Enzymatic Activation of Biotin

BIOTINYL ADENYLATE FORMATION*

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In the course of a study of the mechanism by which biotin is incorporated into holocarboxylases, a soil bacterium was isolated which grows on biotin as the sole carbon source. As reported in 1961 (1, 2), cell-free preparations of this micro-organism oxidize the biotin side chain, and a soluble enzyme was obtained which catalyzes (a) the biotin-dependent exchange of radioactive pyrophosphate with adenosine triphosphate and (b) the formation of biotin hydroxamic acid from d-biotin, adenosine triphosphate, and hydroxylamine. The latter reaction is stimulated by coenzyme A. These findings suggested that d-biotinyl 5'-adenylate and biotinyl-CoA are formed as products of the carboxyl activation of biotin.

The present paper is a full account of such experiments with the bacterial enzyme as well as of the purification and properties of the biotin-activating enzyme from pig liver (3). Synthetically prepared d-biotinyl 5'-adenylate is shown to yield ATP in the presence of the liver activating enzyme, pyrophosphate, and Mg++ ions, and to form biotinyl-CoA in the presence of the enzyme and CoA. The role of these intermediates in biotin oxidation and in the conversion of apocarboxylases to biotin-containing holocarboxylases is discussed below.

EXPERIMENTAL PROCEDURE

Materials

Synthesis of Biotin-α-14C—The methods described by Goldberg and Sternbach (4) were adapted to the synthesis of biotin labeled with 14C in the α carbon. In this procedure, a suspension of L-34-(1',3''-dibenzyl-2'-ketoimidazolido)-1,2-trimethylenethio-phumum d-camphorsulfonate1 in toluene was allowed to react with the sodium derivative of diethyl malonate-2-W. The condensation product was heated with 48% HBr to give d-biotin. The product, purified by chromatography on Dowex 1-acetate and by recrystallization to constant specific activity (3.2 × 10⁴ c.p.m. per mg), was pure as judged by microbiological assay.

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1 We are indebted to Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc., who kindly provided the thiophanium derivative and suggested this procedure for synthesizing radioactive biotin.

with Lactobacillus arabinosus (5) and by paper chromatography (Rf 0.70 in butanol-water-acetic acid, 5:1:1).

Synthesis of d-Biotinyl 5'-Adenylate—A procedure previously described by Berg (6) for the synthesis of an aminoacyl adenylate was adapted to the synthesis of biotinyl-AMP. AMP and d-biotin (2 mmoles each) were dissolved in 10 ml of 75% aqueous pyridine at 0°. A solution of 8 g of dicyclohexylcarbodiimide in 8 ml of pyridine was added, and the mixture was stirred at 0° for 20 hours, at which time there was no further increase in the amount of neutral hydroxylamine-reactive material (7). The product was precipitated by the addition of 150 ml of acetone previously chilled to −15° and recovered by filtration. The precipitate was washed with small portions of acetone-ethanol, 3:2, and then with ether at 0° and dried overnight at reduced pressure over P₂O₅ and paraffin shavings. The dried powder was mechanically dispersed in 25 ml of cold water, and the mixture was adjusted to pH 6.0 with 0.5 M KOH. The solid material was collected on a filter and washed with 10 ml of cold water. The filtrate, which contained the product in about 50% yield, was concentrated to 10 ml in a rotary evaporator and stored at −15°. Further purification was achieved by chromatography of the product at room temperature on a column of DEAE-cellulose. As shown in Fig. 1, biotinyl-AMP, preceded closely by biotin, was eluted by 0.05 to 0.1 M KCl, and AMP was eluted by 0.1 to 0.2 M KCl in 0.05 M HCl.

The solutions in those tubes which contained biotinyl-AMP but no free biotin were pooled, and an aliquot was found to give a single spot, Rf 0.59, on paper chromatography in isobutyric acid-water-concentrated ammonium hydroxide, 66:33:1. The compound was detected by ultraviolet light absorption and by the color produced when the paper was sprayed with neutral hydroxylamine and then with alcoholic ferric chloride. Further indication of the purity of the compound, acyl phosphate, adenine, organic phosphate, and vicinal hydroxyls were found to be present in the ratio 1.00:0.96:1.02:0.98. The acyl phosphate content was determined by hydroxamic acid formation at pH 6.5, adenine by the absorbance at 260 μ, and organic phosphate by the colorimetric determination of P₈ after acid hydrolysis (8). The uptake of periodate, estimated spectrophotometrically (9), served as a measure of vicinal hydroxyl groups. Since biotin reacts readily with an equimolar amount of periodate, presumably to form the sulfoxide, the value given for vicinal hydroxyls has been corrected for the reaction of periodate with the biotin moiety. The results obtained rule out the presence of significant amounts of free biotin, AMP, biotin anhydride, or adenyl anhydride. The presence of the biotinyl
Enzymatic Synthesis of Biotinyl Adenylate

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Enzyme Assay—The biotin-activating enzyme was routinely assayed by photometric determination of biotin hydroxamic acid. The reaction mixture contained 100 μmoles of Tris buffer, pH 7.4, 10 μmoles of MgCl₂, 10 μmoles of the disodium salt of ATP, 20 μmoles of the potassium salt of biotin, 2500 μmoles of salt-free hydroxylamine (16) adjusted to pH 7.4, and the enzyme preparation in a final volume of 1.0 ml. ATP and biotin were saturating at the concentrations employed, but higher concentrations of hydroxylamine gave slightly greater activity. When the bacterial enzyme was assayed, CoA (0.1 μmole) was routinely added, and the tubes were flushed with nitrogen and stoppered. After incubation for 30 minutes at 37°C, the reaction mixture was treated with 1 ml of 40% trichloroacetic acid and 3 ml of 0.67 N HCl containing 10% FeC₃·6H₂O and 5% trichloroacetic acid. The mixture was centrifuged, and the absorbance of the supernatant solution was determined in a Klett-Summerson photometer with a green filter (No. 54). Authentic biotin hydroxamic acid was employed as a standard. Hydroxamic acid formation in the enzyme system was linear up to 1 hour and, as shown in Fig. 2, was linear with respect to enzyme concentration. One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmole of biotin hydroxamic acid per minute under the conditions described, and specific activity is expressed as units of enzyme per mg of protein.

PP₃-ATP Exchange—The bacterial and liver biotin-activating enzymes may also be assayed by the biotin-dependent exchange reaction between PP₃ and ATP. The enzyme was incubated for 30 minutes at 37°C with 100 μmoles of Tris buffer, pH 7.4, 5 μmoles of MgCl₂, 5 μmoles of ATP, 10 μmoles of the potassium salt of biotin, and 1 μmole of PP₃ in a final volume of 1.0 ml. The reaction was terminated with trichloroacetic acid, the precipitate was discarded, and the ATP was assayed on acid-washed Norit A according to the method of Crane and Lipmann (17). The charcoal was washed with water and treated with 1 N HCl at 100°C; after removal of the charcoal, aliquots of the solution were assayed for radioactivity. The total amount of

Fig. 1. Chromatography of biotinyl AMP on DEAE cellulose. A mixture of crude biotinyl-AMP (about 100 μmoles), AMP, and biotin-α-¹⁴C (0.5 μmole, 4000 c.p.m.) was applied to a column of DEAE-cellulose (1.5 × 15 cm) which had previously been washed with 1 liter of 0.1 M potassium phosphate buffer, pH 7.8, and 1 liter of water. The column was eluted with the solutions indicated, and 5.0-ml fractions were collected. Aliquots were taken for the determination of acyl phosphate by hydroxamic acid formation (O—O), biotin by radioactivity (Δ-Δ), and adenine-containing material by absorption at 260 μm (●●●●●).

Fig. 2. Biotin hydroxamic acid formation as a function of enzyme concentration. A 0 to 58% ammonium sulfate fraction of the liver biotin-activating enzyme was used.

group in 2'- or 3'-ester linkage is ruled out by the periodate data and the fact that such esters react slowly and incompletely with hydroxylamine at pH 6.5, whereas with biotinyl-AMP the reaction is complete in less than 3 minutes.

Biotinyl-CoA—The CoA thioester of biotin was prepared by the general method of Wieland and Rueff (10). The mixed anhydride was obtained in low yield because of the poor solubility of biotin in anhydrous tetrahydrofuran and other solvents which were tested. An aliquot of the anhydride solution was taken to dryness, and a solution of reduced CoA in bicarbonate solution was added to the solid residue. The concentration of the biotinyl-CoA formed was estimated by thiol disappearance (11).

Biotin Hydroxamic Acid—Biotin methyl ester was prepared in crystalline form by the procedure of du Vigneaud et al. (12). The ester (0.24 mmole) was added to 5 ml of alkaline hydroxylamine (made from 3 mmoles of hydroxylamine hydrochloride and 5.2 mmoles of KOH) and stirred for 15 minutes after solution was complete. The mixture was acidified to pH 6, concentrated under reduced pressure, and cooled in an ice bath to give white needles. The compound, recrystallized twice from water, had a decomposition point of 191-192°C. This product was used as a standard for estimating enzymatically formed biotin hydroxamic acid.

Other Materials—d-Biotin was obtained from Hoffmann-La Roche, Inc., and l-biotin was a gift from Dr. W. E. Scott of the same company. d-Biotin was hydrolyzed with alkali according to the directions of Hofmann, Melville, and du Vigneaud (13) to prepare the diaminoearboxylic acid. Diethyl malonate-2-¹⁴C was purchased from Schwarz BioResearch, Inc. The purity of ²⁶³⁶P₁, prepared by pyrolysis of ²⁶³⁶P, (14) or purchased from Volk Radiocemical Company, was established by paper chromatography in 2-propanol-water-trichloroacetic acid-ammonia (15). Crystalline yeast pyrophosphatase was a gift from Dr. M. Kunitz, and glucose 6-phosphate dehydrogenase and hexokinase were obtained from Boehringer and Sons.

Methods


P<sub>i</sub> recovered (8) agreed closely with that expected from the amount of ATP used. The results are expressed as percentage of the maximum theoretical exchange. The percentage of exchange in the enzyme system was found to be proportional to the time of the incubation for at least 1 hour, and proportional to the enzyme concentration over a limited range.

Isolation and Growth of Bacteria—A bacterium which grows on biotin as sole carbon source was isolated from soil by enrichment culture technique. This organism, which has been classified as a pseudomonad by Dr. Albert H. Wheeler, is distinct from *Pseudomonas oleovorans* (18) but has not been further classified. Cultures were grown, with shaking, in flasks containing inorganic salts (19) and 1 g of biotin per liter.

**Purification of Bacterial Biotin-activating Enzyme**

Unless stated otherwise, the buffer used was 0.1 M potassium phosphate, pH 7.0, containing 0.01 M EDTA and 0.01 M cysteine. The procedures were carried out at 0°. Protein concentrations were determined according to published procedures (20, 21).

Harvested bacteria (20 g, wet weight) were suspended in 20 ml of 0.05 M KHCO<sub>3</sub> and passed through a French pressure cell at an output pressure of 5000 p.s.i. The material obtained was stirred for 30 minutes with 2 volumes of 0.05 M KHCO<sub>3</sub> and centrifuged for 30 minutes at 100,000 × g. The precipitate was discarded, and the supernatant solution was dialyzed overnight against 50 volumes of buffer. Dihydrostreptomycin sulfate was slowly added to a final concentration of 1%, and the precipitate obtained upon centrifugation was discarded.

The supernatant solution was brought to 0.3 saturation in ammonium sulfate by the slow addition of 17 g of the solid salt during 20 minutes. Stirring was continued for 20 minutes, and the precipitate removed by centrifugation.

The supernatant solution was brought to 0.6 saturation by the addition of 19 g of ammonium sulfate per 100 ml during 20 minutes. The precipitate was removed by centrifugation and dissolved in a volume of buffer equal to one-third that of the original extract. The protein solution was adjusted to pH 5.0 by the dropwise addition of M acetic acid, stirred for 15 minutes, and centrifuged. The precipitate was dissolved in buffer to give a protein concentration of about 10 mg per ml, and was dialyzed overnight against 100 volumes of buffer.

At this stage the enzyme had been purified about 6 fold in 40% yield, with a specific activity of 0.06, and was free of ATPase and inorganic pyrophosphatase. The activating enzyme lost 50% of the activity in 24 hours.

**Purification of Liver Biotin-activating Enzyme**

The steps employed in the purification of the pig liver biotin-activating enzyme are summarized in Table I. The various operations were carried out at 0° unless otherwise noted, and 0.05 M KHCO<sub>3</sub> containing 0.01 M mercaptoethanol was used for dissolving protein precipitates and for dialysis. Centrifugation of protein suspensions was done at 20,000 × g for 20 minutes.

**Extraction**—A 200-g portion of frozen pig liver was cut into small pieces and homogenized in 1.5 volumes of 0.5 M potassium chloride in a Waring Blender for 2 minutes. The resulting suspension was stirred for 30 minutes and then chilled to −15° in a glass beaker in a cold room at −15°. A solution of 0.1 M potassium chloride in 60% ethanol (400 ml) was added with efficient stirring for 1 hour, during which the temperature slowly dropped to −5°. The precipitate removed by centrifugation at −3° was discarded, and the supernatant solution was dialyzed 18 hours against 10 liters of bicarbonate solution, which was replaced at the end of 6 hours.

**First Ammonium Sulfate Fractionation**—The dialyzed solution was taken to 0.4 saturation by the addition of 23 g of solid ammonium sulfate per 100 ml during 20 minutes. Stirring was continued for 20 minutes, and the precipitate removed by centrifugation was discarded. The addition of 19 g of ammonium sulfate per 100 ml of the supernatant solution brought the mixture to 0.7 saturation. The resulting precipitate was removed by centrifugation, thoroughly drained, and dissolved in bicarbonate solution.

**Second Ammonium Sulfate Fractionation**—The protein concentration of the enzyme solution from the above step was adjusted to 20 mg per ml, and the preparation was brought to 0.58 saturation by the addition of 1.38 volumes of saturated ammonium sulfate solution during 1 hour with stirring. The precipitate was then promptly collected by centrifugation, dissolved in a minimal amount of bicarbonate solution, and dialyzed against 100 volumes of the same solution for 18 hours.

**Ethanol Fractionation**—The pH of the solution was adjusted to 6.5 by the addition of M acetic acid, and the solution was made 5 × 10<sup>-2</sup> M in MgCl<sub>2</sub> and 10<sup>-4</sup> M in ATP. Absolute ethanol at −20° was added to a final concentration of 15% (17.6 ml/100 ml, initial volume) while the temperature of the mixture was slowly lowered to −5°. The precipitate collected by centrifugation at −5° was discarded, and the same temperature was maintained while absolute ethanol (21.4 ml/100 ml of supernatant solution) was added to a final concentration of 30%. The mixture was centrifuged at −5°, and the precipitate was dissolved in 40 ml of bicarbonate solution containing ATP (10<sup>-4</sup> M) as well as mercaptoethanol; this solution was dialyzed for 18 hours against 5 liters of 0.005 M bicarbonate containing 0.01 M mercaptoethanol.

**DEAE-cellulose Chromatography**—A 100-g portion of DEAE-cellulose (Schleicher and Schuell, No. 20, with a capacity of 0.83 meq per g) was washed with 2 liters of each of the following solutions in the order given: 1 M NaCl, 0.5 M KOH, 0.1 N HCl in 95% ethanol, and 0.5 M KOH. In each step the DEAE-cellulose

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**Table I**

**Procedure for purification of biotin-activating enzyme from pig liver**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>9200</td>
<td>185</td>
<td>0.02</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (0.40-0.70 saturation)</td>
<td>6500</td>
<td>176</td>
<td>0.03</td>
<td>95</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (0-0.58 saturation)</td>
<td>2900</td>
<td>125</td>
<td>0.04</td>
<td>67</td>
</tr>
<tr>
<td>Ethanol ppt. (15-30%)</td>
<td>1000</td>
<td>80</td>
<td>0.08</td>
<td>43</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (0-0.60 saturation) of DEAE-cellulose eluate</td>
<td>130</td>
<td>90</td>
<td>0.22</td>
<td>16</td>
</tr>
<tr>
<td>Bentonite supernatant fraction</td>
<td>65</td>
<td>18</td>
<td>0.28</td>
<td>10</td>
</tr>
<tr>
<td>Hydroxyapatite eluate</td>
<td>12.4</td>
<td>11</td>
<td>0.89</td>
<td>6</td>
</tr>
</tbody>
</table>

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TABLE II

Oxidation of biotin-α-14C to radioactive carbon dioxide

Tissue slices about 0.5 mm in thickness or bacterial preparations were incubated with 4 amoles of biotin-α-14C (30,000 c.p.m.) in 3.0 ml of buffer-salt solution (22) at 37°C for 2 hours in flasks with center wells containing sodium hydroxide. At the end of the incubation, the reaction mixtures were acidified with H2SO4 and were incubated with 4 pmoles of biotin-cu-14C (30,000 c.p.m.) in 3.0 ml of buffer-salt solution. The protein remaining on the column was eluted by a gradient increase in KHCO3 concentration, achieved with 0.005 M KHCO3 and with 1 liter of the eluting solution, which contained 0.01 M mercaptoethanol, 10-4 M ATP, 10-4 M MgCl2, and 0.005 M KHCO3. Bicarbonate appeared to stabilize the enzyme during DEAE-cellulose chromatography. The potassium bicarbonate solutions were freshly prepared, but no attempt was made to control the pH during the following operations.

A portion of the dialyzed enzyme preparation containing 400 mg of protein was put onto the column, followed by 150 ml of the eluting solution. The protein remaining on the column was eluted by a gradient increase in KHCO3 concentration, achieved by slowly adding 400 ml of 0.2 M KHC03 to 400 ml of the eluting solution. The protein remaining on the column was eluted by a gradient increase in KHCO3 concentration, achieved with 0.005 M KHCO3 and with 1 liter of the eluting solution, which contained 0.01 M mercaptoethanol, 10-4 M ATP, 10-4 M MgCl2, and 0.005 M KHCO3. Bicarbonate appeared to stabilize the enzyme during DEAE-cellulose chromatography. The potassium bicarbonate solutions were freshly prepared, but no attempt was made to control the pH during the following operations.

A portion of the dialyzed enzyme preparation containing 400 mg of protein was put onto the column, followed by 150 ml of the eluting solution. The protein remaining on the column was eluted by a gradient increase in KHCO3 concentration, achieved by slowly adding 400 ml of 0.2 M KHC03 to 400 ml of the eluting solution.

---

<table>
<thead>
<tr>
<th>Tissue or bacterial preparation</th>
<th>Amount of tissue or bacteria (wet wt.)</th>
<th>Radioactivity of carbon dioxide formed (total c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td>Rat heart</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>Guinea pig liver</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>Guinea pig heart</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Guinea pig kidney</td>
<td>0.2</td>
<td>110</td>
</tr>
<tr>
<td>Bacteria (whole cells)</td>
<td>0.1</td>
<td>3790</td>
</tr>
<tr>
<td>Bacteria (cells broken in French press)</td>
<td>0.1</td>
<td>5100</td>
</tr>
<tr>
<td>Bacteria (particle-free extract)</td>
<td>0.1</td>
<td>40</td>
</tr>
</tbody>
</table>

* In this experiment a broken cell preparation was centrifuged at 100,000 X g for 30 minutes, and an amount of the supernatant solution derived from 0.1 g of bacteria was used.

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RESULTS

Oxidation of Biotin-α-14C to 14CO2—Attempts were made to find a mammalian tissue which efficiently oxidizes the valeric acid side chain of biotin. Only small amounts of radioactive CO2 were found upon incubation of biotin-α-14C with slices of various tissues, as shown in Table II. The results with guinea pig kidney slices are similar to those obtained by Baxter and Quastel (23) with biotin-carboxyl-14C. On the other hand, both whole bacterial cells and broken cell preparations readily oxidize the biotin side chain to CO2. Apparently, acetoacetate is also formed in the broken cell preparations, as shown by the recovery of a radioactive mercury-acetone complex (24) from the incubation medium, but the radioactivity of the acetoacetate was only about 5% that of the CO2. From the effect of malonate and other compounds on biotin-carboxyl-14C oxidation in kidney slices, Baxter and Quastel suggested that 2 carbon fragments removed from the side chain are oxidized in the citric acid cycle. Malonate was also found to inhibit the conversion of biotin-α-14C to radioactive CO2 in our broken cell bacterial preparations; for example, 0.03 μm malonate inhibited this conversion about 45%.

As described below, both the bacterial and the liver extracts were found to contain a biotin-activating enzyme which generates biotinyl-CoA. Since the particle-free bacterial extract is unable to convert the biotin side chain to CO2 (Table II), it appears that the citrate cycle enzymes or the enzymes which form acetyl-CoA from biotinyl-CoA are particulate in the pseudomonad.

Requirements for Hydroxamic Acid Formation with Soluble Enzyme Preparations from Bacterium and from Liver—As shown in Table III, the bacterial activating enzyme requires ATP and Mg2+ ions as well as biotin for activity in the hydroxamic acid assay system. Hydroxamic acid formation is markedly stimulated by CoA, which was therefore included routinely in the reaction mixture. The effect of CoA is catalytic; 0.1 μm caused an additional 1.1 amoles of biotin hydroxamic acid to be formed in the experiment described. It seems likely that the biotinyl-CoA formed reacts more readily with hydroxylamine than does the enzyme-bound intermediate, biotinyl-AMP, as described above.

The activity of the liver enzyme is, in contrast, only slightly stimulated by the addition of CoA, which was therefore not added routinely. Larger concentrations of CoA gave at best an additional stimulation in activity of about 15%. The activity of the liver enzyme is clearly dependent upon the presence of biotin, ATP, and MgCl2, but the dependency on Mg2+ ions was not complete unless EDTA was included in the reaction mixture.

Identification of Biotin Hydroxamic Acid—The hydroxamic acid formed in the presence of the bacterial or the liver enzyme was identified by paper and ion exchange chromatography.
On paper the hydroxamic acid was detected by spraying with acidic FeCl₃, and both free biotin and the hydroxamic acid were detected by the brown color developed on spraying with 0.1 N KMnO₄ in 0.1 N sulfuric acid. In butanol-acetic acid-water, 5:1:1, the following Rₑ values were obtained: biotin, 0.69; authentic biotin hydroxamic acid, 0.52; enzymatically formed hydroxamic acid, 0.53.

For identification by ion exchange chromatography, radioactive biotin hydroxamic acid was made from biotin-¹⁴C in a typical reaction mixture containing the bacterial enzyme. The reaction was terminated by placing the tube in a boiling water bath, the precipitate was discarded, and the supernatant solution was desalted with ethanol and taken to dryness. The residue was dissolved in water and put onto a column of Dowex 50-X₈, 0.5 x 17 cm. The biotin hydroxamic acid was eluted by 0.02 M ammonium acetate in a symmetrical peak and contained both the expected radioactivity and total amount of hydroxamic acid as determined colorimetrically. The radioactive biotin was subsequently eluted by 30% acetic acid.

**Biotinyl-CoA Formation**—The stimulation of biotin hydroxamic acid formation by catalytic amounts of CoA suggested that biotinyl-CoA is formed enzymatically. This CoA thioester was found to accumulate when larger amounts of CoA were incubated with biotin, ATP, and the enzyme. As shown in Table IV, the biotin-dependent disappearance of CoA sulphydryl was accounted for by the amount of thioester formed. A mixture of the enzymatically synthesized CoA ester and authentic biotinyl-CoA gave a single thioester spot, Rₑ 0.51, on paper chromatography in ethanol-acetate (25). Similar results were obtained with the liver enzyme.

The dehydrogenation of biotinyl-CoA, predicted by analogy with the corresponding reaction with straight chain fatty acyl-CoA esters (26), was indicated by the assay procedure of Green et al. (27). Biotinyl-CoA reduced triphenyltetrazolium chloride at the rate of 0.18 pmole per hour per mg of enzyme protein (30 to 50% ammonium sulfate fraction of the bacterial extract).

### Table III

*Requirements for synthesis of biotin hydroxamic acid*

<table>
<thead>
<tr>
<th>Bacterial enzyme</th>
<th>Hydroxamic acid formed</th>
<th>Liver enzyme</th>
<th>Hydroxamic acid formed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>System</strong></td>
<td>µmoles</td>
<td><strong>System</strong></td>
<td>µmoles</td>
</tr>
<tr>
<td>Complete</td>
<td>1.67</td>
<td>Complete</td>
<td>0.91</td>
</tr>
<tr>
<td>No CoA</td>
<td>0.56</td>
<td>Complete + CoA</td>
<td>1.06</td>
</tr>
<tr>
<td>No ATP</td>
<td>0.15</td>
<td>No ATP</td>
<td>0.01</td>
</tr>
<tr>
<td>No MgCl₂</td>
<td>0.27</td>
<td>No MgCl₂</td>
<td>0.15</td>
</tr>
<tr>
<td>No biotin</td>
<td>0.14</td>
<td>No biotin</td>
<td>0</td>
</tr>
<tr>
<td>No enzyme</td>
<td>0</td>
<td>No enzyme</td>
<td>0</td>
</tr>
<tr>
<td>No hydroxylamine</td>
<td>0</td>
<td>No hydroxylamine</td>
<td>0</td>
</tr>
</tbody>
</table>

* The complete system contained, in addition to the components described in the text, 0.1 µmole of CoA and bacterial enzyme (30 to 55% ammonium sulfate fraction; 2.3 mg of protein), and was incubated for 1 hour. EDTA (5 µmoles) was included when MgCl₂ was omitted.

† The complete system was that described in the text, contained the liver enzyme (bentonite-treated fraction; 0.2 mg of protein), and was incubated for 30 minutes under nitrogen. EDTA (1 µmole) was included when MgCl₂ was omitted. CoA (0.1 µmole) was present only in the experiment indicated.

### Table IV

**Evidence for enzymatic synthesis of biotinyl-CoA**

The complete reaction mixture contained 100 µmoles of Tris buffer, pH 7.4, 10 µmoles of ATP, 20 µmoles of biotin, 5 µmoles of CoA, and bacterial enzyme (30 to 60% ammonium sulfate fraction; 2 mg of protein). After incubation at 37° under nitrogen for 60 minutes, aliquots were taken for the estimation of CoA sulphydryl (11) and of thioester by conversion to biotin hydroxamic acid and comparison with an authentic standard.

<table>
<thead>
<tr>
<th>System</th>
<th>CoA sulphydryl remaining µmoles</th>
<th>Thioester formed µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>3.37</td>
<td>1.76</td>
</tr>
<tr>
<td>No biotin</td>
<td>4.41</td>
<td>0.67</td>
</tr>
<tr>
<td>Calculated biotin-dependent change</td>
<td>-1.04</td>
<td>+1.09</td>
</tr>
</tbody>
</table>

### Table V

**Stoichiometry of biotin-activating reaction**

The complete reaction mixture contained 100 µmoles of Tris buffer, pH 7.7, 5 µmoles of MgCl₂, 2.5 µmoles of ATP, 20 µmoles of biotin, 2500 µmoles of NH₄OH, crystalline pyrophosphatase (10 µg), and bentonite-treated liver biotin-activating enzyme (Experiment 1, 0.12 mg of protein; Experiment 2, 0.27 mg of protein), in a final volume of 1.0 ml. After 1 hour the tubes were heated in a boiling water bath for 1 minute. AMP was determined with adenylate deaminase (28), ATP with hexokinase and glucose 6-phosphate dehydrogenase, and other components as described in the text.

<table>
<thead>
<tr>
<th>System</th>
<th>Components determined µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td>ATP</td>
</tr>
<tr>
<td>No pyrophosphatase.</td>
<td>No ATP</td>
</tr>
<tr>
<td>No biotin.</td>
<td>Biotin-dependent increase or decrease</td>
</tr>
<tr>
<td>0.79</td>
<td>+0.81</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td>No biotin</td>
</tr>
<tr>
<td>Biotin-dependent increase or decrease</td>
<td>2.21</td>
</tr>
<tr>
<td>-1.29</td>
<td>+1.38</td>
</tr>
</tbody>
</table>

and controls showed that the reduction was not due to thiol liberation from the CoA ester.

**Products of ATP Cleavage and Stoichiometry of Biotin-activating Reaction**—Data were obtained with the liver enzyme indicating that in the biotin-dependent cleavage of ATP, equimolar amounts of biotin hydroxamic acid, AMP, and pyrophosphate are formed (Table V). Similar results were obtained with the bacterial enzyme.

**ATP Exchange Studies**—¹³C-Labeled PP₁ was found to exchange readily with ATP in the presence of biotin, the liver activating enzyme, and MgCl₂, but without added AMP (Table VI). In contrast, neither ³¹P₁ nor ¹³C-labeled AMP gave a significant biotin-dependent exchange. CoA inhibited the PP₁-
The exchange of \(^{32}\text{P}\)P, (1 pmole; 1.5 \(\times\) 10\(^6\) c.p.m.) or \(^{32}\text{Pi}\)P, (1 pmole; 1 \(\times\) 10\(^6\) c.p.m.) with ATP was determined with the bentonite-treated liver enzyme (0.2 mg of protein) by the procedure described in the text. The tubes were flushed with nitrogen and stopped prior to incubation. The experiment with AMP-\(^{3}\text{C}\) (2 pmoles; 6 \(\times\) 10\(^4\) c.p.m.) was similar except that a smaller amount of ATP was present (3 pmoles) and that after adsorption on charcoal the nucleotides were eluted with ethanol-water-3 N NH\(_4\)OH (1:4:1) and separated by paper chromatography in isobutyric acid-water-concentrated ammonium hydroxide (66:33:1). The area containing ATP was eluted with water and the radioactivity was determined. No ADP was detected on the chromatogram.

No MgCl\(_2\) (5 pmoles of EDTA added).
Complete (5 pmoles of EDTA added).

### TABLE VII

#### Synthesis of ATP from biotinyl-AMP and radioactive PP\(_i\)P

The complete reaction mixture contained 100 pmoles of Tris buffer, pH 7.4, 3.4 pmoles of biotin-AMP, 5 pmoles of MgCl\(_2\), 2 pmoles of \(^{32}\text{Pi}\)P (4 \(\times\) 10\(^4\) c.p.m.), and bentonite-treated liver enzyme (0.1 mg of protein) in a final volume of 1.0 ml, and was incubated for 30 minutes at 38°C. The reaction was stopped by the addition of 0.2 ml of 40% trichloroacetic acid; the resulting precipitate was discarded, and the supernatant solution was stirred with 0.2 g of acid-washed charcoal. The charcoal was washed with water, and the adsorbed nucleotides were eluted with ethanol-water-3 N NH\(_4\)OH (1:4:1) and separated by paper chromatography in isobutyric acid-water-concentrated ammonium hydroxide (66:33:1). The area containing ATP was eluted with water and the radioactivity was determined. No ADP was detected on the chromatogram.

### Experiment 2

- **No biotinyl-AMP**
- **No biotinyl-AMP (3.4 pmoles of AMP and 3.4 pmoles of biotin added)**
- **No enzyme**
- **Complete**
- **Complete (5 pmoles of EDTA added)**
- **Complete (5 pmoles of EDTA added)**

### Enzymatic Conversion of Biotinyl-AMP to ATP—When chemically synthesized biotinyl-AMP was incubated with \(^{32}\text{P}\)P, and MgCl\(_2\), the PP\(_i\)-ATP exchange was shown spectrophotometrically in a reaction mixture containing hexokinase and glucose 6-phosphate dehydrogenase as well as the biotin-activating enzyme (Fig. 3). In this system the formation of TPNH was found to be completely dependent upon the presence of biotinyl-AMP, PP\(_i\), and the liver biotin-activating enzyme. The effect of omitting Mg\(^{2+}\) ions was not determined since this metal is required for hexokinase activity.

### Substrate Specificity—The possibility was considered that biotin activation might be catalyzed by known fatty acid-activating enzymes (30, 31). The relative activity of the bacterial enzyme in the hydroxamic acid assay system with various 0.01 \(\mu\) m substrates was as follows: d-biotin, 100; acetate, 23; butyrate, 61; hexanoate, 88; and octanoate, 144. Further identification of the radioactive ATP was established by paper chromatography in isobutyric acid-water-concentrated NH\(_4\)OH (66:33:1). The location of the nucleotides was detected by ultraviolet absorption, and the corresponding areas were eluted with water. The ATP spot was radioactive, whereas the AMP was not. As expected, in control experiments in which biotinyl-AMP or enzyme was omitted from the reaction mixture, or in which equivalent amounts of biotin and AMP were substituted for biotinyl-AMP, no significant amount of ATP could be detected on the paper chromatogram by ultraviolet absorption or by radioactivity.

The formation of ATP from biotinyl-AMP was also shown spectrophotometrically in a reaction mixture containing hexokinase and glucose 6-phosphate dehydrogenase as well as the biotin-activating enzyme (Fig. 3). In this system the formation of TPNH was found to be completely dependent upon the presence of biotinyl-AMP, PP\(_i\), and the liver biotin-activating enzyme. The effect of omitting Mg\(^{2+}\) ions was not determined since this metal is required for hexokinase activity.

### Enzymatic Conversion of Biotinyl-AMP to Biotinyl-CoA

When CoA and chemically synthesized biotinyl-AMP were incubated with the liver activating enzyme, biotinyl-CoA was formed as determined by sulfhydryl disappearance (Table VIII). Over 60% of the biotinyl-AMP was converted to biotinyl-CoA in Experiment 2. The formation of the CoA ester in this reaction mixture occurred at about one-tenth the rate of the ATP-dependent conversion of biotin to biotin hydroxamic acid in the standard assay system. The addition of Mg\(^{2+}\) ions or EDTA had no effect on the reaction.

### Fig. 3. Spectrophotometric evidence for the synthesis of ATP from biotinyl-AMP. The complete reaction mixture contained 100 pmoles of Tris buffer, pH 7.4, 1 pmole of glucose, 0.5 pmole of TPN, 2 pmoles of MgCl\(_2\), 10 pmoles of PP\(_i\), 3.4 pmoles of biotinyl-AMP, bentonite-treated liver biotin-activating enzyme (0.1 mg of protein), and an excess of hexokinase and glucose 6-phosphate dehydrogenase in a final volume of 3.0 ml, and was incubated at 37°C. The increase in absorbance at 340 m\(\mu\) was determined with a Gilford multiple sample absorbance recorder. Curve A indicates the rate of TPNH reduction in the complete reaction mixture, and Curve B indicates the results in experiments in which biotinyl-AMP, PP\(_i\), or the biotin-activating enzyme was omitted.
enzyme purification would be required to determine whether the bacterial preparation contains an enzyme specific for biotin. On the other hand, an extract of the same organism grown on leucine as the sole carbon source activated octanoate efficiently, but had no activity toward biotin.

Chromatography of the liver biotin-activating enzyme on hydroxylapatite (Fig. 4) results in the separation of two enzymes, one highly active with octanoate but having no activity toward biotin, and a second, which exhibits the following relative activities: d-biotin, 100; L-biotin, 49; butyrate, 13; hexanoate, 12; octanoate, 12; oleate, 6; lipoate, 3; and acetate, 0. The diamino-carboxylic acid derived from biotin has no significant activity. Additional evidence that the octanoate- and biotin-activating enzymes of pig liver are distinct was obtained by differential inhibition by N-ethylmaleimide. The bentonite-treated enzyme which had been stored in 0.01 M mercaptoethanol was dialyzed against 0.06 M bicarbonate, and a portion was incubated with 10^{-3} M N-ethylmaleimide at 0° for 4 hours. The untreated enzyme had a specific activity of 0.39 with octanoate and 0.13 with biotin. After the treatment described, the activity with octanoate was only slightly reduced (0.37), but there was no activity with biotin. The results described indicate that the biotin-activating enzyme is apparently distinct from the fatty acid- and lipoic acid-activating enzymes.

The relative activities of various nucleoside triphosphates (0.01 M, final concentration) with the bentonite-treated liver biotin-activating enzyme were as follows: ATP, 100; ITP, 33; GTP, 22; UTP, 11; and CTP, 4. When ADP was substituted for ATP, no hydroxamic acid formation was detected.

**Metal Ion Requirement—**Various metal salts were tested in the hydroxamic acid assay system at 0.01 M concentration with 1 μmole of EDTA added to bind traces of metal ions present in the enzyme (bentonite-treated preparation). The following relative activities were found: Mg^{2+}, 100; Mn^{2+}, 94; Ca^{2+}, 38; Zn^{2+}, 19; Cd^{2+} or no metal, 0. Enzyme activity was optimal at a [Mg^{2+}]:[ATP] ratio of 1.0, and decreased at lower ratios.

**TABLE VIII**

**Conversion of biotinyl-AMP to biotinyl-CoA**

<table>
<thead>
<tr>
<th>System</th>
<th>Total CoA sulphydryl</th>
<th>Biotinyl-CoA formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m mole</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>No enzyme</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>No biotin-AMP</td>
<td>1.66</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Complete + 10 μmoles of MgCl₂</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Complete + 10 μmoles of EDTA</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>No biotin-AMP</td>
<td>1.76</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* Calculated as the biotinyl-AMP-dependent disappearance of CoA sulphydryl.

In such experiments, the ATP concentration was held at 0.01 M while the MgCl₂ concentration was varied.

**Other Properties—**The liver enzyme has activity in the hydroxamic acid assay system over the pH range from 6 to 9, with maximal activity at pH 7.4 to 7.7. In the PPi-ATP exchange assay, the pH optimum is 7.4. The Kₘ values, determined according to Lineweaver and Burk (32), were found to be 1.8 × 10^{-4} M for ATP (with biotin at 0.02 M) and 2.5 × 10^{-4} M for biotin (with ATP at 0.01 M).

**DISCUSSION**

The results presented indicate that the biotin-activating enzyme catalyzes the following reactions.

\[
\text{ATP}^{++} + \text{biotin} \rightleftharpoons \text{biotinyl-AMP} + \text{PPi} \quad (1)
\]

\[
\text{Biotinyl-AMP} + \text{CoA} \rightleftharpoons \text{biotinyl-CoA} + \text{AMP} \quad (2)
\]

The carboxyl activation of biotin is clearly analogous to the enzymatic conversion of acetate to acetyl-CoA (33). In the latter reaction, acetyl-AMP is an intermediate, as shown by Berg (34). Similar reactions have been reported with kinases acting on fatty acids of intermediate (30) and long chain length (31). As described above, the purified biotin-activating enzyme from pig liver is apparently distinct from these enzymes and also from the lipoate-activating enzyme (35). The earlier studies of Baxter and Quastel (23) and our own findings strongly suggest that in the oxidation of the biotin side chain 1 or more units of acetyl-CoA are formed by means of β oxidation. Presumably,
one function of the biotin-activating enzyme is to furnish biotinyl-
CoA as a substrate for this process.3

The role of ATP in the biotin-dependent formation of prop-
ionyl-CoA holocarboxylase has been studied previously (3, 37-
40). Kosow, Huang, and Lane (38) concluded that a carboxyl-
activated biotin was not involved in propionyl-CoA holocar-
boxylase synthesis and stated that synthetically prepared bio-
tinyl-AMP failed to replace ATP and biotin in this synthesis.

Siegel et al. (40) in this laboratory recently presented evidence,
however, that synthetically prepared biotinyl-AMP of high
purity is fully active in replacing ATP and biotin in holocar-
boxylase synthesis and that a holocarboxylase synthetase,2
purified from rabbit liver, catalyzes this reaction. We there-
fore proposed that biotinyl-AMP functions in the final stage
of holocarboxylase synthesis as follows.

\[
\text{Propionyl-CoA apocarboxylase + biotinyl-AMP} \rightarrow \text{propionyl-CoA holocarboxylase + AMP}
\]

Neither the bacterial nor the pig liver biotin-activating enzyme
described in the present paper catalyzes Reaction 3 (39, 40). 
Apparently, the biotin-activating enzyme is not identical with
the holocarboxylase synthetase, or, less likely, an additional
protein removed during the purification of the former is required
for the transfer of the biotinyl group of biotinyl-AMP to the
apocarboxylase. The holocarboxylase synthetase, like the
biotin-activating enzyme, apparently forms biotinyl-AMP from
ATP and biotin, but, in addition, possesses the ability to transfer
the biotin moiety from this intermediate to the apocarboxylase.

Lané and Young (41) have recently reported that biotinyl-AMP
participates in the formation of holotranscarboxylase, and in a
brief report Lynen and Rominger (42) have stated that biotinyl-
AMP is involved in acetyl-CoA holocarboxylase formation in a
yeast preparation. A full report of our studies on the role of
biotinyl-AMP in propionyl-CoA holocarboxylase synthesis will
be presented in a subsequent paper.

SUMMARY

1. Extracts of liver and of a soil organism grown on biotin as
the sole carbon source contain an enzyme which catalyzes (a)
the reaction of biotin with adenosine triphosphate in the presence
of hydroxylamine to yield biotin hydroxamic acid, adenylate,
and pyrophosphate and (b) the biotin-dependent exchange of
\(^{32}\)P-labeled pyrophosphate with adenosine triphosphate.

2. Evidence is presented that the biotin-activating enzyme
catalyzes the following reactions.

\[
\text{Biotin + adenosine triphosphate} \rightarrow \text{biotinyl adenylate + pyrophosphate}
\]

\[
\text{Biotinyl adenylate + coenzyme A} \rightarrow \text{biotinyl-} \quad \text{biotinyl-}
\quad \text{coenzyme A + adenylate}
\]

3. Biotinyl \(\delta\)-adenylate, prepared by the reaction of biotin and
adenylic acid with dicyclohexylcarbodiimide, is enzymati-
ically converted to adenosine triphosphate in the presence
of pyrophosphate and Mg\(^{++}\) ions, and to biotinyl coenzyme A in the
presence of reduced coenzyme A.

4. The purification and properties of the pig liver biotin-
activating enzyme are described.

Acknowledgment—The authors wish to acknowledge the
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Enzymatic Activation of Biotin: BIOTINYL ADENYLATE FORMATION
James E. Christner, Milton J. Schlesinger and Minor J. Coon


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