion of tyrosine to Dopa, the rate-limiting step in the biosynthesis of the catecholamines, dopamine, noradrenaline, and epinephrine (1, 2) and is therefore important in the regulation of in vivo catecholamine metabolism. In recent years, there have been a number of studies which showed that enzymatic phosphorylating conditions activate tyrosine hydroxylase in brain tissues (3–10) and adrenal medulla (9–12). Although the activation of the enzyme required the presence of ATP, Mg2+, cAMP, and protein kinase, the mechanism of the activation of tyrosine hydroxylase by cAMP-dependent protein kinase has remained obscure. Present studies show direct phosphorylation of tyrosine hydroxylase associated with its activation by cAMP-dependent protein kinase. Preliminary accounts of a portion of this work have already appeared (13).

**EXPERIMENTAL PROCEDURES**

**Materials**—Purchased from the following sources were: [γ-32P]ATP (3500 Ci/mmol), Radiochemical Centre, Amersham; Ampholine (pH 3 to 8, 40% solution), LKB; DMF, and 3-iodo-L-tyrosine, Aldrich Chemical Co.; cAMP, ATP, Mes, Hepes, heparin (grade III), and cAMP-dependent protein kinase of beef heart, Sigma Chemical Co.; crystalline catalase of beef liver, bovine serum albumin, chymotrypsinogen of bovine pancreas, and cytochrome c of horse heart, Boehringer Mannheim Corp.; Sepharose 4B, Pharmacia Fine Chemicals; and Bio-Gel P-10, Bio-Rad. All other reagents were of the highest purity commercially available.

**Preparation of Affinity Gels**—The 3-iodo-L-tyrosine-substituted Sepharose 4B gel was prepared according to reports from Kaufman’s laboratory (14, 15) following the general procedure of Cuatrecasas (16). Sepharose 4B, 100 ml, was activated by 15 g of cyanogen bromide at pH 11, and then 1.5 g of 3-iodo-L-tyrosine was added. The mixture was stirred at pH 9.5 for 48 h at 4°C. The extent of coupling was found to be 9% by measurement of the uncoupled ligand which was washed from the gel.

Heparin-substituted Sepharose 4B was prepared by the method of Iverius (17). Sepharose 4B, 25 ml, was activated with 3.75 g of cyanogen bromide at pH 11 (16). The activated gel was mixed with 60 mg of heparin and the volume was made up to 50 ml with 0.1 M NaHCO3. After the mixture was stirred at 4°C for 24 h, 0.4 g of ethanamine/HCl was added and the mixture was stirred for 2 h. The gel was then washed with water. The extent of the coupling was found to be about 50% by measurement of ionic acid of the uncoupled heparin which was washed from the gel.

**Purification of Bovine Adrenal Tyrosine Hydroxylase**—Fresh bovine adrenal glands were obtained at the slaughterhouse. Unless otherwise stated, all subsequent procedures were performed at 4°C. The adrenal medullae, about 50 g, were dissected from the bovine adrenal glands and homogenized in 250 ml of 10 mM Tris/HCl, pH 7.3, containing 0.3 M sucrose with a Potter-Elvehjem homogenizer. The soluble fraction which was obtained from the homogenate by centrifugation at 105,000 × g for 1 h was chromatographed on a 3-iodotyrosine-substituted Sepharose 4B column (2.0 × 21 cm) equilibrated with 20 mM Tris/HCl, pH 7.3, containing 0.23 M sucrose. The column was washed with 150 ml of the same buffer, then 900 ml of the same buffer containing 0.6 M KCl, and eluted with 20 mM Tris/HCl, pH 10, containing 0.5 M KCl. Fractions of about 0.8 ml were collected in tubes each containing 0.8 ml of 0.5 M Tris/HCl, pH 6.0. The active fractions were pooled and applied to 0.2 M potassium phosphate buffer, pH 7.3, containing 1 mM dithiothreitol. The column was washed with 110 ml of the same buffer containing 0.1 M KCl and eluted with a 0.1 M linear gradient of 0.1 to 0.5 M KCl in equilibration buffer. The active fractions were pooled, brought to 40% saturation with solid ammonium sulfate, and centrifuged. The resulting precipitate was dissolved in 2 ml of 20 mM potassium phosphate buffer, pH 7.3, containing 1 mM dithiothreitol and stored frozen at −80°C until used for further purification. Three preparations, which had been purified and stored as described above, were combined, desalted by gel filtration on Bio-Gel P-10 equilibrated with 20 mM potassium phosphate buffer, pH 7.1, containing 0.23 M sucrose and 1 mM dithiothreitol, and then applied to a column (1.6 × 9 cm) of phosphocellulose equilibrated with the same buffer. The column was washed with 100 ml of equilibration buffer and eluted with 20 mM potassium phosphate buffer, pH 7.6, containing 0.23 M sucrose, 1 mM dithiothreitol, and 0.5 M KCl. The active fractions were pooled, brought to 40% saturation with solid ammonium sulfate, and centrifuged. The resulting precipitate was dissolved in 20 ml of 20 mM potassium phosphate buffer, pH 7.3, containing 1 mM dithiothreitol. The enzyme was desalted by gel filtration on Bio-Gel P-10 and small aliquots were stored at −80°C over 3 months without detectable loss of activity. The total purification scheme is summarized in Table I. The purified enzyme was analyzed by SDS-polyacrylamide gel electrophoresis as shown in Figure 1.
Phosphorylation of Tyrosine Hydroxylase

bovine adrenal medulla with an 18% recovery of activity.

Assay of Tyrosine Hydroxylase—Tyrosine hydroxylase was assayed by fluorometric method as described previously (18). During purification, the assays were carried out under the standard assay conditions (18). The standard assay medium contained 200 µM tyrosine, 800 µM DMPH, 40 mM 2-mercaptoethanol, 100 mM Mes buffer, pH 6.1, 100 µg of catalase, and a suitable amount of tyrosine hydroxylase in a final volume of 0.5 ml. When the enzyme activity was measured after exposure to enzymatic phosphorylating conditions, the assay medium contained 200 µM tyrosine, 100 µM DMPH, 40 mM 2-mercaptoethanol, 1 mM EDTA, 100 mM Hepes buffer, pH 6.8, 100 µg of catalase, and a suitable amount of tyrosine hydroxylase in a final volume of 0.5 ml. The phosphorylating conditions led to a shift in the pH optimum of tyrosine hydroxylase from 6.0 to 6.8 (6). The reaction was carried out at 30°C for 10 min with shaking. One unit of tyrosine hydroxylase is defined as the amount which catalyzes the formation of 1 nmol of Dopa/min at 30°C.

Phosphorylation of Tyrosine Hydroxylase—The standard incubation mixtures for the phosphorylation of tyrosine hydroxylase contained 20 mM sodium phosphate buffer, pH 6.8, containing 50 or 100 µM [γ-32P]ATP (60 to 200 cpm/µmol), 9 µM cAMP, 5 mM MgCl2, 5 or 12 µg of protein kinase, and a suitable amount of tyrosine hydroxylase in a final volume of 70 or 100 µl. The incubation was carried out for 5 min at 30°C. Incorporation of [γ-32P]phosphate into protein was estimated by the method of Corbin and Reimann (19) and analyzed by polyacrylamide gel electrophoresis, gel electrofocusing, and sucrose density gradient centrifugation.

Polyacrylamide Gel Electrophoresis—Polyacrylamide disc gel electrophoresis was performed at 4°C with 6.3% polyacrylamide gels containing 0.23% sucrose essentially according to the procedure of Davis (20), except that riboflavin (0.0005%) was added as a catalyst in running and stacking gels (21). Electrode buffer contained 1 mM thioglycolic acid.

SDS-polyacrylamide gel electrophoresis was carried out with 10% polyacrylamide gels in the presence of 0.1% SDS at 8 mA/gel for 5 h according to the procedure of Weber and Osborn (22).

Gel Electrofocusing—Analytical isoelectric focusing was performed on 4% polyacrylamide gels containing 0.23% sucrose and 2% Ampholine (pH 5 to 8) essentially according to the procedure of Wrigley (23). Electrofocusing was carried out at 200 V for 4 h in a cold room.

Sucrose Density Gradient Centrifugation—Sucrose gradient ultracentrifugation was performed in a 5-ml gradient solution from 6 to 26% sucrose in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol with a Hitachi RPS-65T rotor in a Hitachi model 65P ultracentrifuge. After centrifugation at 55,000 rpm (300,000 × g) for 5 h at 4°C, tubes were punctured from the bottom, and the contents were collected in 24 fractions of approximately 220 µl.

Determination of Protein concentrations were estimated by the method of Lowry et al. (24) with bovine serum albumin as a standard. Sucrose and uronic acid were determined by anthrone reaction and by carbazole reaction, respectively (25).

RESULTS

The time course of activation of tyrosine hydroxylase partially purified as summarized in Table I and protein phosphorylation by cAMP-dependent protein kinase is shown in Fig. 1. The activation of tyrosine hydroxylase closely paralleled the increase of protein phosphorylation. About 310 pmol of [γ-32P]phosphate were incorporated into 48 µg of protein after incubation for 10 min as shown in Fig. 1B and the amount corresponded to approximately 4.4% of the total amount of [γ-32P]ATP used in the experiment. Although the maximum activation of tyrosine hydroxylase was approximately 3-fold in the experiment presented in Fig. 1A, the extent of the activation varied to some extent from one preparation of tyrosine hydroxylase to another.

As shown in Table II, both activation of tyrosine hydroxylase and protein phosphorylation required the presence of protein kinase, cAMP, and Mg++. Addition of EDTA inhibited both the activation and the phosphorylation. Thus, there was a good correlation between protein phosphorylation and tyrosine hydroxylase activation, suggesting that cAMP-dependent protein kinase catalyzed the phosphorylation of tyrosine hydroxylase which resulted in a concomitant activation of tyrosine hydroxylase.

In order to ensure that the 32P incorporation was actually into tyrosine hydroxylase, tyrosine hydroxylase preparations activated by exposure to enzymatic phosphorylating conditions were analyzed by sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, and gel electrofocusing. Fig. 2 shows the sucrose density gradient patterns of 32P radioactivity incorporated into protein and tyrosine hydroxylase activity. The single peak of enzyme activity was collocated with the peak of radioactivity and the amount of 32P radioactivity eluted in the active fractions was found to correspond to about 5.3% of the total amount of [γ-32P]ATP used in the experiment. The recovery of activities of both activated and control enzyme was approximately 50%. The activation of the enzyme by exposure to phosphorylating conditions was calculated to be about 4-fold. Analysis of the phosphorylation mixture by polyacrylamide gel electrophoresis revealed that almost all of the radioactivity incorporated into the protein coincided with the band of tyrosine hydroxylase as shown in Fig. 3A. The amount of the radioactivity located in the enzyme band was found to correspond to 6.4% of the total [γ-32P]ATP.

![Fig. 1](http://example.com/fig1.png)

**Summary of purification of tyrosine hydroxylase from 143 g of bovine adrenal medulla**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg</th>
<th>Activity units</th>
<th>Specific Activity units/mg</th>
<th>Recovery %</th>
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<tbody>
<tr>
<td>High speed supernatant</td>
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<td>8750</td>
<td>2.09</td>
<td>100</td>
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<tr>
<td>3-Iodo-L-tyrosine-substituted Sepharose 4B</td>
<td>271</td>
<td>4500</td>
<td>16.1</td>
<td>51</td>
</tr>
<tr>
<td>Heparin-substituted Sepharose 4B</td>
<td>50</td>
<td>2620</td>
<td>52.5</td>
<td>30</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>11</td>
<td>1600</td>
<td>146</td>
<td>18</td>
</tr>
</tbody>
</table>
TABLE II
Requirements for phosphorylation and activation of tyrosine
hydroxylase by cAMP-dependent protein kinase

The reaction was carried out at 30°C in a total volume of 100 μl.
The complete system contained 20 mM sodium phosphate buffer, pH
6.8, 100 μM [γ-32P]ATP (1.69 × 10^6 cpm), 5 μM cAMP, 5 mM MgCl₂,
24 μg of tyrosine hydroxylase, and 5 μg of protein kinase. After incubation
for 5 min, a 10-μl aliquot was assayed for tyrosine hydroxylase as
described under "Experimental Procedures" and a 50-μl aliquot was
assayed for the 32P incorporation into protein according to the method
of Corbin and Reimann (19).

<table>
<thead>
<tr>
<th>Systems</th>
<th>Tyrosine hydroxylase</th>
<th>32P incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>cpm × 10^3/mg protein</td>
</tr>
<tr>
<td></td>
<td>units/mg protein</td>
<td>%</td>
</tr>
<tr>
<td>Complete system</td>
<td>162</td>
<td>312</td>
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<td>-Protein kinase</td>
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<tr>
<td>-MgCl₂</td>
<td>53</td>
<td>102</td>
</tr>
<tr>
<td>+EDTA (10 mM)</td>
<td>58</td>
<td>112</td>
</tr>
</tbody>
</table>

**Fig. 2.** Sucrose density gradient centrifugation of phosphorylated
tyrosine hydroxylase. A, 96 μg of tyrosine hydroxylase was incubated
with 12 μg of protein kinase and 100 μM [γ-32P]ATP (0.573 × 10^6 cpm)
in a total volume of 70 μl. Composition of the reaction mixture and
further experimental details are given under "Experimental Proce-
dures." B, control experiments were carried out without protein
kinase. After incubation for 5 min, 50 μl of the mixture were layered
onto 5 ml of 6 to 26% sucrose gradient, centrifuged as described under
"Experimental Procedures," and fractions of 220 μl each were col-
clected. One aliquot, 50 μl, was taken for determination of tyrosine
hydroxylase activity (O- - -O); a second aliquot, 100 μl, was with-
drawn, and 32P radioactivity was measured in 10 ml of water by Cerenkov
counting (X-X); and a third aliquot was taken for determination of sucrose
concentration (M).

The recovery of activities of activated and control enzyme
was 30 and 46%, respectively. The activation of the enzyme was
approximately 3.5-fold. Analysis of the phosphorylated
sample on gel electrophoresis also revealed that 32P radioac-
tivity incorporated into the protein co-migrated with tyrosine
hydroxylase as shown in Fig. 4. The radioactivity on the gel
was located at the position corresponding to the single band
of tyrosine hydroxylase, the isoelectric point of which was
estimated to be about pH 6.6. The amount of the radioactivity
located in the enzyme band was found to correspond to 7.1%
of the total [γ-32P]ATP. The recovery of activities of activated
and control enzyme was 60 and 82%, respectively. The acti-

**Fig. 3.** Polyacrylamide gel electrophoresis of phosphorylated ty-
rosine hydroxylase. A, tyrosine hydroxylase, 72 μg, was incubated
with 12 μg of protein kinase and 50 μM [γ-32P]ATP (0.728 × 10^6 cpm)
in a total volume of 70 μl as described under "Experimental Proce-
dures." B, control experiments were carried out without protein
kinase. After incubation, 10 μl of 40% sucrose were added to the
mixture and a 50-μl aliquot was subjected to polyacrylamide gel
electrophoresis as described under "Experimental Procedures." After
electrophorosis, the gel was sliced into 2-mm sections which were
subsequently cut longitudinally into equal parts. One part was ho-
mogenized in 0.5 ml of assay mixture of tyrosine hydroxylase and
assayed as described under "Experimental Procedures" (O- - -O).
The other part was placed into a counting vial and 32P radioactivity
was determined in 10 ml of water by Cerenkov counting (X-X).
vation of the enzyme by phosphorylation was approximately 4-fold. When the phosphorylated sample was subjected to electrophoresis on polyacrylamide gels in the presence of 0.1% SDS, only one peak of $^{32}$P radioactivity was observed as shown in Fig. 5. The molecular weight of the phosphorylated protein was estimated to be approximately 60,000 as shown in the inset in Fig. 5. The amount of the radioactivity found in this band corresponded to 5.7% of the total amount of $[\gamma-^{32}\text{P}]$ATP used in the experiment.

**DISCUSSION**

Since the rate of synthesis of catecholamines in vivo is controlled by the initial enzyme in the pathway, tyrosine hydroxylase (2), the mechanism of its regulation has generated great interest. Recently, evidence has accumulated that protein phosphorylation by CAMP-dependent protein kinase and that phosphorylation of proteins other than tyrosine hydroxylase in the enzyme preparation did not occur as a result of protein kinase action. When the phosphorylated enzyme was examined by SDS-polyacrylamide gel electrophoresis, only one peak of $^{32}$P radioactivity coincided with a protein band with a molecular weight of 60,000. From the results described above, it was concluded that bovine adrenal tyrosine hydroxylase was directly phosphorylated by CAMP-dependent protein kinase and that phosphorylation of proteins other than tyrosine hydroxylase in the enzyme preparation did not occur as a result of protein kinase action. When the phosphorylated enzyme was examined by SDS-polyacrylamide gel electrophoresis, only one peak of $^{32}$P radioactivity coincided with a protein band with a molecular weight of 60,000. From the results described above, it was concluded that bovine adrenal tyrosine hydroxylase was directly phosphorylated by CAMP-dependent protein kinase and that phosphorylation of proteins other than tyrosine hydroxylase in the enzyme preparation did not occur as a result of protein kinase action.

**REFERENCES**

In vitro phosphorylation of bovine adrenal tyrosine hydroxylase by adenosine 3':5'-monophosphate-dependent protein kinase.

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