Studies on the Mechanism of Action of Acetyl Coenzyme A Carboxylase

III. ENZYME-BOUND 1'-N-CARBOXYBIOTIN AS THE CARBOXYLATION INTERMEDIATE*

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SUMMARY

Studies presented here, with the criterion of the degree of binding of the biotin derivatives obtained from acetyl coenzyme A carboxylase to avidin, show that these compounds have an intact ureido ring, eliminating the diamine derivative as an intermediate in the carboxylation. With the use of carboxyl-14C-biotin acetyl- and propionyl-CoA carboxylases, it has been possible to show both in vivo and in vitro that no turnover of the ureido carbon of biotin occurs in the carboxylation reaction. The 1'-N-methoxycarbonylbiotin methyl ester was isolated from the acetyl-CoA carboxylase substantiating the hypothesis that the 1'-N-carboxamide of enzyme-bound biotin is an intermediate in these carboxylation reactions.

In our earlier observations (1, 2) on the mechanism of action of acetyl coenzyme A carboxylase-bound biotin, it was found that a 14CO2 ~ biotin-enzyme complex could be isolated in good yield by gel filtration on Sephadex G-25 of a reaction mixture containing H14CO3−, adenosine triphosphate, Mn++, and acetyl coenzyme A carboxylase. The complex, 14CO2 ~ biotin-enzyme, could interact with the acceptor acetyl-CoA forming 14C-malonyl-CoA. It was also found that ATP and Mn++ were absolutely required for the formation of 14CO2 ~ biotin-enzyme complex, and that avidin when incubated with the enzyme prior to its interaction with the substrates prevented the formation of the complex. The isolation of this complex afforded us the opportunity to study the chemical nature of the enzyme-bound biotin ~ CO2 and possibly to verify the hypothesis of Lynen et al. (3) that 1'-N-carboxamide of biotin (Diagram 1) represents the intermediate in the carboxylation reaction. Our first attempts to isolate the CO2 ~ biotin included methylation with diazomethane followed by digestion with papain. In studying various conditions in this isolation procedure, however, it was then found that if the methylation step were omitted, very little loss of radioactivity occurred, contrary to the known properties of the carboxamide of biotin. In addition, if the product of the papain digest was further hydrolyzed by strong acid and autoclaving, the same amount of radioactivity remained present in the solution and hence was not lost as 14CO2. To better understand these unexpected properties, a more thorough study of the interactions of the enzyme and the substrates were undertaken; in consequence, the following observations were made. (a) Acid or alkaline hydrolysis of the 14CO2 ~ biotin-enzyme complex yielded a radioactive product identified as biotin by chromatographic and recrystallization techniques. (b) Biotin-enzyme, when allowed to react with 14C-acetyl-CoA yielded 14C-malonyl-CoA and “decarboxylated enzyme.” (c) ADP and 32P-orthophosphate were coupled in the presence of the biotin-enzyme and yielded ATP32P plus the “decarboxylated enzyme.” (d) Hydrolysis of the “decarboxylated enzyme” yielded a new compound tentatively identified as 3,4-cis-diamino-2-tetrahydrothiophene-n-valeric acid (Diagram 2) (referred to here as DACBi).

These observations led us to postulate the following enzymatic mechanism

\[
\text{DACB-enzyme + ATP + HCO}_3^- \xrightarrow{\text{Mn}^{++}} \text{biotin-enzyme + ADP + P}_i \quad (1)
\]

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The abbreviations used are: DACB, diaminocarboxylic acid derivative of biotin; DACB-enzyme, the carboxylase containing diaminocarboxylic acid derivative of biotin as prosthetic group instead of biotin.
Biotin-enzyme + acetyl-CoA $\rightarrow$

\[ \text{DACB-enzyme} + \text{malonyl-CoA} \]  \hspace{1cm} (2)

\[
\text{Sum: } \text{ATP} + \text{HCO}_3^- + \text{acetyl-CoA} \xrightarrow{\text{Mn}^{++}}
\text{malonyl-CoA} + \text{ADP} + \text{P},
\]

Shortly after publication of our observations, Knappe, Wenger, and Wiegand (4) reported the isolation of the $'^{14}$C-$'^{1}$-N-methoxy-carbonylbicyclin from the $'^{14}$CO$_2$ $\rightarrow$ biotin $\beta$-methylcrotonyl-CoA carboxylase with diazomethane to methylate the complex, followed by papain digestion. In their experiments, further digestion of this compound by biotinidase from hog kidney followed by remethylation with diazomethane yielded $'^{14}$C-$'^{1}$-N-methoxy-carbonylbicyclin methyl ester as shown by chromatographic and recrystalization techniques. These observations were confirmed by Lane and Lynen (5) with propionyl-CoA carboxylase and by Wood et al. (6) with the transcarboxylase, thus substantiating the hypothesis that the carboxamide of biotin is the intermediate in carboxylation reaction involving these enzymes.

Numa, Ringlemann, and Lynen (7) were able to isolate a $'^{14}$CO$_2$ $\rightarrow$ biotin-acetyl-CoA carboxylase with preparations of the acetyl-CoA carboxylase from rat liver after incubation of the enzyme with $3'$-C-malonyl-CoA and subsequent gel filtration through Sephadex. They found this complex to be acid-labile and to yield on methylation and treatment with Pronase, the $'^{14}$C-$'^{1}$-N-methoxy-carbonylbicyclin; this compound, upon digestion with biotinidase (4) was found to yield the $'^{1}$-N-$'^{14}$C-methoxy-carbonylbicyclin. These observations led them to conclude that the acetyl-CoA carboxylase from rat liver behaves in a manner similar to the other enzymes described thus far.

Allen, Stjernholm, and Wood (8) investigated the role of the urido carbon of biotin in the transcarboxylation reaction by growing propionibacteria in a medium containing urido-labeled $'^{14}$C-biotin, by isolating the labeled enzyme from the cells, and by using it to catalyze the transcarboxylation of the carbonyl group from methylmalonyl-CoA to pyruvate. They were unable to demonstrate either the transfer of $'^{14}$C to the oxalacetate formed or loss of the $'^{14}$C-label from the enzyme, thereby concluding that in the case of the transcarboxylase the biotin does not function through the transfer of the urido carbon.

In light of the aforementioned experimental findings, it became imperative to re-examine our earlier observations on the acetyl-CoA carboxylase, and experiments were performed similar to those described by Knappe, Wenger, and Wiegand (4) in an attempt to obtain the carboxamide derivative of biotin of the acetyl-CoA carboxylase as an intermediate. In order to show that the acetyl-CoA carboxylase functions through the transfer of the urido carbon of the acetyl-CoA carboxylase-bound biotin as compared to the role of the urido carbon of propionyl-CoA carboxylase-bound biotin by using carbonyl-labeled and carbonyl-labeled biotin.

The evidence presented below indicates that the acetyl-CoA carboxylase from chicken livers and the propionyl-CoA carboxylase from rat livers behave in a similar manner; i.e., the $'^{1}$-N-carboxamide of biotin, rather than the urido carbon, is involved in the carboxylation reaction. Attempts to repeat experiments on the incorporation of $'^{14}$CO$_2$ into the urido position of biotin have given us cause to concur with others regarding the noninvolvement of the urido carbon in the carboxylation reaction, our recent experiments yielding inconsistent and most often minimal quantities of $'^{14}$C-biotin. Also, with the use of the avidin-biotin binding technique of Greene (9, 10), we have been able to show that the prosthetic group obtained from the enzyme following its treatment with acetyl-CoA is biotin.

**Experimental Procedure**

**Enzyme Preparations**—$'^{14}$CO$_2$ $\rightarrow$ biotin-enzyme was prepared and isolated as described earlier from preparations of acetyl-CoA carboxylase that were concentrated 400- to 500-fold over crude extracts (2, 11, 12). Preparations of biotinidase isolated by the method of Knappe, Brummer, and Biederbich (13) were purified through the second ammonium sulfate fractionation.

**Ureido- or carboxy-$'^{14}$C-biotin acetyl-CoA carboxylase** was prepared from the livers of chicks which were treated as follows. Three dozen newly hatched chicks were placed on a biotin-free diet to which 10% dried egg white was added (both obtained from Nutritional Biochemicals Corporation) until signs of biotin deficiency became apparent (usually within 3 to 4 weeks), after which daily injections of 0.2 ml of $'^{14}$C-biotin (10 µg per ml) were given intraperitoneally over a 5-day period. During the injection period and for 2 days subsequently, the chicks were maintained on biotin-free egg white diet; they were then sacrificed and their livers removed. The $'^{14}$C-acetyl-CoA carboxylase was purified from the homogenized livers through the first ammonium sulfate fractionation.

**Ureido- or carboxy-$'^{14}$C-biotin propionyl-CoA carboxylase** was prepared from the livers of rats which had been raised from weaning age in the same manner as the chicks. The $'^{14}$C-propionyl-CoA carboxylase was purified according to the procedure of Halena et al. (14) through the first ammonium sulfate precipitation, with the exception of a few preparations which were carried through the third ammonium sulfate precipitation. These highly purified preparations were then used to confirm the observations made with the cruder system.

**Microbiological Assay**—Enzyme-bound biotin was quantitated as described earlier (2) by microbiological assay with cultures of Lactobacillus arabinosus 17-5 obtained from American Type Culture Collections.

**Chromatography of Biotin and Its Derivatives**—The products of papain digestion of the methylated $'^{14}$CO$_2$ $\rightarrow$ biotin-enzyme complex were chromatographed on paper with three different solvent systems: butanol-pyridine-acetic acid-$'^{1}$H$_2$O (30: 20: 8: 24, v/v), butanol-acetic acid-$'^{1}$H$_2$O (4: 1: 1, v/v), and pyridine-isoamyl alcohol-$'^{1}$H$_2$O (30: 30: 35, v/v). Paper chromatography of the products of the biotinidase hydrolysis of $'^{14}$C-derivative from the preceding digestion was carried out in three different systems: ether on ethylene glycol-impregnated paper, ethyl acetate-tetrahydrofuran-$'^{1}$H$_2$O (10:59:78, v/v), and isobutyric acid-$'^{1}$H$_2$O (30: 30: 35, v/v).

**Labelled Biotin and Various Nonlabeled Biotin Derivatives**—The ureido-$'^{14}$C-biotin was prepared from $'^{14}$C-phosgene (specific activity of 17.5 µC per µmole, obtained from the New England Nuclear Corporation) by the method of Melville, Pierce, and Partridge (15). Carboxy-$'^{14}$C-biotin was donated by Hoffman La Roche, Inc.; and 1'-N-methoxy-carbonylbicyclin methyl ester supplied by Dr. J. Knappe.
Avidin-Biotin Binding—Avidin-biotin association was studied by the method of Greene (9, 10) with samples of highly purified avidin (10 units per mg of protein) provided by Dr. N. Michael Greene.

RESULTS

Identification of Product by Hydrolysis of Acetyl-CoA-treated Carboxylase—It was reported earlier that a new biotin derivative tentatively identified as DACB (2) was detected on chromatograms of hydrolysates of acetyl-CoA-treated carboxylase. Further studies designed to prove the structure of this compound with hydrolysates of the carboxy-14C-biotin-acetyl-CoA carboxylase were complicated, however, by the appearance of several new compounds on chromatography and by spontaneous changes in standard DACB during the various manipulations employed. To circumvent many of these problems, we have recently utilized the techniques developed by Greene (10) that showed that formation of the avidin-biotin complex was dependent upon the intact ureido ring of biotin (being neither open nor substituted). He found a dissociation constant for the DACB-avidin complex of $3 \times 10^{-7}$ M as compared to $10^{-18}$ M for the biotin avidin complex. The difference in the dissociation constants of the two complexes is sufficient to allow differentiation of the two forms of biotin. Comparison of the dissociation constants of the biotin derivatives from the acetyl-CoA-treated acetyl-CoA carboxylase and avidin with known biotin and avidin was used to indicate possible alteration in the ureido ring of the biotin. Consequently, the degree of binding between avidin and known carboxy-14C-biotin from the carboxylase and carboxy-14C-biotin from acetyl-CoA-treated carboxylase was studied (Fig. 1) with the finding that the labeled compounds from the enzymes and standard biotin were associated with the avidin to the same degree, whereas standard DACB was not appreciably bound by the avidin under the experimental conditions (cf. Fig. 1). These results indicated that the treatment of the enzyme with acetyl-CoA did not yield a derivative of biotin with an altered ureido ring.

Stability of Ureido Carbon of Biotin—14C-Acetyl-CoA carboxylase and 14C-propionyl-CoA carboxylase, prepared from 14C-biotin as described under “Experimental Procedure,” were tested for turnover of the ureido carbon. Table I, which compares propionyl-CoA carboxylase activity and biotin concentrations of enzymes prepared from biotin-deficient rats and rats treated by injection with carboxyl- or carbonyl-labeled biotin, indicates the marked stimulation of specific enzymatic activity and increase in the level of the biotin content which occurred in the enzyme preparations purified through the first ammonium sulfate precipitation. Also, the 14C-content of carboxyl- and ureido-labeled biotins were found to be nearly equivalent (cf. Table I), thereby indicating no loss of the ureido carbon and hence, no turnover of this carbon in vivo.

The ureido-14C-biotin enzyme was then pipetted into solution containing ATP, nonradioactive bicarbonate, MgCl₂, glutathione, and propionyl-CoA and incubated for 5 min in a sealed flask with a center well. When the reaction was completed, the protein was precipitated with acid and the center well filled with Hyamine to trap any CO₂ which might have liberated during the reaction. An aliquot of the reaction mixture was withdrawn from the solution and assayed for the ADP formed as a measure of enzymatic turnover. After allowing all of the CO₂ to be trapped in the Hyamine, the solution was withdrawn from the flask, the denatured protein was removed by centrifugation, and an aliquot of the supernatant fluid was counted in a liquid scintillation counter (as was the Hyamine solution). The protein was hydrolyzed with 4 N H₂SO₄ in the autoclave, neutralized, and an aliquot of the hydrolysate counted. Since the radioactivity from

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**TABLE I**

Relative stability of ureido and carboxyl groups of biotin in propionylcarboxylase in vivo

<table>
<thead>
<tr>
<th>Injections</th>
<th>Specific enzymatic activity</th>
<th>Biotin content of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radioactivity</td>
<td>μmol biotin/mg protein</td>
</tr>
<tr>
<td>None</td>
<td>1.68</td>
<td>8.4</td>
</tr>
<tr>
<td>Carboxy-14C-biotin</td>
<td>4.27</td>
<td>14.0</td>
</tr>
<tr>
<td>Ureido-14C-biotin</td>
<td>3.54</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Values are micromoles of methylmalonyl-CoA formed per hour per mg of protein.

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Fig. 1. Characterization of carboxy-14C-biotin by its binding to avidin. Carboxy-14C-biotin enzyme (1.43 × 10⁴ dpm), 50 μg, was incubated in 5 ml solution containing 0.4 μmole of acetyl-CoA and 100 μmoles of potassium phosphate, pH 7.0. The mixture was incubated at 38° for 5 min. Another incubation was carried out without the addition of acetyl-CoA. At the end of incubation, 1.0 ml of concentrated H₂SO₄ was added and the mixture was hydrolyzed as described earlier (2). The radioactivity recovered in the hydrolysates was over 90% of original. Samples of hydrolysates (O, acetyl-CoA-treated enzyme; △, untreated enzyme), carboxy-14C-biotin (■), and carboxy-14C-DACB (△) containing about 7.5 μg of 14C-biotin (estimated from specific activity of carboxy-14C-biotin of 22.5 μC per μmole) were assayed for biotin by formation of the biotin-avidin complex as described by Greene (9) with the use of the indicated amounts of avidin. The radioactivity in the solution after removal of the biotin-avidin complex was determined by counting an aliquot in the Packard Tri-Carb scintillation spectrometer.
Noninvolvement of ureido group of \(^{14}\text{C}\)-biotin in carboxylation reaction

The reaction mixture, in which the possible turnover of the ureido carboxyl of the propionyl-CoA carboxylase-bound biotin was tested, contained 2.5 mg of propionyl-CoA carboxylase (purified through the second ammonium sulfate step (14)), 10 μmoles of ATP, 10 μmoles of MgCl₂, 1.36 μmoles of propionyl-CoA, 20 μmoles of KHCO₃, 3.0 μmoles of glutathione, and 100 μmoles of Tris buffer, pH 6.0, in a total volume of 2.5 ml. The reaction mixture was incubated for 5 min at 38° in a sealed flask fitted with a rubber cap. Subsequently, 0.5 ml of 1.0 M HClO₄ solution was injected into a center well and the reaction stopped by the injection of 0.5 ml of a 10% solution of trichloroacetic acid. A sample (0.2 ml) of the acidified reaction mixture was withdrawn from the flask and assayed for ADP formation with pyruvate kinase lactate dehydrogenase.

The remaining contents of the flask were shaken for 1 hour to allow complete trapping of the CO₂ by Hyamine after which the reaction mixture was centrifuged, the supernatant fluid withdrawn, and the protein hydrolyzed with 4 N H₂SO₄. The Hyamine solution and samples of the supernatant fluid and the protein hydrolysate were counted for radioactivity. The turnover (millimicromoles of \(^{14}\text{C}\)-Biotin-enzyme recovered from \(^{14}\text{C}\)-Biotin-enzyme added) was determined from the amounts of ADP formed as a function of time. Similar observations were made on preparations of the ureido-labeled acetyl-CoA carboxylase which catalyzed the formation of 249 μmoles of malonyl-CoA without loss of radioactivity from the enzyme (cf. Table II). Thus, the retention of the radioactivity in the ureido carbon of biotin of both propionyl-CoA carboxylase and acetyl-CoA carboxylase, despite their turning over 1100 and 33 times, respectively (Table II), is consistent with the hypothesis that the ureido carbon of biotin is not involved in the carboxylation reactions.

Isolation of \(^1\text{N}\)-Methoxy carbonylbiotin from CO₃ ~ Biotin-Acetyl-CoA Carboxylase—The \(^{14}\text{CO}_{2} ~ \text{biotin-enzyme complex was isolated from the reaction mixture by gel filtration on Sephadex as described earlier, and the amount formed was equivalent to about 70% of the biotin present on the enzyme as determined by microbiological assay (cf. Table III). The isolated enzyme was exposed to trypsin for 5 min followed immediately by methylation with diazomethane and digestion by papain. The hydrolysate was then partially purified by chromatography on Whatman No. 3 paper in the butanol-acetic acid-water system. The major peak of radioactivity had an Rₚ of 0.55 compared to an Rₚ of 0.45 for known \(^1\text{N}\)-methoxy carbonylbioctin. This radioactive material was eluted from the paper,

<table>
<thead>
<tr>
<th>Propionyl carboxylase</th>
<th>Acetyl carboxylase</th>
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<tbody>
<tr>
<td>(^{14}\text{C})-Biotin-enzyme added</td>
<td>0.62</td>
</tr>
<tr>
<td>(^{14}\text{C})-Biotin-enzyme recovered from reaction mixture</td>
<td>0.54</td>
</tr>
<tr>
<td>(^{14}\text{C}) remaining in solution after enzyme precipitation</td>
<td>0</td>
</tr>
<tr>
<td>(^{14}\text{C}) recovered in CO₂</td>
<td>0</td>
</tr>
<tr>
<td>ADP formed</td>
<td>091</td>
</tr>
<tr>
<td>Malonyl-CoA formed</td>
<td>249</td>
</tr>
<tr>
<td>Enzyme turnover</td>
<td>1113</td>
</tr>
</tbody>
</table>

Table II: Noninvolvement of ureido group of \(^{14}\text{C}\)-biotin in carboxylation reaction

Table III: Isolation of \(^1\text{N}\)-Methoxy carbonylbiont from acetyl-CoA carboxylase

The reaction mixture contained 3.0 μmoles of ATP, 0.60 μmoles of MnCl₂, 60 μmoles of potassium phosphate, pH 6.5, 10 μmoles of KH₄CO₃ (3.50 × 10⁶ cpm per pmole), and 8.25 mg of enzyme in a total volume of 1.0 ml. It was then incubated for 1 min at 38° and filtered through a column of Sephadex G-15 as previously described (1, 11). The isolated \(^{14}\text{CO}_{2} ~ \text{biotin-enzyme was exposed to 15 mg of trypsin for 5 min at 35° followed by the addition of diazomethane. The solution was then made 0.10 M with sodium acetate, pH 6.0, and the following were added: EDTA, 29 μmoles; mercaptoethanol, 25 μmoles; papain, 15 mg, in a total volume of 15 ml. The hydrolysate was then boiled at 40° over an 18-hour period. The solution was then boiled for 3 min, followed by lyophilization. The residue was then taken up in methanol and spotted on Whatman No. 3 paper for chromatography in butanol-acetic acid-H₂O system. The radioactive spot on the developed chromatogram was eluted from the paper with methanol. A sample of this eluate was dried, resuspended in H₂O, and hydrolyzed further in a reaction mixture containing 200 μmoles of potassium phosphate, pH 6.0, 20 μmoles of EDTA, and 5.4 mg of biotinidase, in a total volume of 2.0 ml. The mixture was then boiled at 30° for 30 min. It was then boiled for 3 min, lyophilized, and the radioactive material extracted in methanol and methylated with diazomethane. The "C-content of the various samples was then determined by the use of a Packard Tri-Carb liquid scintillation counter. Under the conditions used 100 cpm would have been readily detected.

<table>
<thead>
<tr>
<th>Manipulation</th>
<th>(^{14}\text{C})-Content of CO₂ ~ biotin complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{14}\text{CO}_{2} ~ \text{biotin-enzyme complex (4.81 pmole biotin)}</td>
<td>1.30 × 10⁶</td>
</tr>
<tr>
<td>5 min exposure to trypsin, methylation with diazomethane, and digestion with papain</td>
<td>8.63 × 10⁴</td>
</tr>
<tr>
<td>Chromatography in butanol-acetic acid-water system (4:1), Rₚ 0.55</td>
<td>8.37 × 10⁴</td>
</tr>
<tr>
<td>(^1\text{N})-Methoxy carbonylbiont methyl ester</td>
<td>7.50 × 10⁴</td>
</tr>
</tbody>
</table>
and samples of the eluate were rechromatographed by using three different systems (cf. Fig. 2). In all three of these systems, the radioactive material showed chromatographic behavior different from that of known 1'-N-methoxycarbonylbioctin or of biocytin. The main portion of the 14C-containing material that was eluted from the paper was further hydrolyzed with biotinidase, methylated with diazomethane, and chromatographed with standard 1'-N-methoxy carbonylmethylbiotin, the expected product (cf. Fig. 3). The finding that the product of papain digestion exhibited chromatographic behavior different from that of known 1'-N-methoxy carbonylbioctin could represent a difference in the amino acid moieties of the molecule since biotinidase hydrolysis yielded the expected product, 1'-N-methoxy carbonylbioctin.

In the light of this newer evidence, efforts have been made to reinvestigate our earlier experiments. As a result, we found that the incorporation of 14CO2 into a stable position in the enzyme-bound biotin was insufficient to be considered a part of the mechanism of action of the biotin. However, some factors which improved the formation of 14C-biotin, such as hydrolysis of the complex before its separation from the substrates or concentration of the isolated complex before hydrolysis, were found. Even though this raised the yield somewhat (up to 30% of that predicted by theory on the basis of the amount of 14CO2 ~ biotin-enzyme complex formed), these values remained much lower than those originally obtained (up to 84%).

Although the reasons for this nonreproducibility are unknown, it seems probable that the method of isolation employed in the biotin assay caused the rearrangements observed in the biotin molecule. Schaeffer and Bhargava (16) have shown with model compounds that either the amide or ester of a carboxylated imidazolidone not only can transfer this carbonyl group to an attacking nucleophilic reagent but also can undergo various ring openings at rates they felt too slow to be considered part of the enzymatic turnover. Also, they found that the CO2 complex of DACB (Diagram 3) was formed in good yield upon the interaction of the DACB and CO2. Such a carboxylated form is similar to the intermediate proposed by Hoffmann and Bridgwater (17) in their synthesis of biotin. They found that the diethyl-N,N'-dicarboxylic ester of DACB, when hydrolyzed with Ba(OH)2, would form biotin in high yields. If a slow ring opening or rearrangement occurred as conceived by Schaeffer and Bhargava (16), it would then be possible under the conditions of hydrolysis for the ring to reclose with the 14C-carboxamide carbon becoming the ureido carbon.

Acknowledgments—We wish to thank Hoffman-La Roche for their generous gift of carboxy14C-biotin, Dr. J. Knappe who kindly supplied 1'-N-methoxy carbonylbioctin and 1'-N-methoxy carbonylbioctin methyl ester, and Dr. N. Michael Greene who generously provided us with samples of highly purified avidin.

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