Studies on the Mechanism of Action of Acetyl Coenzyme A Carboxylase

II. ON THE MECHANISM OF ACTION OF ENZYME-BOUND BIOTIN*

Moseley Waite† and Salih J. Wakil

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

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Ever since the identification of biotin with the curative factor (vitamin H) for egg white injury (1, 2), investigators have concerned themselves with the mode and mechanism of action of this vitamin. With the use of both microbiological techniques and animal experiments, early investigators recognized various biochemical roles for biotin, especially as a key component in the metabolism of CO₂, fatty acids, and various dicarboxylic acids. More specifically, biotin was shown somehow to be required for various carboxylation reactions, including the synthesis of oxaloacetic acid from pyruvate plus CO₂ (3–7). The sparing effect of oleic acid on biotin requirements suggested that the latter may play a role in the synthesis of fatty acids (8–11). Biotin deficiency in animals was also known to cause a reduction in the levels of malic enzyme, citrulline synthetase, and other enzymes (12–19).

Melville, Pierce, and Partridge (20) first postulated that biotin may function in carboxylation reactions through the turnover of the carbonyl group of the ureido ring. These workers synthesized ureido-C⁴-biotin and used it as a growth factor for Lactobacillus arabinosus. Biotin was reisolated from both cells and media and its C⁴ content was determined. Approximately 98% of the initial C⁴ was still associated with the biotin; this high recovery seemed to invalidate the aforementioned hypothesis, since if the carbonyl carbon of biotin were the group involved in carboxylation reactions, they would have found little or no radioactivity in biotin.

In 1958 Wakil, Titchener, and Gibson (21) and Wakil and Gibson (22) observed that bicarbonate was required for fatty acid synthesis and that one of the enzymes involved in fatty acid synthesis contained large amounts of biotin. Biotin was tightly bound to the protein and was closely associated with the enzymatic activity throughout the purification steps. In addition, Wakil and Gibson (22) demonstrated that avidin, an egg white protein long known for its high binding affinity for biotin (23), inhibited the carboxylation of acetyl-CoA. Treatment of avidin with free biotin, before its interaction with the enzyme, prevented this inhibition; this strongly suggested that the avidin inhibition was due to its binding of the carboxylase biotin and not due to a nonspecific inhibition. This technique has proved to be a useful diagnostic test for the presence of biotin enzymes.

In 1959, Lynen et al. (24) reported the isolation of β,β-dimethylacrylyl-CoA carboxylase which catalyzed the carboxylation of β,β-dimethylacrylyl-CoA to β-methylglutaconyl-CoA in the presence of adenosine triphosphate and Mg++. This enzyme contained biotin as the prosthetic group and was inhibited by avidin. Kaziro, Leone, and Ochoa (25) as well as Lane et al. (26) found biotin as a prosthetic group of propionyl-CoA carboxylase. Meanwhile Swick and Wood (27) discovered in Propionibacteria a novel transcarboxylation reaction in which a biotin enzyme catalyzed the transfer of CO₂ from methylmalonyl-CoA to pyruvate to form propionyl-CoA and oxaloacetic acid.

Lynen et al. (24) found that the β,β-dimethylacrylyl-CoA carboxylase could carboxylate not only its substrate, β,β-methylacrylyl-CoA, but also free (+)-biotin at relatively high concentration. These authors isolated the carboxylated biotin from the reaction mixture as the stable methyl ester and proved the structure to be N-carboxybiotin (Diagram 1) (28–30).

Diagram 1

Since the carboxylation of free biotin was specific for the (+)-biotin, these authors concluded that they were “dealing with a carboxylation reaction of such marked specificity that it seems justified to consider the thereby generated CO₂ biotin as the model for the binding relationships of the carbonic acid within the CO₂ ~ biotin-enzyme.” Thus they assumed the formation of the N-carboxybiotin-enzyme complex as an intermediate in the carboxylation reactions as shown in Equations 1 and 2, where M⁺⁺ is a divalent metal ion, usually Mg⁺⁺ or Mn⁺⁺, and the acceptor may be β,β-dimethylacrylyl-CoA, acetyl-CoA, propionyl-CoA, etc., depending on the specificity of the enzyme.

\[
\text{HCO}_3^- + \text{ATP} + \text{biotin enzyme} \rightleftharpoons \text{CO}_2 \sim \text{biotin-enzyme complex} + \text{ADP} + \text{P}_1 \quad (1)
\]

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CO₃ ~ biotin-enzyme complex + acceptor ⇄
\[ \text{CO₃ acceptor + biotin enzyme (2)} \]

\[ \text{Sum: HCO₃}^- + \text{ATP} + \text{acceptor} \]
\[ \text{CO₃ acceptor + ADP + Pi (3)} \]

Lynen's hypothesis received further support from the findings of Ochoa and Kaziro (31), Lane et al. (26), and Halenz and Lane (32) that the exchange between labeled inorganic phosphate and ATP that was catalyzed by propionyl carboxylase could occur only in the presence of bcarboxylate and ADP. Thus the earlier assumption of Lynen that ADP ~ biotin enzyme was formed before the formation of the CO₃ ~ biotin-enzyme complex was ruled out.

More recently Kaziro and Ochoa (33) and Waite and Wakil (34, 35) have isolated the C⁴O₂ ~ biotin-enzyme complex of propionyl-CoA carboxylase and acetyl-CoA carboxylase, respectively. The C⁴O₂ content of the complex was shown to be equivalent to the amount of biotin present and could be quantitatively transferred to an acceptor acyl-CoA to form the carboxysuyl-CoA. In a preliminary communication, Waite and Waite (36) presented evidence to suggest that the ureido carbon of biotin, ureido-carboxylase-bound biotin is the "active carbon" of biotin and that it is involved in the carboxylation reactions. This conclusion was based on the following observations. (a) Hydrolysis of the C⁴O₂ ~ biotin enzyme yielded free biotin containing >80% of the radioactivity. (b) The C⁴ of the isolated biotin was located exclusively in the ureido carbon atom of biotin. (c) "Native carboxylase" yielded biotin on hydrolysis whereas enzyme treated with acetyl-CoA or ADP and Pi yielded over 60% of a biotin derivative that has been tentatively identified as "diaminobiotin." These characteristics of the C⁴O₂ ~ biotin-enzyme complex are at variance with those predicted by the hypotheses of Knapp et al. (28), Lynen et al. (29), and Knappe, Ringelmann, and Lynen (30). The present communication deals with detailed experimental accounts that support the aforementioned conclusions. Evidence will also be presented to show, contrary to the conclusion of Melville, Pierce, and Partridge (30), that biotin has been quantitatively recovered from L. arabinosus grown on limited amounts of ureido-C⁴biotin.

EXPERIMENTAL PROCEDURE

C⁴O₂ ~ Biotin-Enzyme Complex—The C⁴O₂ ~ biotin-enzyme complex was prepared from highly purified preparations of acetyl-CoA carboxylase in the presence of HC⁴O₂, ATP, and Mn⁺⁺. The procedure followed in the formation and isolation of this complex was exactly the same as that described by Waite and Waite (34, 35).

Isolation of Enzyme-bound Biotin—Isolation of enzyme-bound biotin was carried out according to the procedure of Wright and Skeggs (37). One to ten milligrams of acetyl-CoA carboxylase were added to 1.0 ml of 4 N H₂SO₄ and the mixture was autoclaved for 1 hour at 121°C. After cooling the hydrolysate to room temperature, enough Ba(OH)₂ was added to neutralize the acid. The mixture was filtered to remove the insoluble salts, the precipitate was washed three times with water, and the filtrate and washings were lyophilized. The residue was extracted with methanol several times, the extracts were combined, and the methanol was removed under vacuum. The residue was dissolved in water and samples were withdrawn for microbiological assays with the use of the organism Lactobacillus arabinosus in a procedure similar to that described by Wright and Skeggs (37).

Chromatography of Biotin—Paper chromatography of biotin was carried out in three different solvent systems (24, 38, 39): butanol-H₂O-acetic acid, 4:5:1; propylmethyl-H₂O, 15:85; and butanol-methanol-benzene-H₂O, 2:1:1:1. An authentic sample of biotin had Rₚ values of 0.75, 0.5, and 0.7 in the three different solvent systems, respectively. The biotin content of the paper chromatograms was assayed by cutting the paper into strips of 2.5 × 1 cm and dropping the strips into 10 ml of biotin-deficient growth media before sterilization of the media. The rest of the procedure was the same as that of Wright and Skeggs (37).

Synthetic Ureido-C⁴biotin—This compound was prepared by the procedure of Melville, Pierce, and Partridge (20). Analysis of the C⁴biotin revealed that the compound was chromatographically pure and at least 98.6% of the radioactivity was located in the ureido carbon.

Lactobacillus arabinosus—Cultures of L. arabinosus 17-5 were obtained from the American Type Culture Collection, Washington, D. C.

RESULTS

Incorporation of C⁴O₂ into Biotin—When the freshly isolated C⁴O₂ ~ biotin-acetyl-CoA carboxylase complex (35, 36) was hydrolyzed in 4 N H₂SO₄ by autoclaving the mixture for 1 hour at 121°C, C⁴biotin was isolated from the hydrolysate as described in "Experimental Procedure." The amount of C⁴ recovered on the biotin was approximately 54% of the C⁴ content of the complex as shown in Table I. Similar results were also obtained if the hydrolysis was performed in 4 N Ba(OH)₂ under the same conditions. The recovery of the C⁴biotin under these conditions was usually lower than that obtained with H₂SO₄. Proteolytic digestion of the C⁴O₂ complex with papain for 24 hours at pH 5.2 in the presence of CN⁻ yielded C⁴biocytin, which contained approximately 80% of the radioactivity of the complex (cf. Table I).

Identification of the radioactivity in the hydrolysate of the C⁴O₂ ~ biotin-enzyme complex as being associated with biotin was made by several independent methods. The methanol extracts of the hydrolysate contained almost all of the radioactivity of the lyophilized residue. An aliquot of the methanol extract used for microbiological assay promoted growth of the L. arabinosus to the equivalence of approximately 90 to 100% recovery of the total biotin of the purified enzyme. This test established the presence of biotin or an equivalent derivative in the methanol extract of the hydrolysates. Under these conditions, extracts of the papain digest of the carboxylase or the C⁴O₂ ~ biotin-carboxylase complex did not promote growth of L. arabinosus until prior acid hydrolysis. Since L. arabinosus does not grow on biocytin (37, 40) but does so after acid hydrolysis, it was reasonable to assume that the product of papain digestion of the carboxylase was biocytin or a closely related derivative.

When the methanol extracts of the acid or alkaline hydrolysates were chromatographed on paper in butanol-H₂O-acetic acid and the paper strips were assayed for radioactivity and bacterial growth, spots corresponding to biotin were detected (Rₚ of 0.75) that contained both radioactivity and bacterial growth-promoting ability as shown in Fig. 1. The chromatography of the methanol extracts of the papain digest on the other
hand yielded biocytin ($R_f$ of 0.3) which contained most of the C$^{14}$ (cf. Fig. 2). Similar results were also obtained in other chromatographic systems (24, 38, 39). In all of these systems, the radioactivity of the sample was associated very closely with biotin, suggesting that the C$^{14}$ content of the C$^{14}$O$_2$ $\sim$ biotin-enzyme complex was tightly bound to biotin or part of the biotin molecule.

Finally, C$^{14}$-biotin was isolated from the methanol extracts

### Table I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>C$^{14}$O$_2$-biotin-enzyme complex formed</th>
<th>Method of hydrolysis</th>
<th>C$^{14}$-biotin</th>
<th>C$^{14}$ biocytin</th>
<th>C$^{14}$ recovered in biotin or biocytin</th>
<th>C$^{14}$ %</th>
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<td>1</td>
<td>3286</td>
<td>H$_2$SO$_4$</td>
<td>2760</td>
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<td>2760</td>
</tr>
<tr>
<td>2</td>
<td>3100</td>
<td>Ba(OH)$_2$</td>
<td>2150</td>
<td>69.4</td>
<td></td>
<td>2150</td>
</tr>
<tr>
<td>3</td>
<td>3200</td>
<td>Papain*</td>
<td>230</td>
<td>80.0</td>
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<td>230</td>
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</table>

*The products of hydrolysis in this case were 90% biocytin and 10% biotin as shown by $R_f$ of 0.30 and 0.75 in butanol-H$_2$O-acetic acid, respectively.

Fig. 1. Chromatography, in butanol-acetic acid-water system, of the C$^{14}$-biotin isolated from C$^{14}$O$_2$-biotin-acetyl-CoA carboxylase complex after hydrolysis in 4 N H$_2$SO$_4$. The C$^{14}$-biotin was prepared as described in Table I. The bars represent the growth response of L. arabinosus on 1-cm paper strips of the chromatogram, and the tracing represents the radioactivity content (cpm) of the same strips.

after addition of nonlabeled biotin as carrier. The C$^{14}$-biotin was crystallized several times from the methanol-H$_2$O solvent system and the specific activity and melting point of the crystalline compound determined. Table II shows that after the first crystallization, the melting point as well as the C$^{14}$ content of the crystalline biotin remained constant. Methylation of the crystals with diazomethane resulted in the formation of methyl biotin which on recrystallization from methanol-H$_2$O gave a compound with a melting point of 166$^\circ$ (compared with a melting point of 166.5$^\circ$ for authentic sample), and a constant specific activity on recrystallization. Moreover, chromatography of the C$^{14}$-methyl derivative in butanol-acetic acid-H$_2$O solvent system yielded a single radioactive compound with an $R_f$ value of 0.85, identical to that of an authentic sample of methyl biotin (Fig. 3).

Hydrolysis of the crystalline C$^{14}$-biotin or its methyl derivative with Ba(OH)$_2$ in a sealed tube at 140$^\circ$ for 24 hours resulted in the formation of diaminobiotin and the liberation of the ureido carbonyl group of biotin as BaCO$_3$ (41). Both the diamino-
biotin and the BaCO₃ were isolated from the reaction mixture and their C¹⁴ content was determined. The diaminobiotin isolated as the crystalline sulfate salt, melting point 251-255° (melting point of 245-255° for this compound was reported by Hofmann, Melville, and duVigneaud (41)), had very little or no C¹⁴ whereas the BaCO₃ contained all the C¹⁴ as shown in Table III. These observations indicated that the C¹⁴O₂ which was incorporated into the C¹⁴O₂-biotin-enzyme complex had become the ureido carbonyl group of the isolated C¹⁴biotin.

Reactions of C¹⁴O₂-biotin-Enzyme Complex—In a previous communication Waite and Wakil (35, 36) demonstrated the quantitative transfer of the C¹⁴O₂ from the C¹⁴O₂-biotin-carboxylase complex to the acceptor acetyl-CoA to form C¹⁴-malonyl-CoA and the decarboxylated biotin enzyme. The "native" acetyl-CoA carboxylase appeared to be almost 100% in the carboxylated form. This was shown by the ability of the native enzyme both to carboxylate acetyl-L-C¹⁴ to form C¹⁴-malonyl-CoA and to yield the decarboxylated form of the enzyme. Acid hydrolysis of the native carboxylase followed by isolation and subsequent chromatography of the prosthetic group yielded only biotin with an Rₚ of 0.75 in butanol-H₂O-acetic acid as shown in Fig. 4. These results together with those obtained from studies on C¹⁴O₂-biotin-enzyme complex of the carboxylated form of the enzyme indicated that the prosthetic group of the carboxylase both in the "native" state and in the C¹⁴O₂-biotin-enzyme complex was biotin.

If biotin itself is the prosthetic group of the carboxylated enzyme, it was of interest to ascertain the nature of the prosthetic group of the decarboxylated form. This was achieved by isolating the decarboxylated form of the enzyme from the native carboxylase by treatment of the latter with acetyl-CoA, followed by gel filtration on a Sephadex G-25 column and hydrolysis with 4 N H₂SO₄ for 1 hour at 121°. The hydrolysate was neutralized with Ba(OH)₂ and the "decarboxylated" form of biotin was isolated from the reaction mixture by methanol extraction similar to that described for biotin. Aliquots of the methanol extract of the decarboxylated biotin were bioassayed and the remainder were examined chromatographically in the three solvent systems previously described. The location of biotin-like activity on the chromatogram was followed by growth response of L. arabinosus in biotin-deficient media when suppl-

### Table II

<table>
<thead>
<tr>
<th>Crystallization</th>
<th>Specific activity</th>
<th>Melting point</th>
</tr>
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<tr>
<td></td>
<td>c.p.m./mg</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>330</td>
<td>231°</td>
</tr>
<tr>
<td>2</td>
<td>360</td>
<td>231°</td>
</tr>
<tr>
<td>3</td>
<td>332</td>
<td>231°</td>
</tr>
</tbody>
</table>

### Table III

**Hydrolysis of C¹⁴-biotin to C¹⁴O₂ and nonlabeled diaminobiotin**

The C¹⁴-biotins used in these experiments were prepared by either chemical synthesis (Experiment 1) or hydrolysis of the C¹⁴O₂-biotin-carboxylase complex (Experiments 2 and 3).

In each experiment the indicated amounts of C¹⁴-biotin were placed in a tube with 10.0 mg of nonlabeled biotin, 200 mg of Ba(OH)₂, and 1.0 ml of H₂O. The tube was sealed and placed in a refluxing xylene bath at 139° for 22 hours. The samples were then withdrawn from the tubes and placed in a flask fitted with a removable center well which contained 500 amoles of Hyamine hydroxide. Tight-fitting rubber caps were then placed over the tops of the samples and 0.2 ml of 12 N H₂SO₄ was injected into the main compartment of the flask. The flasks were shaken in a water bath maintained at 38° for 1 hour after which the center wells were removed and placed in scintillation counting vials and counted in a Packard Tri-Carb liquid scintillation counter. The acidified solution was withdrawn from the flask, made slightly basic with Ba(OH)₂, reneutralized with carbonic acid, and filtered. Aliquots of the filtrate were taken for counting and chromatography in the butanol-water-acetic acid system. The remainder of the solution was reduced in volume and the diaminobiotin sulfate (melting point 251-255°) was crystallized from the solution by the addition of methanol.

![Fig. 3. Chromatography of the C¹⁴-biotin methyl ester in butanol-acetic acid-H₂O system. The C¹⁴-biotin was prepared as described in Table I and was methylated with diazomethane. The bars represent bacterial growth response on 1-cm paper strips of the chromatogram, and the tracing represents the radioactivity of the same strips. The positions of authentic biotin and methyl biotin are also indicated.](http://www.jbc.org/)
Conversion of Diaminobiotin Enzyme to Biotin Carboxylase—

The evidence presented so far demonstrated the conversion of the biotin enzyme to the diaminobiotin enzyme as a result of either transcarboxylating acetyl-CoA to form malonyl-CoA or coupling of the phosphate to ADP to form ATP and bicarbonate. The reconversion of the diaminobiotin enzyme to the biotin enzyme could be achieved only in the presence of HCO₃⁻, ATP, and Mn⁺⁺ as shown in Table V. The amount of C¹⁴O₂ incorporated into the biotin-enzyme complex under the conditions of the experiment (2 minutes at 0°C) was less than half the amount of C¹⁴O₂ incorporated into the diaminobiotin enzyme (cf. Table V). It is also of interest to note that in the absence of ATP, there was always a significant amount of C¹⁴O₂ incorporated into the biotin and that this amount was usually higher in the case of the biotin enzyme than the diaminobiotin enzyme. This observation would indicate that the ureido carbonyl group of biotin-bound enzyme is relatively labile and can exchange with the medium. In all cases, whether the C¹⁴O₂ incorporated into the C¹⁴O₂ ~ biotin-enzyme complex was by exchange or the result of the conversion of the diaminobiotin enzyme to the biotin enzyme, the C¹⁴O₂ could be recovered quantitatively from the ureido carbonyl group of biotin as shown in Table III.

Growth of Lactobacillus arabinosus on C¹⁴-biotin—Since the

amount of P³² incorporated into the ATP was approximately equivalent to the amount of diaminobiotin formed, which indicated that a stoichiometric relationship between the two existed (cf. Table IV). When the decarboxylated enzyme was used instead of the biotin enzyme, there was very little (0.05 mpmole) ATP³² formed and essentially no change in the relative ratio of the biotin to the diaminobiotin occurred.

FIG. 4. Chromatography of the prosthetic groups of the native carboxylase and the decarboxylated enzyme in butanol-acetic acid-water system. The decarboxylated enzyme was prepared as described in Table IV. The native enzyme as well as the decarboxylated enzyme were hydrolyzed in 4 N H₂SO₄ and the methanol extracts of the hydrolysates were prepared as described under “Experimental Procedure.” The broken curve represents the bacterial growth response on 1-cm paper strips of the chromatograms while the solid curve represents the bacterial growth response on similar strips of chromatograms of the decarboxylated preparation. The positions of authentic biotin and diaminobiotin are shown on the graph.

FIG. 5. Chromatography of the prosthetic group of the decarboxylated C¹⁴O₂ ~ biotin-enzyme complex in butanol-acetic acid-water system. The C¹⁴O₂ ~ biotin-acetyl-CoA carboxylase was prepared as described in Table I. The complex was then decarboxylated by treatment with acetyl-CoA and the protein was separated by gel filtration through Sephadex G-50 as described in Table IV. The decarboxylated C¹⁴O₂ ~ biotin-enzyme complex was hydrolyzed in 4 N H₂SO₄ and the methanol extracts of the hydrolysate were prepared as described under “Experimental Procedure.” The solid curve represents L. arabinosus growth response on 1-cm paper strips of the chromatograms, and the broken curve represents radioactivity content of the same strips.
The decarboxylated enzyme was prepared by reacting the biotin enzyme ("native" carboxylase) with 0.50 amole of acetyl-CoA for 5 minutes at 0\(^\circ\) followed by gel filtration on a column of Sephadex G-50. The protein was precipitated with ammonium sulfate (20 g per 100 ml of protein solution), redissolved in a minimal volume of CO\(_2\)-free 0.005 M phosphate buffer, pH 6.5, and dialyzed against 4 liters of the same buffer for 1 hour at 0\(^\circ\). Native carboxylase, 1.55 mg, and decarboxylated enzyme, 4.30 mg, were then added to two separate tubes that contained 2.0 \(\mu\) moles of ADP, 1.25 \(\mu\) moles of phosphate-P\(_2\) (specific activity 2.98 \(\times\) \(10^5\) c.p.m. per \(\mu\) mole), and 50 \(\mu\) moles of Tris, pH 7.0, and the mixture was incubated for 15 minutes at 38\(^\circ\). Then 0.1 ml of 1 N HCl was added to the reaction mixture and the precipitated protein was isolated by centrifugation, washed, and hydrolyzed in 6 N HzSO\(_4\).

Biotin and diaminobiotin were then isolated from the hydrolysates and chromatographed in butanol-acetic acid as described under "Experimental Procedure." The relative amounts of biotin and diaminobiotin were determined by comparing the two areas under the peaks. To the supernatant fluids, 2.0 ml of a 5% Norit suspension were added and the charcoal was separated by filtration and washed thoroughly with 0.01 M phosphate. The nucleotides were then eluted from the charcoal with a 0.5% ammonia-ethanol-water mixture and aliquots were chromatographed in the ammonia-water-isobutyric acid system (1:32:96.1). All the radioactivity was associated with the ATP spot.

**Table IV**

<table>
<thead>
<tr>
<th>Form of carboxylase</th>
<th>ATP(_2) formed</th>
<th>Relative amounts of prosthetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu) moles/mg protein</td>
<td>Biotin</td>
</tr>
<tr>
<td>Native enzyme</td>
<td>0.53</td>
<td>47</td>
</tr>
<tr>
<td>Native enzyme + ADP + P(_2)</td>
<td>0.05</td>
<td>53</td>
</tr>
<tr>
<td>Decarboxylated enzyme</td>
<td>0.05</td>
<td>53</td>
</tr>
</tbody>
</table>

Data presented in this paper indicated that the ureido carbon of biotin might be involved in the carboxylation reaction, we decided to re-examine the experiment of Melville, Pierce, and Partridge (20). Chemically synthesized C\(_4\)-biotin was added to \(L.\) arabinosus growth media in a manner similar to that described by Melville, Pierce, and Partridge (20) except that various levels of biotin were used. Fig. 6 shows the 24-hour growth response of \(L.\) arabinosus to the amount of C\(_4\)-biotin added. The cells were harvested and the biotin content of both cells and media was determined. Under these conditions, little or no biotin was found in the media and almost all of the added biotin was associated with the cells. The biotin was isolated from the cells after hydrolysis with sulfuric acid, and methanol extracts of the hydrolysates were prepared. The C\(_4\) and total biotin content of the extract were determined as mentioned earlier, and a sample was chromatographed on paper as described under "Experimental Procedure." The results as plotted in Fig. 6 show that in the presence of limiting amounts of biotin and when the growth of the cells was proportional to the amounts of biotin added, there was very little C\(_4\) recovered in biotin despite the almost quantitative recovery of the total biotin (cf. Table VI). As the amounts of biotin were increased, more and more radioactivity was recovered in the biotin as shown in Table VI. When the amount of C\(_4\)-biotin added to the medium was relatively high (5.1 \(\mu\) moles per liter as shown in Fig. 6), almost all of the biotin was recovered containing approximately 89% of the radioactivity; thus the amount of biotin used in this experiment was in excess of that needed for optimal cell growth.

Two control experiments were carried on simultaneously in order to measure growth of the cells and recovery of the C\(_4\)-biotin. In these experiments, \(L.\) arabinosus was grown in media containing 0.071 and 1.4 \(\mu\) moles of nonlabeled biotin per liter, respectively. (The growth response of the bacteria to these concentrations of biotin were represented by Points a and b in Fig. 6, respectively.) After harvesting the cells, 0.76 and 1.28 \(\mu\) moles of C\(_4\)-biotin were added, respectively, and the C\(_4\)-biotin and diaminobiotin were separated by chromatography in butanol-acetic acid-H\(_2\)O system. The radioactivity and bioassays of the chromatographic strips were carried out as described previously. The percentages of biotin and diaminobiotin were calculated from bacterial growth values.

**Table V**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Reaction mixture</th>
<th>C(_4)-biotin formed</th>
<th>Prosthetic group</th>
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<tr>
<td></td>
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<td>Percentage of c.p.m.</td>
<td>Biotin</td>
</tr>
<tr>
<td>1</td>
<td>Native enzyme</td>
<td>2503</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Native enzyme + ATP + Mn(^{++}) + HC(_4)O(_3)^-</td>
<td>811</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Native enzyme + ATP + HC(_4)O(_3)^-</td>
<td>1135</td>
<td>100</td>
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<tr>
<td></td>
<td>Native enzyme + HC(_4)O(_3)^-</td>
<td>863</td>
<td>100</td>
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<tr>
<td>2</td>
<td>Decarboxylated enzyme</td>
<td>6607</td>
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<td>Decarboxylated enzyme + HC(_4)O(_3)^-</td>
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<td>Decarboxylated enzyme + HC(_4)O(_3)^-</td>
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</table>

The reaction mixture was incubated for 15 minutes at 38\(^\circ\). Then 0.1 ml of 1 N HCl was added to the reaction mixture and the precipitated protein was isolated by centrifugation, washed, and hydrolyzed in 6 N HzSO\(_4\).

The "decarboxylated" biotin enzyme was prepared by a procedure similar to that described in Table IV. The following components were added to the reaction mixture wherever indicated: 2.0 \(\mu\) moles of ATP, 20 \(\mu\) moles of NaHC\(_4\)O\(_3\) (1.10 \(\times\) \(10^5\) c.p.m. per \(\mu\) mole), 0.6 \(\mu\) mole of MnCl\(_2\), 60 \(\mu\) moles of phosphate buffer, pH 6.5, and water to a final volume of 1.0 ml. The reaction was initiated by the addition of either 7.93 mg of "native" enzyme or 4.55 mg of the "decarboxylated" enzyme as indicated in the table. The reaction mixture was incubated at 0\(^\circ\) for 2 hours followed by the addition of 3 ml of 6 N HzSO\(_4\). The samples were autoclaved for 1 hour at 121\(^\circ\), and the biotin derivatives were extracted in methanol as described under "Experimental Procedure." Biotin and diaminobiotin were separated by chromatography in butanol-acetic acid-H\(_2\)O system. The radioactivity and bioassays of the chromatographic strips were carried out as described previously. The percentages of biotin and diaminobiotin were calculated from bacterial growth values.
have lost its CO₂ under these conditions (29), two alternative explanations were considered for these observations. The first alternative was that the N-carboxybiotin was indeed an intermediate in biotin reactions and that on hydrolysis, some rearrangements occurred that resulted in the displacement of the ureido carbonyl group of enzyme-bound biotin by the N-carboxyl group. Unless such rearrangements were quantitative, it was very difficult for us to conceive of such reactions under all the varied conditions used (acid, alkaline, and enzymatic hydrolysis). Moreover, the high recovery of the incorporated C¹⁴O₂ into the biotin would also exclude the possible partial

**Table VI**

**Recovery of C¹⁴-biotin from culture media of L. arabinosus**

A culture of *L. arabinosus* maintained in an agar medium was transferred to 10 ml of Difco Biotin Assay Medium which contained 1.77 μmole of biotin. This culture was allowed to grow for 20 hours at 38°C, after which the cells were centrifuged, washed with NaCl, recentrifuged, and suspended in NaCl. One-tenth of a milliliter of this suspension was then transferred to each flask containing 1 liter of Difco Biotin Assay Medium and the indicated amounts of ureido-C¹⁴-biotin (3.21 X 10⁶ c.p.m. per μmole). Flasks 7 and 8 each contained 1 liter of the assay medium and the indicated amounts of nonlabeled biotin. The bacteria were allowed to grow for 20 hours at 38°C. Bacterial growth was determined by optical density measurements at 660 nm. The cells were harvested, washed with water, and suspended in 40 ml of 4 N H₂SO₄. C¹⁴-biotin, 76.8 and 1280 μmole, was added to the Control Samples 7 and 8, respectively. The cell suspensions were then autoclaved at 15 pounds of pressure for 1 hour. The hydrolysates were neutralized with Ba(OH)₂ and made slightly basic. Carbon dioxide was then bubbled through each solution until the pH dropped to approximately 6.0. The mixtures were then filtered by suction and the filtrates lyophilized. Each residue was extracted with hot methanol, and aliquots were then taken for biological assay, determination of radioactivity, and chromatography in the butanol-acetic acid-water solvent system.

**DISCUSSION**

The availability of acetyl-CoA carboxylase both in highly purified state and in relatively large quantities has afforded us with a unique opportunity to study the mechanism of action of the enzyme-bound biotin. In studies where stoichiometric amounts of enzyme were used, Waite and Wakil (35, 36) demonstrated the formation of the C¹⁴O₂ ~ biotin enzyme complex by the interaction of the enzyme with HCO₃⁻ in the presence of ATP and Mn²⁺. The formation and isolation of the C¹⁴O₂ ~ biotin complex for acetyl-CoA carboxylase was a further support to the current concept of the action of biotin enzymes as advocated by Lynen et al. (24), Kaziro, Leone, and Ochoa (25), and Lane et al. (26). According to Lynen et al. (29), the CO₂ ~ biotin-enzyme complex is an unstable complex and is very closely related to if not identical with the enzymatically synthesized N-carboxybiotin (Diagram 1) which was obtained by the action of β,β-dimethylacryl-CoA carboxylase on free (+)-biotin.

The "C¹⁴O₂ ~ biotin" complex of acetyl-CoA carboxylase, although relatively unstable at the enzyme surface, can be fixed into the biotin on acid or alkaline hydrolysis of the enzyme. Similar results were obtained by hydrolyzing the complex with papain at pH 5.2. The C¹⁴O₂ incorporated into the biotin was shown to be exclusively in the ureido carbonyl group of the ring system. Since the N-carboxybiotin was unstable and would

**Table VI**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>C¹⁴-biotin added</th>
<th>Total biotin recovered</th>
<th>C¹⁴-biotin recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>Per cent of added</td>
<td>μmoles</td>
</tr>
<tr>
<td>1</td>
<td>38.4</td>
<td>37</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>76.8</td>
<td>69</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>153.6</td>
<td>151</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>512.0</td>
<td>445</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>1280.0</td>
<td>1265</td>
<td>99</td>
</tr>
<tr>
<td>6</td>
<td>5120.0</td>
<td>5056</td>
<td>114</td>
</tr>
<tr>
<td>7†</td>
<td>71.0</td>
<td>138</td>
<td>93</td>
</tr>
<tr>
<td>8†</td>
<td>1420.0</td>
<td>2436</td>
<td>91</td>
</tr>
</tbody>
</table>

* The relatively high percentage of recovery in this experiment may represent errors in counting of samples with very low radioactivity.
† In Experiments 7 and 8 the bacteria were grown on the indicated amounts of nonlabeled biotin and before acid hydrolysis, equivalent amounts of C¹⁴-biotin were added, respectively, and the C¹⁴-biotin was reisolated from the mixture in the same manner as in Experiments 1 to 6. Experiments 7 and 8 demonstrated the high recovery procedure, 91 to 93% recovery of total biotin containing 81 to 100% of the initial radioactivity.
interchange between the carboxyl and the ureido carbonyl carbons of N-carboxybiotin.

The unlikelihood of the N-carboxybiotin as the structure of the CO₂ - biotin-enzyme complex made the second alternative, namely that the ureido carbonyl group of biotin is directly involved in the CO₂ transfer reaction of acetyl carboxylase, more attractive. According to this hypothesis, the ureido carbonyl carbon of the enzyme-bound biotin can be donated to an acceptor such as acetyl-CoA to form malonyl-CoA and the decarboxylated enzyme-bound biotin (such as diaminobiotin). The latter can then be carboxylated in the presence of ATP and Mn⁺⁺ to form the carboxylated enzyme, which would donate the carbonyl group to acetyl CoA, thus regenerating the decarboxylated enzyme-bound biotin. The data presented in this paper appeared to be compatible with these views and to afford strong support for this hypothesis.

Our observations indicated that the C¹⁴O₂ incorporated into the C⁴₀₂ ~ biotin-enzyme complex yielded on hydrolysis ureido-C¹⁴-biotin. The amount of radioactivity recovered in the C⁴⁻⁰₂-biotin was more than 80% of that found in the complex. The "native" enzyme as isolated from the chicken livers appears to be in the carboxylated form as shown by its ability to carboxylate acetyl-1-C¹⁴-CoA to C¹⁴-malonyl-CoA. On hydrolysis, the native enzyme yielded biotin. These observations established the first point, namely, that the prosthetic group of the carboxylated enzyme or the CO₂ - biotin-enzyme complex is biotin.

Hydrolysis of the decarboxylated form of the enzyme yielded small amounts of biotin plus a new compound that was closely related to biotin as shown by its growth-promoting activity of L. arabinosus. The new compound has been tentatively identified as diaminobiotin in three different solvent systems. The diaminobiotin enzyme can be converted to the biotin enzyme by carboxylating the former with H⁺ and CO₂ in the presence of ATP and Mn⁺⁺. If H⁺ and CO₂ was used during this conversion, then ureido-C¹⁴-biotin was isolated from the carboxylated enzyme as would have been predicted by this hypothesis.

A serious objection to this hypothesis was encountered in the work of Melville, Pierce, and Partridge (20) on the high recovery (93%) of the ureido-C¹⁴-biotin from cultures of L. arabinosus grown on media containing ureido-C¹⁴-biotin. On repeating these experiments in our laboratory, we were able to confirm these authors' findings that when high levels of C¹⁴-biotin were used in growth experiments, almost quantitative recovery of the C¹⁴ could be obtained. However, when the amounts of C¹⁴-biotin were limiting (40 to 160 μmole of biotin per liter) and the growth of the organism was proportional to the C¹⁴-biotin content of the media, no C¹⁴ was recovered in the reisolated biotin despite the fact that up to 97% of the biotin was accounted for by microbiological assays. On increasing the amount of C¹⁴-biotin in the media up to 5.9 mmoles per liter, we were able to recover up to 86% of the radioactivity in biotin with a total recovery of almost 100% of the biotin. The loss of C¹⁴ in this experiment, presumably as CO₂, which amounted to 14% of the added radioactivity or the equivalent of 0.83 mmoles, was sufficient for the optimal growth of the bacteria as shown from growth curves, whereas the remaining 86% of the C¹⁴-biotin was stored unaltered within the cells. According to this interpretation therefore, the loss of 7% of the radioactivity, or the equivalent of 4.7 mmoles of biotin per liter of media, in the experiment of Melville, Pierce, and Partridge (20) was enough to account for the optimal growth of the L. arabinosus.

The loss of the radioactivity from the ureido-C¹⁴-biotin during the growth of L. arabinosus suggests that the involvement of the ureido carbonyl group of biotin in the carboxylation reactions may not be limited to the acetyl-CoA carboxylase reaction and that it may reflect a general mechanism for biotin enzymes. The hypothesis for the action of biotin enzymes as revealed by our studies on acetyl-CoA carboxylase can be illustrated in two possible ways (Reactions 4 and 5 or 6 and 7).
that only one bond is formed during the carboxylation of the

diaminobiotin enzyme. This possibility seems more reasonable
since only 1 mole of ATP is required for the carboxylation of the
diaminobiotin enzyme. It will also explain the O$^18$ data of
Kuziro et al. (42), who reported the incorporation of 2 oxygen
atoms of bicarbonate into the CO$_2$ ~ biotin-enzyme complex of
propionic-CoA carboxylase with the third oxygen atom found in
the inorganic phosphate. It seems reasonable to assume that
only one amino group of the biotin is involved, and based on the
results of Lynen et al. (24), this is the nitrogen furthest removed
from the side chain. The CO$_2$ may then be attracted by the
other amino group to a position somewhere between the two
amino groups.

The "open" form of the ureido ring of biotin enzyme may
account for the carboxylation of the free (+)-biotin in the $\beta$-$\beta$
dimethylglyceryl-CoA system by transcarboxylating the nitrogen
toxygen atom furthest removed from the side chain.

The $\alpha$-amino group is involved, and based on the
results of Lynen et al. (24), this is the nitrogen furthest removed
from the side chain of biotin. It may also account for the formation of biotin on hydrolysis of the
diethyl-1,2-dicarboxylic ester of diaminobiotin to biotin by
hydrolysis with Ba(OH)$_2$ (43) as shown in Equation S.

\[ \text{E(O-C} \quad \text{O=O=C-OEt} \]
\[ \text{HN} \quad \text{HN} \quad \text{(CH$_2$)$_4$COH} \quad \text{Ba(OH)$_2$} \]
\[ \text{O} \quad \text{O} \quad \text{HN} \quad \text{(CH$_2$)$_4$COH} \]

The foregoing possibilities of the "closed" and "open" forms
of the carboxylated enzyme are only speculative, and further
experimentation is necessary in order to ascertain the exact
nature of the carboxylated form of the biotin enzyme.

**SUMMARY**

C$_4$-bicarbonate can be incorporated into the acetyl coenzyme
A carboxylase in the presence of adenosine triphosphate and
Mn$^{++}$ to form the C$_4$O$_2$ $\sim$ biotin-enzyme complex. Hydrolysis of the
C$_4$O$_2$ $\sim$ biotin-enzyme complex with 4 $\times$ H$_2$SO$_4$ or 4 $\times$
Ba(OH)$_2$ yields free biotin containing over 85% of the radio-
activity. The C$_4$ of the isolated biotin is located exclusively in
the ureido carbon atom of biotin. Papain hydrolysis of the
intact C$_4$O$_2$ $\sim$ biotin enzyme yields biocytin containing approximately
80% of the radioactivity. The release of biocytin by papain digestion of the enzyme indicates that biotin is bound to the protein of acetyl carboxylase via the amino group of
lysin.

The "native" carboxylase yields biotin on hydrolysis with
H$_2$SO$_4$, whereas enzyme treated with acetyl-CoA or with ADP
and PO$_4$ yields up to 94% of a closely related biotin derivative
that has tentatively identified as "diaminobiotin." This
suggests that "diaminobiotin" is the prosthetic group of the
"decarboxylated" enzyme. The diaminobiotin enzyme can be
reconverted to the biotin enzyme only in the presence of HCO$_3$-,
ATP, and Mn$^{++}$.

Growth of Lactobacillus arabinosus on limiting amounts of
ureido-C$^{14}$-biotin results in the loss of almost all of the radio-
activity from the biotin despite quantitative recovery of bio-
logically active biotin. This indicates that the ureido carbon
of biotin may be involved in biotin-dependent carboxylation
reactions.

These observations suggest that the ureido carbon of the en-
yyme-bound biotin is the active carbon of biotin; a mechanism
for biotin action is proposed.

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