ON THE MECHANISM OF ACTION OF ENZYME-BOUND BIOTIN*

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In 1958, Wakil et al. (1-3) recognized biotin as the prosthetic group of acetyl CoA carboxylase and presented evidence for its direct participation in the carboxylation reaction. Several other biotin-enzymes have been isolated (4-6) and the mechanism of action of this class of enzymes, as represented by acetyl CoA carboxylase, can be depicted as in reactions 1 and 2.

$$HCO_3^- + ATP + \text{Biotin-enzyme} \overset{Mn^{++}}{\rightleftharpoons} CO_2 \sim \text{Biotin-enzyme} + ADP + P_i \quad 1$$

$$CO_2 \sim \text{Biotin-enzyme} + CH_3COSCoA \rightleftharpoons HOOCCH_2COSCoA + \text{Biotin-enzyme} \quad 2$$

The current concept of the nature of the $CO_2 \sim$ biotin-enzyme complex derives from the work of Lynen and his colleagues on β-β-dimethylacrylyl CoA carboxylase (4). These authors employed free (+) biotin as a CO_2 acceptor and isolated a CO_2-biotin compound which they showed to be N-carboxy-biotin (I). By analogy, these workers have concluded that the CO_2-biotin enzyme complex (II) was closely related, or identical, to N-carboxy-biotin (I).

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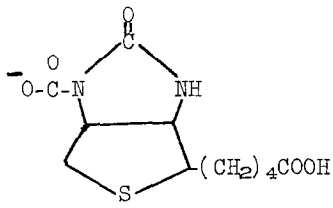
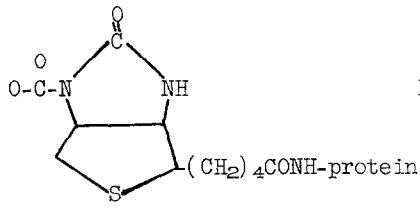
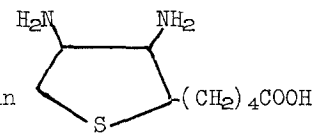


I



II



III

This communication deals with recent observations on the nature of the $CO_2 \sim$biotin complex obtained from acetyl CoA carboxylase. Our studies revealed characteristics of this complex that are at variance with those predicted by Lynen's hypothesis and suggest, rather, that the ureido-carbonyl of biotin is the active carbon of the biotin molecule.

Incorporation of $C^{14}O_2$ into biotin: The $C^{14}O_2 \sim$biotin-enzyme complex was prepared by incubating acetyl CoA carboxylase with $HC^{14}O_3^-$, ATP, Mn^{++} and isocitrate as described in Table I. The amount of $C^{14}O_2$ associated with the complex was equivalent to the amount of biotin present in the enzyme as determined by assay with L. arabinosus (7). Moreover, the $C^{14}O_2$ was transferred quantitatively from the $C^{14}O_2 \sim$biotin complex to the acceptor acetyl CoA to form malonyl CoA. In the absence of ATP, or Mn^{++}, or both, there was little incorporation of $C^{14}O_2$ (less than 20%) into the complex. Addition of avidin to the reaction mixture inhibited the formation of the $C^{14}O_2 \sim$biotin-enzyme complex whereas addition of biotin-treated avidin did not affect the formation of the complex. These observations indicated that the $C^{14}O_2 \sim$biotin complex was formed by enzymatic interaction of the enzyme-bound biotin with the substrates HCO_3^- and ATP.

When the $C^{14}O_2 \sim$biotin complex was hydrolyzed with 4N H_2SO_4 or 4N $Ba(OH)_2$, up to 90% of the carbon 14 was recovered in free biotin as shown in Table I.

Biotin was identified in the hydrolysate by its R_f in several chromatographic systems, by its promotion of growth of L. arabinosus and by isolation of the C^{14}-biotin by dilution techniques and recrystallization of the biotin to constant specific activity. Hydrolysis of the crystalline

TABLE I

Incorporation of $C^{14}O_2$ into the Enzyme Complex and its Recovery in the Isolated Biotin

Expt. No.	$C^{14}O_2$-biotin enzyme complex cpm	Method of hydrolysis	C^{14}-biotin cpm
1	3286	4N H_2SO_4	2760
2	3100	4N $Ba(OH)_2$	2150
3	3200	papain	2300*

* The product of hydrolysis in this case was biocytin as shown by R_f of 0.35 in butanol:H_2O:acetic acid.

In each experiment 2.3 mg of acetyl CoA carboxylase (specific activity 0.4 μmoles of malonyl CoA formed per minute per mg of protein) were added to 0.8 ml of a reaction mixture which contained 2.0 μmoles ATP, 10 μmoles $NaHC^{14}O_3$ (specific activity 1.44×10^3 cpm per mμmoles), 60 μmoles potassium phosphate buffer (pH 6.5), 0.60 μmoles $MnCl_2$ and 40 μmoles isocitrate. The mixture was incubated at 38° for 3 minutes. The $C^{14}O_2$~biotin enzyme complex was separated from the reaction mixture by passing the mixture through a column of Sephadex-25 (2 x 20 cm). To the solution of CO_2~biotin-enzyme complex of experiments 1 and 2 were added H_2SO_4 and $Ba(OH)_2$, respectively, to a final concentration of 4N and the mixtures were autoclaved for 1 hr at 121°. Biotin was isolated from the hydrolysate by extraction in methanol and was identified by chromatography (0.8 in butanol: H_2O:acetic acid, 4:5:1, 0.5 in propanol:H_2O;15:85, and 0.7 in butanol: methanol:benzene:H_2O 2:1:1:1 (4,8,9); and by growth of L. arabinosus. Papain digestion of the CO_2~biotin enzyme complex was carried out in experiment No. 3 in acetate buffer, (at pH 5.2) for 24 hrs. Biocytin was isolated from the hydrolysate and identified by paper chromatography and hydrolysis to free biotin which promoted growth of L. arabinosus.

C^{14}-biotin with $Ba(OH)_2$ according to the procedure of Melville et al. (10) yielded CO_2 (trapped as $BaCO_3$) and the diaminobiotin (III). Over 90% of the radioactivity was found in the $BaCO_3$ whereas the diaminobiotin (III) was devoid of C^{14}. These data indicated that the ureido-carbon of the isolated biotin contained all of the radioactivity. The incorporation of $C^{14}O_2$ into the ureido group of biotin is incompatible with the suggestion that the CO_2 biotin-enzyme complex is N-carboxy-biotin (II), since the latter compound is highly unstable and presumably would lose its CO_2 on hydrolysis (11). The high percentage of incorporation of $C^{14}O_2$ into biotin (>80%) excludes a possible rearrangement of I or II during hydro-

lysis. Had such a rearrangement occurred, no more than 50% of the radioactivity would have been recovered in the isolated C^{14}-biotin.

Product of Transcarboxylation: When the isolated $CO_2 \sim$ biotin-enzyme complex was treated with acetyl-CoA, malonyl-CoA and the decarboxylated form of the enzyme were isolated from the reaction mixture. Hydrolysis of the decarboxylated enzyme with 4N H_2SO_4 yielded a compound that behaved differently from biotin on chromatography in various systems. This compound has been tentatively identified as diaminobiotin both by its R_f values (0.5 in butanol:H_2O:acetic acid, 0.85 in propanol:H_2O and 0.45 in butanol:methanol:benzene H_2O) and by its support of growth of L. arabinosus. As shown in Table II, the prosthetic group of the isolated $C^{14}O_2 \sim$ biotin enzyme complex was 83% biotin and 17% "diaminobiotin" whereas treatment of the $C^{14}O_2 \sim$ biotin enzyme complex with acetyl CoA reduced the amount of biotin to 49% and increased the amount of diaminobiotin to 51%. The "native" carboxylase, isolated by the procedure of Waite and Wakil (2), on hydrolysis yielded 100% biotin as shown in Table II. Treatment of the "native" enzyme with acetyl-1-C^{14} CoA, followed by hydrolysis, yielded 4.4 mμmoles C^{14}-malonic acid, 33% biotin and 67% "diaminobiotin" (cf. Table II). The "diaminobiotin-enzyme" was reconverted to biotin-enzyme in the presence of HCO_3^-, ATP and Mn^{++}. Little or no conversion occurred in the absence of any one of these components, as shown in Table II.

Since the carboxylation reaction is reversible, it was of interest to study the conversion of biotin-enzyme to the diaminobiotin enzyme in the presence of ADP and P_i. This was accomplished in experiments comparable to the ones described for acetyl CoA (cf. Table II). Furthermore, in the presence of P_i^{32}, ADP and Mn^{++}, ATP^{32} was synthesized from biotin-enzyme (native enzyme) but not from "diamino-enzyme".

Growth of L. arabinosus on Ureido-C^{14}-labeled biotin: Earlier workers considered the possibility of the functional involvement of the ureido-carbon of biotin in the biological activity of this vitamin. Melville et al. (10) synthesized ureido-C^{14}-labeled biotin and studied the retention of the

TABLE II

Biotin and "diamino-biotin" content of various forms of acetyl CoA carboxylase

Form of carboxylase	Per cent*	
	biotin	diamino biotin
"Native" enzyme	100	0
" " + AcCoA	33	67
" " + ADP + P_i + Mn^{++}	47	53
$C^{14}O_2$~biotin enzyme complex	83	17
" " + AcCoA	49	51
Decarboxylated-biotin enzyme	12	88
" " + ATP + Mn^{++}		
+ HCO_3^-	80	20
" " + Mn^{++} + HCO_3^-	9	91
" " + ATP + HCO_3^-	26	74
" " + HCO_3^-	13	87

* The percentage of biotin and amino biotin were calculated from the amounts of growth of L. arabinosus after separation of the two compounds on paper chromatography.

The $C^{14}O_2$~biotin enzyme complex was prepared as described in Table I. The various cofactors were added essentially at concentrations comparable to that described in Table I.

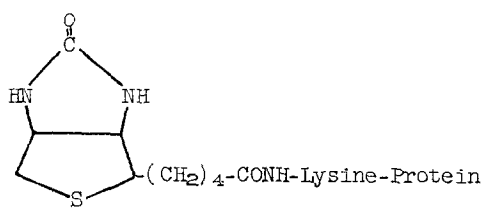
radioactivity in biotin after growth of L. arabinosus. The organisms were grown in the presence of 67 mμmoles of C^{14}-biotin per liter of medium. At the end of the incubation, 93% of the added C^{14}-biotin was reisolated from both media and cells. Such high recovery led them to conclude that the ureido-carbon atom of biotin was not involved in the mechanism of biotin action. However, Melville et al. used levels of C^{14} biotin in their media in excess of that required for optimal growth of this organism.

Ureido-C^{14}-biotin, synthesized by the procedure of Melville et al. (10), was added to growth medium for L. arabinosus in varying amounts. When the amount of biotin was limiting and bacterial growth was proportional to the amount of biotin added (40 to 160 μμmoles of biotin/l), no significant amount of radioactivity was recovered in the reisolated biotin despite the fact that up to 97% of the biotin added was accounted for by subsequent micro-

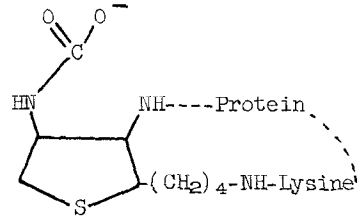
biological assays. However, on increasing the amount of C^{14}-biotin in the media to 5.9 mμmoles per l, up to 86% of the radioactivity in biotin was recovered with a total recovery of 114% of the biotin itself by microbiological assay. The loss of C^{14}, presumably as CO_2, corresponded to 14% of the added biotin (0.83 mμmoles), an amount sufficient for optimal growth of the bacteria. The C^{14} recovered in the biotin represented the excess biotin that was stored within the cells. Similarly, the loss of 7% of C^{14}-biotin (4.7 mμmoles of biotin per liter) observed by Melville et al., sufficed to account for optimal growth of the L. arabinosus, but its significance was not then apparent.

In summary our observations show: (1) $C^{14}O_2$ can be incorporated into the acetyl CoA carboxylase to the extent of one mole of $C^{14}O_2$ per mole of enzyme bound biotin; (2) hydrolysis of the $C^{14}O_2$~biotin enzyme yields free biotin containing <80% of the radioactivity; (3) the C^{14} of the isolated biotin is located exclusively in the ureido carbon atom of biotin; (4) "native" carboxylase yields biotin on hydrolysis whereas enzyme treated with AcCoA or ADP and P_i yields over 60% of a biotin derivative that has been tentatively identified as "diamino-biotin"; (5) the diaminobiotin enzyme can be reconverted to the biotin enzyme only in the presence of HCO_3^-, ATP and Mn^{++}; and (6) biotin which has been quantitatively recovered from L. arabinosus, grown on limited amounts of ureido-C^{14} biotin, lost almost all of its C^{14}.

These observations lead us to conclude that the ureido-carbon of enzyme-bound biotin is the 'active carbon' of biotin. Whether the active form of the enzyme-bound biotin is "closed" (IV) or "open" (V) remains to be determined.



IV



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