It has been difficult to obtain a homogeneous preparation of the biotinyl carboxyl carrier protein for determination of its amino acid sequence but limited proteolysis of transcarboxylase is useful in obtaining a large segment of the biotinyl carboxyl carrier protein for determination of the primary structure. Such studies are in progress.

These studies are but one of many in which useful information has been obtained regarding the structure of enzymes by use of controlled proteolysis (Mihalyi, 1972).

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Evidence that the Two Partial Reactions of Transcarboxylation Are Catalyzed by Two Dissimilar Subunits of Transcarboxylase†

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ABSTRACT: The results presented here show that isolated subunits of transcarboxylase specifically catalyze the two partial reactions of transcarboxylation as shown in eq 1–3. The 12S central subunit is active in the transcarboxylation with methylmalonyl-CoA but inactive with oxalacetate and the peripheral metallo 5S subunit is active in the transcarboxylation with oxalacetate but inactive with methylmalonyl-CoA. These subunits, likewise, are specific for the reverse partial reactions; the central subunit catalyzing transfer from the carboxylated biotinyl group to propionyl-CoA to yield methylmalonyl-CoA and the peripheral subunit to pyruvate to yield oxalacetate. Thus, the central subunit contains the sites for the CoA esters (methylmalonyl-CoA and propionyl-CoA) and the peripheral metallo subunits for the keto acids (oxalacetate and pyruvate). In the overall reaction the biotinyl carboxyl carrier protein acts as a shuttle to carry the carboxyl groups between the two subunits. Biotin and certain biotin analogs are inactive in these partial reactions but the ~40- or ~66-residue biotinyl peptides, which are derived from the carboxyl carrier protein, are active. Transcarboxylase can be reconstituted from its isolated subunits and a comparison was made of the rate of the overall reaction when the subunits were assembled, as in the intact enzyme, with that obtained when the reaction was catalyzed by the nonassembled subunits. In the latter case, since the biotinyl carboxyl carrier subunit must diffuse from one subunit to the other, the overall reaction is much slower than with the assembled subunits. The reaction with trypsinized transcarboxylase from which the ~66-residue and ~40-residue biotinyl peptides have been stripped, likewise, was slow even though the biotinyl peptides were added to the reconstitution mixture. The $12S_H$ and $5S_E$ subunits remain assembled after trypsin treatment but the biotinyl peptides apparently do not combine firmly or properly with the trypsinized enzyme and the biotinyl group apparently must oscillate as a carboxyl carrier between the two sites on the subunits by diffusion.

Transcarboxylase from propionibacteria is a biotinyl enzyme, but unlike the numerous other biotinyl enzymes which catalyze fixation of CO_2 (see Alberts and Vagelos, 1972; Moss and Lane, 1971, for reviews), it catalyzes a carboxyl transfer from one compound to another without involvement of carbon dioxide. Transcarboxylase is made up of three different subunits which have been isolated and purified (Wood et al., 1975; Berger and Wood, 1975) and it is shown here that the subunits of transcarboxylase specifical-

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ly catalyze two partial reactions which make up the overall reaction (eq 1-3). Thus, it is similar to acetyl-CoA carboxylase from *Escherichia coli* which Alberts and Vagelos (1972) and Moss and Lane (1971) have shown to consist of three dissimilar subunits: (a) the biotinyl carboxyl carrier protein, (b) biotin carboxylase which catalyzes the carboxylation of the carboxyl carrier protein with CO_2, and (c) the carboxyl transferase component which catalyzes the carboxyl transfer from the carboxyl carrier protein to acetyl-CoA. Transcarboxylase, likewise, contains a biotinyl carboxyl carrier protein but there are two different transferases; one catalyzes the carboxyl transfer from the carboxyl donor (methylmalonyl-CoA) to the biotinyl carboxyl carrier protein and the other catalyzes the transfer from the carboxylated biotinyl carrier protein to a carboxyl acceptor (pyruvate).

$$\begin{array}{l} {}^{14}COO^- \\ | \\ CH_3CHCOSCoA + \text{biotinyl subunit} \end{array} \overset{12S\ \text{subunit}}{\rightleftharpoons} {}^-OO^{14}C\text{-biotinyl subunit} + CH_3CH_2COSCoA \quad (1)$$

$${}^-OO^{14}C\text{-biotinyl subunit} + CH_3COCOO^- \overset{5S\ \text{subunit}}{\rightleftharpoons} \text{biotinyl subunit} + {}^-OO^{14}CCH_2COCOO^- \quad (2)$$

$$\text{Sum: } \begin{array}{l} {}^{14}COO^- \\ | \\ CH_3CHCOSCoA \end{array} + CH_3COCOO^- \rightleftharpoons CH_3CH_2COSCoA + {}^-OO^{14}CCH_2COCOO^- \quad (3)$$

Experimental Procedure

Materials

Enzymes. Inorganic pyrophosphatase was obtained from Worthington, lactate dehydrogenase, malate dehydrogenase, and galactose dehydrogenase from Boehringer. Carboxytransphosphorylase was purified from propionibacteria as described by Wood et al. (1969a).

Transcarboxylase was purified to near homogeneity from *Propionibacterium shermanii* and assayed spectrophotometrically as described previously (Wood et al., 1969b). Specific activities are in micromoles of products formed per minute per milligram of protein. Protein was determined by the method of Warburg and Christian (Layne, 1957).

40-Residue and 66-Residue Biotinyl Peptides. These peptides were prepared by mild treatment of transcarboxylase with trypsin as described by Ahmad et al. (1975). The resulting peptides were separated from the residual protein by chromatography over a Sephadex G-50 fine column. The leading edge of the peak containing the biotinyl peptides was largely made up of the 66-residue peptide and the trailing edge of the 40-residue peptide (Ahmad et al., 1975). The nanomoles of peptide was assumed to be equal to the nanomoles of biotin, i.e., one biotin/peptide. The biotin content was calculated by dividing the radioactivity of the peptides by the specific radioactivity of the biotin. The specific radioactivity of the biotin was determined by measuring the biotin content of a portion of the enzyme using a modification of the method of Green and Toms (1970) and by dividing its radioactivity by the determined nanomoles of biotin.

1.3S_E Subunit (*Biotin Carboxyl Carrier Protein*). This subunit was isolated as described by Wood et al. (1975) from transcarboxylase dissociated in 6 *M* urea plus 1 m*M* dithiothreitol and by chromatography on Bio-Gel A-1.5m in 6 *M* urea. It also was obtained from transcarboxylase dissociated at pH 9 and by chromatography on Bio-Gel A-1.5m in Tris-HCl at pH 9. The nanomoles of subunit were calculated on the basis of its biotin content, determined as described above.

5S_E Subunit. The majority of the studies were done with the subunit isolated from transcarboxylase that had undergone inactivation during its isolation and was designated "dead" transcarboxylase (Wood et al., 1975). The preparation was homogeneous as judged by its sedimentation profile and by gel electrophoresis in 6 *M* urea. The $5S_E$ subunit also was prepared by dissociation of the $6S_E$ subunit at pH 9 as described by Wood et al. (1975). This preparation contained a small amount of $6S_E$ subunit and perhaps a trace of $6S_H$ subunit. In addition, $5S_E$ subunit was used which was prepared from trypsinized transcarboxylase. The nanomoles of $5S_E$ subunit were calculated on the basis of the protein content and a molecular weight of 120,000.

12S_H Subunit. Most of the studies were done with $12S_H$ subunit isolated from "dead" transcarboxylase dissociated in 0.05 *M* Tris-HCl (pH 8.0) in 20% glycerol (Wood et al., 1975). This material was homogeneous as judged by its sedimentation profile and by gel electrophoresis in 6 *M* urea. The $12S_H$ subunit also was isolated from trypsinized transcarboxylase and contained a small amount of $6S_H$ material and from transcarboxylase that had been complexed with avidin-Sepharose and eluted from the avidin-Sepharose column with Tris-SO_4 at pH 8 in 20% glycerol (Berger and Wood, 1975). The nanomoles of $12S_H$ subunit were calculated on the basis of a molecular weight of 360,000.

Chemicals. Sodium [^{14}C]bicarbonate was obtained from New England Nuclear, coenzyme A from PL Biochemicals, and Sephadex from Pharmacia. Biotinyl acetate, biotin methyl ester, and biotinol were a generous gift from M. D. Lane and the biocytin (N^{ϵ}-biotinyl-L-lysine, L-511, 199-00V02) was the generous gift of Dr. Lewis Mandel, Merck Sharp and Dohme Research Labs, Inc. Other chemicals were the highest grade commercially available.

[4-^{14}C]Oxalacetate was synthesized using carboxytransphosphorylase (Wood et al., 1969a) linked to pyrophosphatase.

$$\text{P-enolpyruvate} + P_i + {}^{14}CO_2 \rightleftharpoons [4\text{-}{}^{14}C]\text{oxalacetate} + PP_i \quad (4)$$

$$PP_i \rightleftharpoons 2P_i \quad (5)$$

In a typical example, the reaction mixture contained in μmol/ml: P-enolpyruvate, 5.0; $NaH^{14}CO_3$ (40 Ci/mol), 10; potassium phosphate buffer (pH 6.5), 16.6; $MgCl_2$, 4.2; $CoCl_2$, 0.08; and in units, inorganic pyrophosphatase, 11; and carboxytransphosphorylase (crystalline, specific activity 15), 25. The mixture was incubated at room temperature for 15 min and then an equal volume of 0.1 *N* HCl was added and the solution was chilled in ice. The oxalacetate and pyruvate were assayed in one portion using malate dehydrogenase followed by lactate dehydrogenase and the P-enolpyruvate in a second portion using carboxytransphosphorylase after the oxalacetate was removed with malate dehydrogenase. The yield per milliliter was 2.66 μmol of oxalacetate and 0.24 μmol of pyruvate. The assay for unused P-enolpyruvate gave a value of 1.64 μmol. The samples from the assays were acidified with acetic acid and evaporated in vials for determination of the radioactivity in the malate and the cpm/nmol of oxalacetate was calculated from these values. The [^{14}C]oxalacetate was used without purification and was assayed prior to use each day. It slowly

decarboxylates and there is about a 25% loss after 3 days. It was neutralized just prior to use.

Propionyl-coenzyme A (unlabeled) was prepared by the procedure of Simon and Shemin (1953).

[3-^{14}C]Methylmalonyl-CoA was synthesized enzymatically using P-enolpyruvate, $^{14}CO_2$, propionyl-CoA, DPNH, galactose, carboxytransphophorylase, transcarboxylase, pyrophosphatase, lactate dehydrogenase, and galactose dehydrogenase. The pyrophosphatase and lactate dehydrogenase pull the reaction by removing PP_i and pyruvate and the galactose dehydrogenase regenerates the DPNH.

The reactions are as follows:

$$\text{P-enolpyruvate} + {}^{14}CO_2 + P_i \rightleftharpoons [4\text{-}^{14}\text{C}]\text{oxalacetate} + PP_i \quad (4)$$

$$PP_i \rightleftharpoons 2P_i \quad (5)$$

$$[4\text{-}^{14}\text{C}]\text{oxalacetate} + \text{propionyl-CoA} \rightleftharpoons [3\text{-}^{14}\text{C}]\text{methylmalonyl-CoA} + \text{pyruvate} \quad (3)$$

$$\text{pyruvate} + \text{DPNH} \rightleftharpoons \text{lactate} + \text{DPN} \quad (6)$$

$$\text{galactose} + \text{DPN} \rightleftharpoons \text{galactonate} + \text{DPNH} \quad (7)$$

The reaction mixture contained in μmol/ml: P-enolpyruvate, 8.0; potassium phosphate buffer (pH 6.6), 10; propionyl-CoA, 5.0; DPNH, 0.2; $MgCl_2$, 12.0; $CoCl_2$, 0.1; $NaH^{14}CO_3$ (0.15 Ci/g), 5; galactose, 23; and in units/ml, carboxytransphosphorylase, 2; transcarboxylase, 2; inorganic pyrophosphatase, 2; lactate dehydrogenase, 2; and galactose dehydrogenase, 2. The mixture was incubated at room temperature for 1.5 hr and then brought to pH 5 with 0.5 *N* HCl. A portion was assayed for methylmalonyl-CoA and for propionyl-CoA using transcarboxylase (Wood et al., 1963b). The yield per milliliter was 3.2 μmol of methylmalonyl-CoA and 1.4 μmol of propionyl-CoA were not utilized. It is likely that a better yield of methylmalonyl-CoA would be obtained if an excess of $NaH^{14}CO_3$ were used.

The coenzyme A thioesters were purified by anion exchange chromatography on DEAE-cellulose using gradients of LiCl (Fung, 1972). The concentrated coenzyme A thioesters were desalted over Sephadex G-10 columns and stored at −20°. Their concentrations were determined enzymatically as indicated previously. The radioactivity of the [3-^{14}C]methylmalonyl-CoA was determined and the cpm/nmol was calculated.

Methods

Radioactivity was measured in a dioxane-based scintillant in a Nuclear Chicago Mark I liquid scintillation counter equipped with an external standard.

Carboxylation of the 1.3S_E Biotinyl Carboxyl Carrier Protein or the Biotinyl Peptide(s) and Their Separation from Excess Substrate. The procedure was similar to that used by Wood et al. (1963b) in studies of the carboxylation of the intact transcarboxylase. The reactions were conducted at 0° for 10 min with either the 1.3S_E subunit or the biotinyl peptides as the carboxyl acceptor and with [3-^{14}C]methylmalonyl-CoA or [4-^{14}C]oxalacetate as the carboxyl donor and with the 12S_H or 5S_E subunits as the catalysts. The 12S_H subunit was considered to have 6 sites, the 5S_E subunit, 2 sites, and the 1.3S_E subunit or biotinyl peptide, 1 site, i.e., 1 site for each constituent peptide. On the basis of sites, an approximately equal amount of each subunit was used in most experiments. The [4-^{14}C]oxalacetate or [3-^{14}C]methylmalonyl-CoA was added in excess. Only the S form of methylmalonyl-CoA is utilized by transcarboxylase (Allen et al., 1963) and presumably by its subunits and thus the concentration of the active isomer was assumed at one-half the total. The determination of methylmalonyl-CoA with transcarboxylase was done in the presence of racemase, thus, both forms were measured. There is a gradual loss of methylmalonyl-CoA during storage at 0 or −20° (Wood et al., 1963b).

After 10 min of incubation the mixture (usually 0.2–0.4 ml) was transferred to a Sephadex G-50 coarse or Sephadex G-25 fine column and eluted with 0.1 *M* phosphate buffer (pH 6.5) at 4° to separate the carboxylated 1.3S_E subunit or carboxylated biotinyl peptide and the 5S_E or 12S_H subunit from the excess ^{14}C-labeled substrate. It was necessary to use Sephadex G-25 fine to accomplish the separation in the experiments with the biotinyl peptides.

The carboxyl-N_1'-biotin linkage (Wood et al., 1963b) is acid-heat labile yielding CO_2 and this property was used to measure the amount of carboxylated biotinyl groups. The total radioactivity was determined by making one portion alkaline so as to retain any $^{14}CO_2$ that might arise by decarboxylation of the carboxylated biotinyl groups. Acetic acid was added to a second portion which was evaporated under a heat lamp (see legend to Figure 1) for determination of ^{14}C in the acid-heat stable compounds. The latter value represents the free or protein bound [3-^{14}C]methylmalonyl-CoA still associated with the protein fraction following the chromatography on Sephadex. The nanomoles of carboxylated biotinyl groups (acid-heat labile) was calculated from the difference between the total and acid-heat stable radioactivity by dividing by the specific radioactivity of the methylmalonyl-CoA.

The procedure was modified in the experiments with [4-^{14}C]oxalacetate because oxalacetate is acid-heat labile yielding pyruvic acid and $^{14}CO_2$ (Krampitz et al., 1943). In these experiments any free or protein bound [4-^{14}C]oxalacetate was converted to malate using malate dehydrogenase and DPNH. The difference then was determined between the total radioactivity and acid-heat stable radioactivity (oxalacetate converted to malate) and the nanomoles of the carboxylated biotin was calculated by dividing this radioactivity by the specific radioactivity of the [4-^{14}C]oxalacetate.

Transfer to Pyruvate or Propionyl-CoA from the Carboxylated 1.3S_E Subunit or the Carboxylated Biotinyl Peptides. The specificity of the subunits was determined using the fractions from the Sephadex columns which contained the highest content of acid-heat labile radioactivity. Since transfer of a biotinyl-COO^- to propionyl-CoA yields methylmalonyl-CoA, which is an acid-heat stable compound, transfer was determined by measurement of the conversion of acid-heat labile radioactivity to the acid-heat stable form. When the transfer was to pyruvate, yielding oxalacetate, the oxalacetate was converted to malate prior to the determination of the acid-heat stable radioactivity.

Measurement of Carboxylation by Determination of Acid-Heat Labile Carboxyl Groups. The carboxylation of biotin and some of its analogs was tested by measurement of the acid-heat labile radioactivity formed from [3-^{14}C]methylmalonyl-CoA following incubation with the 12S_H subunit and biotin or its derivatives (Table III). In similar studies with the 5S_E subunit and [4-^{14}C]oxalacetate the oxalacetate was converted to malate before measurement of the acid-heat labile radioactivity.

Reconstitution of Transcarboxylase from the Subunits. The reconstitution was done in 0.75 *M* phosphate buffer as described by Wood et al. (1975).

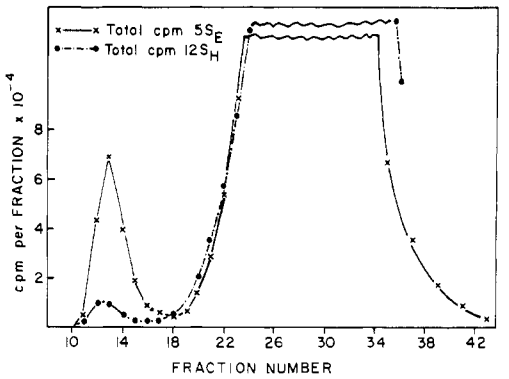

FIGURE 1: Evidence that the carboxylation of the 1.3S$_E$ subunit with [4-^{14}C]oxalacetate is catalyzed by the 5S$_E$ subunit and not by the 12S$_H$ subunit. The 5S$_E$ subunit or 12S$_H$ subunit was incubated with the 1.3S$_E$ subunit and radioactive oxalacetate and then chromatographed on a Sephadex G-50 column. The subunits occur in the void volume (fractions 10–15). *Carboxylation with the 5S$_E$ subunit.* The mixture (0.2 ml) contained 3.5 nmol of 5S$_E$ subunit from "dead" transcarboxylase, 7.0 nmol of 1.3S$_E$ subunit, 54 nmol of [4-^{14}C]oxalacetate (8.5 × 10^4 cpm/nmol) which was neutralized by the addition of 0.05 *M* Tris base, and 20 nmol of phosphate buffer, pH 6.5. Incubation was at 0° for 10 min. *Carboxylation with the 12S$_H$ subunit.* The procedure was the same as above except the 12S$_H$ subunit (1.07 nmol) was substituted for the 5S$_E$ subunit and 35 nmol of [4-^{14}C]oxalacetate (9.3 × 10^4 cpm/nmol) were used. *Separation of subunits from the excess [4-^{14}C]oxalacetate.* The reaction mixture (0.2 ml) was layered on a Sephadex G-50 (coarse) column 0.7 cm in diameter with an 18-ml bed volume and 6-ml void volume. The column had been equilibrated at 4° with 0.1 *M* phosphate buffer (pH 6.5). Elution was with the same buffer and 0.7-ml fractions were collected. Aliquots (0.5 ml) plus one drop of 0.2 *N* KOH, 1 ml of H_2O, and 10 ml of the scintillant were used for determination of the total radioactivity of each fraction. With the 12S$_H$ subunit, 32,700 cpm were present in fractions 9–16, equivalent to 0.35 nmol of [^{14}C]oxalacetate or 0.05 nmol/1.3S$_E$ subunit. With the 5S$_E$ subunit, 202,000 cpm were present in these fractions, equivalent to 2.37 nmol of [^{14}C]oxalacetate or 0.34 nmol/1.3S$_E$ subunit. *Determination of acid-heat labile counts.* Fractions 12 and 13 from the Sephadex column were combined and used for determination of this value by difference between total and acid-heat stable counts. The free or bound oxalacetate was converted to malate by addition of 0.01 ml of malate dehydrogenase (0.4 unit) and 0.01 ml of ~3 m*M* DPNH to 0.20 ml of the combined fractions 12 and 13. The mixture was incubated at 0° and 0.05-ml samples were removed at 5 and 10 min for determination of total and acid-heat stable radioactivity. The total radioactivity was determined as described above. The acid-heat stable radioactivity was determined by adding 4 drops of 1 *N* acetic acid to the sample which was evaporated under a heat lamp before adding the 1 ml of H_2O and 10 ml of scintillant. The values were nearly the same at 5 and 10 min and were averaged. With the 12S$_H$ subunit the average total cpm in fractions 12 and 13 was 13,700 and the average acid stable cpm was 8950; thus, there were 4750 cpm as acid-heat labile counts equivalent to 0.05 nmol of carboxylated biotinyl groups. With the 5S$_E$ subunit the total cpm was 61,620, the acid-heat stable cpm was 13,360. Therefore, there were 48,260 cpm as acid-heat labile counts, equivalent to 0.57 nmol of carboxylated biotinyl groups.

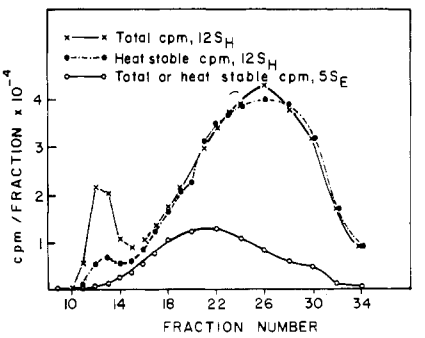

FIGURE 2: Evidence that the carboxylation of the 1.3S$_E$ subunit with [3-^{14}C]methylmalonyl-CoA is catalyzed by the 12S$_H$ subunit and not by the 5S$_E$ subunit. Both the total and heat stable radioactivity were determined in the fractions from the Sephadex G-50 column. The difference between the total and heat labile radioactivity represents the biotinyl carboxyl. The oxalacetate was converted to malate prior to heating the samples. *Carboxylation with the 12S$_H$ subunit.* The mixture (0.3 ml) contained 2.13 nmol of 12S$_H$ subunit from "dead" transcarboxylase, 12 nmol of 1.3S$_E$ subunit, 73 nmol of [3-^{14}C]methylmalonyl-CoA (13,000 cpm/nmol), and about 20 nmol of phosphate buffer (pH 6.5). Incubation was at 0° for 5 min. *Carboxylation with the 5S$_E$ subunit.* The mixture (0.5 ml) contained 2.19 nmol of 5S$_E$ subunit from "dead" transcarboxylase, 4.5 nmol of 1.3S$_E$ subunit, 24.3 nmol of [3-^{14}C]methylmalonyl-CoA, and 43 nmol of phosphate buffer (pH 6.8). Incubation was at 0° for 10 min. *Separation of subunits from excess [3-^{14}C]methylmalonyl-CoA.* In each case 0.3 ml was layered on a Sephadex G-50 column as in Figure 1 and 0.7-ml fractions were collected and total and acid-heat stable radioactivity was determined on an 0.05-ml aliquot of each fraction by addition of 1 drop of 0.2 *N* KOH solution to one sample and 4 drops of 1 *N* acetic acid to the other. The latter was evaporated under a heat lamp before addition of 1 ml of H_2O and 10 ml of scintillant. With the 5S$_E$ subunit, there was no evidence of carboxylated biotinyl groups since the total and acid-heat stable cpm per fraction were the same within experimental error and there was very little protein bound radioactivity. With the 12S$_H$ subunit in fractions 11–15, the total radioactivity was 67,760 cpm and the acid-heat stable radioactivity was 26,670 cpm. Thus, there were 41,090 acid labile counts in these fractions or 3.16 nmol of carboxylated biotinyl groups, equivalent to 0.26 nmol/1.3S$_E$ subunit.

Results

Carboxylation of the 1.3S$_E$ Biotinyl Carboxyl Carrier Protein and the Biotinyl Peptides

Comparison of the Carboxylation of the 1.3S$_E$ Subunit with [4-^{14}C]Oxalacetate by the 5S$_E$ and 12S$_H$ Subunits. The results obtained are shown in Figure 1. There was much more radioactivity in the protein peak when the 5S$_E$ subunit was used as the catalyst than with the 12S$_H$ subunit. Not all of the radioactivity of the protein peak is due to carboxylated biotinyl groups since some [4-^{14}C]oxalacetate was present as free or protein-bound [4-^{14}C]oxalacetate. Fractions 12 and 13, which contained the highest radioactivity, were pooled and used to determine the total and acid-heat labile radioactivity. With the 12S$_H$ subunit there was only 0.05 nmol of carboxylated biotinyl groups formed but with the 5S$_E$ subunit there was 0.57 nmol. Clearly, the 5S$_E$ subunit catalyzed carboxylation of the biotinyl groups with oxalacetate and there was little or no carboxylation with the 12S$_H$ subunit.

Comparison of the Carboxylation of the 1.3S$_E$ Subunit with [3-^{14}C]Methylmalonyl-CoA by the 5S$_E$ and 12S$_H$ Subunits. These results are shown in Figure 2. More radioactivity was found in the protein peak with the 12S$_H$ subunit than with the 5S$_E$ subunit. There was considerable [3-^{14}C]methylmalonyl-CoA (acid-heat stable) associated with the protein fractions with the 12S$_H$ subunit and after correction for this radioactivity the difference was equivalent to 0.26 nmol of carboxylated biotinyl groups/nmol of 1.3S$_E$ subunit. In the experiment with the 5S$_E$ subunit there was no indication of acid-heat labile radioactivity since the total and acid-heat stable radioactivity were the same. Clearly, the 12S$_H$ subunit and not the 5S$_E$ subunit catalyzes carboxylation of the 1.3S$_E$ subunit with methylmalonyl-CoA.

Carboxylation of the Biotinyl Peptides with [3-^{14}C]Methylmalonyl-CoA by the 12S$_H$ Subunit and with [4-^{14}C]Oxalacetate by the 5S$_E$ Subunit. The biotinyl peptides isolated following mild treatment of transcarboxylase with trypsin were active as carboxyl acceptors. The results obtained with the 66-residue biotinyl peptide, [4-^{14}C]oxalacetate, and the 5S$_E$ subunit and also with a mixture of the 40- and 66-residue biotinyl peptides, [3-^{14}C]methylmalon-

yl-CoA, and the $12S_H$ subunit are shown in Figure 3. In these experiments, Sephadex G-25 was used and there was adequate separation of the protein and biotinyl peptides from the ^{14}C-labeled substrates. With the $12S_H$ subunit and [3-^{14}C]methylmalonyl-CoA, 0.3 nmol of the biotinyl groups/nmol of biotinyl peptide were carboxylated and with the $5S_E$ subunit and [4-^{14}C]oxalacetate, 0.18 nmol. Tests with fractions 11, 12, and 13 and malate dehydrogenase in the [4-^{14}C]oxalacetate experiment showed that 97% of the total radioactivity of the protein peak was in the form of carboxylated biotinyl groups (i.e., acid-heat labile). In results not shown, there was no significant carboxylation of the biotinyl peptides when the $5S_E$ subunit was used with [3-^{14}C]methylmalonyl-CoA or when the $12S_H$ subunit was used with [4-^{14}C]oxalacetate, again demonstrating that the $12S_H$ subunit catalyzes partial reaction 1 and the $5S_E$ subunit, partial reaction 2. Also, in results not shown, the 40-residue biotinyl peptide was found to be effective as a carboxyl acceptor with both subunits in the presence of the appropriate substrate.

Transfer from the Carboxylated 1.3S_E Subunit and from the Carboxylated Biotinyl Peptides to Carboxyl Acceptors

For these experiments, carboxylated biotinyl $1.3S_E$ subunits or carboxylated biotinyl peptides were prepared using [3-^{14}C]methylmalonyl-CoA and the $12S_H$ subunit or [4-^{14}C]oxalacetate and the $5S_E$ subunit as in the experiments of Figures 1–3. About 2.5 hr was required for carboxylation of the biotinyl subunit, separation on the Sephadex G-25 column, and completion of the transfer experiments. The half-life of the carboxylated enzyme is about 4.2 hr at 0° (Wood et al., 1963b).

Carboxyl Transfer with the 5S_E Subunit. The results of these experiments are given in Table I. The $5S_E$ subunit from "dead" transcarboxylase was used in experiments 1, 4, 5, from normal transcarboxylase in experiment 2, and from trypsinized transcarboxylase in experiment 3. Column A of Table I lists the total nanomoles of ^{14}C in the fractions used for the carboxyl transfer and consists of the carboxylated biotinyl groups plus the [^{14}C]oxalacetate complexed with the enzyme. It was determined as radioactivity in an alkaline sample. In column B, the ^{14}C in the oxalacetate per se is given (i.e., acid-heat stable radioactivity after reaction with DPNH and malate dehydrogenase). In column C the total ^{14}C in oxalacetate, after transfer from the carboxylated biotinyl group to pyruvate has occurred, is given and was determined as acid-stable radioactivity after reaction with DPNH and malate dehydrogenase. The last column gives the percent of the biotinyl carboxyl transferred to the pyruvate: C − B is the increase in oxalacetate due to carboxyl transfer to pyruvate and A − B is the total carboxylated biotin (acid labile radioactivity other than residual oxalacetate). The transfer varied from 57 to 100% and was very rapid, usually being complete at the time of the first test, ~1 min. When the experiments were done with propionyl-CoA, replacing pyruvate as the acceptor, less than 1% of the acid labile carboxylated biotinyl groups were converted to an acid stable form, i.e., to methylmalonyl-CoA. Clearly, the $5S_E$ subunit uses only the keto acids as substrates.

Carboxyl Transfer with the 12S_H Subunit. The results of these experiments are presented in Table II. The $12S_H$ subunit from "dead" transcarboxylase was used in experiments 1 and 4, from trypsinized transcarboxylase in experiments 2 and 5 and from an avidin-Sepharose preparation in experiment 3. The procedure was the same as in Table I except the substrate used to carboxylate the biotinyl group and the product of the carboxyl transfer to propionyl-CoA, [3-^{14}C]methylmalonyl-CoA, is acid-heat stable; thus, no reaction is necessary to stabilize the compound. The percent transfer to propionyl-CoA varied from 53 to 94%. When pyruvate was substituted for propionyl-CoA and the resulting oxalacetate was stabilized by conversion to malate, 2.5–7.6% of the carboxyls were found to be transferred to pyruvate. It is possible that the $12S_H$ subunits were contaminated with a small amount of $5S_E$ subunit which catalyzed this slow reaction.

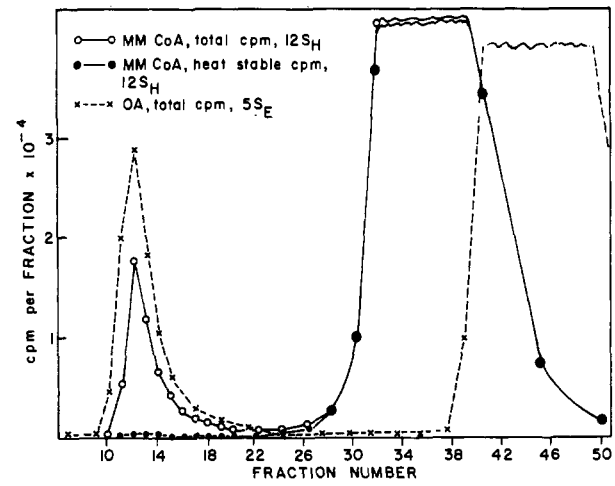

FIGURE 3: Evidence that the biotinyl peptides are carboxylated with [3-^{14}C]methylmalonyl-CoA (MMCoA) by the $12S_H$ subunit and with [4-^{14}C]oxalacetate (OA) by the $5S_E$ subunit. There were no significant acid stable counts in fractions 11, 12, and 13 after conversion of the oxalacetate in these fractions to malate. Chromatography was with Sephadex G-25 in this experiment. *Carboxylation with the 12S_H subunit.* The mixture (0.30 ml) contained 1.10 nmol of $12S_H$ subunit from "dead" transcarboxylase, 13.8 nmol of about an equal mixture of 40- and 66-residue biotinyl peptides, and 65.6 nmol of [3-^{14}C]methylmalonyl-CoA (13,000 cpm/nmol) and 20 nmol of phosphate buffer (pH 6.8). Incubation was at 0° for 10 min. *Carboxylation with the 5S_E subunit.* The mixture (0.34 ml) contained 5.5 nmol of $5S_E$ subunit from "dead" transcarboxylase, 7.5 nmol of 66-residue biotinyl peptide, 48 nmol of [3-^{14}C]oxalacetate (78,000 cpm/nmol) neutralized with 0.1 *M* Tris base just prior to use, and 16 nmol of phosphate buffer, pH 6.8. Incubation was at 0° for 10 min. *Separation of biotinyl peptides and subunits from the excess ^{14}C-labeled substrates.* The mixtures were each layered on Sephadex G-25 (fine) columns 1.7 cm in diameter, 42-ml bed volume, and 18-ml void volume which had been equilibrated at 4° with 0.1 *M* phosphate buffer (pH 6.8). Elution was with the same buffer and 12 ml was collected in the methylmalonyl-CoA experiment and 13 ml in the oxalacetate experiment prior to collection of 0.55-ml fractions; 0.05-ml portions were removed from each of these fractions to determine total and acid-heat stable radioactivity in the methylmalonyl-CoA experiment as described above. Only total radioactivity was determined in the oxalacetate experiment. With the $12S_H$ subunit and [3-^{14}C]methylmalonyl-CoA the total radioactivity of fractions 10–22 was 56,400 cpm and the acid-heat stable radioactivity was 3000 cpm. Thus, the acid-heat labile radioactivity was 53,400 cpm which was equivalent to 4.1 nmol of carboxylated biotinyl groups or 0.3 nmol/biotinyl peptide. With the $5S_E$ subunit and [4-^{14}C]oxalacetate the total radioactivity of fractions 9–21 was 102,400 cpm which was equivalent to 1.3 nmol of [4-^{14}C]oxalacetate or 0.18 nmol/66-residue biotinyl peptide. *Determination of the acid-heat labile radioactivity in the [^{14}C]oxalacetate experiment.* Fractions 11, 12, and 13 were combined and to a 0.3-ml fraction was added 0.05 ml of 0.1 *M* phosphate buffer, pH 6.8; 0.01 ml of 0.03 M DPNH, and 0.005 ml of malate dehydrogenase (0.4 unit). Incubation was at 0° and at 1, 5, and 10 min, 0.05-ml portions were removed for determination of total radioactivity and acid-heat stable radioactivity. The average total radioactivity was 8212 cpm and the acid-heat stable radioactivity 241 cpm, thus, the acid-heat labile radioactivity was 7971 cpm or 97% of the total.

Table I: Catalysis by the $5S_E$ Subunit of the Transfer from the Carboxylated $1.3S_E$ Subunit or Biotinyl Peptides to Pyruvate.[a]

	Total ^{14}C (A) (nmol)	^{14}C in Oxalacetate (B) (nmol)	^{14}C in Oxalacetate after Transfer (C) (nmol)	$^{14}COO^-$ Transfer to Pyruvate [(C − B)/(A − B)] 100 (%)
1. $1.3S_E$ subunit	1.07	0.03	1.05	98
2. $1.3S_E$ subunit	0.80	0.04	0.47	57
3. $1.3S_E$ subunit	0.31	0.02	0.21	66
4. 66-residue peptide	0.61	0.01	0.61	100
5. 40-residue peptide	0.66	0.02	0.66	100

[a] The $1.3S_E$ subunit or biotinyl peptides were carboxylated with [4-^{14}C] oxalacetate using the $5S_E$ subunit and were separated from the excess [^{14}C]oxalacetate by Sephadex filtration as illustrated in Figures 1–3. The fractions with the highest amount of acid-heat labile radioactivity were pooled and aliquot portions were used for the following procedures. To 0.3 ml of the pool, 5 μmol of pyruvate, 0.3 μmol of DPNH, and 0.37 unit of malate dehydrogenase were added (final volume, 0.37 ml) and the mixture was incubated at 0°. 0.05-ml samples were removed usually at 1, 5, and 10 min and the total (column A) and the acid-heat stable radioactivity (column C) determined as described in the legend of Figure 1. C is the amount of biotinyl carboxyl groups transferred to pyruvate yielding oxalacetate plus the subunit bound oxalacetate. The procedure used for the values of column B was as in C except the pyruvate was omitted. The preparation of the carboxylated 1.35_E subunit or biotinyl peptides was done as in Figure 1. The mixtures contained in nmoles: expt 1, $5S_E$ from "dead" transcarboxylase, 3.5; $1.3S_E$ (urea preparation), 7.0; [4-^{14}C] oxalacetate (85,000 cpm/nmol), 54.0; volume, 0.2 ml; expt 2, $5S_E$ subunit (from $6S_E$ subunit), 1.75; $1.3S_E$ subunit (urea prep.), 5.28; and [4-^{14}C]oxalacetate (56,000 cpm/nmol), 62.8; volume, 0.2 ml; expt 3, $5S_E$ subunit (from trypsinized transcarboxylase), 1.27; $1.3S_E$ subunit (urea prep.), 3.52; [4-^{14}C]oxalacetate (56,000 cpm/nmol), 6.28; volume, 0.2 ml; expt $4.5S_E$ subunit (from "dead" transcarboxylase), 5.5; 66-residue biotinyl peptide, 7.5; [4-^{14}C]oxalacetate (78,000 cpm/nmol), 48; volume, 0.34 ml; expt 5, $5S_E$ subunit (from "dead" transcarboxylase), 4.75; 40-residue biotinyl peptide, 6.22; [4-^{14}C]oxalacetate (96,000 cpm/nmol), 44; volume, 0.305 ml.

Table II: Catalysis by the $12S_H$ Subunit of the Transfer from the Carboxylated $1.3S_E$ Subunit or Biotinyl Peptides to Propionyl-CoA.[a]

	Total ^{14}C (A) (nmol)	^{14}C in Methylmalonyl-CoA (B) (nmol)	^{14}C in Methylmalonyl-CoA after Transfer (C) (nmol)	COO^- Transfer to Propionyl-CoA [(C − B)/(A − B)] 100 (%)
1. $1.3S_E$ subunit	2.03	0.08	1.83	89
2. $1.3S_E$ subunit	1.66	0.01	1.38	83
3. $1.3S_E$ subunit	0.55	0.06	0.32	53
4. 40-, 66-residue peptides	2.15	0.03	2.03	94
5. 40-, 66-residue peptides	0.47	0.04	0.43	91

[a] The procedure was the same as described in the legend of Table I except that the $12S_H$ subunit activates the CoA esters and therefore [3-^{14}C]methylmalonyl-CoA was used as the carboxyl donor instead of [4-^{14}C]oxalacetate and propionyl-CoA as the carboxyl acceptor. Therefore, it was not necessary to stabilize the product. The carboxylation mixture contained in nmoles: expt 1, $12S_H$ subunit from "dead" transcarboxylase, 2.13; $1.3S_E$ subunit (urea preparation), 12; [3-^{14}C] methylmalonyl-CoA (13,000 cpm/nmol), 73; volume, 0.3 ml; expt 2, $12S_H$ subunit from trypsinized transcarboxylase, 0.554; $1.3S_E$ subunit (urea preparation), 8.8; [3-^{14}C] methylmalonyl-CoA (13,000 cpm/nmol), 36; volume, 0.30 ml; expt 3, $12S_H$ subunit (avidin-Sepharose preparation), 0.64; $1.3S_E$ subunit (urea preparation), 4.2, [3-^{14}C] methylmalonyl-CoA (13,000 cpm/nmol), 40; volume, 0.32 ml; expt 4, $12S_H$ subunit from "dead" transcarboxylase, 1.105; mixture of 66- and 40-residue biotinyl peptides, 13.8; [3-^{14}C]methylmalonyl-CoA (13,000 cpm/nmol), 65; volume, 0.30 ml; expt 5, $12S_H$ subunit from trypsinized transcarboxylase, 0.553; mixture of 66- and 40-residue biotinyl peptides, 3.84; [3-^{14}C] methylmalonyl-CoA, 35; volume, 0.30 ml.

Attempts to Carboxylate Biotin and Its Derivatives

Transcarboxylase does not carboxylate free biotin but it seemed possible that biotin or its derivatives might be active in the partial reaction with the $5S_E$ or $12S_H$ subunits since there would not be competition by the bound biotinyl groups as in the intact enzyme. The usual test by Sephadex filtration could not be used since the carboxylated biotin is not separated from the [3-^{14}C]methylmalonyl-CoA or [4-^{14}C]oxalacetate by gel filtration. However, the carboxylation reaction can be measured by determination of the decrease in the acid-heat stable radioactivity of the [3-^{14}C]methylmalonyl-CoA. When [4-^{14}C]oxalacetate was used, it was converted to malate prior to the acid-heat treatment. It is seen in Table III that with the $12S_H$ subunit and [3-^{14}C]methylmalonyl-CoA and with the $5S_E$ subunit and [4-^{14}C]oxalacetate, there was a decrease in the acid-heat stable radioactivity in the presence of the $1.3S_E$ subunit or the mixture of the 66- and 40-residue biotinyl peptides but not in the presence of the biotin analogs. Similar results were obtained in tests with biotin per se and with biocytin. The biotin analogs were used because Polakis et al. (1974) have found that these compounds are more active than biotin per se in the transcarboxylation from malonyl-CoA to the biotin derivatives as catalyzed by acetyl-CoA carboxylase of *Escherichia coli*.

Comparison of the Catalysis of the Overall Transcarboxylation Reaction by the Subunits and Biotinyl Peptides before and after Reconstitution

The fact that transcarboxylase can be reconstituted from its subunits permits comparison between the rate of the overall reaction when catalyzed by the unassembled subunits with the rate after partial assembly of the subunits to a reconstituted enzyme. An acid pH and high phosphate and protein concentrations favor reconstitution of the subunits to the intact enzyme (Jacobson et al., 1970). Therefore, the activity of the nonassembled subunits was assayed at pH 7.4 in 0.02 *M* phosphate buffer to reduce the possibility of the assembly of the subunits during the assay. Tests were conducted with the $1.3S_E$ subunit, biotinyl peptides,

Table III: Attempts to Carboxylate Analogs of Biotin.[b]

Substrate	Components		Acid-Heat Stable Radioactivity (cpm)	Acid-Heat Labile Radioactivity (cpm)	Carboxylated Biotinyl Group (nmol)
	Non-Biotinyl	Biotinyl			
[3-^{14}C]Methylmalonyl-CoA	$12S_H$ subunit	None	4730		
	$12S_H$ subunit	$1.3S_E$ subunit	2980	1750	0.39
	$12S_H$ subunit	Biotinyl peptide	3790	1030	0.22
	$12S_H$ subunit	Biotinyl acetate	4740[a]		
	$12S_H$ subunit	Biotin methyl ester	4810[a]		
	$12S_H$ subunit	Biotinol	4860[a]		
[4-^{14}C]Oxalacetate	$5S_E$ subunit	None	7430		
	$5S_E$ subunit	$1.3S_E$ subunit	5490	1940	0.38
	$5S_E$ subunit	Biotinyl peptide	5910	1520	0.30
	$5S_E$ subunit	Biotinol	7490[a]		
	$5S_E$ subunit	Biotin methyl ester	7580[a]		

[a] Not a significant difference from the value with no biotin derivative, the variation is about ±125 cpm in these experiments. [b] The reaction was conducted in vials for scintillation counting and contained in 0.08 ml of 0.05 *M* phosphate buffer, (pH 6.8): 500 nmol of biotinyl acetate or biotin methyl ester or biotinol or 0.58 nmol of the biotinyl peptides, or 0.50 nmol of the $1.3S_E$ subunit, 0.9 nmol of [3-^{14}C] methylmalonyl-CoA (~5100 cpm/nmol), 0.015 nmol of $12S_H$ subunit, and 0.14 units of methylmalonyl racemase. The biotinyl acetate, biotin methyl ester, and biotinol were dissolved in 40% ethanol and the final concentration of ethanol in the reaction mixture was 5%. This concentration of alcohol was included in the reactions with the $1.3S_E$ subunit and with the biotinyl peptides. Control experiments without alcohol showed the alcohol had no effect on the rate of the reaction with either the $12S_H$ subunit or the $5S_E$ subunit. The reactions with the $5S_E$ subunit were the same except the volume was 0.6 ml and 0.026 nmol of $5S_E$ and 1.7 nmol of [4-^{14}C] oxalacetate (4500 cpm/nmol) were used in place of the $12S_H$ subunit and the [3-^{14}C] methylmalonyl-CoA and no racemase was included. Incubation was for 15 min at room temperature with both subunits. In the experiments with [3-^{14}C] methylmalonyl-CoA the reaction was stopped by addition of 2 drops of 1 *N* acetic acid and solution was evaporated to dryness under a heat lamp and with warm air from a hairdryer blowing on the vials. When oxalacetate was the substrate the reaction was stopped by addition of 0.03 ml containing 0.25 unit of malate dehydrogenase, 0.3 unit of lactate dehydrogenase, and 6.8 nmol of DPNH. After 3 min at room temperature, 2 drops of 1 *N* acetic acid was added and the mixture was evaporated as above; 0.4 ml of H_2O and 4.0 ml of scintillant were added for determination of the radioactivity in a Packard TriCarb scintillation counter.

free biotin, and with the trypsin treated enzyme from which the biotinyl peptides had been removed. The results are shown in Table IV. The tests were done under two sets of conditions for each group of subunits, one by assaying under the conditions not favoring assembly of the subunits and the second by assaying after incubation of the mixture under conditions (high phosphate concentration) which favor reassembly of the subunits. The activities are expressed on the basis of the $12S_H$ subunit since it is necessary to have an excess of the $1.3S_E$ and $5S_E$ subunit to cause combination of a substantial part of the $12S_H$ subunit with the peripheral subunits (Wood et al., 1975). The results show that the specific activities were low with all combinations except the mixtures of $5S_E$, $12S_H$, and $1.3S_E$ subunits following reconstitution.

Discussion

The present views of the structure of transcarboxylase and the mechanism of its catalysis are illustrated very schematically in Figure 4. The enzyme ($s_{20,w}$ = 18 S, molecular weight, 790,000) is made up of the $12S_H$ cylindrical central subunit consisting of six peptides each of molecular weight 60,000. To this $12S_H$ subunit are attached three $5S_E$ metallo subunits through six $1.3S_E$ biotinyl carboxyl carrier subunits. The proposed quaternary structure of the enzyme is based on electron microscopy of the enzyme and its subunits (Green et al., 1972). The electron micrographs indicate that the peripheral subunits are at only one end of the cylindrical hexameric subunit and that there is a variable gap between the peripheral $5S_E$ subunits and the $12S_H$ subunit. It, therefore, was proposed that the $1.3S_E$ subunit occurs between the $5S_E$ and $12S_H$ subunits. This prediction was verified by Ahmad et al. (1975) who showed that the $1.3S_E$ subunit is required for binding of the $5S_E$ subunit to the $12S_H$ subunit. However, only a portion of this carboxyl carrier subunit, which does not carry the biotin, is required for the binding and the portion carrying the biotin apparently is in an exposed position and is very susceptible to cleavage by trypsin (Ahmad et al., 1975). The biotinyl peptide is indicated to be flexible so that biotinyl group may be located near the binding sites for the biotinyl ring (indicated by V's), near the keto acid sites on the $5S_E$ subunit indicated by circles and near the CoA ester sites on the $12S_H$ subunit (indicated by squares in Figure 4). There are six biotinyl groups per transcarboxylase of molecular weight 790,000, one per carboxyl carrier protein (Gerwin et al., 1969), and it has been proposed that there are six cobalt plus zinc atoms per mole although the assayed amount was found to be 7 to 8 atoms (Ahmad et al., 1972). These metals are very firmly bound to the $5S_E$ subunit. Northrop and Wood (1969a) on the basis of nuclear magnetic resonance studies proposed that the keto acids may be coordinated with the cobalt. More recently, Fung et al. (1974) have reported that in addition to cobalt and zinc, transcarboxylase contains tightly bound copper and the total Cu, Co, and Zn content is 12 ± 1 g-atoms/790,000 molecular weight. Their studies by electron paramagnetic resonance with [1-^{13}C]- and [2-^{13}C]pyruvate indicate that the keto acid forms a second sphere complex with the metals, possibly with water intervening between the metals.

Previously it was shown by kinetic studies (Northrop, 1969; Northrop and Wood, 1969b) that the transcarboxylase mechanism is "ping pong" with independent binding sites for the keto acids and for the coenzyme A esters. The present results extend these observations and demonstrate that the sites for the keto acids (pyruvate and oxalacetate) are on the peripheral metallo $5S_E$ subunits and those for the coenzyme A esters (methylmalonyl-CoA and propionyl-CoA) are on the central $12S_H$ subunit and that the $12S_H$ central subunit catalyzes partial reaction 1 and the $5S_E$ metallo subunit catalyzes partial reaction 2.

As noted in the introduction, acetyl-CoA carboxylase from *Escherichia coli* (Alberts and Vagelos, 1972; Moss and Lane, 1971) is made up of three subunits with functions

Table IV: Comparison of the Catalysis of the Overall Transcarboxylase Reaction with Subunits and Biotinyl Peptides before and after Reconstitution.[a]

No.	Reconstituted	Non-Biotinyl Component	Biotinyl Component	Specific Activity on Basis of $12S_H$ Subunit
1	No	$5S_E$ subunit and $12S_H$ subunit	$1.3S_E$ subunit	0.38
2	Yes			10.0
3	No	$5S_E$ subunit and $12S_H$ subunit	Biotinyl peptides	0.14
4	Yes			0.29
5	No	Trypsinized transcarboxylase	Biotinyl peptides	0.12
6	Yes			0.08
7	No	$5S_E$ subunit and $12S_H$ subunit	Biotin	0.00

[a] The activities are calculated on the basis of the $12S_H$ subunit which was made limiting in the reconstitution (see experiment 2). With trypsinized transcarboxylase, the activity was calculated on the basis of a $12S_H$ content of ~50%. For the assay of the activity on nonassembled subunits the components were added to the cuvet and readings were made at 340 mμ. The concentrations in micromoles per 0.3 ml were: pyruvate, 1; methylmalonyl-CoA, 0.15; DPNH, 0.06; potassium phosphate buffer (pH 7.4), 5; malate dehydrogenase, 0.28 units, and the following nanomoles of subunits: expt 1, $12S_H$ subunit, 0.09; $5S_E$ subunit, 0.84; $1.3S_E$ subunit, 12; expt 3, $12S_H$ subunit, 0.09; $5S_E$ subunit, 0.84; biotinyl peptides, 3.7; expt 5, trypsinized transcarboxylase, 0.069; biotinyl peptides, 3.7; expt 7; $12S_H$ subunit, 0.54; $5S_E$ subunit, 1.68; *d*-biotin, 21. In expt 1, the $12S_H$, $5S_E$, and $1.3S_E$ subunits were added sequentially and there was no change in optical density until the $1.3S_E$ subunit was added nor was there any significant reaction with any combination of two subunits. In experiment 3, there was a small rate of change in optical density with the combination of biotinyl peptide and $12S_H$ subunit but more than a 90% increase in rate after addition of the $5S_E$ subunit. None of the components had activity alone. A correction was made for this small unexplained activity by the $12S_H$ subunit plus biotinyl peptide. The trypsinized transcarboxylase had some activity in the absence of the biotinyl peptide apparently due to a very small residue of intact transcarboxylase. This value was subtracted from that obtained on addition of the biotinyl peptide. Reconstitutions were done by addition of 0.13 ml of 1.5 *M* phosphate buffer (pH 6.5) to an equal volume containing the following nanomoles of subunits: experiment 2, $12S_H$ subunit, 0.17; $5S_E$ subunit, 1.68, $1.3S_E$ subunit, 12; experiment 4, $12S_H$ subunit, 0.54; $5S_E$ subunit, 2.27; biotinyl peptides, 5.0; expt 6, trypsinized transcarboxylase, 0.44; biotinyl peptides, 5,0. The mixtures were held in ice and aliquot portions assayed as described by Wood et al. (1969b). Maximum activity was reached in 4 days or less. There was no significant activity with any combination of subunits used in experiment 2 except with the complete combination. In experiment 4, a correction was made for a small activity in the absence of the biotinyl peptide and in experiment 6, for a substantial activity (60%) with trypsinized transcarboxylase in the absence of the biotinyl peptide.

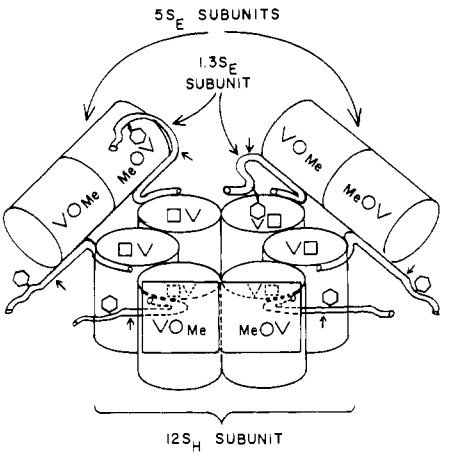

FIGURE 4: Diagrammatic representation of the structure of transcarboxylase and the mechanism of the transcarboxylation reaction. The keto acid and divalent metal sites (indicated by O and Me) occur on the metallo peripheral subunits and the CoA ester sites (□) are on the central subunit. The binding sites for the biotinyl ring (V) are presumed to be near the substrate sites and the biotinyl groups serve as a carboxyl carrier between the different substrate sites. The arrows indicate cleavage by trypsin to yield the biotinyl peptides.

much like those of the three subunits of transcarboxylase. Clearly, since transcarboxylase utilizes either acetyl-CoA or propionyl-CoA as an acceptor (Wood et al., 1963a) the $12S_H$ subunit of transcarboxylase and the transferase component of the acetyl-CoA carboxylase of *E. coli* can catalyze very similar partial reactions.

Acetyl-CoA carboxylase from *E. coli* dissociates very readily and the intact enzyme has never been isolated or reconstituted from its subunits. On the other hand, the acetyl-CoA carboxylase from spinach chloroplasts has been isolated and has been shown to be made up of carboxyl carrier, carboxylase, and transcarboxylase subunits (Kannangara and Stumpf, 1972). The acetyl-CoA carboxylase from wheat germ (Heinstein and Stumpf, 1969) likewise has been isolated but so far only two active components have been obtained by dissociation, one of which is the carboxylase subunit and the other the transcarboxylase subunit; the biotinyl carboxyl carrier protein has not been observed. The enzyme from animals apparently can only be dissociated under denaturating conditions (Gregolin et al., 1968; Inoue and Lowenstein, 1972).

The dissociation of tetrameric pyruvate carboxylase from avian liver by cold treatment has been studied extensively and the dissociation is reversible but the carboxyl carrier protein, if present, apparently is covalently attached to the resulting enzymatically inactive monomer (Scrutton and Young, 1972). Recently, Nakashima et al. (1975) have shown that dissociation of rat liver pyruvate carboxylase yields monomers which retain enzymatic activity, thus, all the components for the two partial reactions are retained in the monomer.

The fact that transcarboxylase can be reconstituted from its subunits permitted comparison between the rate of the overall reaction when the isolated subunits were not assembled with that of the assembled enzyme following reconstitution. Catalysis by the nonassembled subunits requires (1) that the biotinyl group of the $1.3S_E$ subunit be carboxylated by methylmalonyl-CoA through catalysis of the $12S_H$ subunit (reaction 1), (2) that the carboxylated $1.3S_E$ subunit dissociate from the $12S_H$ subunit, (3) that the carboxylated $1.3S_E$ subunit then combine with the $5S_E$ subunit, and (4) that the $5S_E$ subunit catalyze the transfer of the carboxyl from the carboxylated $1.3S_E$ subunit to pyruvate to yield oxalacetate (reaction 2). As expected, when the subunits catalyzing these partial reactions are not combined in the form of the enzyme, the reaction proceeds slowly (experiments 1 and 2, Table IV, 0.38 compared to 10.0). The value 10 is considerably less than the maximum value that has been obtained with reconstituted subunits under the optimum conditions (Wood et al., 1975). The optimum condition involves combination of the $5S_E$ and $1.3S_E$ subunits first and then a second reconstitution using the resulting $6S_E$ subunit and the $12S_H$ subunit. Under these conditions, a specific activity of 50 has been obtained calculated on the basis of the $12S_H$ subunit. It is likely that part of the observed specific activity of 0.38 with the nonassembled $5S_E$, $1.3S_E$, and $12S_H$ subunits resulted from a small amount of

assembly which occurred under conditions of the assay. The biotinyl peptides do not contain the portion of the $1.3S_E$ subunit which binds the $5S_E$ subunit to the $12S_H$ subunit (Ahmad et al., 1975). Therefore, with the biotinyl peptides the subunits probably remain completely dissociated even under conditions suitable for reassembly of the subunits. Thus, in experiments 3 and 4 of Table IV, the specific activity was less than that observed with the $1.3S_E$ subunit and there was only a slight increase in activity under conditions which cause reconstitution. The fact that acetyl-CoA carboxylase from *E. coli* has not been reconstituted from its subunits probably accounts for its low activity in vitro.

The biotinyl peptides were also investigated in combination with the trypsinized transcarboxylase. This inactive form of the enzyme still has the peripheral $5S_E$ subunits linked to the $12S_H$ subunit by the non-biotinyl peptide portion of the $1.3S_E$ subunit (Ahmad et al., 1975). Thus, if the biotinyl peptide combined with the trypsinized transcarboxylase in the proper orientation, enzymatic activity should be high. There was no evidence (experiments 4 and 5, Table IV) that this occurred even under conditions favoring reconstitution. Apparently the biotinyl peptides do not bind with the trypsinized transcarboxylase to form a complex in which the biotinyl group oscillates between the subunits as a direct carboxyl carrier. The higher activity with the $5S_E$ plus $12S_H$ subunits than with trypsinized transcarboxylase may result from the fact that the $5S_E$ subunit was used in a sixfold excess of the $12S_H$ subunit in experiments 3 and 4.

Methylcrotonyl-CoA carboxylase (Lynen et al., 1961) and acetyl-CoA carboxylase (Dimroth et al., 1970) catalyze the carboxylation of free biotin but transcarboxylase does not. Polakis et al. (1974) have reported that biotin per se is an effective carboxyl acceptor for the carboxylase component of acetyl-CoA carboxylase of *E. coli* but it is much less effective in the transcarboxylation reaction as catalyzed by the transcarboxylase component of this enzyme. Derivatives in which the carboxyl of the valeric acid side chain of the biotin was reduced or esterified were much more effective in the transcarboxylation reaction. Thus, it seemed likely that these compounds might be effective in the partial reactions of oxalacetate transcarboxylase as catalyzed by its subunits. However, these compounds were completely inactive under conditions in which the carboxyl carrier protein or the 66- and 40-residue biotinyl peptides were carboxylated. The biotin analogs were tested at concentrations of about 6 m*M* (about 1000 times the molar concentration of the biotinyl peptides) in an attempt to overcome possible differences in their K_m's.

It was evident during the study of the partial reactions that [3-^{14}C]methylmalonyl-CoA and [4-^{14}C]oxalacetate bind to the subunits and remain bound during the gel filtration (Figures 1–3). Attempts made to determine the number of binding sites for methylmalonyl-CoA and propionyl-CoA on the $12S_H$ subunits by equilibrium dialysis using [3-^{14}C]methylmalonyl-CoA and [1-^{14}C]propionyl-CoA were not successful because the $12S_H$ subunit catalyzes a slow decarboxylation of methylmalonyl-CoA as well as deacylation of the propionyl-CoA. A method which permits rapid measurement of binding such as that employed by Colowick and Womack (1969) may prove useful for these studies.

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