TRANSCARBOXYLASE: ROLE OF BIOTIN, METALS, AND SUBUNITS IN THE REACTION AND ITS QUATERNARY STRUCTURE*

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I. INTRODUCTION

Transcarboxylase [methylmalonyl-CoA:pyruvate carboxytransferase (E.C. 2.1.3.1)] catalyzes the following reaction:

$$CH_3 \cdot CH(COO^-) \cdot COSCoA + \quad (1)$$

$$CH_3 \cdot CO \cdot COO^- \rightleftharpoons CH_3 \cdot CH_2 \cdot COSCoA +$$

$$^-OOC \cdot CH_2 \cdot COCOO^-$$

This enzyme occurs in the propionic acid bacteria and is one of the key enzymes in the metabolic pathway of propionate formation. The enzyme was discovered in 1960 by Swick and Wood[1] and is the only enzyme which is known to catalyze the transfer of a carboxyl group from one compound to another. Thus far there is only one report of its presence in other organisms.[2] Transcarboxylase belongs to the family of biotin enzymes, but all other biotin enzymes, in one way or another, involve CO_2 (or HCO_3^-) either in the catalysis of the fixation of CO_2 or in a decarboxylation (see Moss and Lane[3] for a comprehensive review of the biotin enzymes and Utter et al.[4] for a very recent review of pyruvate carboxylase).

Transcarboxylase is of particular interest because it has a unique flexible complex quaternary structure. It is made up of a central subunit to which peripheral subunits are attached by means of the third type of subunits which contain the biotinyl groups. We will see that one part of the amino acid sequence of these biotinyl subunits is involved in binding together the central and peripheral subunits, and another part is involved in placement of the biotinyl groups adjacent to the substrate sites so that they may serve as carboxyl carriers between the sites. The flexibility of the polypeptide chain of the biotinyl subunit permits transcarboxylase to assume a variety of shapes, thus differing from most enzymes which are held together by multiple weak bonds in a rigid and compact structure. The CoA ester sites are on the central subunit and the keto acid sites are on the peripheral subunits; the transfer of the carboxyl group via the biotinyl group is from one subunit to another. In this respect, transcarboxylase resembles acetyl CoA carboxylase of *E. coli* which, likewise, is made up of three different types of subunits. There is a biotinyl subunit and there are specific substrate sites on each of two other subunits (see Alberts and Vagelos[5] for a comprehensive review of this enzyme and of other acyl CoA carboxylases).

Transcarboxylase has the fortunate property of being readily reconstituted from its subunits, which is in contrast to acetyl CoA carboxylase of *E. coli* which has been neither isolated intact nor reconstituted from its subunits. Because the subunits of transcarboxylase are readily isolated, this enzyme provides a good opportunity for study of

the subunit-subunit interactions involved in the assembly of a complex enzyme.

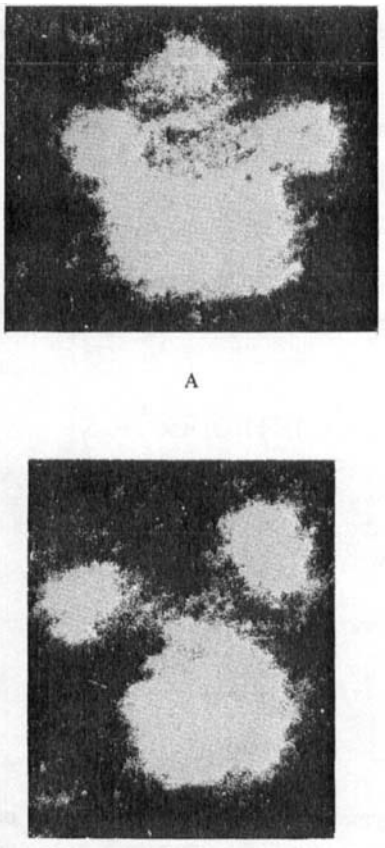
Reviews on transcarboxylase have appeared in 1972[6,7] and a short review is in press.[8] The background of the discovery of transcarboxylase, its role in the fermentation, and the proof that the biotin is covalently linked as an amide to the ϵ-amino group of a lysyl residue of a subunit and that carboxyl transfer is by carboxy amide formation on the 1'N of the biotin have been previously reviewed[6] and will not be dealt with in detail here. The reviews[6,7] should be consulted for a more complete citation of literature prior to 1972. The principal emphasis of this presentation will be a critical review of the quaternary structure of the enzyme in relation to the structure of the subunits. In addition, it has been found that the enzyme dissociates during the usual procedures of isolation, and the native form is more complex than that used in past studies which have been done with a dissociated form. The quaternary structure of the enzyme will be analyzed in relation to this more complex structure. In addition, we shall delve into the chemical mechanism of the catalysis in relation to the subunits and the quaternary structure.

II. QUATERNARY STRUCTURE OF TRANSCARBOXYLASE

The majority of the information on the quaternary structure of transcarboxylase has been derived from electron micrographs and from the subunit composition of the enzyme. We will first confine the considerations to the form of transcarboxylase which has usually been isolated. The structure of the more complex (and apparently the native) form of the enzyme will be considered in later sections.

A. Electron Micrographs

Transcarboxylase presents many profiles in electron micrographs since it has a flexible structure and readily dissociates to its subunits. Selected profiles from publications by Green et al.[9] and Green[10] are presented in Figure 1. The peripheral subunits are seen at variable distance from the large compact central subunit; in addition, during preparation of the sample on the grid, some of the peripheral subunits become detached. The enzyme with three peripheral subunits (Figure 1A) has a sedimentation coefficient of 18S and is referred to as the 18S form of transcarboxylase. Profile B, showing two peripheral subunits, looks like the head of Mickey Mouse, and for that reason the peripheral subunits have been called "ears" and the central subunit, the "head." The enzyme with two peripheral subunits has a sedimentation



A

B

FIGURE 1. Electron micrographs of transcarboxylase and its subunits. (A) Transcarboxylase with three peripheral subunits. (B) Transcarboxylase with two peripheral subunits (the Mickey Mouse form). (C) Avidin complex of transcarboxylase with three peripheral subunits. Note the smaller avidin molecule between each pair of peripheral subunits. The hole in the central subunit is seen in this top view of the enzyme. (D) Avidin complex of transcarboxylase with two peripheral subunits. Note the avidin at each end and between the peripheral subunits. (E) Central subunit, showing the circular top view with a central hole (100 A) and the rectangular side view (70 × 100 A). (F) Peripheral subunit, showing the elongated horizontal view (55 × 90 A). (From Green, N. M., Valentine, R. C., Wrigley, N. G., Ahmad, F., Jacobson, B., and Wood, H. G., *J. Biol. Chem.*, 147, 6284, 1972. With permission.)

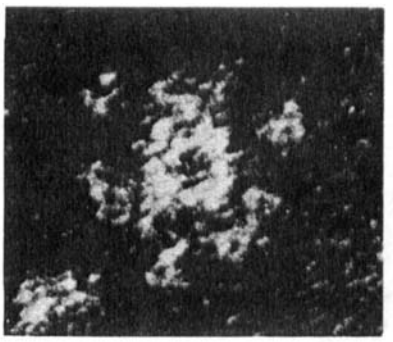


FIGURE 1C

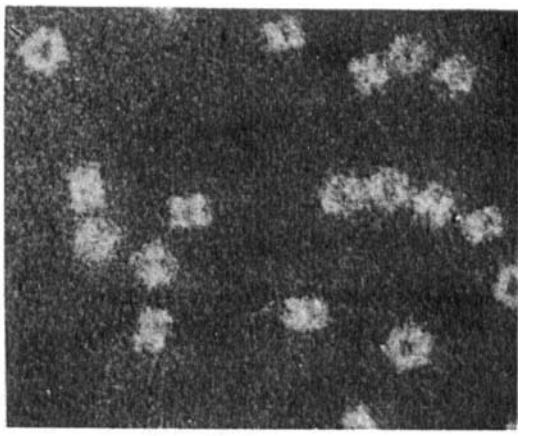


FIGURE 1E

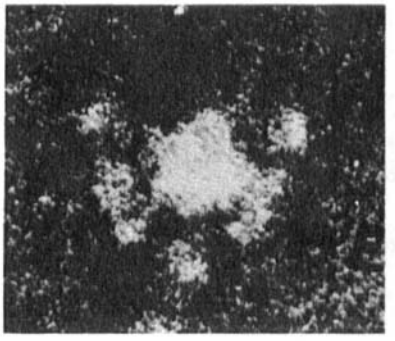


FIGURE 1D

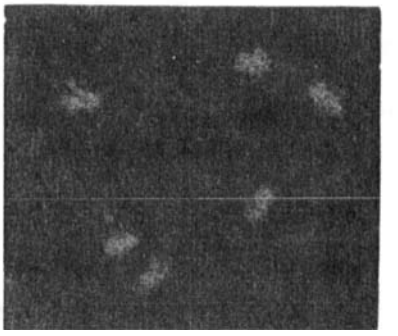


FIGURE 1F

coefficient of 16S. In addition to profiles such as A and B, profiles are seen of the central subunit with one peripheral subunit attached, the central subunit without "ears" or with partly detached "ears," and the "ears" only.[9,10]

The most informative electron micrographs were those obtained by use of avidin as a marker for the biotin. Avidin combines tightly with biotin. Not only did the avidin give an indication of the location of the biotinyl groups, the complex also presented a view of the molecule which was different from that seen with the uncomplexed molecule. For some unknown reason, the complexed enzyme adsorbs to the grid so that a top view is presented, whereas the uncomplexed enzyme presents a side view. In the top view, shown in Figures 1C and D, a hole in the circular large central subunit becomes evident, and the lengthwise aspect of the "ears" is seen instead of the dense end-on view which is presented when the enzyme is on its side (as in Figures 1A and B). The profile in C shows three ears, and between each there is a smaller avidin molecule. Profile D shows the two-eared specimen with avidin between the two ears and one avidin at each end of the ears. Thus, the results indicate that there is a biotinyl group at each end of the peripheral subunits. There is a space between the avidin and the peripheral subunits which is taken to indicate that the biotinyl group is on an extended peptide chain which is not visible with the negative stain.

The electron micrographs of the isolated subunits are also presented. Figure 1E is of the $12S_H$ subunit. Both the top circular view with the central hole and the rectangular side or edgewise views are seen. The isolated peripheral subunit is shown in Figure 1F. The lengthwise aspect is seen since the free subunit adsorbs to the grid in the

horizontal position, whereas the end-on view is seen when the subunit is bound to the enzyme.

B. Dissociation and Subunit Composition of Transcarboxylase

1. Dissociation to the 16S Form

When isolated, transcarboxylase often consists of a mixture of two forms of the enzyme which, on ultracentrifugation, give a split peak such as shown in Figure 2A. The peak with a sedimentation coefficient of 18S is the three-eared specimen (Figure 1A), and the 16S peak is the two-eared or Mickey Mouse specimen (Figure 1B). Occasionally, transcarboxylase, which consists almost exclusively of the 18S form, is isolated as shown in the top frame of Figure 2B. A small shoulder on the edge of the peak is evident as the 16S form of the enzyme. The 18S form of the enzyme readily dissociates at pH 8 with the loss of one peripheral subunit, yielding the 16S form of the enzyme.[9] The lower frame of Figure 2B shows the sedimentation profile after such dissociation. The main peak now is the 16S form with a slight shoulder on the leading edge due to a residual small amount of the 18S species which was not dissociated. In addition, a second peak is observed with a sedimentation coefficient of about 6S, which is due to the peripheral subunit which arises during the dissociation.

2. Biotin Content and Location of the Biotin

From the above type of experiment, the 16S enzyme and the peripheral $6S_E$ subunit were isolated by glycerol gradient centrifugation after dissociation of the 18S enzyme, and the biotin content of each was determined. Approximately 4 mole of biotin was found per mole in the 16S species, 2 moles/mole in the peripheral $6S_E$ subunit, and 6 moles/mole in the original 18S enzyme.[9] The biotin content is consistent with a structure in which each peripheral $6S_E$ subunit possesses two biotins; the 16S form of the enzyme consists of a central subuhit with two peripheral subunits attached to it, and the 18S form consists of a central subunit with three peripheral subunits. The 16S enzyme had a lower enzymatic activity than the 18S species, which is in accord with its biotin content.

3. Subunit Composition and the Molecular Weights

The complete dissociation of transcarboxylase to its subunits and to its constituent peptides is illustrated in Figure 3 using models.* When transcarboxylase is dissociated for several hours at room temperature at pH 8, the peripheral subunits are completely removed from the central subunit; if this is done in 20% glycerol, the further

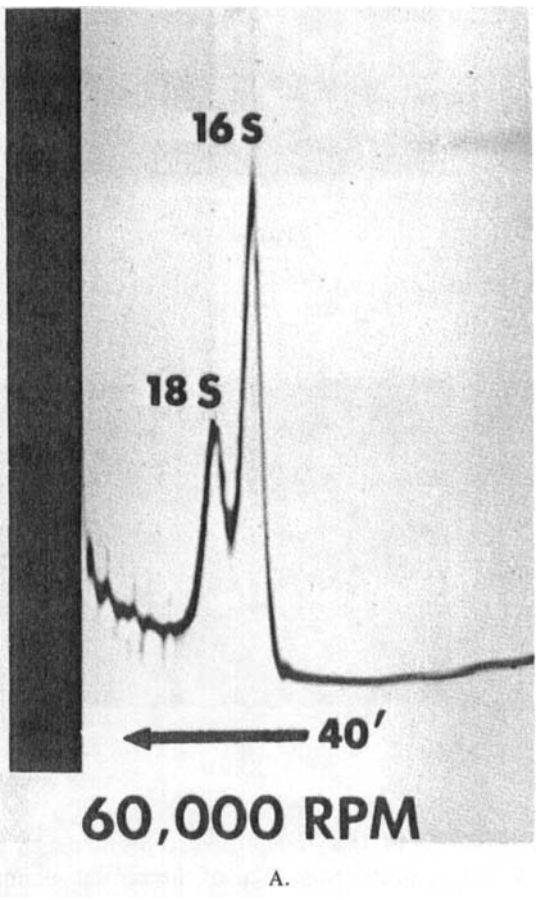


FIGURE 2. Sedimentation velocity profiles of transcarboxylase (sedimentation from right to left). (A) Split peak resulting from the 18S and 16S forms in a purified preparation of transcarboxylase. (From Wood, H. G., Ahmad, F., Jacobson, B., Green, N. M., and Wrigley, N. G., in *Enzymes Structure and Function*, Vol. 29, Drenth, J., Osterbaan, R. A., and Veeger, C., Eds., North-Holland, Amsterdam, 1972, 201. With permission.) (B) Top frame: Sedimentation of enzyme which was predominantly the 18S form. Bottom frame: Sedimentation after dissociation of the 18S form at 4° in 0.1 *M* tris SO_4, pH 8. A small shoulder from the remaining 18S form is observed, and the enzyme has largely been converted to the 16S species. The slow moving peak is the peripheral subunit which dissociated from the 18S form of the enzyme. Sedimentation was at 60,000 rpm. (From Green, N. M., Valentine, R. C., Wrigley, N. G., Ahmad, F., Jacobson, B., and Wood, H. G., *J. Biol. Chem.*, 247, 6284, 1972. With permission.)

*A more complete discussion of the quaternary structure of transcarboxylase and models representing the structure will be presented in Section VIII.B.3.

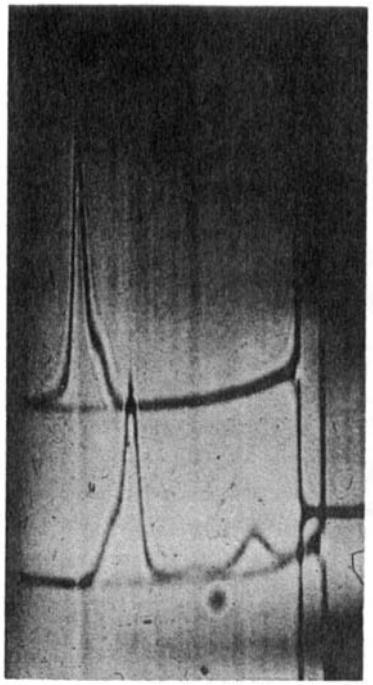


FIGURE 2B

dissociation of the central subunit and the peripheral subunits to their constituent subunits is retarded. The intact 18S form of the enzyme has a molecular weight of ~790,000. The central subunit has a sedimentation coefficient of 12S and a molecular weight of 360,000. This subunit is referred to as the $12S_H$ subunit, the H standing for head. Each of the three peripheral subunits has a sedimentation coefficient of 6S, a molecular weight of 144,000, and is referred to as the $6S_E$ subunit or "ear."

At pH 9 there is further dissociation. The central subunit dissociates to three dimers, each of molecular weight 120,000 with a sedimentation of 6S, which are called the $6S_H$ subunits. At this same pH, the $6S_E$ subunits dissociate to a dimer and two subunits, each of molecular weight 12,000, which contain a biotin and have an $s_{20,w}$ = 1.3S. They are referred to as the $1.3S_E$ subunits or carboxyl carrier proteins. The dimer has a molecular weight of 120,000 and is referred to as the $5S_E$ subunit, although its sedimentation coefficient is about 5.8S.[11] In denaturants, such as sodium dodecylsulfate or urea, the dimeric $5S_E$ and $6S_H$ subunits dissociate to their constituent polypeptides, each with a molecular weight of about 60,000. Thus, the 18S form of the enzyme contains 18 constituent polypeptides: six of the $1.3S_E$ carboxyl carrier proteins (two for each peripheral subunit), six of the $2.5S_E$ polypeptides from the three peripheral $6S_E$ subunits, and six of the $2.5S_H$ polypeptides from the central $12S_H$ subunit.

4. Metal Content and Location of Metals

Transcarboxylase is a metalloenzyme containing cobalt and zinc;[12,13] recently, it has been reported to contain copper.[14] To locate the Co^{++} and Zn^{++} in the transcarboxylase, Ahmad et al.[13] grew the propionic acid bacteria in media containing ^{60}Co and ^{65}Zn, respectively, and isolated the labeled transcarboxylase. The two types of labeled transcarboxylase were dissociated to the central $12S_H$ subunit, the peripheral $5S_E$ subunits, and $1.3S_E$ subunits (see Figure 3). The subunits were separated by glycerol gradient centrifugation and the radioactivity of the fractions determined. Very little radioactivity was found in the 12S and 1.3S regions, and practically all the radioactivity was in the 6S region. Thus, the Co^{++} and Zn^{++} are associated with the $5S_E$ dimeric subunit.

The ratio of Co^{++} to Zn^{++} was found to vary from 0.5 to 10 and apparently is dependent on the Zn^{++} and Co^{++} content of the growth medium. Seven to eight gram atoms of Zn^{++} plus Co^{++} per mole of the 18S form of the enzyme were estimated by atomic absorption to be present. Recently, Fung et al.[14] have presented evidence that transcarboxylase contains not only the Co^{++} and Zn^{++}, but also Cu^{++} and that the sum of these three metals is about 12 g-atom/790,000 g of protein varying from 10.4 to 13.5. The Co^{++} plus Zn^{++} varied from 7.9 to 9.2 g-atom/790,000 in three preparations and was 11.1 in a fourth; the Cu^{++} varied from 1.8 to 2.4 in three preparations and was 4.8 in a fourth. The preparation containing 4.8 g-atom of Cu^{++} contained 7.9 g-atom of Co^{++} plus Zn^{++} (see Section VI.E.2, Table 2).

Northrop and Wood[12] had previously reported that the Co^{++} plus Zn^{++} content varied between 6.5 and 6.7 g-atom/mol of enzyme, but their calculations were based on a molecular weight of 670,000 rather than 790,000. When calculated on the basis of a molecular weight of 790,000, these values become 7.7 to 8.0.

One of the difficulties of estimating the metal content of transcarboxylase is that the Co^{++} and Zn^{++} are in the peripheral subunit. Therefore, if the metal is to be calculated on the basis of a

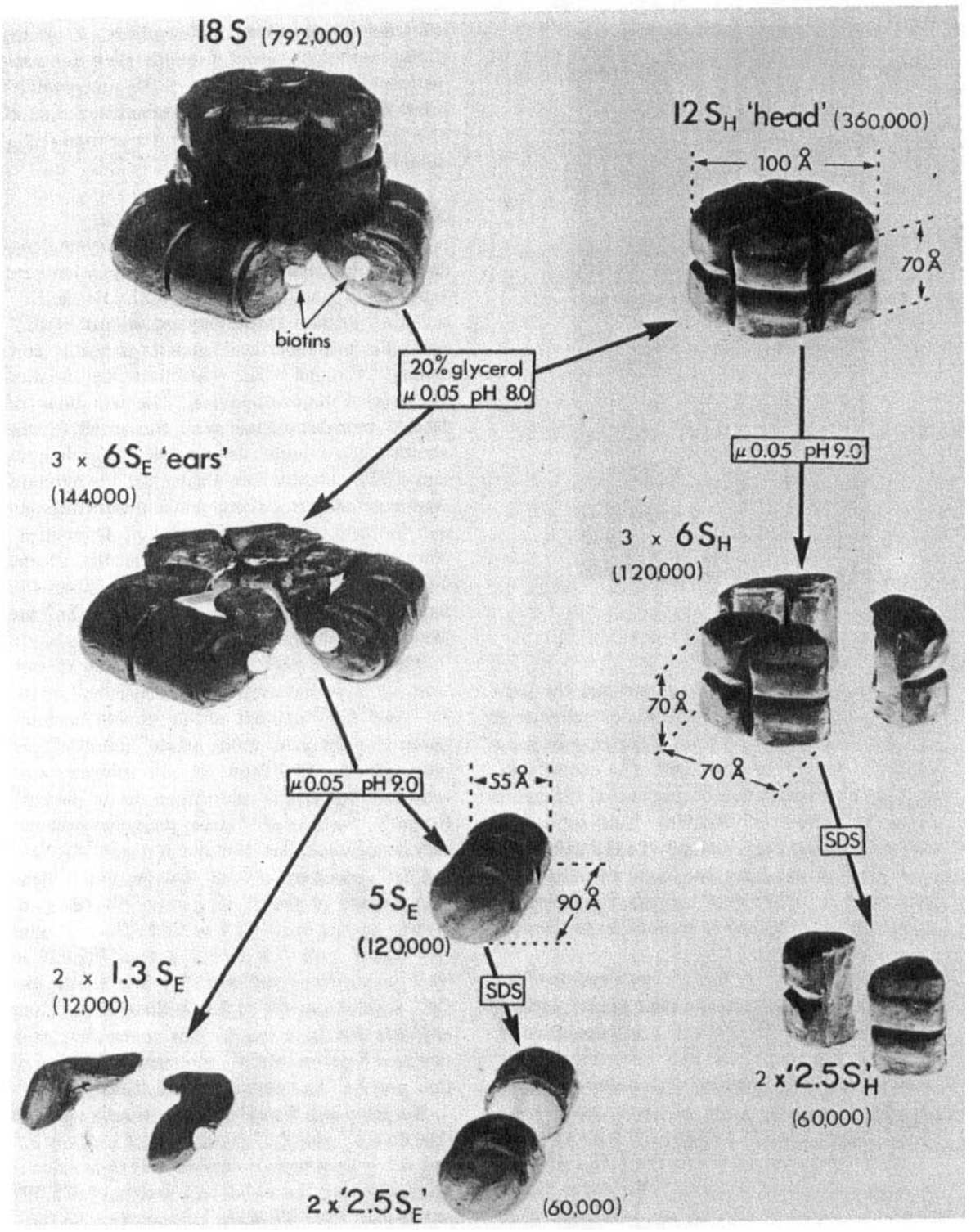


FIGURE 3. Illustration of the dissociation of transcarboxylase. The molecular weights are shown in parentheses. The sedimentation coefficient is shown with a subscript H or E. H signifies the subunit was derived from the central subunit or "head" and E from the peripheral subunit or "ears." The indentations on the $12S_H$ and $5S_E$ subunits indicate that there was stain penetration in these regions. The location of the $1.3S_E$ subunit is deduced from the position of the avidin (Figure 1D); the shape is arbitrary. The white spot indicates the biotinyl group. (From Green, N. M., Valentine, R. C., Wrigley, N. G., Ahmad, F., Jacobson, B., and Wood, H. G., *J. Biol. Chem.*, 247, 6284, 1972. With permission.)

molecular weight of 790,000, it is necessary to know the relative proportion of 16 and 18S forms of the enzyme. Ahmad et al.[13] evaluated this proportion from the area under the sedimentation peaks and made a correction to obtain the values on the basis of the 18S form. One of the enzyme preparations of Fung et al.[14] with the highest content of metal contained 7.1 biotins/790,000. This preparation may have had more than three peripheral subunits per mole of enzyme (see Section VII.C.2), and a correction should perhaps have been made to account for this fact. A further possible source of error is from contamination by proteins with a higher content of Zn^{++} than the carboxylase. Northrop and Wood[12] demonstrated that during the purification of transcarboxylase, proteins were present which contained more Zn^{++} than the transcarboxylase, and two purification steps (gel filtration using Sepharose 6B), in addition to those usually employed, were required to obtain a constant ratio of ^{65}Zn to transcarboxylase activity. In view of the above uncertainties, Ahmad et al.[13] proposed that the 18S form of transcarboxylase might contain 6 atoms of Co^{++} and Zn^{++}, i.e., two per $5S_E$ subunit. Fung et al.[14] did not include the extra steps of purification nor did they, as was done by Ahmad et al.,[13] pass the enzyme through an EDTA Sephadex column to remove absorbed metals from the protein.* There is no loss of enzyme activity during this treatment. It is the authors' opinion that the question of the total metal content of transcarboxylase must remain open at present. The occurrence of 12 metals raises very interesting questions concerning the role of the metals. We will consider the role of Co^{++} and Zn^{++} in catalysis in Sections VI.E and G.4.

Ahmad et al.[13] demonstrated by a variety of experiments that the metals are tightly bound to the $5S_E$ subunit. A number of metal chelators were tested, and only oxalate, an analogue of pyruvate, caused inhibition of the enzyme. The metals are not removed when the enzyme is passed over Sephadex equilibrated with 10 m*M* *o*-phenanthroline, 8-hydroxyquinoline 5-sulfonate, or EDTA. All three reagents are strong chelators and would be expected to remove the metal if it were not tightly bound to the subunit. Dialysis at neutral or alkaline pH does not remove the metals. At pH's of 5, 4.5, and 4.0 there is some release and exchange of metal, but this appeared to be due to the denaturation of the enzyme with the concomitant formation of inactive subunits and their constituent polypeptides. Under conditions where the enzyme is irreversibly dissociated to its constituent peptides, i.e., dialysis against 0.1% sodium dodecylsulfate or 4 to 6 *M* urea, the metals are released.

It is of interest that pyruvate carboxylase and transcarboxylase are the only biotin enzymes which have been found to be metalloenzymes, and both of these catalyze the carboxylation** of pyruvate. The metal components of various pyruvate carboxylases differ. For example, the pyruvate carboxylase from chicken liver contains Mn^{++}, that from yeast contains Zn^{++} rather than Mn^{++}, and others (such as the carboxylase from calf liver) contain a mixture of Mg^{++} and Mn^{++}; it has been shown that by depleting the Mn^{++} level of the chicken's diet, pyruvate carboxylase which contains only Mg^{++} can be obtained (see Utter et al.[4] for references).

III. ISOLATION OF SUBUNITS

Although it is a fortunate property of transcarboxylase that it is readily reconstituted from its subunits, this is an impediment to the isolation of the subunits. Under conditions in which the $12S_H$ and $6S_E$ subunits are relatively stable (e.g., at neutral pH in the presence of polyvalent ions), recombination to the intact enzyme occurs. Thus, if one dissociates the enzyme at pH 8 and then attempts to separate the resulting $12S_H$ and $6S_E$ subunits by gel filtration at neutral pH, the yield is poor because a major part of the subunits recombine; in addition, the $6S_E$ subunits dissociated somewhat at pH 8. Thus, the recovered $6S_E$

*Subsequent to completion of this review, Fung and Mildvan (in a personal communication) state: "Storage or treatment with EDTA (0.1–0.2 m*M*), passage through an EDTA-Sephadex column or through Chelex-100 proportionally reduced the content of all metals (Zn, Co, and Cu) by 22–43%." This decrease in metal content would reduce the values to nearly the same as observed by Ahmad et al.[13] The question of whether there are structural metals, as discussed in Section VI.E.3, remains open, however.

**No differentiation is made in this review between carboxylation by formation of a carboxyl group from CO_2 and transfer of a carboxyl from a carboxyl donor to other compounds.

subunit does not have its full complement of two biotinyl subunits per $6S_E$ subunit.[11] Methods have been developed, however, for isolation of the biotinyl $1.3S_E$ subunit and the $12S_H$ and $5S_E$ subunits.

A. Isolation of the $1.3S_E$ Biotinyl Subunit (Carboxyl Carrier Protein)

The $1.3S_E$ subunit was first isolated by Gerwin et al.[15] but more convenient procedures have been developed more recently. The most frequently used procedure has been to dissociate transcarboxylase at pH 9 to the $6S_H$, $5S_E$, and $1.3S_E$ subunits followed by chromatography on Bio-Gel A 1.5 *m* at pH 9 (Figure 4). The recovery of the biotinyl subunit is almost quantitative in this procedure.[11] Frequently, two forms of the carboxyl carrier protein are observed, one of ~123 residues and one of ~118 residues. The smaller polypeptide probably arises by proteolysis during the isolation of the $1.3S_E$ subunit, even though precautions are taken to prevent such degradation.[11] The $1.3S_E$ subunit contains one biotinyl group and has a molecular weight of about 12,000.[15]

The $1.3S_E$ subunit was purified further for investigations of the amino acid sequence (Section VIII.A) by step-wise elution from DEAE cellulose using 0.01 *M* tris·HCl pH 8.8 containing 0.05 *M* and 0.15 *M* KCl. The two forms of the $1.3S_E$ biotinyl carboxyl carrier protein can be separated from each other by either of two methods: elution from Bio-Gel A 0.5 *m* (200 to 400 mesh) using 0.05 *M* tris·HCl pH 9.0 containing 0.2 *M* KCl or elution from DEAE-Sephadex A-25 with 0.03 *M* tris·HCl pH 7.5 containing a 0 to 0.2 *M* KCl linear gradient.[94]

A highly purified biotinyl carboxyl carrier protein from acetyl CoA carboxylase of *E. coli* has been isolated by Vagelos and co-workers. In a

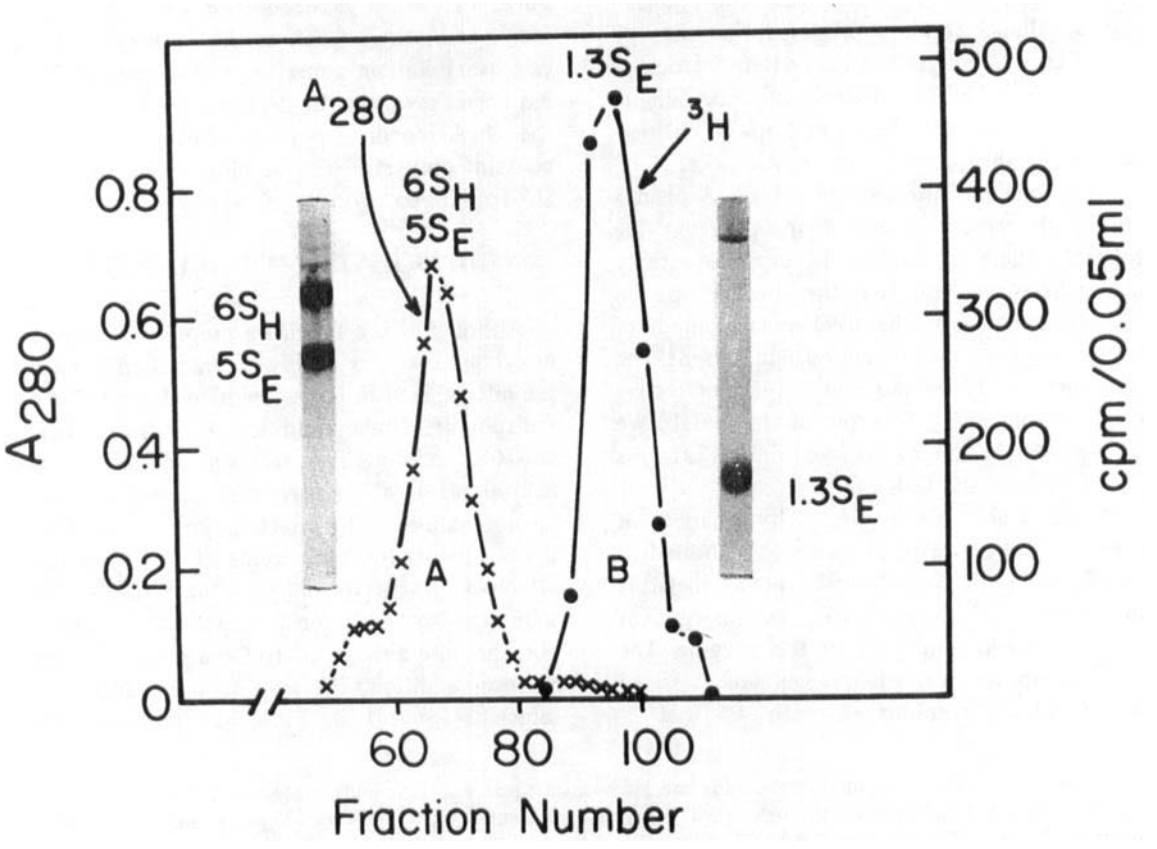


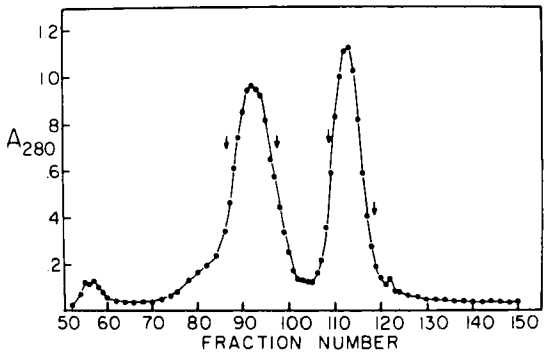
FIGURE 4. Separation on Bio-Gel A 1.5 *m* of $6S_H$ and $5S_E$ subunits from the $1.3S_E$ subunit of transcarboxylase dissociated at pH 9. The enzyme contained tritium in the biotinyl group. The $6S_H$ and $5S_E$ subunits were monitored by the adsorption at 280 nm and the $1.3S_E$ subunit by radioactivity. The electrophoresis patterns of the $6S_H$ and $5S_E$ subunits in standard gel and of the $1.3S_E$ preparation with sodium dodecysulfate are shown in the insets. (From Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M., and Brattin, W., *J. Biol. Chem.*, 250, 918, 1975. With permission.)

series of publication (see Alberts and Vagelos[5] for references) they have described multiple forms of the protein with molecular weights of 9,065, 10,267, ~22,500, and ~45,000. They propose that the protein of molecular weight 45,000 is a dimer and is the native form of the carboxyl carrier; forms smaller than ~22,500 arise by proteolysis during the isolation.[16] We have found no evidence of a carboxyl carrier protein from transcarboxylase with a molecular weight greater than 12,000. It is interesting that Ahmad and Ahmad[17] have recently reported that they have isolated an enzyme from the propionic acid bacteria which catalyzes the carboxylation of acetyl CoA and propionyl CoA with CO_2 and that the biotinyl carboxyl carrier protein for this enzyme is considerably larger than the carboxyl carrier protein of transcarboxylase. In addition, Fall et al.,[17a] by treatment of *P. shermanii* with SDS, have detected a minor species of biotinyl peptide of molecular weight 75,000 to 80,000 in addition to the major species of molecular weight 10,000 to 13,000.

B. Isolation of the Central $12S_H$ Subunit and Peripheral $5S_E$ Subunit

The most satisfactory procedure for isolation of the $12S_H$ and $5S_E$ subunits is by affinity chromatography using avidin Sepharose.[18] The advantage of this procedure is that it removes the $1.3S_E$ subunit as an avidin complex from the dissociated transcarboxylase; under these conditions the $12S_H$ and $5S_E$ subunits do not recombine when placed under conditions in which they are stable. In this procedure the transcarboxylase is complexed with the avidin Sepharose, then placed on a column, and dissociated using first tris-SO_4 at pH 8 in 20% glycerol, which yields the $12S_H$ subunit, leaving the $6S_E$ subunit attached to the avidin. However, there is some dissociation of the $6S_E$ subunit to the $5S_E$ subunit, so the resulting eluate does contain a mixture of subunits. The eluate is collected in phosphate buffer at pH 6.5, thus preventing dissociation of the $12S_H$ subunit to the $6S_H$ subunit. The column is then washed with tris-Cl at pH 9, which dissociates the attached $6S_E$ subunit to the $5S_E$ and the $1.3S_E$ subunits. The latter remains attached to the avidin Sepharose. The $5S_E$ subunit is collected in phosphate buffer at pH 6.5. The fractions are purified by further chromatography on Bio-Gel A 1.5 *m*, and the separation of the subunits is excellent (Figure 5.I) since the subunits do not interact during passage down the column. The electrophoresis patterns of the subunits which have been purified by the chromatography or by glycerol gradient centrifugation are illustrated in Figure 5.II. For comparison, the bands obtained from pure transcarboxylase (A) and transcarboxylase dissociated at pH 9 to the $6S_H$ and $5S_E$ subunits (B) are shown. The transcarboxylase dissociates during the gel electrophoresis at pH 8.9 to the $12S_H$, $6S_H$, $5S_E$, and $1.3S_E$ subunits: thus, three bands are seen in A. The $1.3S_E$ subunit is present in such a low concentration that it is not seen. When the transcarboxylase is dissociated at pH 9, only the $6S_H$ and $5S_E$ bands are observed (B). The purified $12S_H$ subunit gave two major bands corresponding to $6S_H$ and $12S_H$ subunits (Figure 5.II.D). In addition, very weak bands were evident, indicating that there was a small amount of contaminant in the $12S_H$ subunit. The $5S_E$ subunit (Figure 5.II.C) was quite pure, giving only a single band.

This procedure may also be applied directly to



5A

FIGURE 5. Purification of the $5S_E$ and $12S_H$ subunits. (I) Separation of $12S_H$ and $5S_E$ subunits by chromatography on Bio-Gel A 1.5 *m*. The $5S_E$ and $12S_H$ subunits were obtained from crude extracts by avidin-Sepharose chromatography, as described in the text. (II) Polyacrylamide gel electrophoresis at pH 8.9 of purified preparations: (A) 45 μg of transcarboxylase; (B) 30 μg of transcarboxylase dissociated to the $6S_H$ and $5S_E$ subunits at pH 9; (C) 30 μg of the purified $5S_E$ subunit; (D) 30 μg of the purified $12S_H$ subunit. There is dissociation of the transcarboxylase (A) and $12S_H$ subunit (C) since the electrophoresis is done at pH 8.9. (III) Polyacrylamide gel electrophoresis at pH 8.9 of fractions obtained by avidin-Sepharose chromatography of crude extracts: (A) 48 μg of crude extract; (B) 64 μg of the eluate with phosphate buffer; (C) 64 μg of the eluate with tris-SO_4 (pH 8) in 20% glycerol; (D) 78 μg of the eluate with tris-Cl (pH 9). (From Berger, M. and Wood, H. G., *J. Biol. Chem.*, 250, 927, 1975. With permission.)

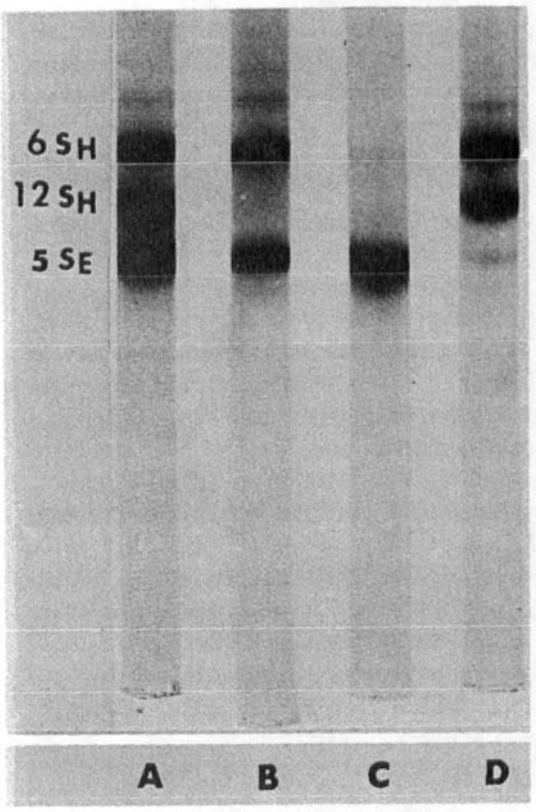


FIGURE 5B

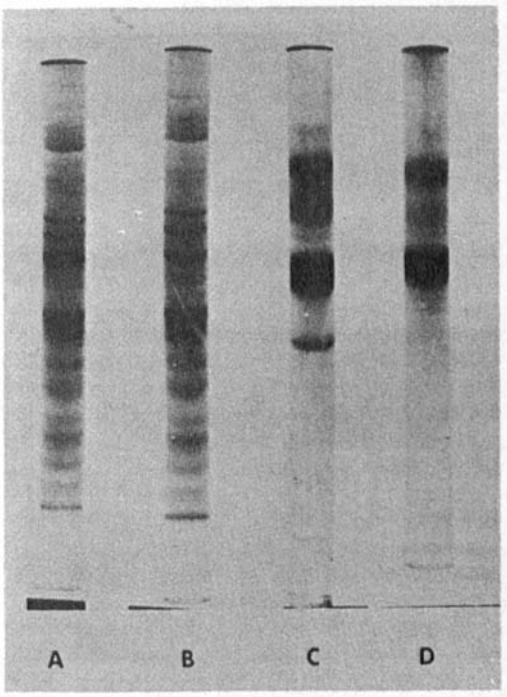


FIGURE 5C

crude extracts since there are only very small amounts of biotinyl enzymes other than transcarboxylase in propionibacteria. The extract is mixed with avidin Sepharose and then placed on a column. The column is washed with phosphate buffer at pH 6.5 to remove nonbiotinyl proteins. The column is then treated with pH 8 and pH 9 buffers as described above. It is evident from the electrophoresis patterns on polyacrylamide gel at pH 8.9 that a very significant purification was obtained (Figures 5.III.C and D), but both fractions contained a mixture of $12S_H$ and $5S_E$ subunits, compared with C and D of Figure 5.II.

The biotin-containing $1.3S_E$ subunit can be recovered from the avidin-Sepharose resin by heating the resin at 100° for 10 min in 0.02 *M* sodium phosphate buffer (pH 7.0) containing 2.0% sodium dodecyl sulfate.[18] The slurry, which consists of resin and "treatment buffer," is centrifuged. Forty-six percent of the $1.3S_E$ subunit was recovered from the clear supernatant solution. Subsequent studies have shown that a second treatment of the avidin-Sepharose resin with sodium dodecyl sulfate gave an additional 34 to 39% of the formerly bound $1.3S_E$ subunit. Using the above procedure, it is possible to recover 80 to 85% of the biotinyl $1.3S_E$ subunit from the avidin-Sepharose resin. Polyacrylamide gel electrophoresis of the $1.3S_E$ subunit isolated by the above procedure showed the characteristic two bands for the long (123 residues) and short (118 residues) forms of the $1.3S_E$ subunit (Sections III.A and VIII.A.1). In addition, a third major band is observed; most likely, this is the dissociated avidin monomer which has a molecular weight of 15,800.[18a] It appears likely that the sodium dodecyl sulfate and the contaminating avidin monomer could be removed by a combination of dialysis in the presence of free biotin followed by gel filtration. Under the proper conditions of dialysis, it is expected that the monomeric avidin subunit would renature, bind the free biotin, and form the avidin tetramer (molecular weight 63,000).[18a] The avidin tetramer could then be removed from the $1.3S_E$ subunit by gel filtration.

The affinity of the monomeric form of avidin for biotin is much lower than the usual tetrameric form. Guchhait et al.[19] have developed a procedure for preparation of monomeric avidin Sepharose. They first prepare tetrameric avidin Sepharose under conditions which minimize the

number of cross links per avidin, so that the tetrameric avidin can subsequently be dissociated. Prior to use, the tetrameric form is dissociated to the monomeric form by use of 6 *M* guanidine HCl. They found that about 60% of the carboxyl carrier protein could be recovered from the monomeric avidin-Sepharose complex, but the remainder was bound almost irreversibly. Of the recovered carboxyl carrier protein, about 30% was obtained by elution with 10 m*M* biotin in 0.5 *M* KCl at pH 9; however, for elution of additional material, 6 *M* guanidine HCl was required. Recently, Libor et al.[19a] have used the method of Guchhait et al.[19] to purify the biotinyl enzyme, pyruvate carboxylase, from *Bacillus stearothermophilus*. They obtained a 25% yield with a high specific activity.

The goal of producing an affinity absorbent, which would readily exchange bound biotinyl enzymes or peptides when excess free biotin is added and of avoiding the need for strong denaturants to elute the enzyme, has not yet been achieved. As demonstrated by Green and Toms,[19b] the avidin monomers, even though covalently linked to Sepharose, can reassociate because of the mobility of the agarose matrix to form tetramers with characteristic tight binding. If one coupled the avidin to a matrix that was rigid enough to prevent mobility and consequent formation of the avidin tetramer, the experimental procedures described above would be more successful. Green and Toms[19b] have greatly decreased the formation of the avidin tetramer by using Sepharose that had been cross linked with divinyl sulfone.

C. Isolation of the $6S_H$ Subunit

The $6S_H$ subunit may be generated from the $12S_H$ subunit by dissociation at pH 9. It is seen in Figure 5.II that the $6S_H$ and $5S_E$ bands are well separated by electrophoresis. Thus far, the only procedure found for preparative separation of the $6S_H$ subunit is preparative disc gel electrophoresis.[20] The mixture of $6S_H$ and $5S_E$ subunits resulting from the isolation of the $1.3S_E$ subunit (Figure 4) has been used for this purpose, and from 19 mg of the mixture, for example, 8.4 mg of $5S_E$ and 5.3 mg of the $6S_H$ subunits have been recovered with good purity.[20]

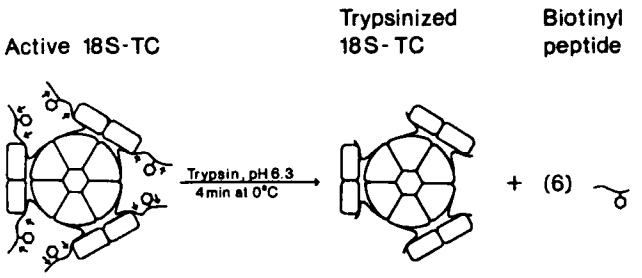
IV. ACTION OF TRYPSIN ON TRANSCARBOXYLASE AND ISOLATION OF BIOTINYL PEPTIDES AND NONBIOTINYL PEPTIDES ORIGINATING FROM THE $1.3S_E$ SUBUNIT

Trypsin has proven to be a very effective reagent for the study of the structure of transcarboxylase. Through its action on the native enzyme, two very useful biotinyl peptide fragments from the 1.3 S_E subunit have been obtained for determination of the amino acid sequence of this subunit (see Section VIII.A.1). The biotinyl peptides contain the amino acid sequence which directs the binding and the orientation of the biotinyl group so that it may function as a carboxyl carrier at the substrate sites on the $5S_E$ and $12S_H$ subunits (see Section VI.B). In addition, trypsinized transcarboxylase has provided a source for isolation of a nonbiotinyl peptide fragment of the $1.3S_E$ subunit which carries the amino acid sequence that directs the assembly of the peripheral $5S_E$ subunits with the central $12S_H$ subunit (see Section V.A).

A. The Structure of Trypsinized Transcarboxylase and the Isolation of the Biotinyl Peptides

The action of trypsin on transcarboxylase is illustrated in Figure 6. In the diagram of Figure 6, the carboxyl carrier protein is indicated by the curved line and the hexagon indicates the biotinyl group. The arrows indicate the exposed portions of the carboxyl carrier protein which are extremely susceptible to cleavage by trypsin and thus cause the release of 65- and 46-residue biotinyl peptides.[21] The main protein remains intact* after

*Comparison of the amino acid composition of the $12S_H$ subunit isolated from trypsinized transcarboxylase with that isolated from the untreated enzyme indicates that the trypsinized $12S_H$ subunit has about 20% fewer residues than the normal $12S_H$ subunit. However, both the $12S_H$ and $5S_E$ subunits of trypsinized transcarboxylase are active in catalyzing the partial reactions (see reactions 4 and 5 below) and as components for reconstitution of the active enzyme (see Section V.B.).



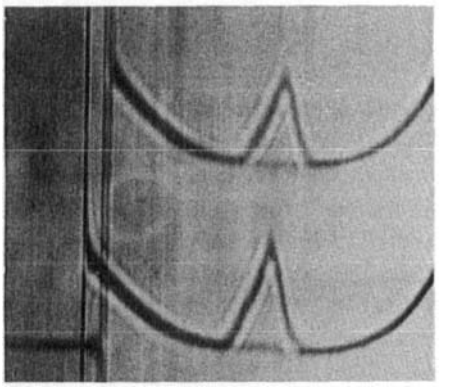


FIGURE 6. Illustration of the action of trypsin on transcarboxylase. The sedimentation velocity profiles are of the transcarboxylase before (top frame) and after (bottom frame) treatment with trypsin. Biotinyl peptides of 65 and 46 residues are cleaved from the enzyme by the trypsin. The treatment was at pH 6.5 for 5 min at room temperature (ratio of trypsin to enzyme, 1:100). The reaction was terminated by addition of diisopropylfluorophosphate. The $s_{20,w}$ of the protein before (top) and after (bottom) treatment was unaltered and was ~ 18S. (Reprinted with permission from Ahmad, F., Jacobson, B., Chuang, M., Brattin, W., and Wood, H. G., *Biochemistry*, 14, 1606, 1975. Copyright by the American Chemical Society; Wood, H. G., Ahmad, F., Jacobson, B., Green, N. M., and Wrigley, N. G., in *Enzymes Structure and Function*, Vol. 29, Drenth, J., Osterbaan, R. A., and Veeger, C., Eds., North-Holland, Amsterdam, 1972, 201. With permission.)

treatment with trypsin, the $s_{20,w}$ value being about 18S before and after the treatment (Figure 6). The trypsinized protein is, of course, inactive since it no longer contains the biotinyl group to serve as the carboxyl carrier.

An example of the isolation of the biotinyl peptides and the trypsinized enzyme by filtration on a Sephadex G-50 column is shown in Figure 7. The biotinyl group contained ^3H, the leading portion of the radioactive peak (fractions 50 to 51) contained a 65-residue radioactive peptide, and the trailing fractions (62 to 64) contained a 46-residue radioactive peptide. The inserts are illustrations of the gel electrophoresis patterns. The separation, purification, and use of these two peptides for determination of the amino acid sequence of the $1.3S_E$ subunit will be described in Section VIII.A.2.

B. Isolation of the Nonbiotinyl Peptide

It had been proposed, on the basis of the electron micrographs (Figure 1), that the $1.3S_E$ subunit, through its polypeptide chain, provides a flexible bond between the peripheral $5S_E$ subunit and the central $12S_H$ subunit, thus accounting for the observed space between the subunits.[9] Since the main protein remained intact after trypsinization, it seemed likely that the portion of the $1.3S_E$ subunit which links the $5S_E$ subunit to the $12S_H$ subunit still was present in the trypsinized enzyme.[21] Isolation of this portion of the $1.3S_E$ subunit (the nonbiotinyl peptide) was, therefore, undertaken, and for this purpose the propionic acid bacteria were grown on a medium containing ^3H lysine so that the nonbiotinyl peptide would be labeled. It was isolated by a rather complex procedure.[21] The lysine-labeled transcarboxylase was isolated and then treated with trypsin, and the trypsinized enzyme was isolated by filtration on Sephadex G-50, as in the experiment of Figure 7. Then the trypsinized transcarboxylase was dissociated at pH 8 to the $12S_H$ and the $6S_E^-$ subunits ($6S_E$ indicates that the subunit lacks the biotinyl peptides). The $6S_E^-$ subunit was in turn separated from the $12S_H$ subunit by chromatography on Bio-Gel A 1.5 *m*. Then the $6S_E^-$ subunit was treated at pH 9, to convert it to the $5S_E$ subunit and the free nonbiotinyl peptide. The resulting nonbiotinyl peptide was then separated from the $5S_E$ subunit by chromatography on Bio-Gel. The results of the last step are shown in Figure 8. The nonbiotinyl peptide had little radioactivity because there are only three lysyl groups in this 42-residue peptide (see Section VIII.A.3). The role of this nonbiotinyl peptide in the assembly of $5S_E$ and $12S_H$ subunits is considered in the next section.

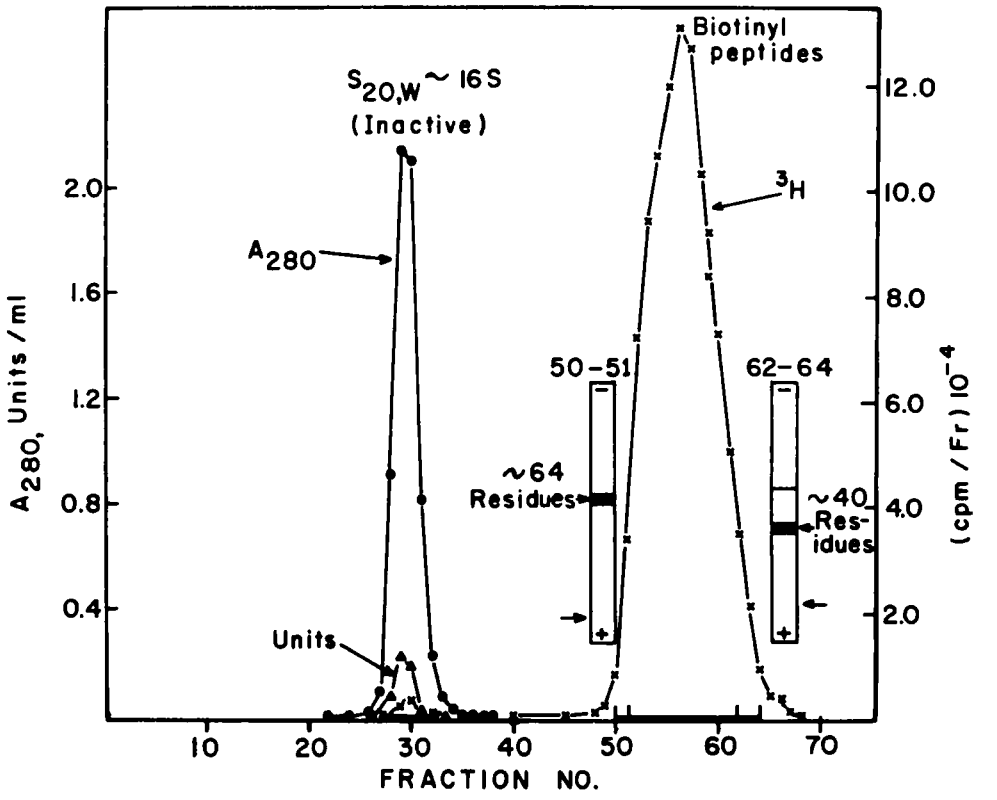


FIGURE 7. Separation of trypsinized transcarboxylase and the resulting biotinyl peptides by chromatography on Sephadex G-50. The biotinyl groups were labeled with ^3H. The trypsinization was as in the experiment of Figure 6. Diagrams of the gel electrophoresis of the biotinyl peptides of fractions 50 and 51 and 62 to 64 are shown in the insets. (Reprinted with permission from Ahmad, F., Jacobson, B., Chuang, M., Brattin, W., and Wood, H. G., *Biochemistry*, 14, 1606, 1975. Copyright by the American Chemical Society.)

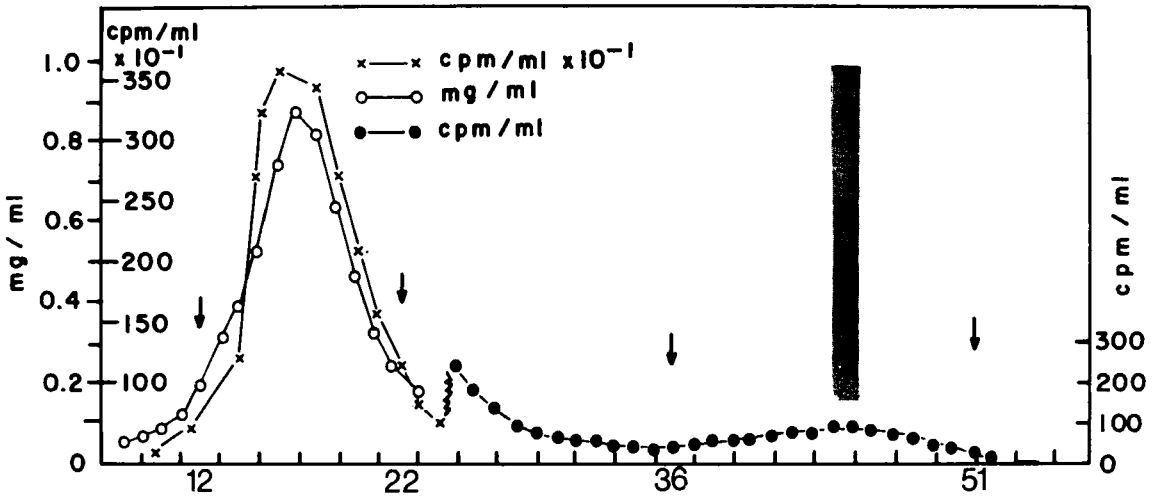


FIGURE 8. Dissociation at pH 9 of the $6S_E$ subunit isolated from trypsinized transcarboxylase (see text) and separation of the resulting $5S_E$ subunit (fractions 12 to 20) from the nonbiotinyl peptide (fractions 36 to 51). The lysyl group contained ^3H. The inset shows an acrylamide gel electrophoresis in sodium dodecylsulfate of the nonbiotinyl peptide. (Reprinted with permission from Ahmad, F., Jacobson, B., Chuang, M., Brattin, W., and Wood, H. G., *Biochemistry*, 14, 1606, 1975. Copyright by the American Chemical Society.)

V. RECONSTITUTION OF TRANSCARBOXYLASE FROM ITS SUBUNITS

A. Role of the $1.3S_E$ Subunit and Nonbiotinyl Peptide in the Assembly of the Subunits

According to the structure postulated for transcarboxylase, as illustrated in Figure 3, the $1.3S_E$ subunit should be required for assembly of the $12S_H$ and $5S_E$ subunits. According to the rationale presented above in Section IV.B, the nonbiotinyl subunit should substitute for the requirement of the $1.3S_E$ subunit in this assembly. Ahmad et al.[21] have demonstrated that this is the case, as shown in the experiment of Figure 9. The $5S_E$ and $12S_H$ subunits were mixed in the absence and presence of the $1.3S_E$ subunit or nonbiotinyl peptide (NBP) under conditions which cause assembly; the products were then sedimented. It is seen that with a mixture of $5S_E$ and $12S_H$ subunits alone, only two peaks were observed corresponding to the $s_{20,w}$ of the added subunits. When the $1.3S_E$ subunit was added, in addition, not only were there 5S and 12S peaks, but there was also a third peak with a sedimentation coefficient of about 16S, due to the assembly of the subunits in the form of an active enzyme. When the nonbiotinyl peptide (NBP) was substituted for the $1.3S_E$ subunit, identical results were obtained, but the product was without enzymatic activity. These results show that the nonbiotinyl peptide has the amino acid sequence and conformation required to cause assembly of the $5S_E$ and $12S_H$ subunits. The amino acid sequence of the nonbiotinyl peptide will be considered in relation to this function of the peptide in Section VIII.A.3.

B. Effectiveness of the Isolated Subunits in the Reconstitution of Active Enzyme

The most effective reconstitution of transcarboxylase from the isolated $12S_H$, $5S_E$, and $1.3S_E$ subunits is accomplished using a two-step process.[11] First, the $1.3S_E$ carboxyl carrier protein and $5S_E$ subunit are combined; then this product is complexed with the $12S_H$ subunit. Thus far, reconstitution of the $12S_H$ subunit from an isolated $6S_H$ subunit has not been accomplished, but there is evidence that this should be possible. For example, transcarboxylase, which has been dissociated to the point where very little $12S_H$ subunit remains intact, when subjected to conditions for reconstitution, sometimes yields transcarboxylase in amounts beyond that predicted from the prior content of the $12S_H$ subunit as judged by the size of the peaks on

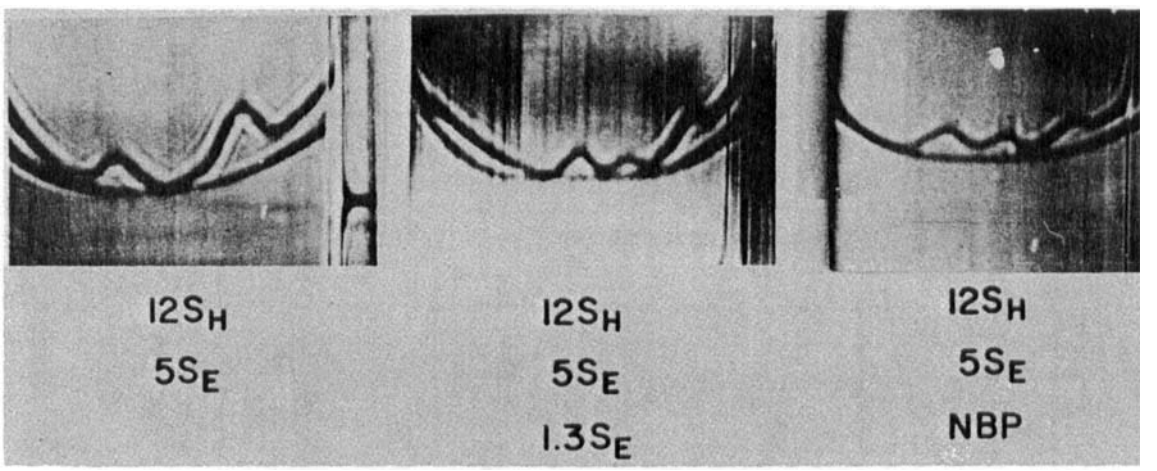


FIGURE 9. Evidence by sedimentation velocity profiles that the $1.3S_E$ carboxyl carrier subunit or the nonbiotinyl peptide portion is required for combination of the $12S_H$ and $5S_E$ subunits. Sedimentation was from right to left. NBP is nonbiotinyl peptide. Recombination of the subunits was done in 0.75 *M* phosphate buffer (pH 6.5) at 4° for 24 to 40 hr using 1.3 mg each of the $12S_H$ and $5S_E$ subunits in 0.5 ml. In addition, 0.21 mg of the $1.3S_E$ subunit was added where indicated. In the experiment with the nonbiotinyl peptide, 0.9 mg each was used, and the volume was 1.04 ml. After the incubation at 4° the protein was precipitated with 80% saturated $(NH_4)_2SO_4$ and taken up to 1.1 ml of 0.1 *M* phosphate buffer (pH 6.5). Centrifugation was at 48,000 rpm at 4°. (Reprinted with permission from Ahmad, F., Jacobson, B., Chuang, M., Brattin, W., and Wood, H. G., *Biochemistry*, 14, 1606, 1975. Copyright by the American Chemical Society.)

centrifugation. Thus, the unresolved $6S_H$ subunit apparently retains the properties which permit it to reconstitute to a $12S_H$ subunit and then combine with the $6S_E$ subunit to yield active enzyme.

Reconstitution of the $5S_E$ and $1.3S_E$ subunits to the $6S_E$ subunit and of the $12S_H$ and $6S_E$ subunits to the 16 and 18S forms of transcarboxylase is facilitated by a high concentration of phosphate buffer (~0.75 *M* at pH 6.5 to 6.8) or acetate buffer (~0.1 *M* at pH 5 to 5.2). An example of the reconstitution of the $6S_E$ subunit and the isolation of the reconstituted $6S_E$ subunit is shown in Figure 10. The reconstitution was not complete since, on the average, only about one $1.3S_E$ subunit was complexed per $5S_E$ subunit, whereas the full complement is two. Better results might have been obtained if a larger excess of the $1.3S_E$ biotinyl subunit compared to $5S_E$ subunit had been used, but this has not been established as yet.

An example of the reconstitution of active enzyme from the reconstituted $6S_E$ subunit in combination with the $12S_H$ subunit is shown in Figure 11. The results show that an excess of the complementary subunit is necessary to obtain maximum conversion of a subunit to the active enzyme. The effect of increasing the amount of $12S_H$ subunit relative to the reconstituted $6S_E$ subunit is shown in Figure 11A, and the reverse situation, i.e., increasing the $6S_E$ subunit relative to the $12S_H$ subunit, is shown in Figure 11B. The maximum observed specific enzymatic activity based on the $6S_E$ subunit was about 50 and on the $12S_H$ subunit, about 40. These values are estimated to be equivalent to about 60 and 50%, respectively, of the specific activities of these subunits in the 18S form of the enzyme. These values are calculated on the basis of a specific activity of about 45 for the fully active 18S form of the enzyme. (For example, 790,000 × 45/144,000 × 3 = 82. The 790,000 is the molecular weight of the 18S form of the enzyme and the 144,000 is for the $6S_E$ subunit. Since the observed specific activity of the $6S_E$ subunit (Figure 11A) was ~50, this is about 60% of the theoretical value of 82.)

An activity of the subunits which is less than theoretical may be accounted for in part by the fact that the assay of the $6S_E$ subunit is done using an excess of the $12S_H$ subunit. This procedure may yield an enzyme with a single peripheral subunit. If there is cooperativity, a single $6S_E$ subunit on the enzyme would be less effective per $6S_E$ subunit than those of the enzyme which had its full complement of three $6S_E$ subunits. In addition, the reconstituted $6S_E$ subunit on the average contained only one instead of two $1.3S_E$ subunits. Thus, when the $12S_H$ subunit was made limiting, three peripheral $6S_E$ subunits which were deficient in the $1.3S_E$ subunit may have formed a complex with the $12S_H$ subunit. In this case, half of the $12S_H$ substrate sites might be ineffective in the transcarboxylation reaction because they would lack the biotinyl carboxyl carrier protein which is essential for the activity of the site to become evident.

When electron micrographs were made of reconstituted enzyme,[9] the Mickey Mouse form of the enzyme was seen, but its ears were extended, indicating that the peripheral subunits were attached at only one end. Only the two-eared specimen was observed. Further studies using a reconstituted $6S_E$ subunit with a full complement of the $1.3S_E$ subunits will be required to determine whether or not an enzyme with a normal structure and full activity can be made from the isolated subunits.

VI. MECHANISM AND CATALYSIS OF THE TRANSCARBOXYLATION

A. A Nonclassical "Two-site" Bi (Uni-Uni) Ping-Pong Mechanism

The studies by Wood et al.[22] established that the transcarboxylase reaction, like that of several other biotin enzymes, occurs via two partial reactions (see reviews[3-6]). The partial reactions are as follows for transcarboxylase.

$$CH_3 \cdot CH(COO^-) \cdot COSCoA + \text{Enz.-biotin} \rightleftharpoons CH_3 \cdot CH_2 \cdot COSCoA + \text{Enz.-biotin-}COO^- \quad (2)$$

$$\text{Enz.-biotin} \cdot COO^- + CH_3 \cdot CO \cdot COO \rightleftharpoons \text{Enz.-biotin} + OOC \cdot CH_2 \cdot CO \cdot COO^- \quad (3)$$

$$\text{Sum } CH_3 \cdot CH(COO^-) \cdot COSCoA + CH_3 \cdot CO \cdot COO^- \rightleftharpoons CH_3 \cdot CH_2 \cdot COSCoA + {}^-OOC \cdot CH_2 \cdot CO \cdot COO^- \quad (4)$$

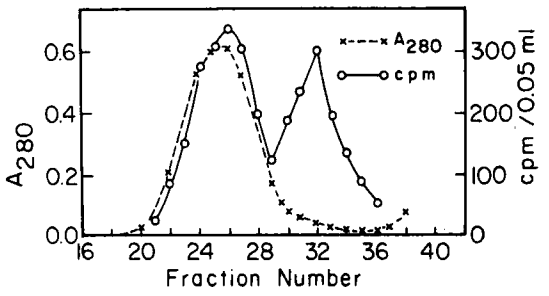


FIGURE 10. Reconstitution of the $6S_E$ subunit from (^{3}H) biotinyl $1.3S_E$ and $5S_E$ subunits. The reconstitution was for 65 hr at 4° in 0.8 ml of 0.25 *M* acetate buffer at pH 5.2 containing 59 nmol of $1.3S_E$ subunit (based on biotin) and 21 mnol of $5S_E$ subunit. The mixture was subjected to glycerol gradient centrifugation, and fractions 21 to 28 were used for the studies of Figure 11. (From Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M., and Brattin, W., *J. Biol. Chem.*, 250, 918, 1975. With permission.)

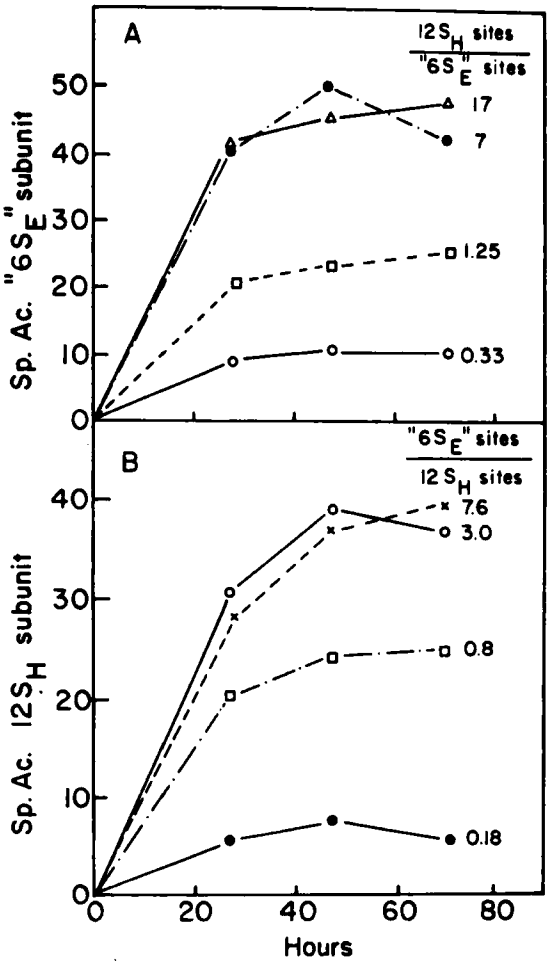
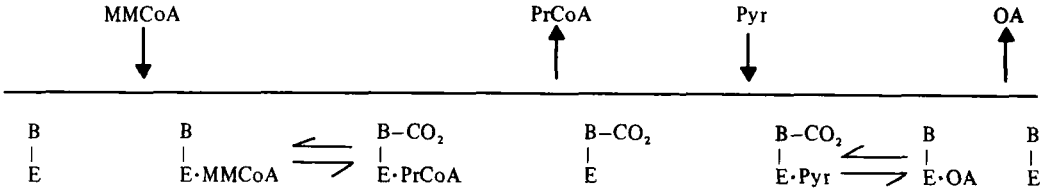


FIGURE 11. Reconstitution of transcarboxylase with the $12S_H$ subunit and the reconstituted $6S_E$ subunit. (A) $12S_H$ subunit in excess of $6S_E$ subunit. (B) $6S_E$ subunit in excess of $12S_H$ subunit. The ratios of sites were calculated on the basis of six sites per $12S_H$ subunit (1 nmol = 0.360 mg. thus nmol site = 0.060 mg) and a bound $1.3S_E$ subunit equal one site on a $6S_E$ subunit (1 bound $1.3S_E$ subunit equal one site on a $6S_E$ subunit (1 nmol $6S_E$ = .144 mg, thus 1 site = 0.072 mg). The required combinations of $12S_H$ and $6S_E$ subunits were made in 0.75 *M* phosphate buffer at pH 6.5 and held in ice. Aliquots were assayed for enzymatic activity at intervals. The specific activities are calculated on the basis of micromoles of oxalacetate formed per milligram of $6S_E$ subunit per minute in (A) and on the basis of the milligrams of $12S_H$ subunit in (B). (From Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M., and Brattin, W., *J. Biol. Chem.*, 250, 918, 1975. With permission.)

It was shown that transcarboxylase is carboxylated by [3-^{14}C] methylmalonyl CoA, yielding an amide with the 1'N of the biotin.[22] Furthermore, incubation of the carboxylated enzyme with pyruvate yielded ^{14}C-oxalacetate equivalent to the enzyme-bound ^{14}C (see the previous review[6] for references and background). Such reactions, in which an enzyme acts alternately as an acceptor and donor of a transferred group, have been designated by Cleland as Ping-Pong mechanisms. There are two types of Ping-Pong mechanisms. The first, the classical Ping-Pong mechanism, involves a single catalytic site and has been demonstrated for such enzymes as transaminase and transaldolase. The second, the nonclassical Ping-Pong mechanism, involves two or more functionally independent catalytic sites, one for each partial reaction. The nonclassical Ping-Pong mechanism was first described by Northrop[23] as a result of his studies with transcarboxylase. Northrop demonstrated that the nonclassical Ping-Pong mechanism may be differentiated from the classical Ping-Pong mechanism by product inhibition studies. The first rule demonstrated by Northrop is that a product of the same partial reaction, i.e., involving the same site, will be competitive with the substrate for that site (for example, propionyl CoA competitive with methylmalonyl CoA). The second rule is that substrate and product of a different partial reaction will give noncompetitive or uncompetitive inhibition (for example, pyruvate noncompetitive with propionyl CoA). The predictions for product inhibition for the classical Ping-Pong mechanism are quite different from those of the nonclassical mechanism.

Northrop[23] demonstrated that transcarboxy-

lase meets all the requirements of the nonclassical Ping-Pong mechanism. It is a nonclassical "two-site" Bi (Uni-Uni) Ping-Pong mechanism, i.e., one substrate combines with the enzyme and one product is released (Uni-Uni), followed by a second substrate on and a product off, thus, Bi (Uni-Uni). The mechanism is illustrated by the line diagram below, where MMCoA is methylmalonyl CoA, PrCoA is propionyl CoA, Pyr is pyruvate, OA is oxalacetate, – is a covalent bond, · is a Michaelis-Menten complex, E is enzyme, and B is the attached biotinyl group. The mechanism is nonclassical since the CoA ester site and keto acid site are different.



It has been shown by Barden et al.[24] that pyruvate carboxylase from chicken liver, likewise, utilizes a "two-site" nonclassical mechanism, but that it is Bi-Bi Uni-Uni Ping-Pong. In this case, two substrates combine with the enzyme (ATP and HCO_3^-) and two products are released (ADP and Pi) during one of the partial reactions, followed by one substrate on (pyruvate) and one product off (oxalacetate) in the second partial reaction. It is of interest that the first example of a nonclassical "three-site" Tri (Uni-Uni Ping-Pong) mechanism has been demonstrated with the enzyme pyruvate phosphate dikinase, which occurs in the propionic acid bacteria.[25]

The above information does not locate the sites; they all could be on one subunit or each on separate subunits. We shall see that each site is on a separate subunit in transcarboxylase, the keto acid site being on the $5S_E$ subunit and the CoA ester site on the $12S_H$ subunit.

B. Catalysis of the Partial Reactions and Location of the Substrate Sites on the Subunits

The availability of the three subunits of transcarboxylase in relatively pure states permitted study of the role of these subunits in the partial reactions.[26] It was known (see References 3 and 5 for reviews and references) that acetyl CoA carboxylase consists of three dissimilar subunits:

(1) the biotinyl carboxyl carrier protein;
(2) the biotin carboxylase, which, with utilization of ATP, catalyzes the carboxylation of the carboxyl carrier protein by HCO_3^-; and
(3) the carboxyl transferase component, which catalyzes the carboxyl transfer from the carboxyl carrier protein to acetyl CoA.

Thus, the partial reactions of this conversion are catalyzed by dissimilar subunits. It seemed likely that transcarboxylase might be similar, i.e., that each of the two functionally independent sites of the nonclassical Bi (Uni-Uni) Ping-Pong mechanism would be on dissimilar subunits, with the biotinyl subunit serving as a carboxyl carrier between the subunits. This was found to be the case, as illustrated below.

$$\mathrm{CH_3 \cdot {}^{14}CH(COO^-) \cdot COSCoA} + \underset{12S_H\ \text{subunit}}{\text{biotinyl subunit}} \rightleftharpoons \mathrm{{}^{-}OO{}^{14}C\text{-biotinyl subunit}} + \mathrm{CH_3 \cdot CH_2 \cdot COSCoA} \quad (5)$$

$$\mathrm{{}^{-}OO{}^{14}C\text{-biotinyl subunit}} + \underset{5S_E\ \text{subunit}}{\mathrm{CH_3 \cdot CO \cdot COO^-}} \rightleftharpoons \text{biotinyl subunit} + \mathrm{{}^{-}OO{}^{14}C \cdot CH_2 \cdot CO \cdot COO^-} \quad (6)$$

$$\text{Sum: } \mathrm{CH_3 \cdot {}^{14}CH(COO^-) \cdot COSCoA} + \mathrm{CH_3 \cdot CO \cdot COO^-} \rightleftharpoons \mathrm{CH_3 \cdot CH_2 \cdot COSCoA} + \mathrm{OO{}^{14}C \cdot CH_2 \cdot CO \cdot COO^-} \quad (7)$$

Evidence substantiating this mechanism is shown in Figure 12. In this experiment, the biotinyl peptides isolated as described in the experiment of Figure 7 were used as the carboxyl acceptor. The $12S_H$ subunit was the catalyst for transcarboxylation with [3-^{14}C] methylmalonyl CoA and $5S_E$ subunit for transcarboxylation with [4-^{14}C] oxalacetate. Other experiments showed that there was no carboxyl transfer if the $5S_E$ subunit was used with the methylmalonyl CoA or the $12S_H$ subunit with the oxalacetate. The carboxylated biotinyl peptides and $12S_H$ or $5S_E$ subunit were separated from the excess ^{14}C-substrate using Sephadex G-25 columns. It is evident (Figure 12) that ^{14}C from the oxalacetate and the methylmalonyl CoA was present in fractions from 10 to 15, which contained the carboxylated biotinyl peptides, and that the peptides and proteins were well separated from the ^{14}C-substrates. That the ^{14}C associated with these fractions was in the form of carboxylated biotin, and not as ^{14}C-bound substrates, was demonstrated by the fact that the carboxylated biotinyl group is unstable to heat, whereas [3-^{14}C] methylmalonyl CoA is stable. It is seen in Figure 12 that there was practically no heat-stable ^{14}C associated with the protein peak. Oxalacetate is heat labile; therefore, malate dehydrogenase and DPNH were added to convert the oxalacetate to a heat-stable form, i.e., malate. However, all the ^{14}C remained heat labile, showing that no ^{14}C-oxalacetate was associated with the protein fractions. When propionyl CoA was added to the mixture containing the $12S_H$ subunit and the ^{14}C-carboxybiotinyl peptide, the ^{14}C was converted to methylmalonyl CoA and became heat stable. When pyruvate was added to

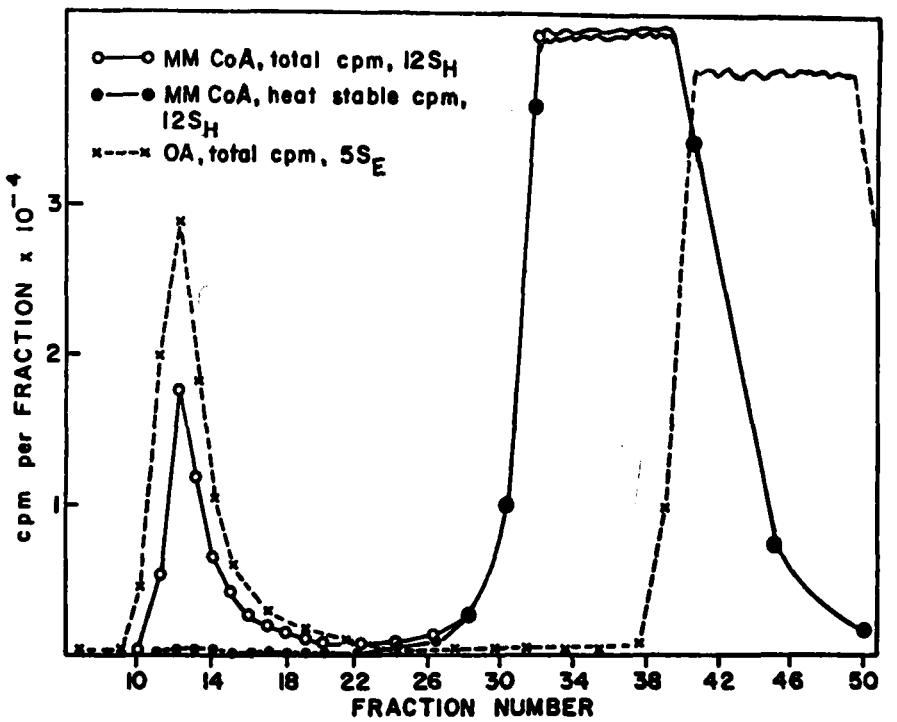


FIGURE 12. Evidence that the partial reactions of transcarboxylase are catalyzed by different subunits. The mixture (0.3 ml) for carboxylation with the $12S_H$ subunit contained 1.10 nmol of $12S_H$ subunit, 13.8 nmol of about an equal mixture of the 46- and 65-residue biotinyl peptides, 65.6 nmol of [3-^{14}C] methylmalonyl CoA (13,000 cpm/nmol), and 20 nmol of phosphate buffer (pH 6.8). The mixture for carboxylation with the $5S_E$ subunit was similar except for the substitution of the $5S_E$ subunit for the $12S_H$ subunit and [4-^{14}C] oxalacetate for the [3-^{14}C] methylmalonyl CoA. Incubation was at 4° for 10 min, and then the mixture was placed on a Sephadex G-25 column. (Reprinted with permission from Chuang, M., Ahmad, F., Jacobson, B., and Wood, H. G., *Biochemistry*, 14, 1611, 1975. Copyright by the American Chemical Society.)

this fraction along with malate dehydrogenase and DPNH, the radioactivity remained heat labile since there was no transfer of the carboxyl to the pyruvate-yielding oxalacetate. Under the same conditions, with the mixture containing the $5S_E$ subunit and the carboxylated biotinyl subunit, the ^{14}C was transferred to the pyruvate and became heat stable in the form of malate. On the other hand, none of the heat labile radioactivity was converted to the heat-stable form if propionyl CoA was added as the carboxyl acceptor. These results show clearly that the CoA ester site is on the $12S_H$ subunit and the keto acid site is on the $5S_E$ subunit.

C. Requirement of a Biotinyl Peptide for the Partial Reaction

The discovery by Lynen et al.[27] that free biotin is carboxylated by β-methylcrotonyl CoA carboxylase opened the door to elucidation of the role of biotin in enzyme catalysis. However, transcarboxylase, in contrast to a number of biotin enzymes, does not carboxylate free biotin. It seemed likely that free biotin could not compete effectively with the biotinyl carboxyl carrier protein in the intact enzyme. Therefore, the carboxylation of free biotin was tested in the partial reactions using the subunits since there is no protein-bound biotin to compete with the free biotin. However, even in the partial reactions, carboxylation of free biotin did not occur.

It was known that the carboxylase component of acetyl CoA carboxylase carboxylates free biotin effectively with ATP and HCO_3^- as substrates; with the transcarboxylase component of this enzyme and malonyl CoA as the carboxyl donor, free biotin is not an effective carboxyl acceptor.[28] However, Polakis et al.[28] found, using derivatives of biotin in which the carboxyl of the valeric acid side chain is reduced or esterified, that these compounds are effective in the transcarboxylation reaction. Thus, it seemed likely that these compounds might be effective in the partial reactions of transcarboxylase with methylmalonyl CoA since this reaction is quite comparable to that with malonyl CoA as catalyzed by the transcarboxylase of acetyl CoA carboxylase. In fact, malonyl CoA is used quite effectively by transcarboxylase. Therefore, biotinyl acetate, biotin methyl ester, and biotinol were tested. They were ineffective (as was biocytin), whereas both the 65- and 46-residue biotinyl peptides were effective. The biotin derivatives were inactive even though they were tested at 1,000 times the molar concentration of the biotinyl peptides. Thus, it is clear that some part of the amino acid sequence of the biotinyl peptide is essential for orienting the biotinyl group so that it may function as a carboxyl acceptor, whereas with the transcarboxylase of acetyl CoA carboxylase, there is a site for binding and activation of the biotin as such. It remains to be determined which portion of the amino acid sequence of the biotinyl peptide is important for the function in transcarboxylase (see Section VIII.A.4).

D. Comparison of the Rate of the Overall Transcarboxylase Reaction by Assembled and Nonassembled Subunits

The fact that transcarboxylase can be assembled from its subunits permits comparison between the rate of the overall reaction as catalyzed by a mixture of the subunits with that of the assembled subunits following reconstitution. The results of this comparison[26] are shown in Table 1. The tests were done under two sets of conditions, first by assaying immediately under conditions not favoring assembly of the subunits and the second by assaying after holding the mixture under conditions favorable for the assembly of the subunits (high concentration of phosphate buffer). In all tests, the $12S_H$ subunit was limiting, and the $5S_E$ and $1.3S_E$ subunits or biotinyl peptide was present in excess. The specific activities, therefore, are calculated on the basis of the $12S_H$ subunit, as was done in the experiments of Figure 11B. The results show that the specific activities were low in all cases, except in the presence of the $1.3S_E$ subunit under conditions favoring reconstitution of the enzyme. The results are as might be predicted, since catalysis by the nonassembled subunits requires the following.

1. That the biotinyl group of the $1.3S_E$ subunit be carboxylated by methylmalonyl CoA through catalysis of the $12S_H$ subunit (reaction 5).
2. That the carboxylated $1.3S_E$ subunit dissociate from the $12S_H$ subunit.
3. That the carboxylated $1.3S_E$ subunits then combine via diffusion with the $5S_E$ subunit.
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 6).

TABLE 1

Comparison of the Catalysis of the Overall Transcarboxylase Reaction with Subunits and Biotinyl Peptides Before and After Reconstitution[a]

No.	Reconstituted	Biotinyl component	Specific activity on basis of $12S_H$ subunit
1	No	$1.3S_E$ subunit	0.38
2	Yes	$1.3S_E$ subunit	10.0
3	No	Biotinyl peptides	0.14
4	Yes	Biotinyl peptides	0.29
5	No	Biotin	0.00
6[b]	No	Biotinyl peptides	0.12
7[b]	Yes	Biotinyl peptides	0.08

[a]The activities are calculated on the basis of the $12S_H$ subunit which was made limiting in the reconstitution.

[b]Trypsinized transcarboxylase was substituted for the $5S_E$ and $12S_H$ subunits in these experiments. In experiments 1, 3, 5, and 6 (not reconstituted) the subunits, biotinyl peptides, and trypsinized transcarboxylase, as indicated, were added to the components for assay of the forward reaction (i.e., pyruvate, methylmalonyl CoA, DPNH, and malate dehydrogenase and phosphate buffer, pH 7.2), and the rate of formation of oxalacetate was determined. In experiments 2, 4, and 7 (reconstituted) the subunits, trypsinized transcarboxylase, and biotinyl peptides, as indicated, were placed in 0.75 *M* phosphate buffer and held in ice. Aliquots were assayed for the forward reaction until maximum activity was reached in 4 days or less. Corrections were made for any activity by the incomplete mixtures of subunits. The $5S_E$, $1.3S_E$ subunits and biotinyl peptides were added in at least threefold excess of the $12S_H$ subunit, assuming two sites for $5S_E$, six for $12S_H$, and one for the $1.3S_E$ subunits or biotinyl peptides.

We shall see (Section VI.F) in the assembled enzyme that the biotinyl group of the $1.3S_E$ subunit perhaps is no more than 4 Å from either substrate site on the two subunits. Thus, there is a possibility of direct transfer of the carboxyl from the carboxyl donor to the biotinyl group and from the carboxylated biotinyl group to the carboxyl acceptor without the necessity of a significant movement of the biotinyl group. Even if transfer is not direct, removal of the necessity of transfer by diffusion serves to illustrate why enzymes are such marvelous catalysts.

The specific activity of 10 for the reconstituted mixture in Experiment 2 of Table 1 is considerably less than the value of 40 to 50 that has been obtained with reconstituted subunits under optimum conditions, such as used in the experiments of Figure 11. To obtain the higher values, the $5S_E$ and $1.3S_E$ subunits are combined first, and then the resulting $6S_E$ subunit is combined with the $12S_H$ subunit. It is likely that part of the observed specific activity of 0.38 with the non-assembled subunits in Experiment 1 resulted from a small amount of assembly which occurred under the conditions of the assay.

Trypsinized transcarboxylase (obtained as in the experiment of Figure 7) was substituted in Experiments 6 and 7 of Table 1 for the $5S_E$ and $12S_H$ subunits. The trypsinized form of the enzyme still has the peripheral $5S_E$ subunits linked to the $12S_H$ subunit by the nonbiotinyl peptide portion of the $1.3S_E$ subunit. Thus, if the biotinyl peptide combined with the trypsinized transcarboxylase in the proper orientation, enzymatic activity should be high. There was no evidence, however, that this occurred even under conditions favoring reconstitution. The failure to obtain good activity with the biotinyl peptides in combination with the trypsinized transcarboxylase was somewhat unexpected because it is known that both the $12S_H$ and $5S_E$ subunits, when isolated from trypsinized transcarboxylase, are active,[11] and the biotinyl peptides are active in the partial reactions. However, for effective carboxyl transfer, which is

not diffusion limited, the biotinyl peptide needs to be anchored to both the $5S_E$ and $12S_H$ subunits. When the peptide is cleaved from the nonbiotinyl peptide, it loses this anchor and apparently does not bind simultaneously to both subunits.

E. Metals in Relation to the Keto Acid Site

We have seen in Section II.B.4 that Ahmad et al.[13] found 7.3 to 8.0 g-atom of Co^{++} plus Zn^{++} per mole of the 18S form of the enzyme (790,000 mole wt), but proposed that the actual value might be 6. They found that the Co^{++} and Zn^{++} were confined to the peripheral subunits (none or very little being present in the $12S_H$ or the $1.3S_E$ subunit). Thus, the six atoms would be equivalent to two per dimeric $5S_E$ subunit. Fung et al.[14] later reported that transcarboxylase not only contains Co^{++} and Zn^{++} but also Cu^{++}, and the total atoms per molecular weight of 790,000 is 12. We shall consider here the evidence that Co^{++} and Zn^{++} are near the keto acid site of the $5S_E$ subunit, the evidence that Cu^{++} is at the keto acid site but inactive catalytically, and the possibility that there may be two types of metals, catalytic and structural.

1 Evidence that Co^{++} is at the Keto Acid Site

Northrop and Wood[12] presented the first preliminary evidence that Co^{++} might be at the keto acid site of transcarboxylase. They used the method that Mildvan and Scrutton[29] had employed to study the interaction of pyruvate with the Mn^{++} of pyruvate carboxylase. Co^{++} like Mn^{++} is paramagnetic. It was shown[12] that transcarboxylase causes a substantial broadening of the nuclear magnetic signal arising from the methyl protons of pyruvate and that this effect is reversed by the addition of increasing concentrations of oxalate. The reversal by oxalate was considered to occur because it chelates with the Co^{++} and displaces the pyruvate from Co^{++}.

Fung et al.,[14] using sophisticated electron and nuclear magnetic techniques, have now completed an extensive investigation of the distances and geometry of the interactions of Co^{++} with pyruvate in transcarboxylase. As background for their studies with transcarboxylase, a brief consideration of the investigations with pyruvate carboxylase will be given (see Utter et al.[4] and Mildvan[30] for references and a more complete discussion). Extensive NMR studies with the pyruvate carboxylase from chicken liver led to the view that the tightly bound Mn^{++} of this enzyme plays a catalytic role by forming a direct complex with the pyruvate. It was considered that the metal aided in the carboxylation because of its electron withdrawing capacity. The proposal was based on the observation that an approximately fourfold enhancement of the relaxation rate of the protons of water was induced by the Mn^{++} bound to the enzyme as compared to $Mn(H_2O)_6$. This enhancement was decreased when a substrate, pyruvate, or oxalacetate was added to the enzyme and by α ketobutyrate and oxalate, which are inhibitors of the pyruvate-oxalacetate partial reaction.[31] The deenhancement was considered to occur because the substrate or inhibitor complexed with Mn^{++} of the enzyme and displaced some of the water. The view that the coordination of Mn^{++} and pyruvate was direct was reinforced when Mildvan and Scrutton[29] investigated the NMR spectrum of the methyl protons of the pyruvate-Mn enzyme complex and estimated the Mn^{++}-to-methyl distance to be about 5.0 Å, which is consistent with an inner sphere complex. Recently, however, with more refined methods and by use of [^{13}C] pyruvate, it has been found that the pyruvate does not form an inner sphere complex but, instead, a second sphere coordinate.[32] In addition, recalculation of the data of the proton relaxation of water, using an experimentally determined value for the correlation time (3×10^{-9} sec) rather than an assumed value, has shown that the maximum number of rapidly exchanging water molecules of the Mn^{++} coordination sphere of the enzyme is 0.33.[33] Thus, the previous hypothesis that pyruvate forms an inner sphere complex by coordination with the Mn^{++} of the enzyme with accompanying displacement of a water molecule is no longer tenable.

Mn^{++} has such a high magnetic moment that the second sphere ligand obscures the signals from the inner sphere ligand in NMR experiments.[32] Therefore, Fung et al.[14] initiated experiments with transcarboxylase. It was expected that the Co^{++} of transcarboxylase, being less paramagnetic than Mn^{++}, would permit observations of the inner sphere complex. The demonstration of such a complex would have major significance for interpretation of the mechanism of the reaction. The interactions of [1-^{13}C] and [2-^{13}C] pyruvate and the methyl protons of pyruvate with Co^{++}-transcarboxylase and the effect of Co^{++}-transcarboxylase on the relaxation rates of the protons

of water were investigated. Since Cu^{++} was found to be present in the enzyme, corrections were made for its paramagnetic effects. We will consider the results of the measurements with Cu^{++} separately.

The longitudinal $1/fT_{1p}$ and transverse $1/fT_{2p}$ relaxation rates of water for Co^{++}-transcarboxylase were determined at 24.3 and 100 MHz, and the longitudinal relaxation rates were used to calculate, by the Solomon-Bloembergen equation, the number of rapidly exchanging water molecules in the coordination sphere of Co^{++}-transcarboxylase. The calculations yielded two water molecules per Co^{++}. Similar measurements and calculations for the ternary pyruvate-Co^{++}-transcarboxylase complex and ternary oxalate complex gave one molecule of water. The decrease is presumed to be due to occlusion of one molecule of water near the metal by the pyruvate or oxalate.

The K_D values of pyruvate and oxalate binding to Co^{++}-transcarboxylase were determined by titration with pyruvate or oxalate and monitoring the $1/T_{1p}$ decrease. The K_D values were calculated with the assumption that the number of pyruvate- or oxalate-binding sites is equivalent to the number of biotins per 790,000 g of transcarboxylase. The K_D for oxalate was found to be 20 μM, which agrees well with the K_I determined kinetically by Northrop and Wood.[34] Since oxalate is a competitive inhibitor with pyruvate[34] and the K_D and K_I values were the same, this is good evidence that oxalate binds at or near the keto acid sites of transcarboxylase. In contrast, the K_D for pyruvate was found to be 7.0 mM, which is about nine times the K_m of pyruvate.[34] However, a similar discrepancy of K_D and K_m was found with pyruvate carboxylase.[31] These authors determined that this discrepancy can be accounted for in Ping-Pong kinetics.

The interaction of Co^{++} with pyruvate containing 70% ^{13}C in the carboxyl and carbonyl position was also determined, and the difference between relaxation rates of these nuclei with Co^{++} and Co^{++} bound to the enzyme was determined. Similar experiments were done to measure the interaction of the Co^{++} in the enzyme with the methyl protons of pyruvate. The addition of a nearly saturating concentration of oxalate caused a marked and similar reduction in the relaxation rates for carbonyl and carboxyl carbons and the methyl protons of pyruvate. These results, therefore, are taken to indicate that the paramagnetic effects with pyruvate are due to interaction at the keto acid site of transcarboxylase.

The longitudinal relaxation of the methyl protons of pyruvate in the transcarboxylase complex was dependent on frequency. This result permits estimation of the correlation time for the Co^{++}-pyruvate dipolar interaction. It was found to be 2.2×10^{-12} sec. Using this value and the relaxation rates (corrected for Cu^{++}), the distances between the Co^{++} and carbon atoms and methyl protons were calculated and are shown in Figure 13. It was found that the only position for the bound Co^{++} consistent with the distances from the carboxyl, carbonyl, and methyl protons of the pyruvate is when the Co^{++} is placed about 1 Å out of the plane defined by the carbon atoms of the

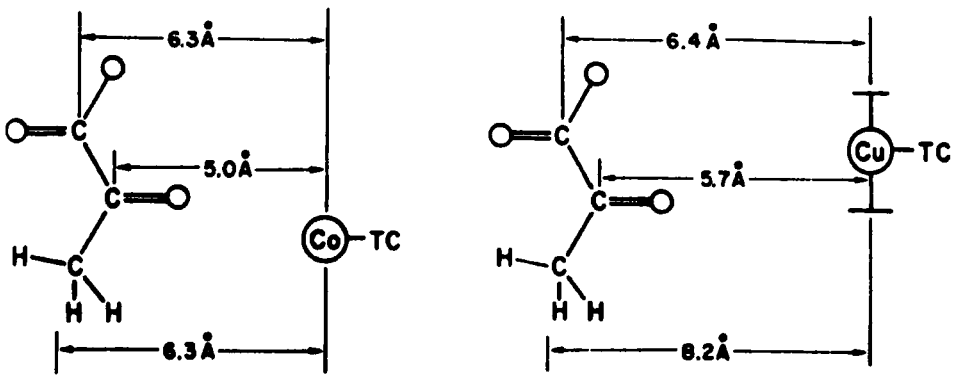


FIGURE 13. Distances of bound pyruvate from the Co^{++} and Cu^{++} of transcarboxylase as determined by Fung et al.[14] The uncertainty of the positioning of Cu^{++} is indicated by the error bars. (Reprinted with permission from Fung, C. H., Mildvan, A. S., and Leigh, J. S., Jr., *Biochemistry*, 13, 1160, 1974. Copyright by the American Chemical Society.)

bound pyruvate molecule. The shortest distance to the cobalt was from the carbonyl carbon (5.0 Å) which is 2.1 Å greater than the maximum distance (2.9 Å) for an inner sphere complex. Thus, the Co^{++} of the enzyme forms a second sphere complex with the pyruvate, which is the same situation that was observed between pyruvate and the Mn^{++} of pyruvate carboxylase.[32]

The role of Co^{++} as related to the mechanism of transcarboxylation will be considered in Section VI.G.4.

2. Evidence that Cu^{++} is at the Keto Acid Site but Inactive

Fung et al.[14] have concluded that Cu^{++} can replace Co^{++} and Zn^{++} at the pyruvate acid site, but it is catalytically inactive. They give four lines of evidence supporting this view.

1. The Cu^{++}:Zn^{++} and Cu^{++}:Co^{++} ratios vary from preparation to preparation, but the total number of metals is constant (see Table 2).

2. The specific activity appears to be proportional to the sum of the Co^{++} and Zn^{++} in the enzyme.

3. Pyruvate and oxalate decrease the effect of enzyme-bound Co^{++}, but not of enzyme-bound Cu^{++}, on the relaxation rates of the protons of water.

4. The distances between Co^{++} of transcarboxylase and Cu^{++} of transcarboxylase and the nuclei of pyruvate are similar (see Figure 13).

We consider that the four points cited above are inconclusive evidence that Cu^{++} is inactive (or active). The evidence relative to the relaxation rates of the protons of water will be considered first. Pyruvate and oxalate were found to displace a molecule of water from the Co^{++} of the enzyme but not from the Cu^{++}; pyruvate and oxalate had no effect on the relaxation rates of protons of the water bound to the Cu^{++} of the enzyme, whereas they did on the protons of water bound to the Co^{++} of the enzyme. However, only 0.1 molecule of water was found bound to the Cu^{++} enzyme; thus, it is doubtful that this water plays any part in the activity of Cu^{++}, if it is active. Since the ligands of Cu^{++} have very different preferred orientations than those of Co^{++}, the Cu^{++} would not be expected to respond to a pyruvate or oxalate ligand in the same manner as Co^{++}.* Nevertheless, pyruvate binds very near to the Cu^{++} of the enzyme (Figure 13), but is oriented somewhat differently than when bound to the Co^{++} of the enzyme. It seems to us that the fact that pyruvate binds near Cu^{++} of the enzyme, but does not have the same effect on the protons of water as when the pyruvate binds near the Co^{++} of the enzyme, is not evidence that the Cu^{++} is inactive. Cu^{++} would not necessarily have the same effect even if it were active. The point is that the pyruvate is bound near the Cu^{++} and the question remains, is it active or not.

The crux of the evidence that Cu^{++} is inactive thus rests on the relationship of the specific activity of the enzyme and its Cu^{++} content. It was not shown that there is a direct relationship between the Cu^{++} content and the specific activity of the enzyme. Instead, it was shown that there is

*Listed below are the preferred and most common coordination numbers and stereochemical orientations of small ligands interacting with the metals Zn^{++}, Co^{++}, and Cu^{++}.

Metal	Electron configuration	Coordination number	Stereochemistry
Zn^{++}	d^{10}	4	Tetrahedral
		6	Octahedral
Co^{++}	d^{7}	4	Tetrahedral
		6	Octahedral
Cu^{++}	d^{9}	4	Square
		6	Distorted octahedral

Zn^{++} and Co^{++} can also form square complexes, and Cu^{++} can form distorted tetrahedral complexes; such examples, however, are rare and not the preferred orientation of the ligands.[36] Fung et al.[14] interpret the EPR spectrum for bound Co^{++} as being that of a distorted octahedral environment, while that of bound Cu^{++} is symmetric. This observation is consistent with the Jahn-Teller theorem which predicts that electronic configurations such as Cu^{++} (d^9, t_{2g}^6 eg^3) will form distorted octahedral complexes, and the distortion will stabilize that conformation.[36,37] In a number of Cu^{++} compounds the distortions of the octahedra around the cupric ion are so extreme that the coordination is best regarded as virtually square.[36] It is difficult to rationalize an explanation for the enzyme incorporating a metal which does not have the proper electronic configuration to give the needed stereochemical orientations.

TABLE 2

Metal and Biotin Content of Transcarboxylase

	TC from 50 μM Zn medium (Zn-TC)	TC from 0.2 mM Co medium (Co-TC)	TC from a defined medium containing Co(II)	
			0.3 mM Co	0.1 mM Co
Specific activity	34.0	45.0	37.3	33.6
Biotin	6.6	7.1	5.4	5.4
Zn^{2+}	7.7	5.1	5.6	5.6
Co^{2+}	0.2	6.0	3.6	2.8
Cu^{2+}	4.8	2.4	2.2	1.8
Total metal	12.7	13.5	11.4	10.2
Total metal/biotin	1.9	1.9	2.1	1.9
Specific activity/biotin	5.2	6.3	6.9	6.2
Specific activity/Zn and Co	4.3	4.1	4.1	4.0
Specific activity/total metal	2.7	3.3	3.3	3.3

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a direct relationship between the Co^{++} and Zn^{++} content and the specific activity of the enzyme preparations. Thus, when the content of Co^{++} and Zn^{++} decreases, the specific activity decreases. Since the Cu^{++} is believed to replace the Co^{++} and Zn^{++}, the decrease in enzyme activity is ascribed to the inactivity of the Cu^{++}.

We do not consider convincing the evidence that the variation of specific activities of the four preparations of enzymes shown in Table 2 is due to Cu^{++} replacing Co^{++} and Zn^{++}. The specific activity of transcarboxylase is related to the number of peripheral subunits (see Section II.B.1, Figure 2); since the peripheral subunits contain the biotin, Co^{++}, and Zn^{++}, there is a relationship between the Co^{++} plus Zn^{++} and activity. In all cases the Co^{++} plus Zn^{++} was greater than six. There probably are only six pyruvate sites, so there is enough Co^{++} plus Zn^{++} to occupy each pyruvate site. It is to be noted (Table 2) that the enzyme with 7.1 biotins and with the highest enzymatic activity may have had more than three peripheral subunits (see Section VII concerning the 26S form of the enzyme). It also is to be noted that the enzyme with 1.8 Cu^{++} atoms had a specific activity of 33.6 and that with 4.8 atoms of Cu^{++} had a specific activity of 34.0.

We find that very pure preparations of transcarboxylase differ in activity because they vary in stability. Transcarboxylase preparations often lose activity, and the loss almost certainly is not due to loss of Co^{++} or Zn^{++} through replacement of Co^{++} or Zn^{++} by Cu^{++}. Ahmad et al.[13] found that the Co^{++} and Zn^{++} are bound extremely firmly to the enzyme; in spite of many tests, they were unable to remove or exchange ^{60}Co or ^{65}Zn from the enzyme. In addition, we have found that some preparations that have lost activity can be reactivated by warming the enzyme in high ionic strength $(NH_4)_2SO_4$ solutions.[35]

There is little doubt that there is a relationship between the specific activity of transcarboxylase and the content of biotin or Co^{++} plus Zn^{++}, but the relationship between Cu^{++} and enzymatic activity, in our opinion, is not established.

3. The Possibility of Two Types of Metals in Transcarboxylase, Catalytic and Structural

It was proposed by Ahmad et al.[13] that there are six cobalt plus zinc atoms in the 18S form of transcarboxylase, but all investigators[12-14] have found values higher than six when the metal content is calculated on the basis of 790,000 g. The calculation of the distance of pyruvate from the Co^{++} or Cu^{++} of the enzyme (Figure 13) was made on the basis of coordination of one metal ion to one pyruvate.[14] This assumption implies there are 12 keto acid sites, i.e., two per protomer of the $5S_E$ subunit. Although the possibility of two sites per $2.5S_E$ polypeptide is not excluded, it seems more likely that there would be one pyruvate site per polypeptide, i.e., six. If all 12 metals were at six pyruvate sites, there would be two metals per site. Fung et al.[14] have recognized this

problem and have discussed two possible situations. (1) One of each pair of metals is located very far from the pyruvate, and it exerts a negligible paramagnetic effect on the relaxation rates of pyruvate nuclei. (2) Two metals are equidistant from the pyruvate site, and each exerts an equal effect on the relaxation rates of pyruvate. The second case was not considered very likely because it is not very feasible geometrically. Fung et al.[14] point out that the distances which were calculated would not be altered very much by the above circumstances. They state that the distances given in Figure 13 should be divided by about 1.12 to correct the distances for either case. A difference of 12% in the distances would not require an alteration in any of their conclusions. A more important aspect of these considerations, which was not discussed, is whether or not there are metals which are different distances from the keto acid site and what their roles are. It is obvious that further study is needed to determine the role of metals in transcarboxylase and whether there are both structural and catalytic metals. In addition, the number of pyruvate sites per $5S_E$ subunit should be determined, and the total metal content of transcarboxylase should be carefully reexamined.

F. Measurement of Distances with the CoA Esters and Determination of the Number of CoA Ester Sites

We have seen that the studies of EPR and NMR by Fung et al.[14] indicate that enzyme-bound pyruvate is very close to the Co^{++} of transcarboxylase, but not close enough to form an inner sphere complex with the Co^{++}. Fung et al.[38] have extended these studies by measurement of the distance of enzyme-bound propionyl CoA from the Co^{++} and also have used [^{13}C] pyruvate and a paramagnetic analogue of propionyl CoA to obtain information about the distance between propionyl CoA and pyruvate.[39] Such measurements are extremely important in relation to the quaternary structure of transcarboxylase. These measurements should give an indication of the distances between the subunits since the CoA ester site is on the $12S_H$ subunit and the keto acid site is on the $5S_E$ subunit (Section VI.B). Likewise, since the biotinyl group must transfer the carboxyl between these sites, the results should give an indication of distance which may be involved in this transfer. In addition, the measurements may define the orientation of the two substances to each other and also give an indication of the symmetry relationships of the two subunits to which they are bound (VIII.B.3.c). Due to the complexity of transcarboxylase, an analysis of the structure by X-ray crystallography is likely to be difficult and not available for some time to come. Therefore, electron microscopy, EPR and NMR measurements, and possibly cross linking of the subunits offer the best opportunity at present to obtain some detail concerning the quaternary structure of this enzyme.

1. Distance between the Co^{++} of Transcarboxylase and Propionyl CoA

Fung et al.,[38] using procedures similar to those described for measurement of the distance of pyruvate from the Co^{++} of transcarboxylase (VI.E.1), have measured distances between Co^{++} and propionyl CoA. They were able to estimate seven distances, two to the protons of the methyl and methylene positions of the propionyl group, two to the protons of the phosphopantetheine group, and three to the protons of the adenosine diphosphate group. In addition, for those protons for which no paramagnetic effects of Co^{++} were detected, lower limit distances were calculated from the error limits of the relaxation rates. These distances were determined for eight positions, including the three phosphorous atoms and five of the protons of the phosphopantetheine. The seven distances for which actual values were obtained were used for a computer program to determine the optimum geometry of propionyl CoA which satisfies the measured distances and which minimizes van der Waal's overlaps of the atoms of the propionyl CoA. The computer search was carried out by simultaneous rotation of portions of the molecule about the N(9)–C(1′), C(5′)–o(5′), and C(1)–0(1) bonds in increments of 10° and searching for those conformations with less than 0.5 Å total van der Waal's overlap. Out of the 46,656 rotamers examined, the computer generated 21 conformations which were consistent with the seven distances and a 0.5-Å (or less) overlap. By use of three of the lower limit distances, the remaining structures were then ranked by tabulating deviation from the lower limits. Two "best fit" conformations were found in which the mean deviation from the eight lower limit distances was 0.423 Å. The two were indistinguishable, and one structure is shown in Figure 14. Also included are the distances

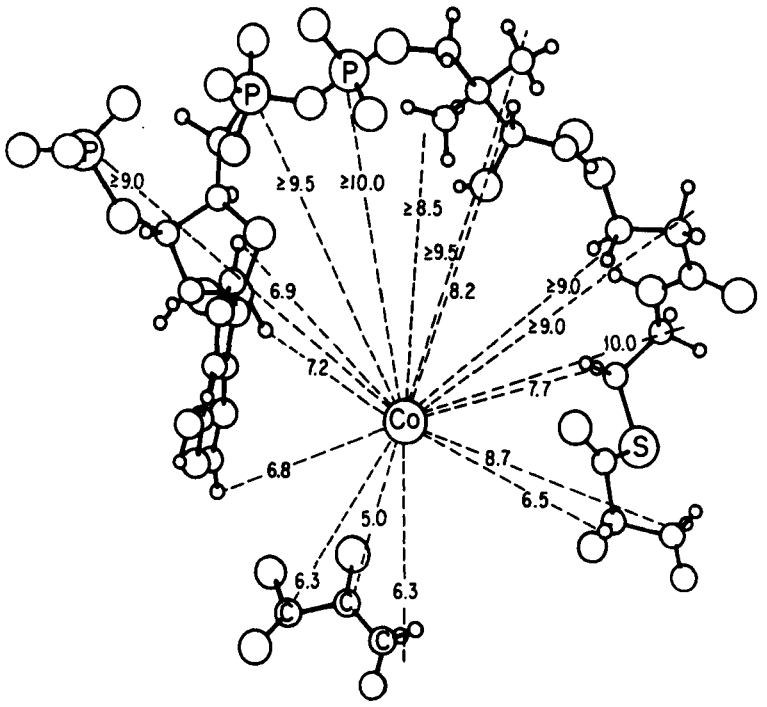


FIGURE 14. Distances of bound propionyl CoA and of bound pyruvate from Co^{++} of transcarboxylase as determined by Fung et al.[14,38] (Reprinted with permission from Fung, C. H., Feldman, R. H., and Mildvan, A. S., *Biochemistry*, 15, 75, 1976. Copyright by the American Chemical Society.)

measured previously with pyruvate (Section VI. E.1).

The conformation of the propionyl CoA is seen to be U-shaped around the Co^{++}, and it is estimated to be approximately 22% unfolded when compared to the conformation of free propionyl CoA. The Co^{++} atom is 4.5Å above the plane defined by the adenine H(2), the pantetheine C9, and the propionyl methylene carbon. All of the distances measured indicated that the propionyl CoA does not coordinate or interact directly with the Co^{++}, methylene protons of the propionyl group are estimated to be very close to the Co^{++} (6.5 Å). This is of interest because one of the two methylene protons is the pro-R proton which is removed from the propionyl group during its carboxylation (Section VI.G.1).

Included in this study were measurements of the relaxation rates of the nuclei of free propionyl CoA and the effects of varying content of Zn^{++}, Co^{++}, and Cu^{++} on the relaxation rates of the 12 protons and 3 phosphorous atoms, as well as a host of other information for backup of this study.

2. Distance between the Cu^{++} of Transcarboxylase and Propionyl CoA

These measurements were made as described above for Co^{++} and propionyl CoA. Each distance was larger than that calculated for Co^{++}, with the exception of the pantetheine CH_2 group at position 9. The differences are approximately 1.3 to 2.3 Å greater for Cu^{++} than Co^{++}. The methylene protons of the propionyl group were calculated to be 8.1 Å from the Cu^{++}.

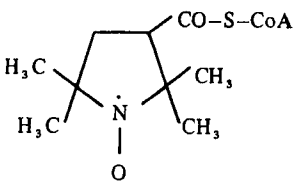
The fit obtained by the computer selection was poorer than that observed with Co^{++}. The mean deviation from the lower limit distance was 1.5 Å, but the local conformations of individual portions of propionyl CoA at Cu^{++} were indistinguishable from those at the Co^{++}. The propionyl CoA was more unfolded than with Co^{++}. The Cu^{++} is considered to be catalytically inactive with the propionyl CoA.

3. Distances between Propionyl CoA and Pyruvate on Transcarboxylase

We have noted previously (Section VI.E.3) that part of the metals of transcarboxylase may have a

structural rather than a catalytic role. Thus, it is possible that propionyl CoA and pyruvate bind near different types of Co^{++}. In that case, the measurements with these two compounds might not indicate the distances between the two substrates per se. Fung et al.[39] have attempted to make measurements to overcome this problem by use of an analogue of propionyl CoA containing a nitroxide radical, thus permitting direct measurements between it and [^{13}C] pyruvate.

The paramagnetic CoA analogue used in these studies was 3-carboxy-2,2,5,5-tetramethyl-1-pyrolidinyl-1-oxy CoA thioester, which will be referred to as R·CoA; its structure is illustrated below.



When Zn^{++}-enriched enzyme was added to R·CoA there was a marked decrease in the three EPR resonance lines of free R·CoA, with a concomitant appearance of two new signals due to binding of R·CoA to the enzyme. This EPR spectrum was found to be reversed by the addition of propionyl CoA, indicating the R·CoA is displaced from the enzyme. From this type of experiment it was calculated that propionyl CoA binds to the enzyme with a dissociation constant which is 1.32-fold lower than that of R·CoA. No effect on the EPR spectrum of free and enzyme-bound R·CoA was detected in the presence of pyruvate. The above results indicated that the R·CoA binds specifically to the propionyl CoA sites on the enzyme. Therefore, R·CoA was judged to be suitable for determination of the distances between the nitroxide radical and enzyme-bound pyruvate. We shall see (in Section VI.F.5 below) that the more important question is whether pyruvate was bound to the $12S_H$ subunit as well as to the $5S_E$ subunit. The paramagnetic effects of enzyme-bound R·CoA on the longitudinal relaxation rates ($1/fT_{1p}$) of the methyl protons and the [^{13}C] carboxyl carbon atoms of pyruvate were determined, as was the correlation time for dipolar effects, τ_c. These values were used to calculate the distance between the R·CoA and the pyruvate nuclei. The theoretical requirements for the measurements were carefully controlled. It was demonstrated that the ($1/fT_{1p}$) values were not exchange limited and were equal to the relaxation rate of the bound ligand ($1/T_{1m}$). The important τ_c value was determined by three separate methods, and the values all agreed within experimental error. The resulting distance measurements were as follows: methyl protons, 7.9 ± 0.7 Å; carbonyl carbon, 10.3 ± 0.8 Å; carboxyl carbon, 12.1 ± 0.9 Å.

By use of the EPR spectral data for the interaction of the Co^{++} of the enzyme with the bound R·CoA complex, Fung et al.[39] attempted to calculate the distance from the nitroxide of R·CoA to the Co^{++} by applying the theoretical line width equation of Drott.[40] Because of the very weak interaction of the Co^{++} with the nitroxide, it could only be established that the distance was equal to or greater than 9.0 Å.

The distances measured from the nitroxide radical of R·CoA to the pyruvate nuclei are shown in Figure 15. A composite model of the pyruvate and propionyl CoA was constructed using the distances measured from Co^{++} to the pyruvate nuclei and the distances measured from Co^{++} to the protons and phosphorous atoms of propionyl CoA; a distance of approximately 7 Å between the active centers of the two substrates (methyl carbon of pyruvate and methylene carbon of propionyl CoA) was calculated on the basis of the model. Fung et al.[39] conclude that these experiments establish the following.

1. That the pyruvate and propionyl CoA bind near the same Co^{++} metal.
2. That the $12S_H$ subunit (binding site of propionyl CoA) and the $5S_E$ subunit (binding site of pyruvate) are in close proximity.
3. That the conformation, orientation, and distances of the substrates at the active site in the enzyme are established.

The possible location of the biotinyl group of the carboxyl carrier protein as related to these sites on the respective subunits is shown in Figure 16. The reliability of their measurements will be considered in Section VI.F.5 below.

4. Number of CoA Ester Sites

In addition to the above use of R·CoA, Fung et al.[39] have attempted to use R·CoA for measurement of the number of CoA ester sites. We shall

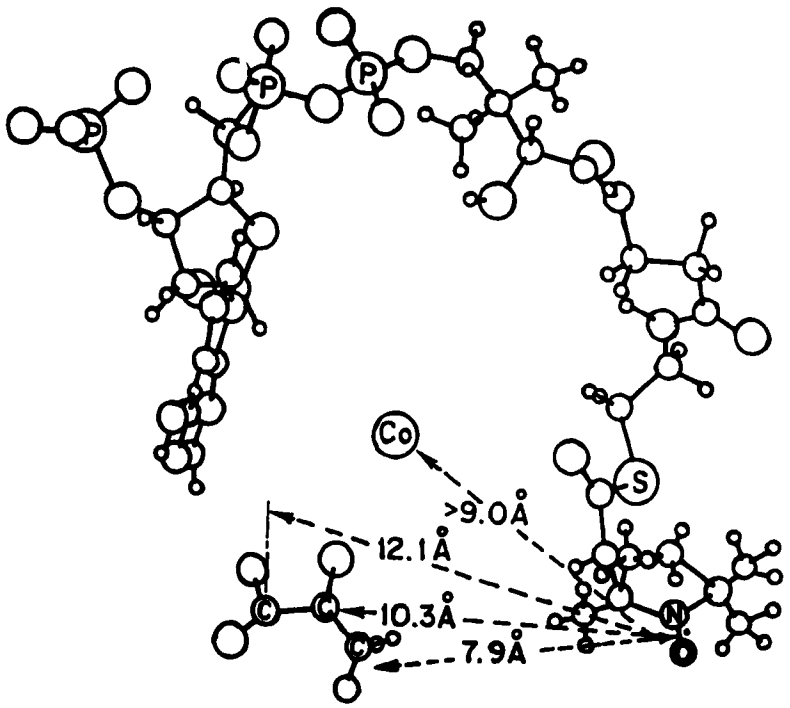


FIGURE 15. Distances of the nitroxide radical of a bound analogue of propionyl CoA from bound pyruvate on transcarboxylase as determined by Fung et al.[39] The conformation of the pyruvate and CoA ester near the Co^{++} of transcarboxylase is based on other measurements of Fung et al.[14,38] (Reprinted with permission from Fung, C. H., Gupta, R. K., and Mildvan, A. S., *Biochemistry*, 15, 85, 1976. Copyright by the American Chemical Society.)

see in Section VIII.B.3.c that this becomes an important question relative to the quaternary structure of transcarboxylase.

The number of binding sites was estimated by titrating the enzyme with R·CoA and measuring the concentration of free R·CoA by EPR. A Scatchard plot of these data gave a biphasic curve, which is indicative of more than one class of binding sites for R·CoA on the enzyme. The number and dissociation constants of both kinds of sites were calculated from an equation which relates concentration, binding site number, and dissociation constants for two thermodynamically noninteracting binding sites. It was calculated that there are 0.7 ± 0.2 tight binding sites (K_D = 0.33 ± 0.12 m*M*) and 2.3 ± 1.0 loose binding sites (K_D = 8.0 ± 3.0 m*M*) per mole of biotin for the R·CoA molecule. Since propionyl CoA displaces R·CoA from the tight site, it was stated[39] that "the tight sites (0.7 ± 0.2/mole biotin) probably represent the active sites for propionyl CoA." In addition, the K_D values determined for R·CoA (0.33 m*M*) and propionyl CoA (0.25 m*M*) by EPR measurements were compared to the inhibitor constants determined kinetically by Northrop[23] for CoA (0.6 m*M*) and propionyl CoA (0.4 m*M*). It is to be noted, however, that the value sited for propionyl CoA is for substrate inhibition and not for product inhibition. It is the authors' opinion that this comparison is not valid since the propionyl CoA substrate inhibition probably is at the keto acid site rather than the CoA site (see Section VI.F.5 below). The K_I for product inhibition should be used for this comparison, which for propionyl CoA is 0.049 m*M*,[23] considerably lower than the K_D values determined by Fung et al.[39]*

It is to be noted that the upper limit of precision of the calculation indicates that there is about one CoA ester binding site per biotin (or six per enzyme), while the lower limit indicates one CoA ester site per 2 mol of biotin (or three per enzyme). Fung et al.[39] appear to favor the six

*Mildvan, in a private communication, has informed us that the K_I of product inhibition by propionyl CoA is 0.150 ± 0.050 m*M* under the conditions used in their[39] experiments, i.e., 0.22 *M* KCl at pH 6.7.

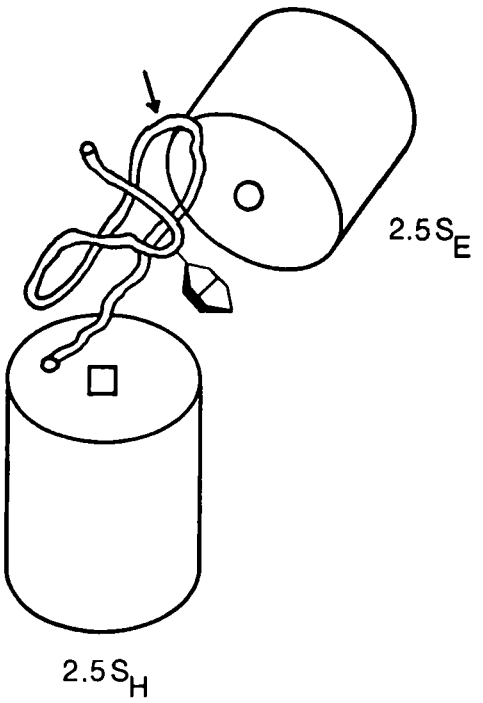


FIGURE 16. Illustration based on the results of Fung et al.,[14,38,39] Chuang et al.,[26] and Zwolinski et al.[69] This illustration shows the linking of the constituent polypeptides of the central subunit and peripheral subunits by the 1.3S_E carboxyl carrier protein. The square on the 2.5S_H polypeptide represents the CoA ester binding site; the circle on the 2.5S_E polypeptide represents the keto acid binding site; biotin, which is attached to the ε-amino group of lysine number 87 on the 1.3S_E subunit, is represented by a hexagon. The arrow indicates the position at which trypsin cleaves the arginyl-alanyl bond at position 42 of the 1.3S_E subunit, thereby removing the biotinyl portion of the 1.3S_E subunit from the intact enzyme.

binding sites and have rationalized the deviation from six in terms of the specific activity of the enzyme preparation used in the titration experiments. The enzyme had a specific activity 87 ± 9% of that of three other enzyme preparations and 73% of that of the enzyme with highest specific activity. The rationalization implies that the observed lower specific activity is due to loss of binding sites for the CoA ester, thus rendering the enzyme less active. This may be a valid assumption, but other factors influence the specific activity. Therefore, the number of CoA binding sites remains in question. More accurate information is needed relative to the number of CoA ester sites.

5. Possible Sources of Error in the Measurement of Distances

The measurements of the distances between the substrates and the Co^{++} of transcarboxylase and between propionyl CoA and pyruvate bound to the enzyme are exceedingly important for the interpretation of the quaternary structure of transcarboxylase (see Section VIII.B.3.c for an explanation of the importance). For this reason, we have examined the measurements very carefully for possible sources of error, which include the following.

1. The measurements were made on the uncarboxylated form of the enzyme, whereas it is the carboxylated form of the enzyme with which both pyruvate and propionyl CoA react. This situation probably is most critical in the case of the pyruvate-to-Co^{++} measurements. If there was a conformational change accompanying carboxylation of the enzyme, it is possible that this change might move the pyruvate site closer to the Co^{++} on the enzyme. In this case, the distance between the Co^{++} and pyruvate might fall within the limits of an inner sphere complex. The question of whether or not pyruvate forms an inner sphere complex with the Co^{++} is of considerable importance in evaluating the mechanism of the catalysis. There is precedent for considering that carboxylation of a biotinyl enzyme may influence conformation. For example, Gregolin et al.[41] have shown that the protomer-polymer equilibrium of acetyl CoA carboxylase from chicken liver is shifted toward the protomer form by converting the enzyme to its carboxylated form.

2. Another possible source of error is that the metals may have two functions, catalytic and structural, and are at different locations in the 5S_E subunit (Section VI.E.3). The situation relative to the measurements with pyruvate has been considered by Fung et al.,[14] as discussed in Section VI.E.3, for the two extremes. First, the two types of metal are equidistant from the pyruvate; second, the one metal is close to the pyruvate and one metal is far removed. They have shown that the distance as determined would only be in error by 12% in either case. The question of the location of the two types of metals (if there are two types) is much more critical for the propionyl CoA measurements. It is possible that the distance measured is to a structural metal of the 5S_E subunit which is quite far removed from the keto acid site. In this case, the measurements

would indicate the distance between the $12S_H$ and $5S_E$ subunits (assuming the propionyl CoA is on the $12S_H$ subunit, see 3 below), but it would not give a direct indication of how far the biotinyl group might have to travel for the transcarboxylation from the propionyl CoA site on the $12S_H$ to the keto acid site on the $5S_E$ subunit.

3. Potentially, the most critical error is that the measurements with propionyl CoA may have been from the CoA ester at the keto acid site to the Co^{++} and not from the CoA ester site on the $12S_H$ subunit to Co^{++} on the $5S_E$ subunit. This possibility arises because Northrop[23] has demonstrated that substrate inhibition occurs with both propionyl CoA and pyruvate at a concentration about ten times the K_M values. Reciprocal plots at these higher concentrations showed that the inhibition is competitive. The K_I for substrate inhibition by propionyl CoA is 4×10^{-4} *M* in the reaction with oxalacetate; for pyruvate it is 3.2×10^{-2} *M** in the reaction with methylmalonyl CoA. In discussing the substrate inhibition, Northrop states, "At high concentrations, propionyl CoA must bind to the keto acid site and pyruvate must bind to the CoA ester site."**

Fung et al.'s[38] determinations of relaxation rates with propionyl CoA were performed at a concentration of 10 m*M*, which is 25 times the K_I value (0.4 m*M*) for substrate inhibition by propionyl CoA. At 25 times the substrate K_I, a major portion of the keto acid sites on the $5S_E$ subunit would be occupied by propionyl CoA, if Northrop's conclusion is correct. Therefore, the distances measured from propionyl CoA to Co^{++} may be of propionyl CoA bound to the keto acid site on the $5S_E$ subunit rather than from the CoA ester on the $12S_H$ subunit to Co^{++} of the $5S_E$ subunit.

The experiments with the spin-labeled analogue of propionyl CoA, in which the propionyl portion of the molecule was replaced with the nitroxide radical, were done as a control on this problem. In this case, the measurements were made between the nitroxide and [^{13}C] pyruvate. However, the concentration of [^{13}C] pyruvate used in the determination of the relaxation rates was about equal to the K_I for substrate inhibition by pyruvate. At these concentrations there is a high probability that a substantial part of the CoA ester sites on the $12S_H$ subunit are occupied by pyruvate.*** Thus, it is possible that the relaxation rate enhancement was with nuclei of pyruvate on the $12S_H$ subunit, i.e., between two sites on the $12S_H$ subunit instead of from nitroxide on the $12S_H$ subunit to pyruvate nuclei on the $5S_E$ subunit. We have no information to indicate how far the two CoA ester sites might be from each other on the $12S_H$ subunit.

There is some reason to consider that the distance between the $12S_H$ and $5S_E$ may not be as close as 7 Å. First, electron microscopy indicates that these subunits may be as far as 30 Å apart. Second, avidin combines with the biotinyl group and, therefore, must have access to it. If the biotinyl group is in such close quarters between the $5S_E$ and $12S_H$ subunits, it seems remarkable that it reacts rapidly with avidin which has dimensions of 41 × 55 × 41 Å.[42] The biotinyl peptide no doubt migrates out of the channel between the subunit, but 7 Å is a narrow gap for rapid escape.

The measurements made by Mildvan's group on transcarboxylase are exceedingly important, if accurate. It is the authors' opinion, however, that the measurements should be repeated under conditions which eliminate the possibility that the

*Recalculation of the K_I for substrate inhibition by pyruvate using the data of Northrop[23] gave a value of 3.2×10^{-2} rather than the published value of 1.5×10^{-2} *M*. We confirmed his calculated value of 4×10^{-4} *M* for substrate inhibition by propionyl CoA. Our preliminary experiments using D_2O and buffers, as employed by Fung et al.,[39] gave a K_I for the substrate inhibition by pyruvate of 5.1×10^{-2} *M*. This value has been used in our calculation of the pyruvate which might be bound on the $12S_H$ subunit (see footnote).

**Northrop, in a private communication, stated: "By default it became necessary to invoke a second binding site to account for substrate inhibition since substrate inhibition cannot be accounted for within the two site ping-pong mechanism by the binding of a substrate to its proper site, regardless of the form of the enzyme. The inhibition is competitive and the simplest explanation is that the keto acids bind to the CoA site and vice-versa. It is equally possible that propionyl CoA binds only near to the keto acid site in such a way that only a small part obstructs the keto acid site. This would allow for competitive inhibition, yet not set the inhibitory propionyl-CoA as close to cobalt as the pyruvate. The main point is not so much the 'correct' identification of the second binding site but the existence of a second site. Such existence is independently confirmed by Mildvan biphasic Scatchard plot with R·CoA."

***Footnote appears on next 2 pages.

***A calculation illustrating the amount of pyruvate which might be on the $12S_H$ subunit at the CoA ester sites using the data of Experiment 2 in Table 2[39] is as follows.

$[12S_H]_T$ = 0.260 m*M*, the concentration of CoA ester sites on the $12S_H$ subunit, the number of CoA ester sites is assumed equal to the number of biotins

[PYR] = 29.4 m*M*, the concentration of pyruvate

$[12S_H\ RCoA]$ = 0.040 m*M*, concentration of bound nitroxide (RCoA) (at the CoA ester site)

[RCoA] = 0.200 – 0.040 = 0.160 m*M*, concentration of free nitroxide (RCoA), $[RCoA]_T$ = 0.200 m*M*

$$K_{D,\ RCoA} = \frac{[12S_H]_{free}\,[RCoA]}{[12S_H\ RCoA]} = \text{apparent dissociation constant of RCoA} \qquad (A)$$

$$K_{I,\ PYR} = \frac{[12S_H]_{free}\,[PYR]}{[12S_H\ PYR]} = 51\ mM, \text{ substrate inhibition constant for pyruvate} \qquad (B)$$

$$K_{D,\ RCoA} = \frac{[12S_H]_{free}\,[0.160]}{[0.040]}\ ;\ [12S_H]_{free} = 0.250\ K_{D,\ RCoA} \qquad (C)$$

The concentration of pyruvate bound at the keto acid site on the $5S_E$ subunit can be neglected in the calculation because it is very small, approximately 113 times smaller than the total pyruvate concentration.

$$[12S_H]_{Total} = [12S_H\ RCoA] + [12S_H\ PYR] + [12S_H]_{free}$$

$$[12S_H\ PYR] = [12S_H]_{Total} - [12S_H\ RCoA] - [12S_H]_{free} \qquad (D)$$

$$K_{I,\ PYR = 51} = \frac{[12S_H]_{free}\,[PYR]_{Total}}{[12S_H]_{Total} - [12S_H\ RCoA] - [12S_H]_{free}}\ ;\ [12S_H]_{free} = 0.250\ K_{D,\ RCoA}$$

$$51 = \frac{[0.250\ K_{D,\ RCoA}]\,[29.4]}{[0.260 - 0.040 - 0.250\ K_{D,\ RCoA}]}$$

$$K_{D,\ RCoA} = 0.558\ mM$$

From (C)

$$12S_{H\ free} = 0.250\ (0.558) = 0.140\ mM$$

Substituting all the known values into (D), the following is obtained.

$$[12S_H\ PYR] = [0.260] - [0.040] - [0.140] = 0.080\ mM$$

Therefore, the concentrations of the CoA ester sites are as follows.

		Distribution per 0.260 site	Sites per $12S_H$ subunit, i.e., per 6 sites
$[12S_H\ RCoA]$	=	0.040 m*M*	0.92
$[12S_H\ PYR]$	=	0.080 m*M*	1.85
$[12S_H]_{free}$	=	0.140 m*M*	3.23

This calculation has been applied to the data of Tables III and IV of Reference 39, and the following is a summary of these calculations.

Estimated Bound RCoA and Bound Pyruvate per $12S_H$ Subunit

Table	Experiment			Distribution of sites	Sites per $12S_H$ subunit, i.e., per 6 sites
2[39]	2	$[12S_H\ RCoA]$	=	0.040 m*M*	0.92
		$[12S_H\ PYR]$	=	0.080 m*M*	1.85
		$[12S_H]_{free}$	=	0.140 m*M*	3.23
		Sum	=	0.260 m*M*	6.00
4[39]	2	$[12S_H\ RCoA]$	=	0.176 m*M*	4.26
		$[12S_H\ PYR]$	=	0.029 m*M*	0.70
		$[12S_H]_{free}$	=	0.043 m*M*	1.04
		Sum	=	0.248 m*M*	6.00
4[39]	3	$[12S_H\ RCoA]$	=	0.213 m*M*	5.24
		$[12S_H\ PYR]$	=	0.012 m*M*	0.29
		$[12S_H]_{free}$	=	0.019 m*M*	0.47
		Sum	=	0.244 m*M*	6.00
4[39]	4	$[12S_H\ RCoA]$	=	0.017 m*M*	0.43
		$[12S_H\ PYR]$	=	0.087 m*M*	2.18
		$[12S_H]_{free}$	=	0.135 m*M*	3.39
		Sum	=	0.239 m*M*	6.00

distance from propionyl CoA to Co^{++} was not from propionyl CoA bound to the $5S_E$ subunit to Co^{++} on that same subunit and that the distance from the CoA nitroxide to pyruvate was not from nitroxide on the $12S_H$ subunit to pyruvate on the same subunit.

G. Stereochemistry and Hydrogen Transfer and Their Relationship to the Mechanism of Transcarboxylation

The stereochemistry of two reactions which are closely related to transcarboxylase, one catalyzed by propionyl CoA carboxylase[43,44] and the other by pyruvate carboxylase,[45] has been found to occur with retention of configuration during the carboxylation. With propionyl CoA carboxylase, there is transfer of a carboxyl from the biotinyl group to propionyl CoA, forming methylmalonyl CoA, which is equivalent to Reaction 5 (Section VI.B); with pyruvate carboxylase there is transfer of a carboxyl from the biotinyl group to pyruvate, forming oxalacetate, which is equivalent to Reaction 6 (Section VI.B). It had previously been shown by Allen et al.[46] that transcarboxylase utilizes and produces the same chiral form of methylmalonyl CoA as propionyl CoA carboxylase. Later, Retey and Lynen[47] and Sprecher et al.[48] showed that this isomer of methylmalonyl CoA has the S configuration.

Recently, Cheung et al.[49] have undertaken an investigation of the stereochemistry of the two half reactions catalyzed by transcarboxylase. Their results show that both reactions occur with retention of configuration, just as with propionyl CoA carboxylase and pyruvate carboxylase. More recently, Rose et al.[50] have made the intriguing discovery that there is transfer of hydrogen between the substrates of the two partial reactions: tritium from [3-^{3}H] pyruvate to propionyl CoA and tritium from [2-^{3}H] propionyl CoA to pyruvate. These results and their interpretation will be considered in this section.

1. Stereochemistry of Transcarboxylation to Propionyl CoA

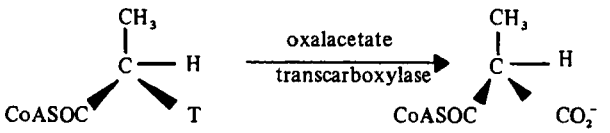
The proof of the stereochemistry of this partial reaction by transcarboxylase included two parts. The first involved comparison of the rate of removal of the C-2 hydrogens of (R,S)-[2-^{3}H] propionyl CoA with the rate of formation of methylmalonyl CoA during the carboxylation with oxalacetate. The rate of formation of methylmalonyl CoA was determined by assaying the rate of pyruvate formation using lactate dehydrogenase and DPNH and monitoring the oxidation of DPNH at A_{340}. If there was ^3H formation from only one of the C-2 hydrogens, the ^3H_2O produced should have half the specific radioactivity of the (R,S)-[2-^{3}H] propionyl CoA. A plot of ^3H_2O released against the micromoles of methylmalonyl CoA formed gave values which were in good agreement with this theoretical rate. The procedure was also tested with propionyl CoA carboxylase, and the results were the same as with the transcarboxylase. These results established: (1) that transcarboxylase removes only one proton at C-2 during the carboxylation and (2) the proton removal is not the slow step, and there is not a preequilibrium enolization of propionyl CoA. This latter statement is based on the fact that if the removal of a proton were the rate-determining step, the nontritiated propionyl CoA would react faster than the tritiated propionyl CoA due to an isotope effect; thus, the H_2O would contain less tritium than predicted. Likewise, if there were enolization of the propionyl CoA prior to the reaction, there would be loss of tritium independent of the formation of methylmalonyl CoA.

In order to definitely establish the stereochemistry of the carboxylation of propionyl CoA, additional experiments were performed individually with (S)-[2-^{3}H] propionyl CoA and (R)-[2-^{3}H] propionyl CoA. These chiral molecules were snythesized by a combination of enzymatic and chemical methods, and their chiral purity was checked by carboxylation and tritium released with propionyl CoA carboxylase. Their respective purities are listed below.

Sample	Chiral content
(S)-[2-^{3}H] propionyl CoA	(S) 77% (R) 23%
(R)-[2-^{3}H] propionyl CoA	(S) 20% (R) 80%

The chiral propionyl CoA samples were individually carboxylated with transcarboxylase, and the rates of tritium release were plotted against the rate of methylmalonyl CoA formation. A line with a slope of 17% was determined with the (S) sample and with a slope of 80% with the (R) sample. The results of the above experiments show that the

CoA ester half reaction of transcarboxylase proceeds with retention of configuration and replacement of the R hydrogen at C-2 by a carboxyl as illustrated below.



(R)– {2-^{3}H} propionyl CoA (S)–methylmalonyl CoA

2. Stereochemistry of Transcarboxylation to Pyruvate

For this determination, Cheung et al.[49] used the method which Rose[45] had employed for the analogous situation with pyruvate carboxylase. This determination is based on utilization of a known chiral species of pyruvate, discrimination against the heavier isotopes of hydrogen in the carboxyl transfer to pyruvate, and assay of the retention or loss of tritium in the product, oxalacetate. The chiral species of pyruvate, 3 (S)-[3-^{2}H-^{3}H] pyruvate, was prepared by the action of phosphoglycerate mutase, enolase, and pyruvate kinase on 3 (S)-[3-^{2}H-^{3}H] phosphyglycerate which was obtained from Rose. The method of assay of the retention or loss of the tritium in the oxalacetate formed in the reaction was as described by Rose.[45] It involves trapping the oxalacetate as malate by using malate dehydrogenase. Then, the tritium at the C(3-S) and C(3-R) positions of the malate is determined by use of fumarase, which stereospecifically removes the pro-R hydrogen at C-3 of malate in the formation of fumarate, converting it to H_2O.

A kinetic isotope effect against the heavier isotopes of hydrogen at C-3 of pyruvate is the crux of this procedure. It is to be noted that there was no isotope discrimination in the carboxylation of (R,S)-[2-^{3}H] propionyl CoA. Without such an isotope selection, pyruvate molecules with chiral methyl groups would not yield [3-^{3}H] malate with a stereoselective enrichment which could be related to the stereochemistry of proton displacement by the incoming carboxyl group. To establish that there is an isotope effect, the tritium or deuterium release from [3-$^3H_3$] pyruvate and [3-$^2H_3$] pyruvate was tested. The tritium release from [$^3H_3$] pyruvate during the reaction was measured by the recovered ^3H_2O. The relative rate of [$^1H_3$] pyruvate utilization was about three times faster than that of [$^3H_3$] pyruvate, but a precise number was not obtained due to enolization of the [3-^{3}H] pyruvate during storage. The kinetic deuterium isotope effect was determined to obtain a more exact measure of the discrimination. For this purpose, the V_{max} of the formation of oxalacetate with [3-^{1}H] pyruvate and [3-$^2H_3$] pyruvate was determined, the $k_{^1H}/k_{^2H}$ was found equal to 2.1 and both compounds had an identical K_m value equal to 0.55 m*M*.

Based on the ratio of $k_{^1H}/k_{^2H}$ = 2.1, it was predicted with chiral 3 (S)-[3-^{2}H-^{3}H] pyruvate that there would be a 68:32 partition of the ^{3}H in the two enantiotopic positions at C-3 of oxalacetate (or malate). Thus, if the carboxylation took place with retention of the configuration, 68% of the ^{3}H at C-3 of malate would be removed by the fumarase, indicating pro-R placement, and 32% would be retained in the fumarate.

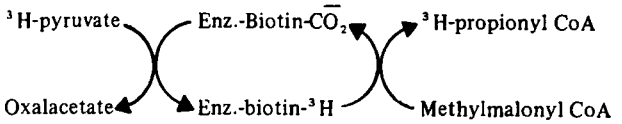
The amount of ^{3}H that was retained in malate was determined by the addition of a small amount of ^{14}C-malate to the completed reaction. From a comparison of the $^3H/^{14}C$ ratio in the malate before and after fumarase treatment, the retention of ^{3}H in malate could be calculated. The actual experiments indicated that 65% of the initial tritium was released from the malate, while 34% was retained. These results are in excellent agreement with the theoretical values predicted for the retention of configuration of 68:32 (pro-R:pro-S). The reciprocal experiment with 3 (R)-[^{2}H,^{3}H] pyruvate was not performed because its chiral purity was not sufficient to yield unambiguous results.

The results of these stereochemical studies of the carboxylation at the CoA ester site and keto acid site, thus, are identical to those with propionyl CoA carboxylase[43,44] and pyruvate carboxylase.[45]

3. Intramolecular Hydrogen Transfer during Transcarboxylation

Rose et al.[50] have made a very interesting

discovery. They noted that the transcarboxylase reaction allows the possibility of proton transfer between the pyruvate and propionyl CoA as follows.



Transcarboxylase presents a unique opportunity to study such transfers, in contrast to carboxylases such as propionyl CoA carboxylase or pyruvate carboxylase in which the substrate-derived hydrogen must terminate as a medium proton.

To test the above hypothesis, [3-^{3}H] pyruvate was carboxylated with methylmalonyl CoA using transcarboxylase. Ten percent of the tritium was labelized and 89% of the tritium was isolated in the oxalacetate. The reason for the small amount of labilized tritium is the kinetic heavy isotope effect, as observed by Cheung et al.[49] and described above, and also because only one out of three of the hydrogens of the methyl group contained tritium. Of the labilized counts, 4.8% were in the pro-R position at C-2 of the propionyl CoA, which had a specific activity 3×10^4 times greater than that of the solvent. The remainder of the labilized counts were found in the reaction solvent. The radioactivity was shown to be in the pro-R position of C-2 propionyl CoA by subjecting the isolated radioactive propionyl CoA to carboxylation with oxalacetate using transcarboxylase. All of the tritium formerly in the propionyl CoA was found in the water. This liberation of the tritium from the propionyl CoA was inhibited about 94% by avidin and was dependent on oxalacetate. To further verify that the tritium was in the propionyl CoA, the propionyl CoA was chromatographed on a cellulose plate and the UV-absorbing spot was eluted with water. The specific radioactivity was unchanged. In addition, the propionyl CoA was hydrolyzed, and the resulting propionic acid, after addition of carrier, was isolated using a silica gel column. The specific activity was within 4% of that expected if all the counts were in the propionyl portion of propionyl CoA.

The transfer of tritium from (2R)-[2-^{3}H] propionyl CoA to pyruvate was accomplished by incubating the labeled propionyl CoA with oxalacetate, transcarboxylase, lactate dehydrogenase, and DPNH. All counts were recovered in the lactate and water with lactate, accounting for 0.39 to 0.63% of the counts found in the water. Although the amounts of tritium transfer are small in both this case and with [3-^{3}H] pyruvate, they were found to be real and reproducible.

The transfer of tritium between pyruvate and propionyl CoA during transfer of the carboxyl from methylmalonyl CoA or oxalacetate indicates that there is a proton carrier which functions between the two substrate sites on the $5S_E$ and $12S_H$ subunits. Rose et al.[50] believe that a transfer between two bases, one on the $5S_E$ subunit and one on the $12S_H$ subunit, is unlikely. They propose that the transfer is more likely to be via a single base on the $1.3S_E$ carboxyl carrier protein. They point out that Guchhait et al.[51] have shown that the carboxyl transferase component of acetyl CoA carboxylase of *E. coli* catalyzes carboxyl transfer from malonyl CoA to free biotin. The fact that free biotin is active suggests that the biotin per se is the proton carrier rather than some amino acid of the carboxyl carrier protein.

In order to evaluate how much of the enol form of biotin might be present, Rose et al.[50] have estimated the pK_a of the proton carrier from the absolute exchange rate. Since about 0.4% of the tritium from the (2R)-[2-^{3}H]propionyl CoA was recovered in the pyruvate and the remainder in the water, they conclude that the rate of dissociation of the tritiated carrier was about 250 times the rate of transfer of tritium from propionyl CoA to pyruvate (100/0.4 = 250). There would be an isotope effect in the transfer of tritium from the enolate of biotin to pyruvate. They corrected for this effect on the basis of the discrimination observed in the incorporation of tritium into pyruvate from ^{3}HOH during transcarboxylation. The pyruvate has only 0.18 times the specific activity of the water, and a correction for this isotope effect gives the rate of dissociation of tritium as about 50 times the V_{max} of the reaction (0.18×250). The V_{max} of the 18S form of transcarboxylase has been observed by us to be

about 40 μmol/min/mg. The molecular weight is 790,000, and since there are 6 biotins, the molecular weight per functional unit is 132,000. The turnover, therefore, is 88 sec^{-1}. Thus, the dissociation rate is 50 X 88 sec^{-1} = 4.4 X 10^3 sec^{-1}. Rose et al.[50] assumed that the reprotonation of the dissociated carrier is diffusion controlled, which would give a rate constant equal to 10^{10} M^{-1} sec^{-1} (Jencks[52]). Thus, $K_a = \frac{4.4 \times 10^3\ sec^{-1}}{10^{10}\ M^{-1}\ sec^{-1}} = 4.40 \times 10^{-7} M$ and the pK_a is 6.4. Rose et al.,[50] using slightly different values, estimated the turnover to be 80 sec^{-1}, also giving a pK_a = 6.4. It is, of course, inherent in these calculations that the loss of tritium and the isotope effect is really due to an essential role of enol biotin in the transcarboxylation. On the above basis, Rose et al.[50] concluded that in spite of the rapid equilibrium with the medium, a significant proportion of the biotin would remain in the enol form for the next transfer step. Once the proton has dissociated from the enol, it would be expected that the reprotonation would favor the ureido form thermodynamically. Rose et al.[50] note that hydrogen bonding of the enol form of biotin may be required to maintain a sufficiently high concentration of the enol for the carboxylation reaction. Likewise, anything that weakens the ureido N-1 to hydrogen bond would raise the concentration of enol. Contributions to both of these effects may be via the $5S_E$ and $12S_H$ subunits when they catalyze the partial reactions.

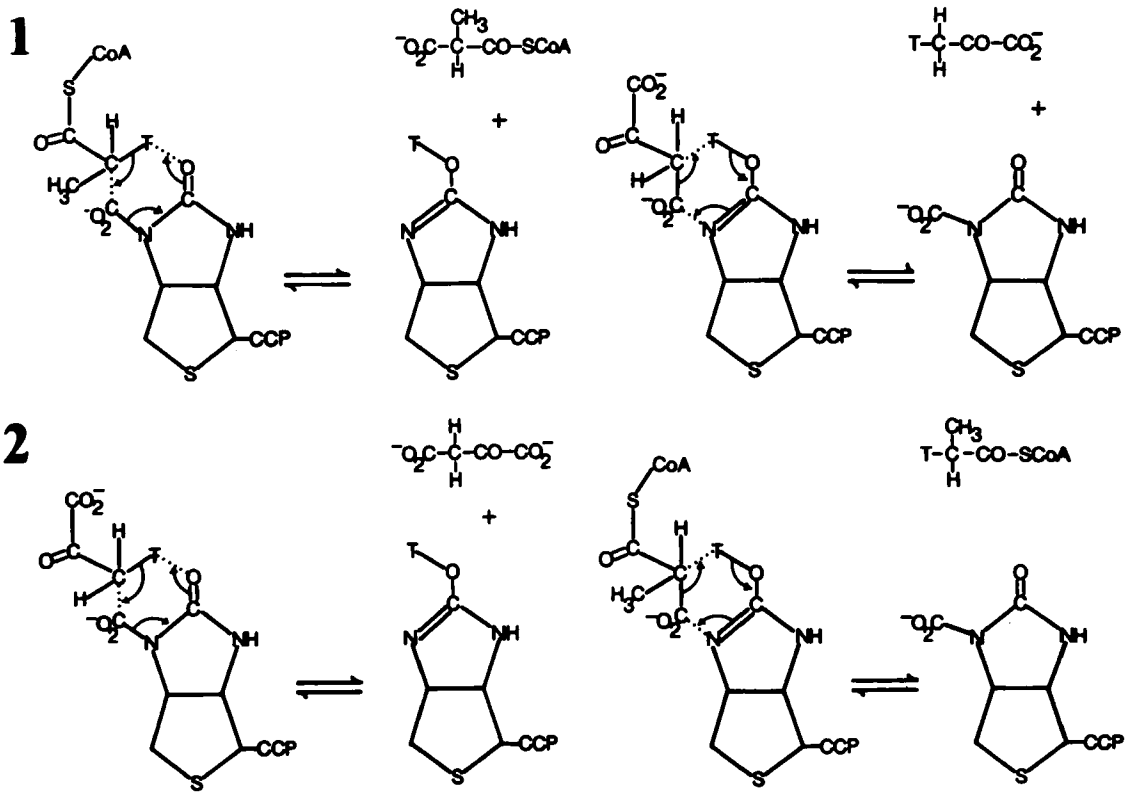
4. The Mechanism of Carboxyl and Hydrogen Transfer by Biotin

The question of whether or not carboxyl transfer occurs via the 1′N or the O ureido group of biotin was a subject of debate for some time, but now has been settled in favor of the 1′N. Bruice and co-workers[53-55] proposed that the carboxyl transfer was by ureido-O-carboxylation rather than 1′N carboxylation and that the 1′N-carboxyl derivative arose by rearrangement of the carboxy-O-ureido-biotin to the stable 1′N-substituted compound as a sequence of the conversion of the carboxybiotin to the methyl ester prior to its isolation. However, Guchhait et al.[51] recently have shown quite conclusively, in excellent and well-documented experiments, that the 1′N-carboxybiotin is the reactive form in catalysis. Using authentic 1′N-carboxy-*d*-biotin, ADP, P_i, and the biotin carboxylase component of acetyl CoA carboxylase, they found that ATP was synthesized. In addition, using 1′N-carboxy-*d*-biotinol with the carboxytransferase component, they demonstrated there is efficient carboxylation of acetyl CoA to malonyl CoA.

Based on present evidence, the concerted mechanism via a cyclic six-membered ring complex, as proposed by Retey and Lynen,[47] for propionyl carboxylase is also the most attractive mechanism for carboxyl transfer in transcarboxylation. It provides for the hydrogen transport observed by Rose et al.[50] and at the same time accounts for the retention of configuration as observed by Cheung et al.[49] This mechanism is illustrated in Figure 17. In this mechanism, carboxybiotin is the base which abstracts the proton, and the enol form of biotin is the species which abstracts the carboxyl group to form carboxybiotin. The imide-nitrogen of the enol of biotin has been shown to be ~10^{10} times more reactive as a nucleophile than the same nitrogen in the ureido form;[56] therefore, the imide N is attractive as the carboxyl acceptor. It is to be noted that the carboxylation of both propionyl CoA (Reaction 1 of Figure 17) and pyruvate (Reaction 2 of Figure 17) is proposed to occur by similar concerted mechanisms. The carboxylation of propionyl CoA occurs on the $12S_H$ subunit, which does not contain Co^{++} or Zn^{++}. Clearly, metal activation is not required for this carboxylation. This observation is in accord with the fact that there have been no reports of enzyme-bound divalent metals in propionyl CoA carboxylase or other acyl carboxylases, although addition of Mg^{++} is required for the partial reaction involving carboxylation of biotin with HCO_3^- and ATP.[3-5]

Much attention has been paid to the role of metals in the partial reaction involving carboxylation of pyruvate (see Section VI.E.1) in part because the firmly bound paramagnetic Mn^{++} of pyruvate carboxylase of chicken liver and the Co^{++} of transcarboxylase present convenient tools for study of the reactions by NMR and EPR. The other biotin enzymes do not present an opportunity for such studies because they lack firmly bound paramagnetic metals.

We have seen that, contrary to early indications with pyruvate carboxylase and transcarboxylase, the coordination of pyruvate with the metal is not direct, but is by a second sphere complex, perhaps with water intervening in the complex (Section VI.E.1). This raises anew the question of the role of metals in the partial reactions. The inner sphere



Reaction I

$$CH_3\cdot\overset{\overset{T}{\cdot}}{C}\cdot COSCoA + {}^-OOC{-}CCP \rightleftharpoons CH_3\cdot\overset{\overset{H}{\cdot}}{C}(COO^-)\cdot COSCoA + T{-}CCP$$

$$T{-}CCP + {}^-OOC\cdot CH_2\cdot CO\cdot COO^- \rightleftharpoons {}^-OOC{-}CCP + T\cdot CH_2\cdot CO\cdot COO^-$$

$$CH_3\cdot\overset{\overset{T}{\cdot}}{C}\cdot COSCoA + {}^-OOC\cdot CH_2\cdot CO\cdot COO \rightleftharpoons CH_3\cdot\overset{\overset{H}{\cdot}}{C}(COO)\cdot COSCoA + \overset{\overset{H}{\cdot}}{T}C\cdot CO\cdot COO^-$$

Reaction II

$$\overset{\overset{H}{\cdot}}{T}\cdot C\cdot CO\cdot COO^- + {}^-OOC{-}CCP \rightleftharpoons {}^-OOC\cdot CH_2CO\cdot COO^- + T{-}CCP$$

$$T{-}CCP + {}^-OOC\cdot\overset{\overset{T}{\cdot}}{C}H(CH_3)\cdot COSCoA \rightleftharpoons {}^-OOC{-}CCP + CH_3\cdot\overset{\overset{H}{\cdot}}{C}\cdot COSCoA$$

$$\overset{\overset{H}{\cdot}}{T}\cdot C\cdot CO\cdot COO^- + {}^-OOC\cdot\overset{\overset{T}{\cdot}}{C}H(CH_3)\cdot COSCoA \rightleftharpoons {}^-OOC\cdot CH_2\cdot CO\cdot COO^- + CH_3\cdot\overset{\overset{H}{\cdot}}{C}\cdot COSCoA$$

FIGURE 17. A mechanism of transcarboxylation which would provide for hydrogen transfer as well as retention of configuration during transcarboxylation (CCP is carboxyl carrier protein).

complex was attractive because of the numerous model reactions directly involving metal-catalyzed decarboxylation and enolization of pyruvate.[57,58] However, a discrete enolization of pyruvate followed by a carboxylation is practically eliminated as a possibility by the experiments of Rose et al.[50] which show there is transport of hydrogen during transcarboxylation. Enolization would be expected to yield a free proton and not involve hydrogen transport. In addition, there would be loss of configuration unless the enzyme directed the carboxylation so that the addition of CO_2 is restricted to a front-side attack.[49]

Fung et al.[14] and Mildvan[30] have considered the possibility that the metal ion promotes the acidity of an inner sphere water ligand which can then protonate the carbonyl oxygen of pyruvate and thereby catalyze the enolization process. They point out that this possibility is suggested by the orientation of pyruvate in which the carbonyl oxygen is pointed toward the metal (Section VI.E.1, Figure 13). If there is such activation of the pyruvate, the potential enolization is concerted with the carboxylation, and the enolate of pyruvate does not occur as such. Exclusion of discrete enolization is necessary to account for the hydrogen transfer. Fung et al.[39] have illustrated this type of role of Co^{++} as shown in Figure 18. They have proposed two models in which the metal plays a direct catalytic role. In one of the models (Figure 18A) the carboxyl transfer is parallel to the ureido ring, and in the other (B) it is perpendicular to the ureido ring. It is proposed that mechanism B could operate with the carboxybiotin immobilized if the 7 Å space between the two substrates is correct (see Section VI.F.5). It could, however, operate with migration. Mechanism A, in which the carboxyl is parallel with the ring, would require rotation in order to carry the carboxyl through the 7 Å.

An expanded version of mechanism B[95] with the biotin ring immobilized is illustrated in Figure 19. It differs from the mechanism shown in Figure 17 and mechanism A of Mildvan in several features.

1. The 1′-nitrogen which abstracts the carboxylate is a secondary amine rather than an imide nitrogen (structure 3).

2. The mechanism for the carboxylate abstraction involves the reaction of five electron pairs (structure 3) rather than the three electron pairs of Figure 17.

3. The 1′-nitrogen of carboxybiotin (structure 4) is a tetravalent nitrogen rather than trivalent (see Figure 17 for comparison).

4. Since the biotin does not migrate, a step is required to move the carboxylate group to the opposite face of the plane of the ureido ring. Thus, it can donate the carboxyl to pyruvate at the site on the $5S_E$ subunit from the front side of the ring. The site of methylmalonyl CoA on the $12S_H$ subunit is on the back side of the ring (Figure 18). It is proposed that this movement is accomplished by the proton leaving the 1′-nitrogen and

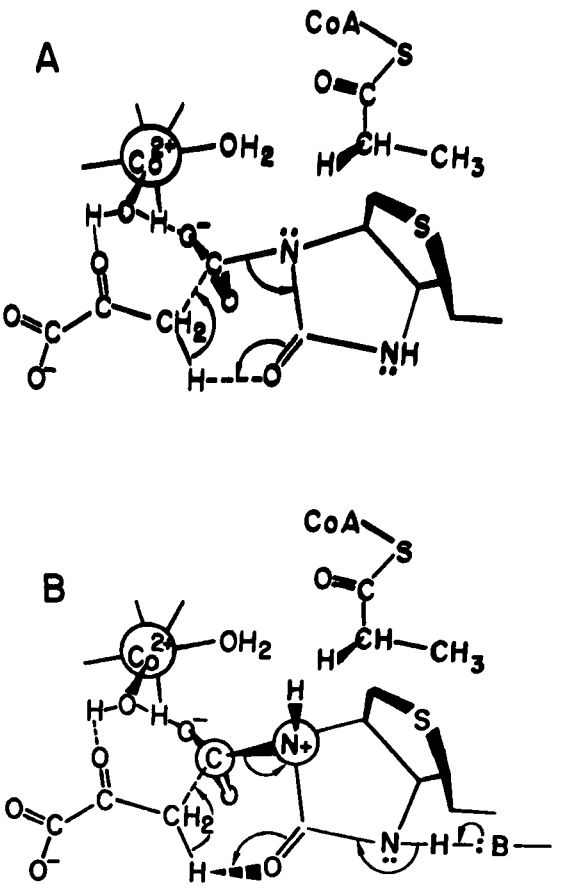


FIGURE 18. Alternative mechanisms of carboxylation of pyruvate by second sphere cobalt complex as proposed by Fung et al.[39] Mechanism A involves a carboxyl transfer parallel to the plane of the biotin ring and thus requires oscillation of the biotin ring between the keto acid site on the $2.5S_E$ polypeptide to the CoA ester site on the $2.5S_H$ polypeptide. Mechanism B can occur with carboxylation perpendicular to the biotin ring and does not necessitate movement of the ring for the transcarboxylation. (Reprinted with permission from Fung, C. H., Gupta, R. K., and Mildvan, A. S., *Biochemistry*, 15, 85, 1976. Copyright by the American Chemical Society.)

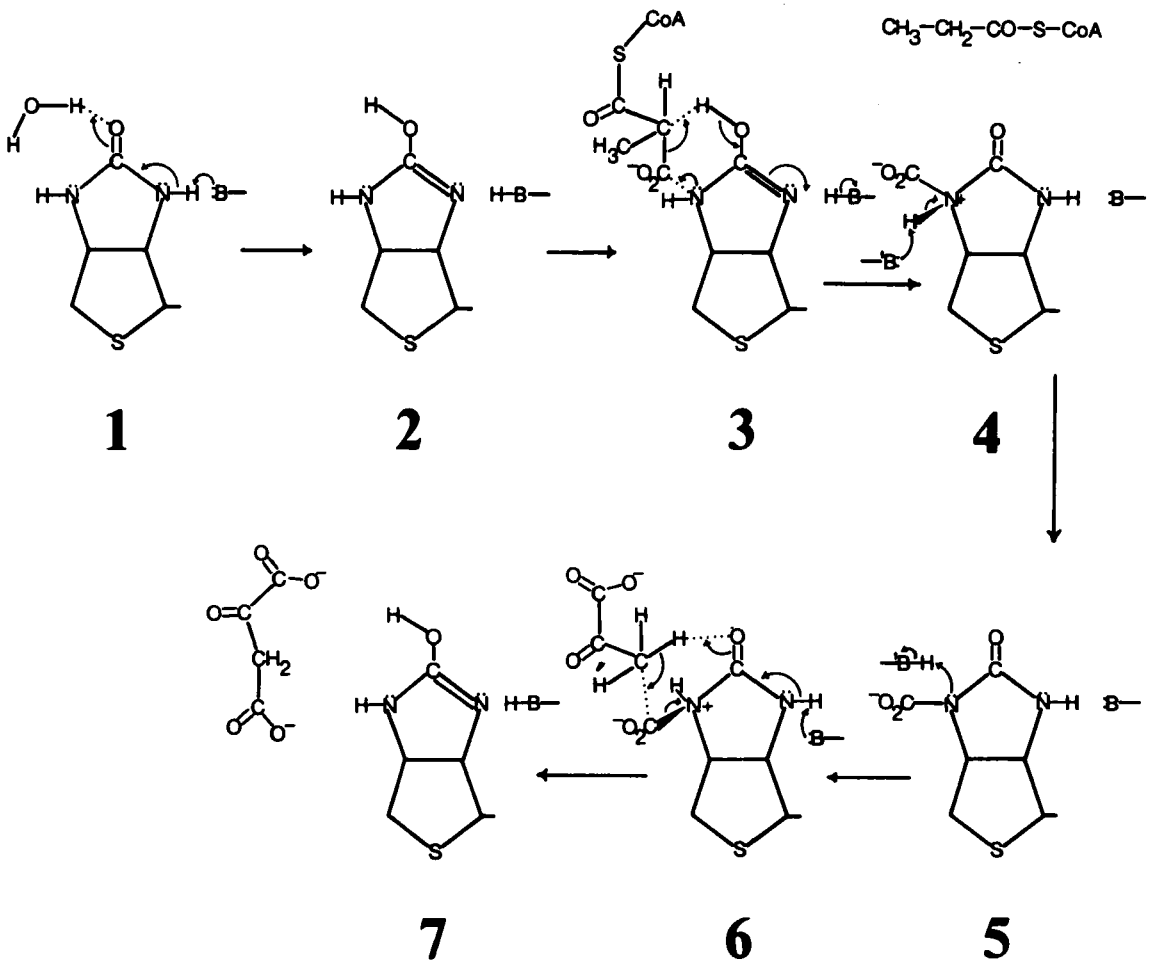


FIGURE 19. A more detailed presentation of the steps involved in transcarboxylation by Mechanism B.[95]

rebinding on the front side of the 1′-nitrogen (structure 5).

5. The carboxylation of pyruvate then takes place by a reaction involving five electron pairs.

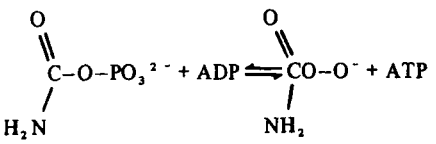
6. Structure 6 is the same as structure 2, and this species can recycle and account for the transfer of hydrogen between pyruvate and propionyl CoA and vice versa.

The concept that there might be carboxyl transfer by the biotinyl group without movement of the biotinyl ring is very interesting. However, Model A and the expanded version of Model A without implication of HOH coordinated to Co^{++} (Figure 17) are more attractive than Model B. Because the imide nitrogen is $\sim 10^{10}$ times stronger as a nucleophile than the ureido nitrogen (secondary amine),[56] it, therefore, would be expected that the 3′-imide nitrogen would abstract the carboxyl from the methylmalonyl CoA or oxalacetate. Also, the mechanism involves shifting of five electron pairs and racemization of the carboxyl at the 1′-nitrogen (Figure 19) and is a much more complicated mechanism than that of Figure 17. In defense of Mildvan's mechanism B, it could be argued that the conformation and orientation of the 3′-nitrogen relative to the substrates are such that the 3′-nitrogen is not near the carboxyl groups of the substrates; thus, even though the imide 3′-nitrogen is more reactive, it cannot serve as the carboxyl acceptor.

Since the carboxylation of the acetyl CoA thio esters does not require a metal and the mechanisms of carboxylation of the CoA thio esters and pyruvate may both be by similar concerted reactions (as indicated in Figure 17), one is led to ask whether a metal is really required for the keto acid carboxylation. Perhaps the CoA

esters become oriented properly at the substrate site because of their size and the numerous groups for binding, whereas for pyruvate and oxalacetate, coordination with a metal is required to properly orient them with respect to the biotinyl group. Thus, the metal may have no direct role as an activator as such of the keto acid, but only serve in orientation of the keto acid.

Our interest in CO_2 fixation leads us to comment on a recent observation which indicates that carbonyl phosphate may be an intermediate in reactions catalyzed by biotinyl carboxylases. Polakis et al.[59] have demonstrated that the biotin carboxylase component of acetyl CoA carboxylase catalyzes a rapid biotin-dependent phosphoryl transfer from carbamyl phosphate to ADP to form ATP.



(8)

The biotin appears to fulfill a conformational requirement of the enzyme rather than participate directly in the reaction. Polakis et al.[59] have proposed that the binding of biotin to the biotin site on the biotin carboxylase may induce a conformational change which reorients the functional groups at the adjacent binding sites for HCO_3^- and ATP, thereby actuating phosphoryl transfer. This arrangement would forestall cleavage of the ATP until the biotin was in the carboxylation site and prevent hydrolysis of the unstable carbonyl phosphate, since it would be utilized immediately.

The following reactions are proposed for formation of 1′-N-carboxy-d-biotin.

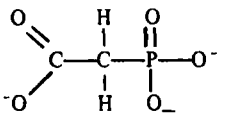
$$HO{-}CO_2^- + ATP + \text{Enzyme} \xrightarrow[[ADP]]{Me^{2+}\ [HO{-}CO_2{-}PO_3^{2-}]} \text{Enzyme} \qquad (9)$$

$$\underset{\text{Enzyme}\ [ADP]}{[HO{-}CO_2{-}PO_3^{2-}]} + d\text{-biotin} \longrightarrow \underset{d\text{-biotin}}{1'\text{-N-carboxy-}} + \qquad (10)$$

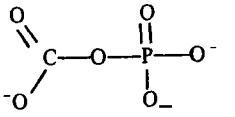
$$ADP + P_i(OH) + \text{Enzyme}$$

$$\text{Net: } HO{-}CO_2^- + ATP + d\text{-biotin} \xrightarrow[\text{Enzyme}]{Me^{2+}\ 1'\text{-N-carboxy-}} d\text{-biotin} + ADP + P_i(OH) \qquad (11)$$

Ashman and Keech[60] have very recently shown that pyruvate carboxylase from sheep kidney, likewise converts carbamyl phosphate and ADP to ATP. They extended the evidence that carbonyl phosphate might be a transitory intermediate in the carboxylation reaction by use of phosphonoacetic acid, an analogue of carbonyl phosphate.



Phosphonoacetic Acid



Carbonyl Phosphate

They showed that the overall reaction catalyzed by pyruvate carboxylase is inhibited by this analogue. A K_I value of 0.5 m*M* was obtained with ATP as the varied substrate. Polakis et al.[59] likewise have reported in a footnote that acetyl CoA carboxylase from avian liver catalyzes the transfer of the phosphoryl group of carbamyl phosphate to ADP.

These events bring us full circle. Twenty years ago, Ochoa and co-workers,[61] using propionyl carboxylase, presented the exciting possibility that carbonyl phosphate is an active form of CO_2 formed from CO_2 and ATP. With crude preparations in the absence of propionyl CoA but in the presence of fluoride, a CO_2-dependent reaction between ATP and fluoride was observed, yielding ADP and monofluorophosphate. This was called the fluorokinase reaction.[62] It was considered that the fluorokinase might be carbonokinase, yielding carbonyl phosphate as an initial product. If the acceptor propionyl CoA were present, methylmalonyl CoA would be formed, but in its absence, if fluoride were present, the carbonyl phosphate would react with fluoride to give fluorophosphate. However, when the fluorokinase[63] and propionyl carboxylase were highly purified, it was found that the two reactions were independent of each other. Fluorokinase proved to be pyruvate kinase.[63]

It is quite certain that the present day studies of phosphoryl transfer by the biotinyl enzymes are

a property of the biotin carboxylases per se. Polakis et al.[59] have carefully purified the biotin carboxylase and have tested its activity for other kinases.

The requirement for ATP in the carboxylation of biotin by HCO_3^- apparently is because the energy barrier is too great for the direct carboxylation of the biotinyl enzyme with HCO_3^-. However, the energy is sufficient for the carboxylation using oxalacetate or methylmalonyl CoA without utilization of ATP. The formation of carbonyl phosphate could provide the means of overcoming this barrier and permitting carboxylation of the biotin, which then could be transcarboxylated by the concerted mechanism as described above in Figure 17.

VII. TRANSCARBOXYLASE WITH A MOLECULAR WEIGHT OF ~1,200,000 AND AN $S_{20,w}$ ~ 26S*

Transcarboxylase as usually isolated has a molecular weight of ~790,000, and the electron micrographs indicate that there are peripheral subunits bound to only one face of the central cylindrical subunit. Recent results indicate, however, that the native form of transcarboxylase may have peripheral subunits bound to both faces of the central subunit and have a molecular weight of 1.2 million. The results also indicate that the binding strengths of the peripheral subunits on the two faces of the central subunit differ and that one set of peripheral subunits is very easily dissociated from the transcarboxylase. Thus, one set is lost during the isolation of the enzyme unless special care is taken.

A. Discovery of the ~26S Form of Transcarboxylase and its Formation from the 18S Enzyme Plus $6S_E$ Subunits

The 26S form of the enzyme was first observed in 1970[35] using the following procedure. Transcarboxylase was dissociated in 0.05 *M* tris-Cl buffer (pH 8) in the absence of glycerol to a mixture of $12S_H$, $6S_H$, $5S_E$, and $1.3S_E$ subunits which had a very low enzymatic activity (Figure 20A). The mixture was then brought to pH 5 by addition of 0.5 *M* acetate buffer (pH 4.4) and was held at 4° until the maximum enzymatic activity was reached; then, any precipitate which formed was removed by centrifugation. The specific activity of the reconstituted material often was about 20 without correction for the considerable

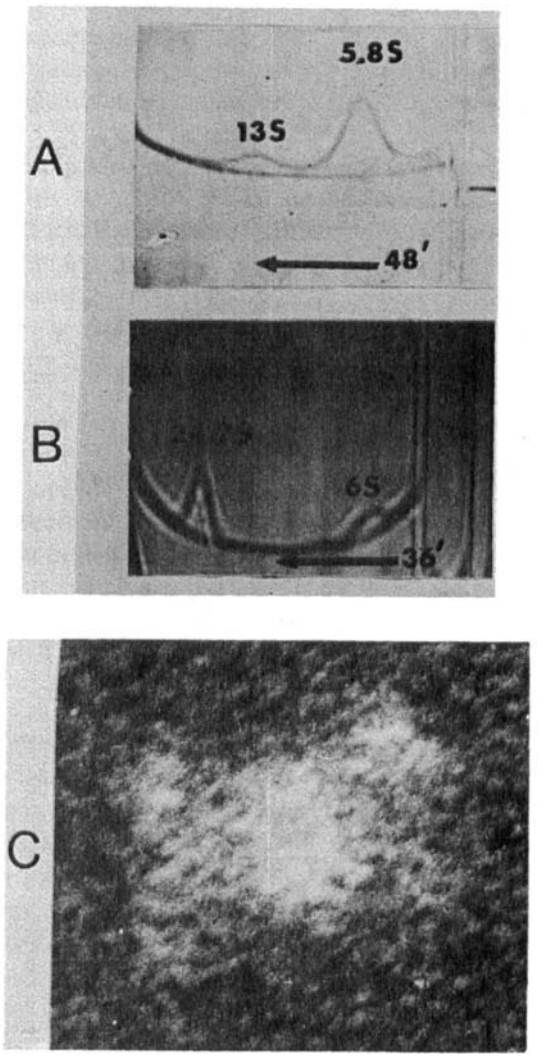


FIGURE 20. Formation of 26S form of transcarboxylase from dissociated transcarboxylase. Sedimentation velocity profiles. (A) Dissociated transcarboxylase. (B) After reconstitution. (From Jacobson, B., Gerwin, B L., Ahmad, F., Waegell, P., and Wood, H. G., *J. Biol. Chem.*, 245, 6471, 1970. With permission.) (C) Electron micrograph of reconstituted 26S transcarboxylase. (From Green, N. M., Valentine, R. C., Wrigley, N. G., Ahmad, F., Jacobson, B., and Wood, H. G., *J. Biol. Chem.*, 247, 6284, 1972. With permission.) See text for method of dissociation and reconstitution.

*Values as high as 29S have been observed for the large form of transcarboxylase, but most frequently the values are about 26S. We, therefore, will refer to the large form as the 26S enzyme.

amount of protein that remained in the 6S form. The surprising finding was that the reconstituted enzyme differed from any forms previously observed. On sedimentation, two peaks were observed: first, the 26S form of the enzyme, and second, protein which had not reconstituted and sedimented at s_{20} ~6S (Figure 20B). Electron micrographs of the 26S form of the enzyme[9] showed that, in contrast to the 18S form of the enzyme, this form had peripheral subunits on both faces of the central $12S_H$ subunit (Figure 20C).

It was then found that when the transcarboxylase is dissociated in tris-Cl buffer at pH 8 containing 20% glycerol, the usual 16S and 18S forms of the enzyme are observed following reconstitution.[35] It, therefore, was considered that the 26S form of the enzyme arose because of some alteration of the $12S_H$ subunit during the dissociation in the absence of glycerol.[9]

The first clue that the 26S form of the enzyme might be a normal form of the enzyme came from experiments with trypsinized transcarboxylase. It was observed when a mixture of trypsinized transcarboxylase and isolated $6S_E$ subunits were placed in acetate buffer at pH 5.2 at 4° that there was a very substantial increase in enzymatic activity (from specific activity 0.1 to ~13). At first it was considered that this formation of active enzyme might occur because the normal $6S_E$ subunit had a stronger affinity for the $12S_H$ subunit than the $6S_E^-$ subunits and had displaced the $6S_E^-$ subunits yielding active enzyme ($6S_E^-$ subunit is a $6S_E$ subunit from which the biotinyl peptide has been cleaved, Figure 6). Sedimentation velocity profiles of the product showed that this probably was not the case; two peaks were observed, one at ~26S and another at ~6S.[64] It then became evident that the active enzyme probably was being formed by addition of $6S_E$ subunits to the $12S_H$ subunit on the face opposite the $6S_E^-$ subunits (see model in Figure 3). These results were indeed interesting because they indicated that there are CoA ester sites which are active in carboxyl transfer with $6S_E$ subunits attached at either face of the $12S_H$ subunit. The possibility also became evident that the $6S_E$ subunit might combine with the untreated 18S form of the enzyme and that the 26S form of the enzyme might be a normal form of the enzyme.

The latter possibility was tested, and it was found that when the 18S form of the enzyme and the $6S_E$ subunit are combined under conditions favorable for reconstitution, a 26S enzyme is formed (Figure 21). The specific activity of the 18S transcarboxylase (Figure 21A) was ~30 and that of the product was ~43 when calculated on the basis of the amount of protein of the added 18S enzyme. These results indicate that the addition of peripheral subunits to the 18S form of the enzyme increases the specific activity of the enzyme. However, the results are somewhat ambiguous since the specific activity of the 18S form of the enzyme decreased from 39 to the value of 30 during the incubation in the acetate buffer. However, the results from the reconstitution with the trypsinized transcarboxylase strongly suggest that peripheral subunits on the opposite face from the $6S_E$ subunits do have the potential to be active.

The explanation of the above-described accidental discovery of the 26S form of the enzyme is most likely the following. It has been observed that the $6S_H$ subunit denatures more easily than the $6S_E$ and $5S_E$ subunits. It thus is likely that the precipitate which forms when the enzyme is dissociated and reconstituted in the

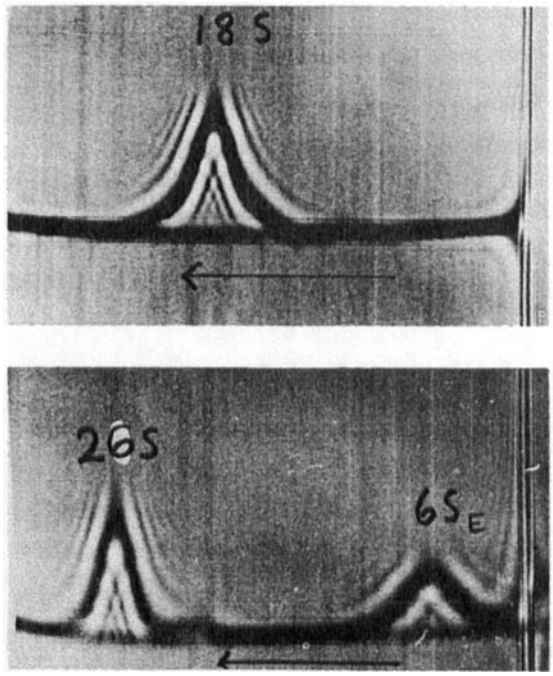


FIGURE 21. *Sedimentation velocity profiles (A) of the 18S form of transcarboxylase and (B) after conversion to the 26S form by combination of the 18S form with $6S_E$ subunits in 0.25 M sodium acetate buffer at pH 5.2 for 20 hr at 4°.*

absence of glycerol is denatured $6S_H$ subunit. Thus, there is an excess of $6S_E$ subunits sufficient to combine at both faces of the remaining $12S_H$ subunit. The denaturation does not occur to the same extent in the presence of glycerol; thus, the 16S and 18S forms result under these conditions.

B. Isolation of the 26S Form of the Enzyme

Purification of the 26S form of transcarboxylase by the usual procedures of ion exchange chromatography is not possible because the 26S form dissociates to the 18S form during these procedures. Thus far, acid pH (about 5.5) is the only condition which has been found to stabilize the 26S form. Therefore, all steps in the purification are done in acetate buffer at pH 5.5. The procedure as presently developed[64] and the purification obtained is shown in Table 3. Purification of the enzyme by this procedure is largely on the basis of size by use of a Bio-Gel column. The chromatography on cellulose phosphate involves elution in a single step with 0.30 *M* phosphate buffer at pH 6.3. This step removes the enzyme quickly. It is considered that if the enzyme dissociates somewhat, it is likely that the $6S_E$ subunits will be eluted and precipitated along with the enzyme during the subsequent precipitation with $(NH_4)_2SO_4$. Under these conditions, the $6S_E$ subunit would most likely recombine with the enzyme to yield the 26S form either during the precipitation or when it is dissolved in acetate buffer at pH 5.5. Enzyme preparations of specific activity 30 to 40 have been obtained more or less routinely by this method. These values are not significantly different from those observed with the 18S form of the

TABLE 3

Purification of the 26S Form of Transcarboxylase

Fraction	mg	Units	Specific activity
I. Crude extract		30,650	~1.0
II. Treated with streptomycin	~17,578	26,749	~1.5
III. 40–60% saturated $(NH_4)_2SO_4$	5,087	26,044	5.1
IV. Bio-Gel A 1.5 *m*	306	9,858	32.0
V. Cellulose-PO_4 eluate	218	7,765	35.6

Note: The procedure for preparation of the crude extract from *Propionibacterium shermanii* (300 g wet weight, grown on a glycerol, yeast extract medium) is as previously described,[67] except the cells are ground in 0.25 *M* sodium acetate buffer (pH 5.5) instead of a phosphate buffer at pH 6.8. The crude extract (Fraction I) is mixed with an equal volume (450 ml) of cold 10% streptomycin sulfate; after stirring for 15 to 20 min, the precipitate is spun down and discarded. The supernatant solution (Fraction II) is brought to 60% saturation with $(NH)_2SO_4$, and the precipitate is spun down at 4° and is resuspended in 0.25 *M* acetate buffer, pH 5.5 (Fraction III). It is applied in two equal parts to two Bio-Gel A 1.5 *m*, 100 to 200 mesh columns (5 × 180 cm) which have been equilibrated with 0.25 *M* acetate buffer (pH 5.5). The fractions containing transcarboxylase are combined in four pools, and each pool is examined by sedimentation. The two pools from the leading portion of the peak of transcarboxylase usually contain only 26S material (Fraction IV). The third and fourth pools usually contain a mixture of 16, 18, and 26S transcarboxylase of specific activity ~20. The combined first and second pools are applied to a 4.5 × 15 cm cellulose phosphate column which has been equilibrated with 0.4 *M* acetate buffer (pH 5.5). The column is then washed with the same buffer until the absorption at 280 nm is 0.1 or less. The buffer then is changed to 0.3 *M* phosphate buffer (pH 6.3), and the enzyme is eluted in a rather small volume. After precipitation with 65% saturated $(NH_4)_2SO_4$, the protein is suspended in a very small volume of 0.25 *M* acetate buffer at pH 5.5 and the suspension is kept at 4° (Fraction V). All buffer solutions (except the final solution of the enzyme) contained 10^{-4} EDTA and 10^{-4} phenylmethyl-sulfonylfluoride; those buffers used on the Bio-Gel columns contained 2×10^{-4} *M* sodium azide in addition.

enzyme. The sedimentation coefficient is ~26S (Figure 22A). On electrophoresis with standard polyacrylamide gel at pH 8.9, the major bands are similar to those of the 18S form (Section II.A, Figure 5.II), but the $5S_E$ band is more dense (Figure 22B). Electron micrographs (Figure 22C) show the presence of forms similar to those of Figure 20C. Thus far, we have not obtained a 26S form with an enzymatic activity high enough to indicate that the second set of peripheral subunits has an enzymatic activity equal to those of the 18S form of the enzyme. If the second set of peripheral subunits were as active as those of the 18S form and the activity was additive, the specific activity would be about 53. It is possible that purification at pH 5.5 denatures some of the enzyme.

It will be shown below (Section VII.C.2) that there are 12 biotinyl groups on the 26S enzyme and that all 12 biotinyl groups can be carboxylated. Therefore, all the biotinyl groups have potential activity. There is no compelling reason to assume that the rate of the reaction should be directly proportional to the number of biotinyl groups. Thus far, we have been unable to determine the number of CoA ester sites (see Section VIII.B.4); there might be 6, and in this case, the 12 biotinyl groups would be competing for the 6 sites. Even if there are 12 CoA ester sites, it is not certain that the specific activity would increase proportionally with the number of potential sites.

C. Properties of the 26S Enzyme

1. Molecular Weight

The molecular weight of the 26S form of transcarboxylase as isolated by the method given in Table 3 has been determined by high-speed meniscus depletion in the Model E ultracentrifuge.[64a] The data are presented in Figure 23, and the calculated molecular weight is 1.22×10^6 d. These results, the symmetrical sedimentation peak and the results of polyacrylamide gel electrophoresis in the experiment of Figure 22, indicate that the enzyme is quite pure; judging from the gel electrophoresis, however it still may contain some contaminant.

2. Biotin Content of the 26S Enzyme and Evidence of the Potential Activity of the Biotinyl Groups

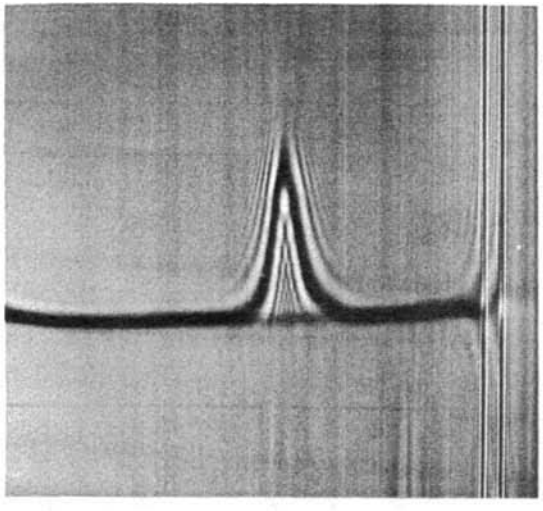
If the 26S form of transcarboxylase has six peripheral subunits (three on each face of the $12S_H$ subunit), then since there are two biotins per subunit, there should be a total of 12 biotinyl groups per mole of enzyme (1.22×10^6 g). The biotin content of a number of the preparations of the 26S enzyme has been determined by a modification of the colorimetric method of Green.[68] The values varied from about 11 to 13 per mole of enzyme,[64] which is considered to be within experimental error of a value of 12 for the enzyme.

The question of whether or not all of these biotinyl groups have a potential for enzymatic activity is of considerable interest in relation to the structure of the enzyme. If all of the biotinyl groups are capable of being carboxylated, then the biotinyl groups of the $6S_E$ subunits on both faces of the $12S_H$ subunit must be able to contact a CoA ester site on the $12S_H$ subunit. This might indicate that there are 12 substrate sites per $12S_H$ subunit, i.e., two for each of the six constituent $2.5S_H$ polypeptides. The question of the structure of the $12S_H$ subunit will be considered in detail in Section VIII.B.

Wood et al.[22] demonstrated by incubation of the 16S and 18S form of transcarboxylase with [3-^{14}C] methylmalonyl CoA that all of the biotins of this enzyme are carboxylated. Similar experiments have been done with the 26S form of the enzyme, and it has been found, as mentioned above, that all 12 of the biotinyl groups are carboxylated.[64] The procedure involves incubation of the enzyme with [3-^{14}C] methylmalonyl CoA and determination of the decrease in heat-stable counts. Since the carboxylated biotinyl groups of the enzyme are heat labile and the methylmalonyl CoA is heat stable, the decrease in the stable radioactivity is a measure of the carboxylation of the enzyme. The reaction was conducted at 4° and was complete almost immediately, there being no significant further decrease in the stable radioactivity during 15 min. The total heat-labile counts divided by the counts per minute per nanomole of the [3-^{14}C] methylmalonyl CoA are considered to be the nanomoles of carboxylated biotinyl enzyme. A value of ~12 per nmole of enzyme of molecular weight 1.22×10^6 was obtained consistently.

3. Association-dissociation of the 26S Form of Transcarboxylase and Relationship to the Native Form of the Enzyme

Numerous electron micrographs of the 16S and 18S forms of transcarboxylase have been made



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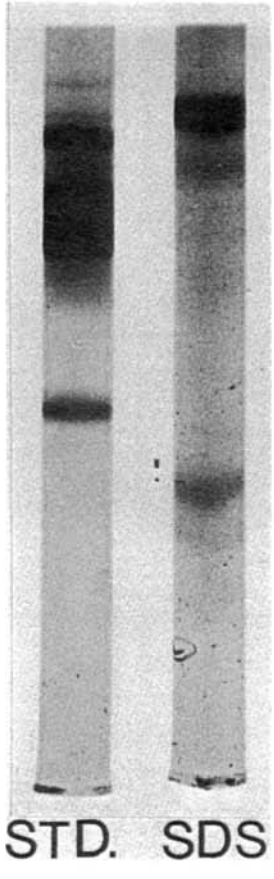

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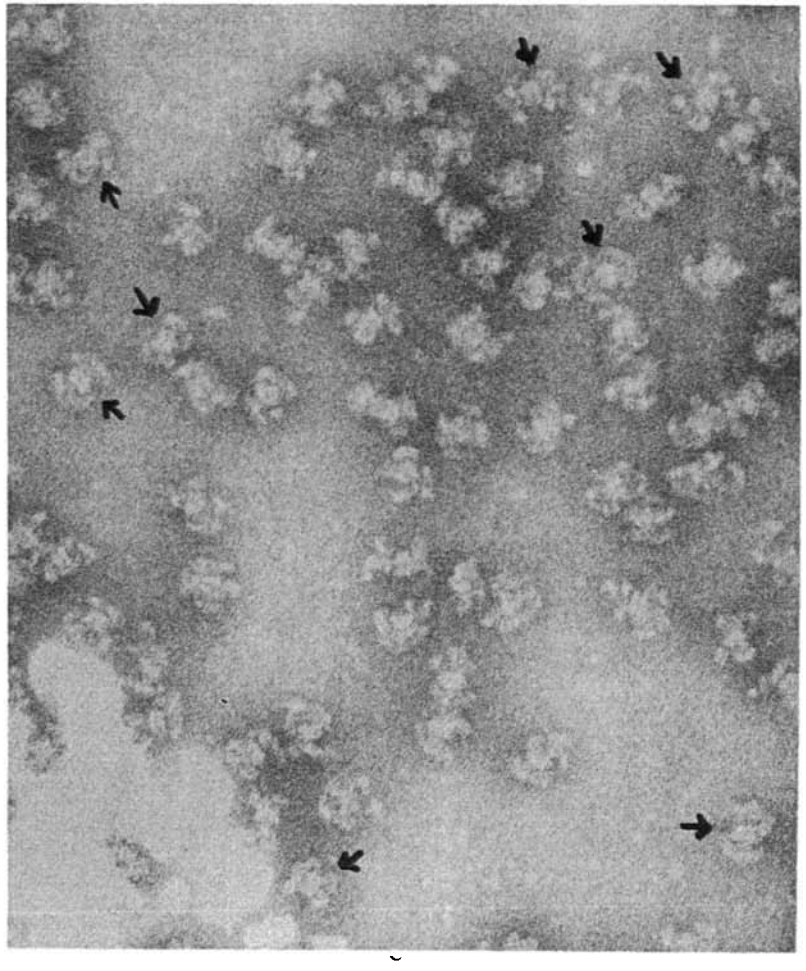


C

FIGURE 22. Sedimentation velocity profile, polyacrylamine gel electrophoresis and electron micrographs of isolated 26S enzyme. (A) Ultracentrifugation, at 48,000 rpm in 0.1 *M* acetate pH 5.5 at 4°, photographed at 28 min, $s_{20,w} = 26.5$. (B) Gel electrophoresis. (C) Electron micrograph of the isolated 26S form of the enzyme.

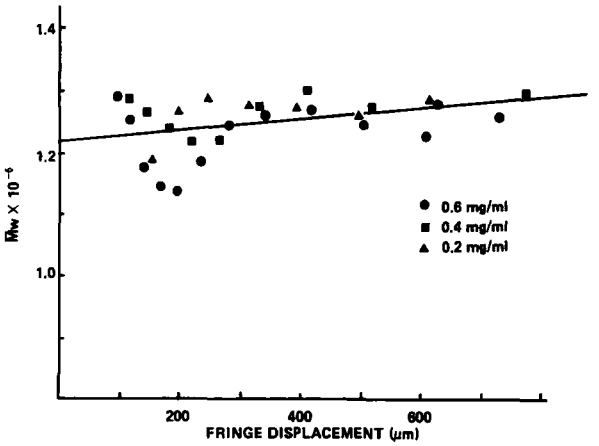


FIGURE 23. Yphantis sedimentation equilibrium of the 26S form of transcarboxylase. Centrifugation was with 0.2, 0.4, and 0.6 mg/ml of the enzyme in 0.05 *M* acetate buffer at pH 5.5. The temperature was maintained at 4°. The rotor speed at equilibrium was 6,800 rpm.

during the course of our studies, but peripheral subunits invariably have only been seen on one face of the $12S_H$ subunit. If the 26S form dissociated with loss of $6S_E$ subunits at random from the two faces, one would expect that peripheral subunits would be seen on both faces. This form of transcarboxylase is seldom, if ever, seen in electron micrographs.[9,100] The dissociation of the 26S enzyme to the 18S form apparently involves loss of peripheral subunits from only one face, yielding a series of intermediates, as diagramed in Figure 24.

a. Reacting Enzyme Sedimentation

To obtain information on the type of enzyme present in the cell, Poto et al.[96] examined crude extracts using the technique of "reacting enzyme sedimentation." This technique[65,66] is an excellent procedure for determining the $s_{20,w}$ values of reacting forms of an enzyme, even in crude preparations which contain a large amount of extraneous protein. A small amount of the preparation to be tested is layered on top of an assay mixture. On centrifugation, the enzyme sediments as a band through the assay mixture; as it passes through the mixture, reaction occurs. For transcarboxylase, the assay mixture contained methylmalonyl CoA, pyruvate, malate dehydrogenase, and DPNH. The oxalacetate which is formed is reduced by the DPNH, and the optical density change is followed at 350 nm. Thus, one is measuring DPNH oxidation rather than protein concentration. Fortunately, crude extracts of the propionic acid bacteria do not contain enzymes other than transcarboxylase, which cause significant oxidation of the DPNH. In addition, transcarboxylase has a broad pH optimum,[67] and it is possible to conduct the experiment at both pH 5.5 and 6.8.

The results of the experiments of Poto et al.[96] are presented in Figure 25. The following is a description of the curves shown in the figure. The centrifugation was from left to right, and the time when the scan was made is indicated by the number preceding each curve. The numbers on the abscissa indicate the distance from the reference hole at the bottom of the cell, and the optical density is plotted on the ordinate. The DPNH of the assay mixture is oxidized in that portion of the assay mixture through which the enzyme has passed; thus, there is little absorption in this region. The absorption increases as the scan reaches the region in the cell through which the enzyme has not passed. If there are two reactive species of the enzyme, one of which sediments faster than the other, there will be maximum oxidation (lowest optical density) in the portion of the cell through which both species have passed. When the scan reaches the region where only the fastest moving species has sedimented, less DPNH will be oxidized and the optical density will increase. Thus, with time as the species separate there will be two inflection points on the curves, the first representing the slower moving component and the second the faster moving component (as in Figure 25D). The log of the radius a calculated from the inflection points of the curv is plotted against time for calculation of the sedimentation coefficients.

The results obtained with purified 18S transcarboxylase are shown in Figure 25A and with 26 transcarboxylase in Figure 25B which was prepared as in the experiment of Figure 20 from dissociated 18S transcarboxylase. The sedimentation coefficients were 20.5 and 26.7S after corrections were made for the effect of the 50% D_2O on the sedimentation. When the sedimentation coefficient was determined using Schlieren optics in 50% D_2O and the 26S form of the enzyme, a value of 26.8S was obtained after correction for the D_2O effect.

The tests of Figures 25C to 25F were done with two preparations. The cells for one preparation were broken in phosphate buffer at pH 6.8; the

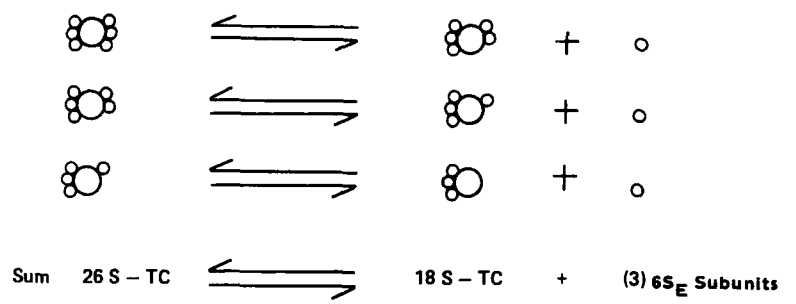


FIGURE 24. *Postulated dynamic equilibrium of forms intermediate between the 26 and 18S forms of the enzymes. The 18S form is relatively stable and does not dissociate to the 12S subunit and peripheral subunits under conditions in which the above dissociation occurs.*

cells for the other preparation were broken in acetate buffer at pH 5.5. The crude extracts were treated with streptomycin and fractionated with ammonium sulfate (45 to 65% saturated). The result obtained with a crude extract prepared in phosphate buffer at pH 6.8 is shown in Figure 25C; the result with the extract prepared at pH 5.5 in acetate buffer is shown in Figure 25D. The preparation made at pH 6.8 had a sedimentation coefficient of 20.9S, but that prepared at pH 5.5 gave two inflections, indicating that two active species of enzyme were present. The one species had an $s_{20,w}$ = 28.3S and the other, 21.1S. Clearly, large forms of the enzyme were present in crude extracts prepared at pH 5.5. However, when the crude extract prepared at pH 5.5 was simply diluted in the assay mixture at pH 6.8 and sedimented (Figure 25E), the larger form disappeared and only a single species was observed with an $s_{20,w}$ of 21.4S. Likewise, when the crude extract, which was prepared at pH 6.8, was sedimented in the assay mixture at pH 5.5, two species were observed (Figure 25F), the larger giving an $s_{20,w}$ = 27.6S.

The results of this experiment are most readily interpreted in relation to the association-dissociation equilibria shown in Figure 24. Viewed in this context, it is obvious that the crude extract from the cells contained $6S_E$ subunits in excess of those in the 18S form of the enzyme; under acid conditions which favor association of the 18S enzyme and $6S_E$ subunit, the equilibria shifted toward the 26S form of the enzyme. Under conditions favorable for dissociations (pH 6.8), the equilibria shifted towards dissociation to the 18S form of the enzyme and $6S_E$ subunits. The results will be considered further in Section VII.C3.c. The $6S_E$ subunits, of course, are not evident by the reacting enzyme technique since they have no enzymatic activity.

b. Factors Influencing the Dissociation of the 26S and 18S Forms of the Enzyme

The properties of the dissociation of the three peripheral subunits from the 26S form of the enzyme differ from those of dissociation of the remaining three subunits from the 18S form of the enzyme.[64] The 26S form dissociates readily at neutral pH[64] whereas the 18S form is quite stable at this pH, and dissociation to the $12S_H$ and $6S_E$ subunits occurs at a more alkaline pH.[35] A second factor which influences association-dissociation is ionic strength, particularly of divalent ions such as sulfate or phosphate. A third factor is the concentration of the enzyme and its subunits. The effect of the concentration of phosphate buffer and the protein concentration of protein on the dissociation of the 26S form of the enzyme is shown in Figure 26. When the 26S form of the enzyme was centrifuged in 0.05 *M* phosphate at pH 7, two peaks were observed, one with an $s_{20,w}$ of 17.3S and the other of 6.1S. Clearly, at this pH and phosphate concentration, the 26S form of the enzyme dissociated to the 18S form and $6S_E$ subunits. With increasing concentration of the phosphate buffer there was an increase in association; at 0.3 and 0.5 *M* phosphate buffer, the $6S_E$ subunit was no longer evident and the larger form of the enzyme predominated. The occurrence of split peaks during the centrifugation will be considered in Section VII.C.3.c.

The effect of protein concentration on the dissociation is illustrated in Figure 26B. These experiments were done with 0.3 *M* phosphate

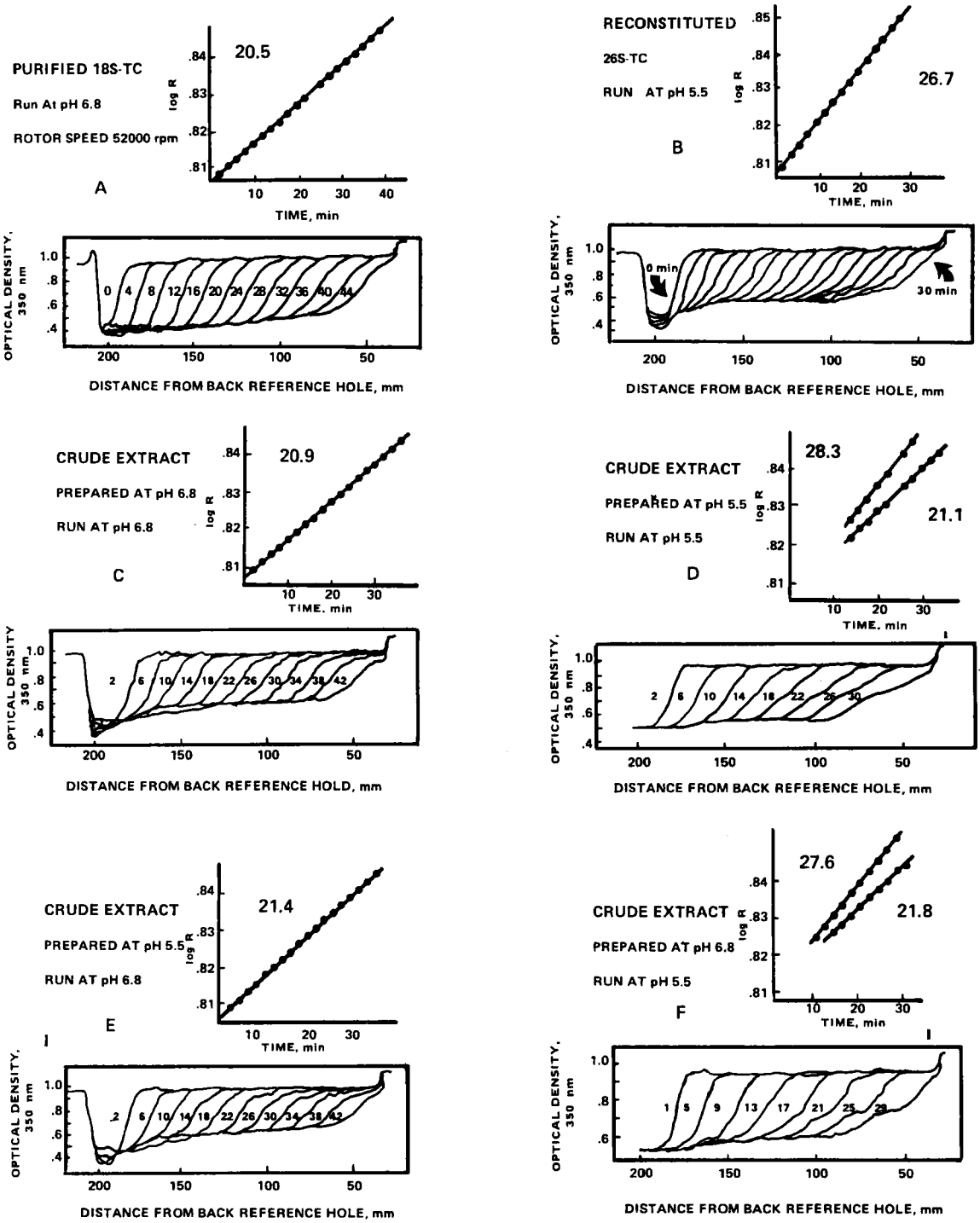


FIGURE 25. Determination of the sedimentation coefficient of transcarboxylase in crude extracts by "reacting enzyme sedimentation." The crude extracts were prepared in 0.2 *M* phosphate buffer at pH 6.8 or in 0.25 *M* acetate buffer at pH 5.5. The crude extract was treated with an equal volume of 10% streptomycin solution and the precipitate spun down after 20 min. A 45 to 65% saturated $(NH_4)_2SO_4$ cut was made on the supernatant solutions to obtain somewhat purified enzymes (specific activity 4). The assay mixture used in the centrifugation contained 0.17 *M* phosphate buffer at pH 6.8 or 0.11 *M* acetate buffer at pH 5.5, each in 50% D_2O at 19°. The other conditions were as described previously.[6,7] The D_2O was included in the solution to reduce diffusion of the sedimenting band of the enzyme preparations. The 18S transcarboxylase had a specific activity of ~30 and 0.016 mg/ml was used in the centrifugation. The 26S form of the enzyme was prepared from the 18S enzyme as described in the experiment of Figure 20 and 0.076 mg/ml was used. The latter contained 6S material that did not reconstitute, but is enzymatically inactive. Centrifugation was at 60,000 rpm except in A, which was at 52,000 rpm.

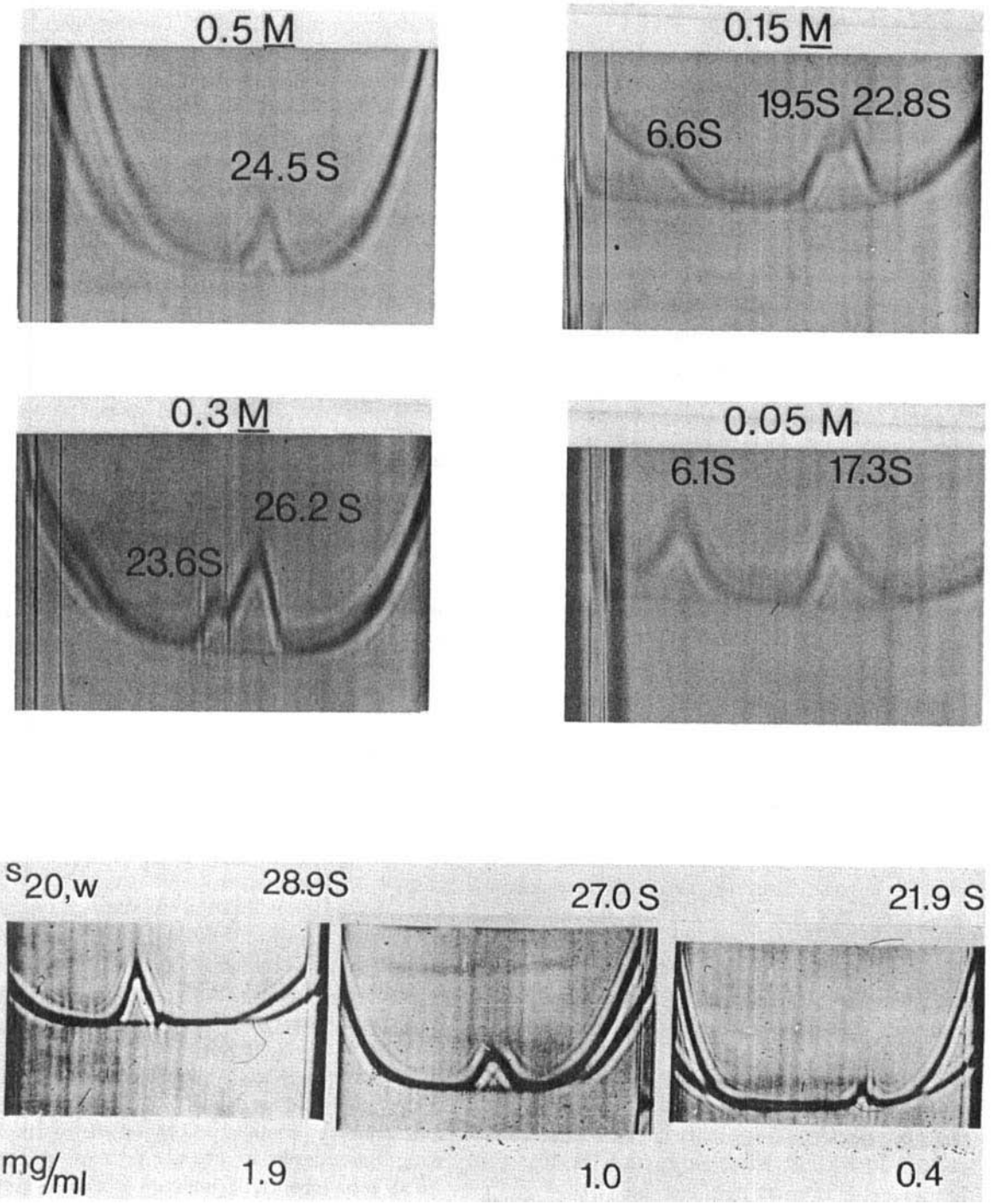


FIGURE 26. The effect of change in concentration of phosphate buffer pH 7.0 (A) and the concentration of the 26S form of the enzyme (B) on sedimentation profiles. (A) 26S transcarboxylase of specific activity 25 (13 mg/ml) was dialyzed for 16 hr at 4° against the indicated buffer and diluted in the same buffer to 1.8 mg/ml. Centrifugation was started after 1 hr and was at 4° at 48,000 rpm. Photographs are at 56 min. (B) The enzyme of specific activity of ~35 at the indicated concentration was sedimented in 0.3 *M* phosphate buffer (pH 6.3). Centrifugation was at 52,000 rpm at 4°, and the photographs are at 36 min.

buffer at pH 6.3. The sedimentation coefficient shifted from 28.9S when the protein concentration was 1.9 mg/ml to 21.9S when it was 0.4 mg/ml. The decrease in the sedimentation coefficient indicates that there is increased dissociation with decrease in the protein concentration. The sedimentation coefficient of 28.9S is unusually high, but such values are occasionally observed. The occurrence of single peaks under the conditions of this experiment and the failure to see evidence of the $6S_E$ subunit are most likely consequences of a rapid equilibria between the components of the dissociation, as discussed in Section VI.C.3.c.

The properties of the 18S enzyme differ greatly from the 26S enzyme. The $s_{20,w}$ value of the 18S form is not altered significantly with change in protein concentration in phosphate buffer at pH 6.8. It is only at a more alkaline pH that this effect becomes evident.[35] The results of Figure 25A confirm this observation. The concentration of protein in the "reacting enzyme sedimentation" was 0.016 mg/ml in the solution used in making the band, and there is further dilution due to diffusion during the sedimentation. Nevertheless, activity was observed at 20.5S, indicating that the dissociation at pH 6.8 in phosphate buffer did not proceed to the $12S_H$ and $6S_E$ subunits. Clearly, the binding of the peripheral subunits on the 18S form of the enzyme differs from that of three of the six peripheral subunits on the 26S form of dissociate from the 26S form almost certainly arise from only one face of the $12S_H$ subunit; apparently, this face of the $12S_H$ subunit has different binding properties than the other face. The facts that the 18S form is quite stable in phosphate at pH 7.0 (Figure 26A) and a pH of 8 is required for its dissociation are evidence of the difference between the binding to the two faces. It is still possible, however, that there is no difference in the two faces of the $12S_H$ subunit and that there is negative cooperativity, so that loss of a peripheral subunit from either face facilitates further loss of subunits from that face.

c. Equilibria and the Association and Dissociation of the 26S Form of Transcarboxylase

The dissociation of the 26S form of transcarboxylase to $6S_E$ subunits and the 18S form of transcarboxylase involves intermediate steps (Figure 24). If the association and dissociation of each step is sufficiently rapid, only a single peak is seen during ultracentrifugation; however, if conditions are such that the rate of association-dissociation is not sufficiently rapid, the rate of sedimentation of the different species will outstrip their equilibration, thus resulting in multiple sedimentation peaks.[68a] Accordingly, evidence of the equilibration between the different forms of transcarboxylase, as illustrated in Figure 24, might be detected by centrifugation at different speeds. Results of such tests,[96] as well as the effect of temperature on the sedimentation patterns, are shown in Figure 27. The upper patterns show the results when the 26S enzyme was in 0.1 *M* phosphate buffer at pH 6.3; the lower patterns show the results in 0.05 *M* acetate buffer at pH 5.5. At 30,000 rpm at 3° (Figure 27A) the equilibration was sufficiently rapid so as to maintain equilibria of the different forms at pH 5.5 and pH 6.3, thus giving single major peaks. At pH 6.3 there was evidence of dissociation since a small peak was observed with a sedimentation coefficient of 6.5S. The equilibria at pH 6.3 are toward dissociation but clearly not completely to the 18S enzyme and $6S_E$ subunits since the $s_{20,w}$ value was 22.6S. At pH 5.5, which favors association, the equilibria are shifted towards the 26S form of the enzyme giving an $s_{20,w} = 25.8$.

When the centrifugation was at 60,000 rpm (Figure 27B) split peaks were observed,* showing that the equilibration of the respective species was not rapid enough to prevent their separation during the rapid sedimentation. The peak of 26.1S at pH 5.5 may predominantly represent the species with six peripheral subunits, and the peak of 24.7S probably is an equilibrium mixture of species with six and five peripheral subunits. At pH 6.3 there obviously was more dissociation; the 21.6S peak may represent equilibration in which the species with four peripheral subunits predominate, and 23.5S may represent equilibration between forms with four and five peripheral subunits. It is interesting that when the temperature was raised to 21° (Figure 27C), the dissociation at pH 6.3 was more complete and the equilibration apparently was

*Note that in Figures 22A and 25B there was no separation of the peaks in acetate buffer at pH 5.5. In addition, during sedimentation equilibrium (Figure 23) in acetate buffer at pH 5.5 there was no indication of dissociation. The 26S enzyme of these experiments was stored in acetate buffer at pH 5.5 and was freshly prepared, whereas the 26S enzyme of Figure 27 was stored in 0.1 *M* potassium phosphate buffer, pH 6.0, plus 10% glycerol. Apparently some change in the enzyme occurred during storage.

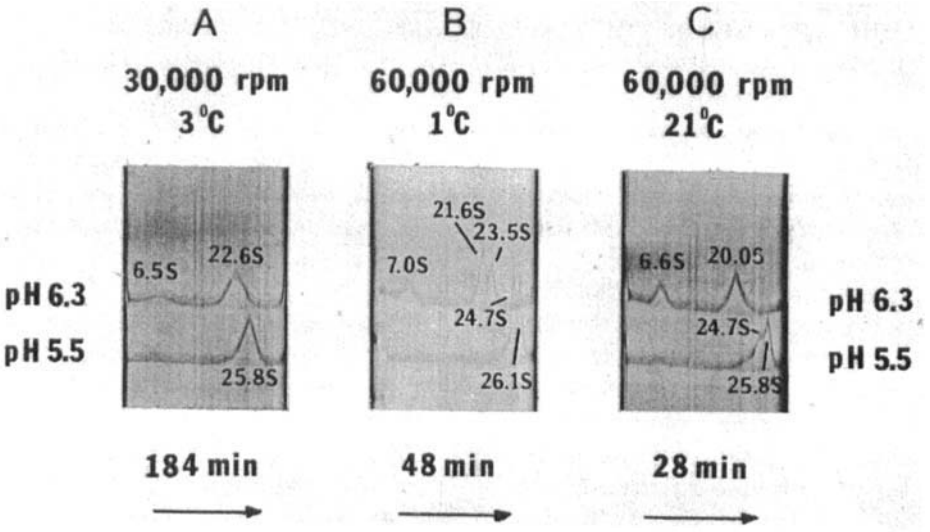


FIGURE 27. Sedimentation profiles demonstrating effect of the rate of centrifugal separation and temperature on reversible equilibria of transcarboxylase components. Centrifugation was done in 12-mm double-sector wedge cells at speeds and temperatures indicated. Purified 26S transcarboxylase was dialyzed overnight, diluted to ~5 mg/ml and centrifuged in 0.1 *M* potassium phosphate buffer, pH 6.3 (top) and 0.05 *M* sodium acetate buffer, pH 5.5 (bottom). The $s_{20,w}$ values are shown on the sedimentation profile.

rapid, giving single peaks with $s_{20,w}$ values of 20.0S and 6.6S. There also was some evidence of dissociation at pH 5.5, as is evidenced by the small peak of 7.1S and split peaks of 24.7S and 25.8S.

Tests have been done with oil layered over the enzyme solution[68b] to determine whether or not the observation of multiple peaks is due to the increased pressure at 60,000 rpm. Multiple peaks appeared at the same time at 60,000 rpm with or without the oil layer, thus demonstrating that pressure was not responsible for the appearance of the different species. It is to be noted that Jacobson et al.[35] observed dual peaks of 18S and 16S at 60,000 rpm with the 18S form of the enzyme and a single peak of 17S at 30,000 rpm. They found that the double peak persisted at 60,000 rpm when the enzyme was centrifuged in 30% sucrose, and the time required for sedimentation was twice that required for sedimentation at 30,000 rpm. It was concluded that the equilibrium involves forms of transcarboxylase which are sensitive to pressure. It is possible that the rate of equilibration of the 18S and 16S forms is not the same in 30% sucrose as in its absence, and the two forms did not equilibrate in 30% sucrose even though the rate of sedimentation was slow. Further studies are necessary to evaluate the effect of pressure on this system.

It is noted that the reacting enzyme sedimentations of Figure 25 were done at ~19°. At pH 6.8, a single species was observed with $s_{20,w}$ values of 20.9 and 21.4, thus resembling the results at pH 6.3 of Figure 27C. At pH 5.5 of Figure 25 two species were seen in each case, which is in accord with the split peaks of Figure 27C. The $s_{20,w}$ values were substantially higher in the experiment of Figure 25. This result may in part be due to the effect of D_2O on the equilibria of the association-dissociation and to exchange of D_2O with the protein.

The results of Figure 26, likewise, can be interpreted in terms of association and dissociation equilibria. In the case of the experiment of Figure 26B, the conditions were apparently favorable for rapid equilibria of the different forms, and single peaks were observed throughout.

It is apparent from the above results that the species of transcarboxylase which will be present in the cells of propionibacteria will depend on the pH, concentration of divalent ions, concentration of 18S enzyme and $6S_E$ subunits, as well as other factors within the cell. It is possible that the activity of the enzyme modulates with the change in the transcarboxylase species, and this modulation may function as part of the mechanism of control of the propionic acid fermentation.

VIII. STRUCTURE OF THE SUBUNITS AND RELATIONSHIP TO THE QUATERNARY STRUCTURE OF TRANSCARBOXYLASE

A. Structure of the $1.3S_E$ Subunit (Biotinyl Carboxyl Carrier Protein)

We have seen (Section V.A, Figure 9) that a nonbiotinyl polypeptide arising from a portion of the $1.3S_E$ subunit provides the amino acid sequence which links together the $12S_H$ and $5S_E$ subunits. In addition, it has been observed that the 65- and 46-residue biotin peptides which are derived from the $1.3S_E$ subunit are effective as carboxyl acceptors in the partial reactions (Section VI.B and C, Figure 12), but biotin per se or derivatives such as biocytin are ineffective. Thus, it is evident that a portion of the amino acid sequence of the $1.3S_E$ carboxyl carrier protein is essential in the combination and orientation of the biotinyl groups relative to the $12S_H$ and $5S_E$ subunits, so that it may serve as a carboxyl transfer agent between these subunits. As a first step in the further elucidation and definition of the properties and requirements for these interactions, the amino acid sequence of the $1.3S_E$ carboxyl carrier protein is being determined.[69]

1. Amino Acid Composition of the $1.3S_E$ Subunit

The isolation and purification of the $1.3S_E$ subunit has been described in Section III.A. Two forms of the carboxyl carrier protein were isolated, purified, and separated from each other. Their respective amino acid compositions appear in Table 4.* The longer 123-residue form always predominates and accounts for 75 to 95% of the total isolated and purified $1.3S_E$ subunit. Although the same precautions are used against proteolysis and oxidizing agents in every preparation, the amount of the 123-residue polypeptide has varied as noted above. The similarity in the amino acid composition and the fact that one unique sequence is obtained by use of a mixture of the short and long forms proves that the peptides differ only in their length. It has not been established as yet whether the difference is at the amino or carboxyl terminal or both.

The amino acid composition is rather unique. There are no cysteine or tryptophan residues (Table 4), and there are a very small number of aromatic residues. Comparing the percentage of each specific amino acid in this protein with the average percentage of each amino acid found in 307 proteins[70] of known composition, structure, and sequence leads to the following observations.

1. There is an extremely high glycine content and a high content of valine and alanine.
2. The protein has a higher than average content of hydrophobic residues, while the basic and aromatic residue content is lower than average.

2. Determination of the Amino Acid Sequence of the $1.3S_E$ Subunit

The amino acid sequence was determined by automated sequential analysis at the University of Washington, Seattle. At the present time, the sequence has been determined from residue 1 through 109, and work is in progress on the determination of the sequence of the remaining 14 residues at the C-terminus. Figure 28 diagrammatically illustrates the sequential overlap, the positions of the various peptide fragments used in determining the sequence to position 109, and the amino acid sequence as thus far determined.[69] All peptide fragments and the intact protein were sequenced at least twice, and the amino acid sequence as given is considered to be reliable. At the present time there are three residues at positions number 35, 79, and 108 which have not been identified in the sequence of 1 through 109.

The sequence was determined on the intact carboxyl carrier protein, the 65- and 46-residue biotinyl peptides obtained by trypsinization of transcarboxylase (Section IV.A, Figure 7), a cyanogen bromide cleavage product, and a chymotrypsin cleavage product. The trypsinization of transcarboxylase yields not only the 65- and 46-residue peptides but also a 19-residue biotinyl peptide.** The three biotinyl peptides were separated from each other in very high purity by

*Gerwin et al.[15] reported that the carboxyl carrier protein contained 97 residues and the amino acid composition was at variance with that reported here, particularly with respect to proline.

**Variation in the reaction time and temperature of the trypsin hydrolysis can be used to produce a predominance of any of the three biotinyl peptides. Low temperature (0°) and short reaction times (2 to 3 min) lead to a predominance of the 65-residue biotinyl peptide, while digests at room temperature and longer times (10 min) lead to a predominance of the 46- and 19-residue biotinyl peptides.

TABLE 4

Amino Acid Composition of the $1.3S_E$ Subunit and Derived Fragments

Amino acid	$1.3S_E$ subunit		Tryptic Biotinyl Peptides			CNBr biotinyl peptide	Chymotryptic biotinyl peptide	Nonbiotinyl peptide
	Long	Short	1	2	3			
Lysine	11	10	7	5	2	4	2	2
Histidine	1	1	0	0	0	0	0	1
Arginine	2	2	1	1	0	1	0	2
Aspartate	10	10	3	3	2	3	2	6
Threonine	9	9	3	3	2	2	2	3
Serine	3	3	1	1	1	1	0	2
Glutamate	12	12	7	7	2	6	3	4
Proline	6	6	4	2	1	1	1	2
Glycine	21	20	12	6	2	6	1	6
Alanine	14	14	9	5	2	3	2	4
Valine	14	13	8	6	2	4	2	5
Methionine	4	3	1	1	1	0	1	1
Isoleucine	6	5	4	3	1	3	1	2
Leucine	8	7	5	4	1	2	1	2
Tyrosine	1	1	0	0	0	0	0	2
Phenylalanine	1	1	0	0	0	0	0	2
Total residues	123	117	65	47	19	36	18	46

preparative disc gel electrophoresis using an upper buffer of 0.005 *M* tris-0.038 *M* glycine (pH 8.3) and a lower and elution buffer of 0.06 *M* triethanolamine·HCl (pH 8.1). The cyanogen bromide biotinyl peptide from the $1.3S_E$ subunit was separated from the other peptides of the mixture by gel filtration using Bio-Gel P-6 and P-10 columns and elution with 0.05 *M* tris·HCl (pH 8.0). The amino acid compositions of these peptides are given in Table 4. In all cases the peptides isolated from $1.3S_E$ subunits contained ^3H biotin and could be identified by their radioactivity.*

Residues 1 through 55, with the exception of 35, 51, and 54, were sequenced on the intact carboxyl carrier protein. The 65-residue biotinyl peptide arises by cleavage at arginyl 42 and lysyl 107, and the first 23 residues of this polypeptide were sequenced. The overlap was from residue 43 to residue 55. The 46-residue biotinyl peptide arises by cleavage at lysyls 51 and 97, and 38 residues of this peptide were sequenced. The biocytin was found to be at residue 87. The overlap was from residue 52 to 65. The cyanogen bromide biotinyl peptide arises by cleavage at methionyl residue 86, and 23 of the 36 residues were sequenced. An overlap of three residues was obtained, but the residue at position 108 was not identified. In order to obtain better sequential overlap in the region of biocytin at position 87, the 46-residue biotinyl peptide was digested with chymotrypsin. An 18-residue biotinyl peptide was separated and purified from the resulting mixture by elution from a Bio-Gel P-10 column with 0.05 *M* tris·HCl (pH 8.0), and its amino acid composition appears in Table 4. Six residues were sequenc-

*In the course of preparing the above peptide fragments of the carboxyl carrier protein for sequencing, several interesting aspects arose concerning the susceptibility of particular residues to enzymatic and chemical cleavage. The arginyl-alanyl bond at positions 42 to 43 is not only very susceptible to trypsin hydrolysis, but is cleaved to a large extent when the intact carboxyl carrier protein is subjected to CNBr in 70% formic acid for 24 hr at room temperature. The lysyl-alanyl bond at positions 51 to 52 is more resistant to trypsin hydrolysis than the arginyl-alanyl bond at 42 to 43. The longer tryptic biotinyl peptide, which has alanyl 43 as its N-terminus, is sometimes isolated as a 58- or 61-residue peptide, indicating that the lysyls at positions 100 and 103 are susceptible to trypsin, but not as readily as 107. Within experimental error, the amino acid composition of the tryptic 19-residue biotinyl peptide, which is produced only during very long exposures to trypsin, matches the sequence from positions 72 through 90. The lysyls at positions 67 and 76 are not cleaved by trypsin even after prolonged treatment. Cyanogen bromide cleavage at the methionyl-biocytin bond has given low yields (20%). Steric hindrance problems due to the large biotin group attached to the lysine may be a factor influencing the generally poor cleavage at this methionine.

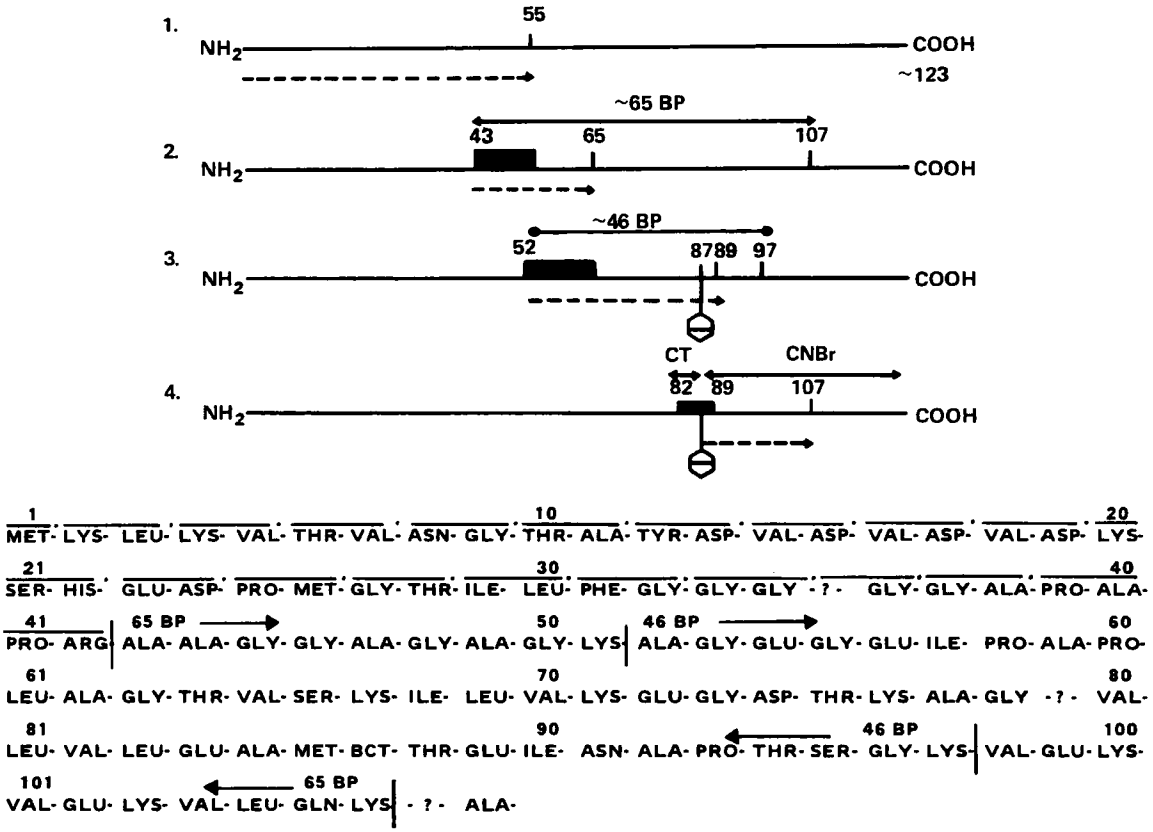


FIGURE 28. Sequence of the $1.3S_E$ subunit (biotin carboxyl carrier protein) residues 1 through 109 and a diagrammatic representation of the sequential overlap of the peptides used in the sequencing. (Scheme 1) Intact $1.3S_E$ subunit. (Scheme 2) 65-residue biotinyl peptide. (Scheme 3) 46-residue biotinyl peptide. (Scheme 4) CNBr and chymotryptic biotinyl peptide. In the diagrammatic representation, the following symbols are used. (↔) Length and position of the fragment in the total sequence. (– – →,) Number of residues sequenced with the respective peptide. (▬) Position and number of residues involved in the overlap of sequence between the given sequence and the previously determined peptide. (– · – · –) Over residues 1 through 42, indicates the nonbiotinyl peptide in the total sequence.

ed from this peptide, starting at position 82 and ending at biocytin.

3. Nonbiotinyl Peptide

The isolation of the nonbiotinyl peptide is described in Section IV.A. Comparison of the amino acid composition of this peptide (Table 4) with the known sequence of the carboxyl carrier protein revealed that the nonbiotinyl peptide is made up of residues 1 through 42. Examination of this particular sequence in terms of amino acid type, i.e., hydrophobic, hydrophilic, and polar, reveals several unique patterns (Figure 29).

1. Residues 1 through 5 are alternating hydrophobic and basic-hydrophilic.
2. Residues 13 through 19 are alternating acidic-hydrophilic and hydrophobic in which all residues are aspartic acid and valine.
3. Residues 32 through 41 are all neutral hydrophobic.
4. Two areas (6 through 12 and 21 through 31) of this linking peptide contain residues of serine, threonine, and asparigine. These neutral polar residues are often involved in intermolecular hydrogen bonding.[71] In addition, a tyrosine and a phenylalanine are found in these respective areas, and these residues have the ability to interact with similar residues in separate chains through π electron clouds.[71]

The Chou-Fasman method[72,73] for the prediction of protein conformation was applied to the nonbiotinyl peptide, and the method predicts the following potential conformations (Figure 29).

1. Residues 1 through 4 – random.
2. Residues 5 through 12 – β-sheet.

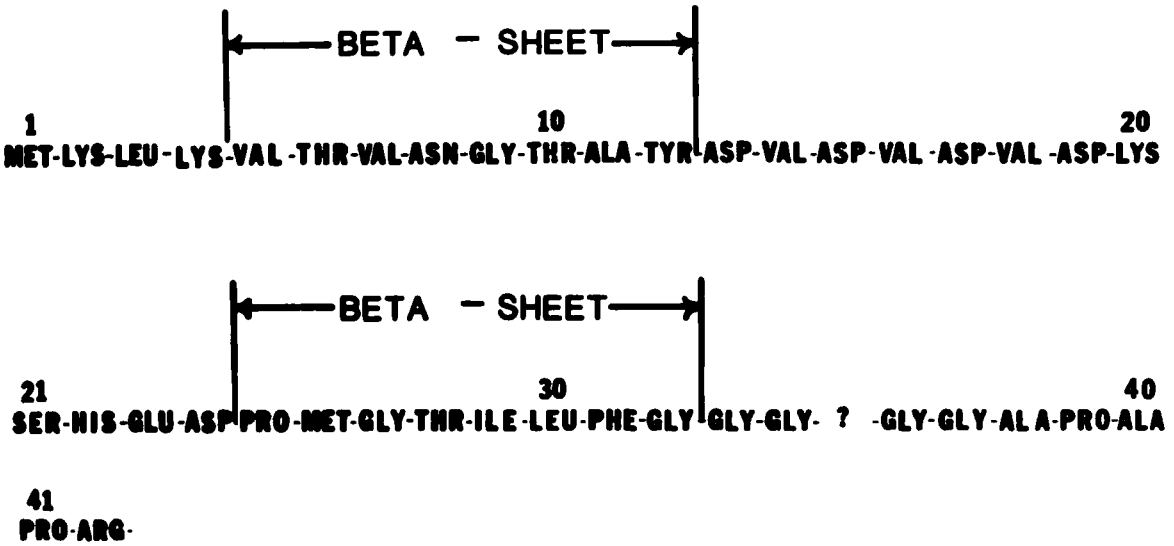


FIGURE 29. Sequence of the nonbiotinyl peptide of the $1.3S_E$ subunit with potential conformations calculated by the Chou-Fasman method.[72,73]

3. Residues 13 through 24 – random.
4. Residues 25 through 32 – β-sheet.
5. Residues 33 through 42 – random.

In the Chou-Fasman formulation random means any conformation that is not α-helix or β-sheet. Unfortunately, there have been no circular dichroism investigations made on the carboxyl carrier protein to either support or refute these potential conformation predictions.

If the above predictions are correct, the two most likely major binding portions of the peptide chain are the two β-sheet regions, one interacting with the $12S_H$ (central subunit) and the other with the $5S_E$ (peripheral subunit). The residues 13 through 24 likewise are probably involved in the linking of the $12S_H$ and $5S_E$ subunits. Fung et al.[38,39] (Sections VI.F.1 and 3) have reported that the distance between the pyruvate on the $5S_E$ subunit and propionyl CoA on the $12S_H$ subunit is approximately 7 Å. Electron micrographs[9] of the enzyme indicate that the distances between the $5S_E$ subunits and the $12S_H$ subunit vary from 0 to 30 Å. These variations in distance may be accounted for through the breakdown of the ionic interactions of residues 13 through 24 by anionic disruption due to the negative staining procedure (2 to 4% sodium silicotungstate) used for the electron micrographs.[74,75] It seems likely that residues 13 through 24 would provide a much weaker ionic interaction and be disrupted, rather than the hydrogen-bonded β-sheet regions.

There are histidyl and glutamyl residues at positions 22 and 23, respectively. These residues may form coordinate bonds with the Co^{++} or Zn^{++} of the $5S_E$ subunit near the binding sites of the keto acids. The interactions of histidyl and glutamyl residues with metal ions have been observed in many proteins (carboxypeptidase A,[76] thermolysin,[77] insulin[78]). These interactions often dictate structure and conformation near the active site. This interaction is also compatible with the dissociative properties of transcarboxylase. The portion of the carboxyl carrier protein which links the $6S_E$ subunit to the $12S_H$ subunit can be disrupted by raising the pH to 8 in 0.05 *M* tris·HCl buffer. The pH must be raised to 9 in order to dissociate the $1.3S_E$ subunit from the $5S_E$ subunit, indicating the $1.3S_E$ subunit is linked much more firmly to the $5S_E$ subunit than it is to the $12S_H$ subunit. The combined binding via the β-sheet and the histidyl and glutamyl interaction with the metal may account for the greater stability of the $5S_E$ and $1.3S_E$ subunit bonding.

4. Biotinyl Peptides

The following potential conformations were determined using the Chou-Fasman method[72,73] for the biotinyl portion of the $1.3S_E$ carboxyl carrier protein (Figure 30).

1. Residues 43 through 60 – random.
2. Residues 61 through 72 – α-helix.
3. Residues 73 through 79 – random.

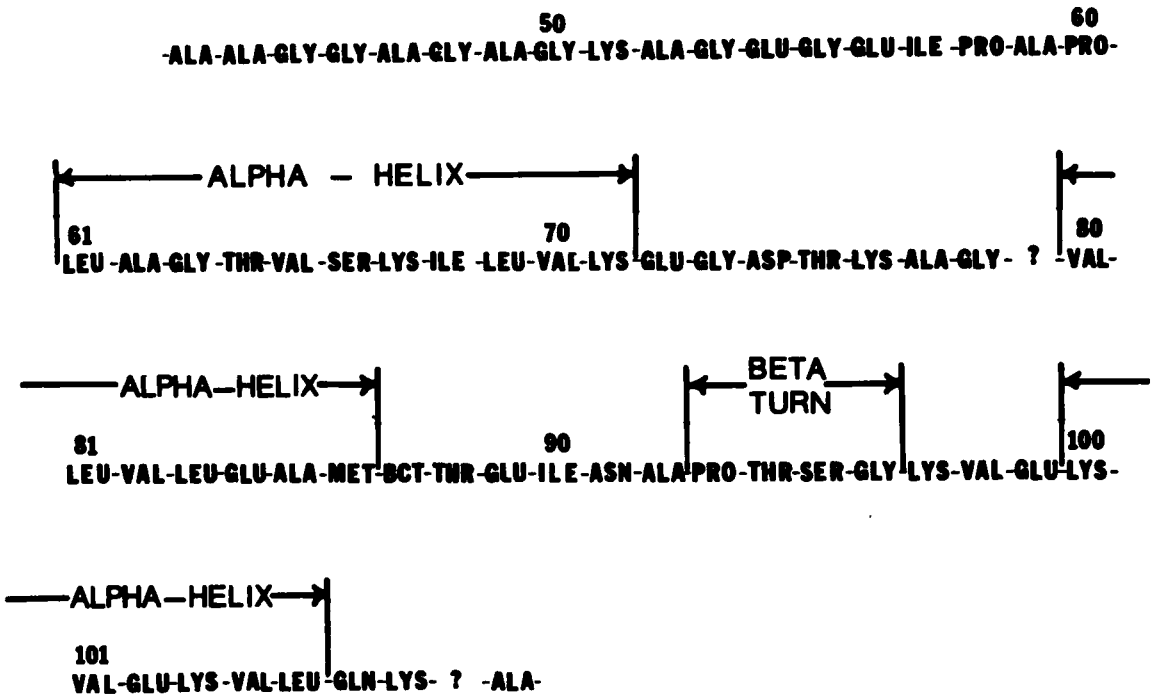


FIGURE 30. Sequence of the biotinyl portion of the $1.3S_E$ subunit with potential conformations as calculated by the Chou-Fasman method.[72,73]

4. Residues 80 through 86 – α-helix.
5. Residues 87 through 92 – random.
6. Residues 93 through 96 – β-turn.
7. Residues 100 through 104 – α-helix.

Also of interest are the two PRO-ALA-PRO patterns which are located at the end of the nonbiotinyl portion of the protein (39 to 41) and in the biotinyl portion at position 58 to 60. It is well documented that prolines produce bends in the peptide chain;[71] thus, these two proline sequences may bring the polypeptide chain back on itself. The residues between the PRO-ALA-PRO sequences are predominantly alanine and glycine, while the remainder of the known sequences is estimated to contain three potential α-helical regions. The helical regions could serve to reduce the length of the peptide chain while adding a degree of rigidity to the structure. The combination of these conformational factors may place the biotin at the specific position between the keto acid site on the $5S_E$ subunit and the CoA ester site on the $12S_H$ subunit (see Figure 16).

Bradshaw and co-workers of Washington University[97] have partially sequenced a portion of the biotinyl carboxyl carrier protein of acetyl CoA carboxylase from *E. coli*. For these studies they used the polypeptide (molecular weight 8,900) obtained from the carboxyl carrier protein (molecular weight ~22,500) by treatment with subtilisin.[16] This polypeptide is very similar to the carboxyl carrier protein of transcarboxylase in the region near the biocytin. If methionine is omitted as indicated in Figure 31, then 10 of the 17 amino acid residues in the same relative position to biocytin are identical (assuming that Glx is Glu), six are similar, and one is different. The term similar is used to indicate that the amino acids could arise by change in one of the three bases that make up the codon. There are two plausible explanations for this high degree of identity and similarity.

1. The two proteins were derived from a common gene, and this gene had undergone subsequent mutation.
2. The enzyme which attaches the biotin to the ε-amino group of the lysine in the biosynthesis of biotin enzymes may require specific residues or conformations in this region in order to recognize which lysine is to become biocytin.

Clearly, sequences which are as similar as those surrounding the biocytin in the *E. coli* and *P.*

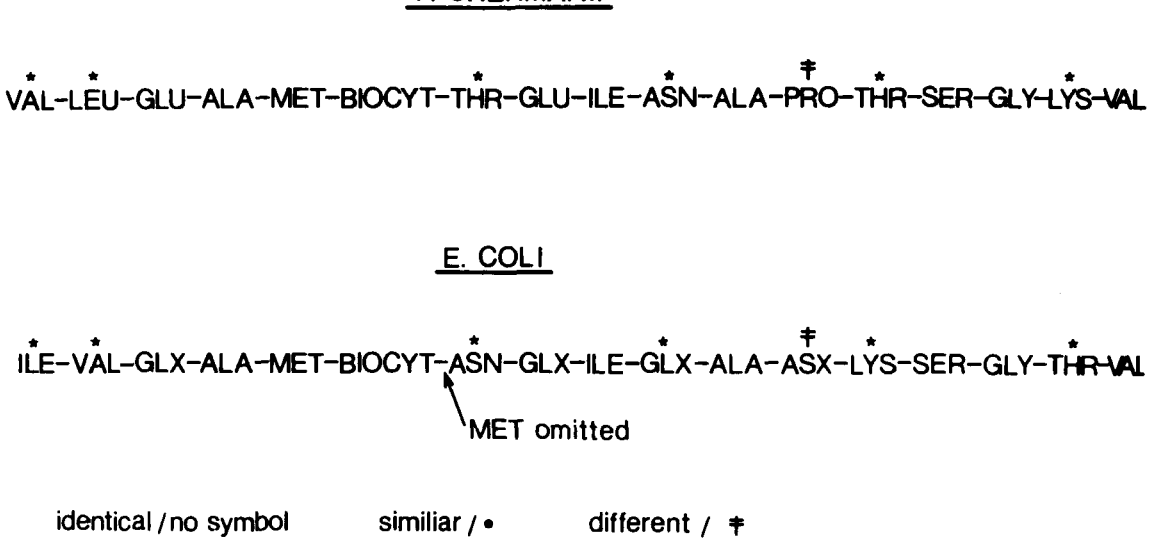


FIGURE 31. Comparison of the sequence near biocytin in the biotin carboxyl carrier protein of transcarboxylase (*P. shermanii*) and acetyl CoA carboxylase (*E. coli*).

shermanii enzymes would have similar conformations and might be recognized by synthetases which biotinylate an apoenzyme. Cross reactivity has, in fact, been observed. McAllister and Coon[79] have shown that synthetases of rabbit liver, yeast, and *P. shermanii* are active with propionyl CoA apocarboxylase from rat liver, β-methylcrotonyl CoA apocarboxylase from an *Achromobacter*, and apotranscarboxylase from *P. shermanii*. The one exception was that of synthetase from rabbit liver which did not biotinylate apotranscarboxylase. These results were interpreted as indicating that the various biotinyl enzymes may possess similar structural features in the region of the specific lysine residues to which biotin is attached by the synthetase.[3,79] The comparison between the carboxyl carrier proteins from *P. shermanii* and *E. coli* in the region of biocytin provides the first evidence that this is indeed true.

There also are other similarities. There are two PRO-ALA-PRO sequences which are positioned at 46 and 27 residues away from the biocytin in the sequence of the $1.3S_E$ subunit from transcarboxylase. In the subunit from acetyl CoA carboxylase, there is one PRO-SER-PRO sequence that is 46 residues from the biocytin and another potential PRO-X-PRO sequence that is 21 residues from the biocytin. In both cases the PRO-X-PRO sequences are located between the N-terminal and the biocytin. The number of residues between the PRO-ALA-PRO sequences of transcarboxylase is 16, while the number of acetyl CoA carboxylase is approximately 23. There is also one histidine residue in both proteins, and it is located between the N-terminal and the first PRO-X-PRO sequence. The histidine is 17 residues from the first PRO-X-PRO sequence in the transcarboxylase subunit and 14 residues from the sequence in the acetyl CoA carboxylase subunit. Both proteins have a small percentage of aromatic amino acids: the *P. shermanii* subunit has one phenylalanine and one tyrosine, while the *E. coli* subunit has three phenylalanines and one tyrosine. In the *P. shermanii* subunit the phenylalanine and tyrosine are located on each side of the histidine residue at a distance of nine and ten residues, respectively. In the *E. coli* subunit, a phenylalanine and tyrosine are adjacent to each other, and they are ten residues from the histidine. These similarities are most likely the consequence of the fact that both proteins have the same active catalytic component (biocytin) and catalyze similar reactions. Nevertheless, in our hands[98] the 10,267 mol wt fragment of the *E. coli* subunit[16] has been found to be ineffective as a replacement for the $1.3S_E$ subunit or biotinyl peptides in the partial reactions catalyzed by the $12S_H$ and $5S_E$ subunits, as described in Section VI.C. On the other hand, Vagelos and co-workers[99] have found that the $1.3S_E$ subunit is effective in the reactions catalyzed by the *E. coli* enzyme, but this is not surprising because biotin per se is effective in these reactions.

B. Structure of the $12S_H$ and $5S_E$ Subunits and their Relationship to the Quaternary Structure of Transcarboxylase

A detailed consideration of the quaternary structure of transcarboxylase will be presented in this section. As background, we will review briefly the rationale which led Green et al.[9] to propose the model of Figure 3 (Section II.B.3) for the quaternary structure of transcarboxylase. The electron micrographs of the 18S form of the enzyme showed that the three peripheral $6S_E$ subunits are attached to only one face of the hexagonal central $12S_H$ subunit. Green et al.[9] proposed that the $1.3S_E$ biotinyl peptide provides a flexible link between the $5S_E$ and $12S_H$ subunits (Figure 6, Section IV.A), thus accounting for the variable gap seen in the electron microscope between the subunits (Figure 1). It was known that the $12S_H$ subunit is made up of six $2.5S_H$ polypeptides and that the three peripheral $6S_E$ subunits consist of dimeric $5S_E$ subunits with two $1.3S_E$ biotinyl carboxyl carrier proteins attached to each. Since the $12S_H$ subunit consists of six $2.5S_H$ polypeptides and there are two $1.3S_E$ biotinyl peptides on each of the three $6S_E$ peripheral subunits, it seemed logical that each of the biotinyl peptides would be bonded to a $2.5S_H$ polypeptide. The electron micrographs of avidin complexed with transcarboxylase indicated that the biotinyl groups were at each end of the $5S_E$ subunit (Figure 1, Section II.A). To provide the six binding sites on one face of the $12S_H$ subunit, it was proposed that the constituent $2.5S_H$ polypeptides all point in the same direction. However, it was known that a ~26S form of the enzyme is formed under certain conditions of reconstitution,[35] and the electron micrographs showed that with this form, there are peripheral subunits on both faces of the $12S_H$ subunit.[9] This fact posed a dilemma. If the $2.5S_H$ polypeptides are all pointed in one direction, how could binding sites be provided for the biotinyl peptides on the opposite face of the $12S_H$ subunit? As a possible explanation, Green et al.[9] suggested that there might be a repeating homologous amino acid sequence in the $2.5S_H$ polypeptides as a consequence of gene duplication and fusion which could thus provide for similar, but not necessarily identical, binding sites for the $1.3S_E$ subunit at both faces of the $12S_H$ subunit. This proposal is illustrated in Figure 32.[7] At that time, the 26S transcarboxylase was thought to be an artifact arising because of some denaturation during the reconstitution of the enzyme (Section VII.A).

It was against this background that the studies described below were undertaken in an attempt to answer whether or not there is a duplicating sequence in the polypeptide of the $12S_H$ subunit. Furthermore, it had not been established by chemical means whether or not the $2.5S_H$ polypeptides were identical. The $2.5S_H$ and $2.5S_E$ subunits had been analytically separated[7,9] by acrylamide gel electrophoresis with urea, but had not been characterized.

1. Peptide Maps, Amino Acid Composition, and N-Terminal Determinations of the $2.5S_H$ and $2.5S_E$ Polypeptides as Tests for Sequence Homology

Purified transcarboxylase containing ^{3}H-biotin

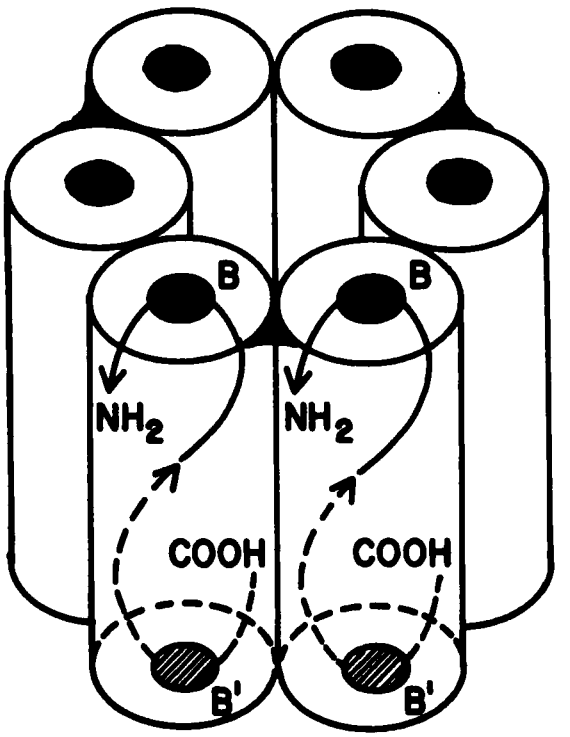


FIGURE 32. Schematic representation of the $12S_H$ central subunit of transcarboxylase. The six $2.5S_H$ polypeptides are shown with two regions of homologous sequence (indicated by solid and dashed lines) which form two similar sites. One site B binds peripheral subunits more firmly than the second site B'. (From Wood, H. G., Ahmad, F., Jacobson, B., Green, N. M., and Wrigley, N. G., in *Enzymes Structure and Function*, Vol. 29, Drenth, J., Osterbaan, R. A., and Veeger, C., Eds., North-Holland, Amsterdam, 1972, 201. With permission.)

was dissociated at pH 9, and the mixture of $6S_H$ and $5S_E$ subunits was separated by gel filtration from the ^{3}H-biotin containing $1.3S_E$ subunit, as described in Figure 4, Sections III.A, C. The $6S_H$ and $5S_E$ subunits were separated from each other by preparative disc gel electrophoresis. This method of separation yields very pure subunits with which the chemical studies were performed. The results will be presented in terms of the constituent $2.5S_H$ and $2.5S_E$ polypeptides since the dimeric $6S_H$ and $5S_E$ each contained duplicate polypeptides.

a. Amino Acid Composition of the $2.5S_H$ and $2.5S_E$ Polypeptides

The amino acid compositions of the $2.5S_H$ and $2.5S_E$ polypeptides of the $6S_H$ and $5S_E$ subunits are given in Table 5. Comparisons have been made between the amino acid distribution of the two polypeptides and the average amino acid composition and amino acid type of 307 proteins of known sequence as given in the *Atlas of Protein Structure and Sequence*.[70] The $2.5S_H$ polypeptide is characterized by a lower than average amount of basic and aromatic residues and a higher than average amount of hydrophobic residues. With respect to individual amino acids, the $2.5S_H$ polypeptide has a very large amount of alanine, methionine, and glutamic acid plus glutamine. The $2.5S_E$ polypeptide is characterized by a lower than average aromatic residue content and a slightly higher than average amount of hydrophobic residues. Individually, the methionine, alanine, and combined glutamic acid plus glutamine residues are present in proportionally large quantities.

The N-terminal residue of the $2.5S_H$ polypeptide was determined by the dansylation procedure[80] and found to be alanine, while that of the $2.5S_E$ polypeptide appears to be blocked.

b. Peptide Maps of the $2.5S_H$ and $2.5S_E$ Polypeptides and the Lack of Evidence of a Homologous Sequence

Three types of peptide maps were made from both the $2.5S_H$ and $2.5S_E$ polypeptides from the $6S_H$ and $5S_E$ subunits: one by tryptic digestion giving cleavage at both the lysyl and arginyl residues, a second by tryptic digestion following succinylation (thus giving cleavage only at arginyl residues), and a third by cleavage with AL-1 protease II of a Myxobacter, which Wingard et al.[81] have shown cleaves specifically at the amino

TABLE 5

Amino Acid Composition of $2.5S_H$ $2.5S_E$ Polypeptides of Transcarboxylase

Amino acid	$2.5S_H$ molar ratios	$2.5S_H$ residues per mole	$2.5S_E$ molar ratios	$2.5S_E$ residues per mole
Lysine	3.44	30	2.82	34
Histidine	1.00	9	1.00	12
Arginine	2.82	24	2.15	26
Aspartate	7.05	61	4.45	53
Threonine	3.15	27	2.52	30
Serine	2.85	25	2.03	24
Glutamate	8.28	71	6.15	73
Proline	3.13	27	2.76	33
Glycine	5.80	50	3.45	41
Alanine	7.15	62	4.67	56
Half cystine	0.77	7	0.40	5
Valine	4.87	42	3.30	40
Methionine	2.31	20	2.33	28
Isoleucine	3.15	27	1.55	19
Leucine	4.31	37	3.12	37
Tyrosine	1.23	11	1.30	16
Phenylalanine	2.49	22	1.33	16
Tryptophan	0.56	5	0.33	4
Total residues		557		547

side of lysyl residues. The peptide mapping was performed on thin-layer plates by electrophoresing in one direction and using solvent chromatography in the perpendicular direction. When cleavage was both at the lysyl and arginyl residues, the chromatography was repeated to obtain clean separation of the peptides. The peptide maps and the experimental conditions used in their determinations are given in Figure 33. The results show that within experimental error, the number of peptides obtained by all three procedures was in accord with the number of arginyl and lysyl residues (Table 5) of both $2.5S_H$ and $2.5S_E$ polypeptides. It is concluded:

1. The two $2.5S_H$ polypeptides that comprise the $6S_H$ subunit are identical.
2. The two $2.5S_E$ polypeptides that comprise the $5S_E$ subunit are identical.
3. There are no exactly duplicated peptides within each respective $2.5S_H$ or $2.5S_E$ polypeptide.

If either the $2.5S_H$ or $2.5S_E$ polypeptide had consisted of two types of polypeptide, more peptides would have been obtained than predicted.

Clearly, by this procedure, no evidence was obtained of a duplicating amino acid sequence in support of the proposal of Green et al.[9] However, these results do not necessarily exclude the possibility of a duplicating homology in the peptides. Although peptide mapping has been used successfully in several cases for demonstration of sequence homology (for example, transferrin,[82] leucyl-tRNA synthetase,[83] methionyl-tRNA synthetase,[84,85] and valyl-tRNA synthetase[84]), there also are examples of homologous sequences, which apparently were derived by gene duplication, in which the homology would not be detected by peptide mapping.

We have examined several proteins of known sequence which contain two or three extensive regions of homology and which are reported to have been derived by gene duplication.[70] The proteins examined were cytochrome C_3 of *Desulfovibrio vulgaris*, the γ heavy chain of immunoglobin of rabbit, the protease inhibitor from lima bean, the β chain of human hemoglobin, and the E chain of alcohol dehydrogenase (horse). If a hypothetical tryptic digest (cleavage at lysyl and arginyl residues) is applied to these proteins, the exact or almost exact number of peptides, based on the number of lysyl and arginyl residues, is obtained. Thus, if only the peptide maps were used, no evidence would be obtained of sequence homology. It is necessary in these cases to know the amino acid sequences to demonstrate the homology. Thus, even though none of the tryptic peptides of the $2.5S_H$ polypeptide of transcarboxylase has an exact residue for residue identity, there may be regions in this protein that do contain very similar sequences (hydrophobicity and charge type) which are responsible for binding the peripheral $6S_E$ subunits to both ends of the $2.5S_H$ subunit to form 26S transcarboxylase. This would be analogous to Rossmann's[86] finding that there is little sequence homology in the NAD-binding portions of several dehydrogenases even though their conformational structural domains are the same. Unfortunately, such regions of similar sequence cannot be determined by peptide mapping.

The problem with peptide mapping is that it can provide positive evidence of a duplicated gene only if it has not undergone extensive substitution by mutation. If the $2.5S_H$ polypeptide originally consisted of two large duplicated sequences, it would need a minimum of 27 substitutions in the appropriate lysyl and arginyl peptides to give the full number of tryptic peptides expected of a nonduplicated sequence. A more realistic figure for random substitutions is probably around 84 (15%). With this amount of substitution, enough of the original structure can still remain to retain its function.[70]

2. Experimental Facts to be Considered and the Formulation of Models of Transcarboxylase

The following facts have been established and should be considered in relation to the quaternary structure of transcarboxylase:

1. Electron microscopy shows that three $6S_E$ peripheral subunits are observed on one face of the central $12S_H$ subunit with the 18S form of the enzyme (Figure 1, Section II.A).
2. Electron microscopy of avidin complexes with the enzyme shows that avidin reacts with a biotinyl group at each end of the peripheral subunits (Figure 1, Section II.A).
3. Electron microscopy also shows that the 26S form of the enzyme has two sets of peripheral subunits, one on each face of the $12S_H$ subunit

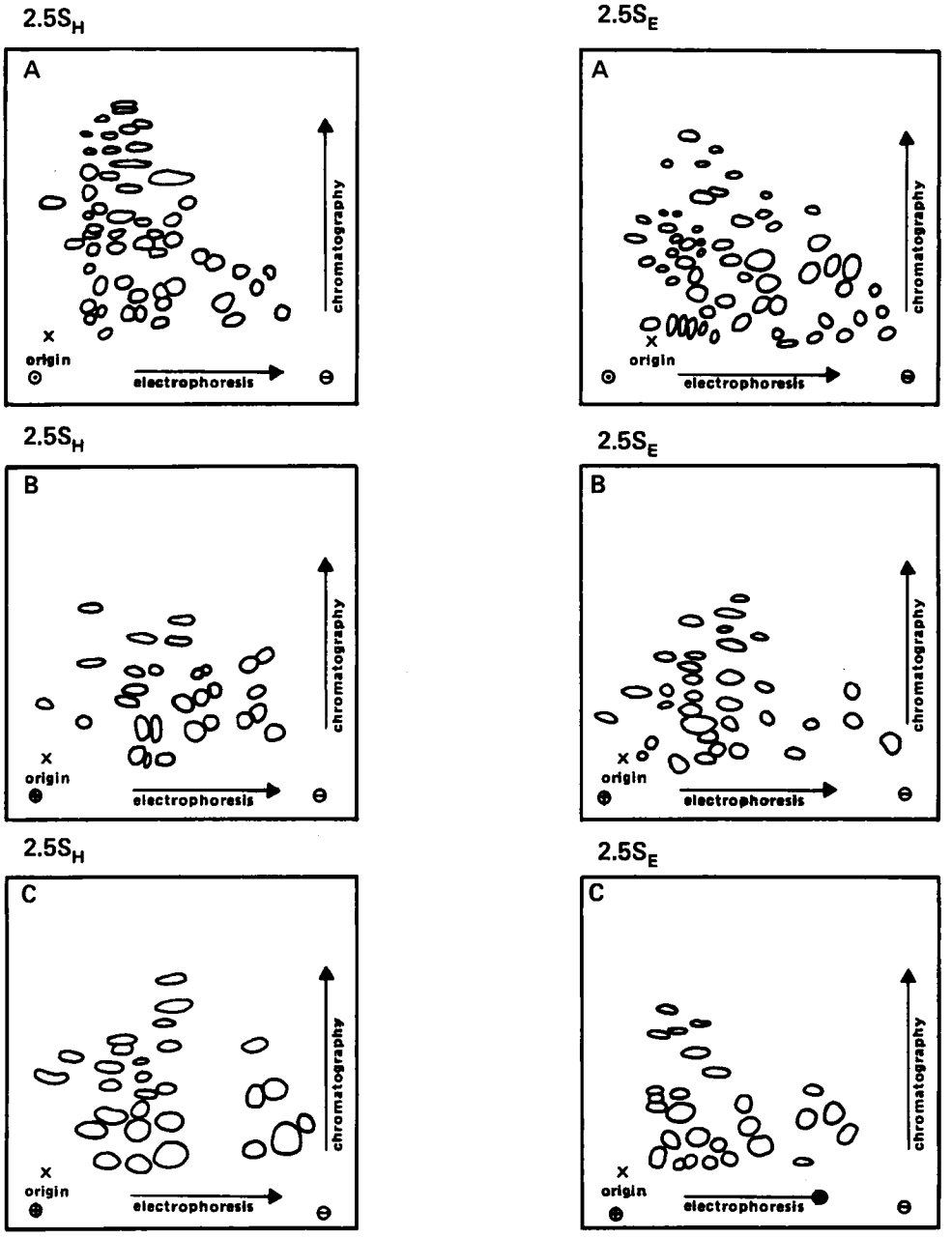


FIGURE 33. Peptide maps of the 2.5S$_H$ and 2.5S$_E$ polypeptides. Silica-gel thin-layer plates were used in mapping the digested polypeptides. Electrophoresis was in the horizontal direction and ascending solvent chromatography in the perpendicular direction. The electrophoresis buffer was 10% acetic acid titrated with pyridine to pH 3.6, and the solvent for the ascending chromatography was *n*-butanolacetic acid-water (3:1:1 v/v) pH 2.35. (A, 2.5S$_H$ polypeptide.) The sample was digested with trypsin, and electrophoresis was for 5½ hr. It was then subjected to a double chromatographic development. The peptide map indicated 55 lysyl and arginyl peptides. (B, 2.5S$_H$ polypeptide.) The sample was digested with AL-1 protease II of myxobacter. Electrophoresis was for 7 hr. It then was chromatographed in the perpendicular direction. The peptide map indicates 29 lysyl peptides. (C, 2.5S$_H$) The ϵ-amino groups of the lysyl residues were succinylated, digested with trypsin, and treated as in (B, 2.5S$_H$ polypeptide). Twenty-eight peptides were observed. (A, 2.5S$_E$ polypeptide.) The sample was digested with trypsin. The electrophoresis was for 5½ hr. It was then subjected to a double chromatographic development. The peptide map indicates 59 lysyl and arginyl peptides (B 2.5S$_E$ polypeptide). The sample was digested with Al-1-protease II of myxobacter. Electrophoresis was for 5½ hours and then chromatographed in a perpendicular direction. The peptide map indicates 34 lysyl peptides. (C, 2.5S$_E$ polypeptide.) The sample was succinylated and then digested with trypsin and treated as in (B, 2.5S$_E$ polypeptide). The peptide map indicates 27 arginyl peptides.

(Figure 20, Section VII A; Figure 22, Section VII.B).

4. A portion of the $1.3S_E$ biotinyl subunit serves as a bond between the $12S_H$ and $5S_E$ subunits (Figure 9, Section V.A), and since there are two $1.3S_E$ subunits per $6S_E$ peripheral subunit, there apparently are six bonding sites on each face of the $12S_H$ subunit (Section II.B.2).

5. The affinity of the $6S_E$ subunits at the two faces of the $12S_H$ subunit differs, since the 26S form of transcarboxylase dissociates readily to the stable 18S form (Section VII.C.3.b and 3.c).

6. The 26S form of the enzyme contains 12 biotinyl groups, all of which can be carboxylated (Section VII.C.2).

7. The CoA ester sites are on the $12S_H$ subunit and the keto acid sites are on the $5S_E$ subunits (Figure 12, Section VI.B). At present it is not known how many CoA ester sites there are per $12S_H$ subunit, only that all 12 biotinyl groups have access to substrate sites since they are carboxylated with methylmalonyl CoA (Section VII.C.2).

8. The $12S_H$ subunit dissociates to three $6S_H$ dimers, and the dimeric $5S_E$ subunit is stable (Figure 5, Sections III.B and C).

9. The six $2.5S_H$ polypeptides which make up the $12S_H$ subunit are identical, and there is no evidence by peptide mapping with trypsin or with Myxobacter AL-1 protease II that there is an exact residue for residue homology (Figure 33, Section VIII.B.1.b).

10. The two $2.5S_E$ polypeptides of the dimeric $5S_E$ subunit are identical, and by peptide mapping there is no evidence of residue for residue homology (Figure 33, Section VIII.B.1.b).

From these ten facts, three important criteria evolve which are the most significant in evaluation of the models we wish to consider.

1. The $12S_H$ hexamer and $5S_E$ dimers are stable, i.e., they do not aggregate, and these components as well as the $6S_H$ dimer can be isolated from transcarboxylase.

2. The $1.3S_E$ subunits interact with sites on both the $5S_E$ and $12S_H$ subunits, and the biotinyl groups are observed by electron microscopy at both ends of the $6S_E$ subunits.

3. There are six binding sites for the carboxyl carrier protein at each face of the $12S_H$ subunit (two per $6S_E$ subunit), but the affinity of the binding differs at the two faces.

3. Evaluation of the Models for Transcarboxylase

Four models are shown in Figure 34 which we will consider in relation to these criteria. None of the models fits all the criteria, and some rationalization is required in each case. The use of cylinders to represent the polypeptides is for the purpose of illustration of the concepts but, of course, does not imply anything about the actual shape of the structures.

In constructing the models, the following procedures have been adopted.

1. Since the $2.5S_H$ and $2.5S_E$ polypeptides are not identical in primary structure and there is not a residue for residue identity in either of the polypeptides as determined by the mapping of each subunit, an arbitrary assignment of top (+) and bottom (-) has been made to indicate a parallel or antiparallel association of subunits.

2. "Domains of bonding" of the subunit interactions are indicated by letters A, B, C for the polypeptides of the $12S_H$ subunit and a, b, c for the polypeptides of the $5S_E$ subunit. The binding sites for the $1.3S_E$ subunit on the $12S_H$ subunit are indicated by X and on the $5S_E$ subunit by X'.

3. The models of the $5S_E$ subunit have been constructed so that there is retention of symmetry between the $12S_H$ and $5S_E$ subunits, e.g., if $12S_H$ was isologous, parallel the $5S_E$ was given the same orientation. This requirement may not be essential, but it is esthetically more satisfactory. If the distance measurements shown in Figure 14 (Section VI.F.1) are correct, we shall see that the assumption becomes necessary.

The electron micrographs of transcarboxylase cannot be considered an accurate indication of distances, but they do appear to indicate a flexible structure with a variable distance between the $5S_E$ and $12S_H$ subunits.

The models as indicated in Figure 34 are described (1) by their type of binding (isologous, e.g., A,A binding or heterologous, e.g., A,B binding), (2) by the orientation of the polypeptides (parallel or antiparallel), and (3) by the symmetry of the $12S_H$ subunit (i.e., number of rotations within 360° which transpose components on themselves).[87] Models A and B of Figure 34 were constructed such that six $2.5S_H$ polypeptides of the $12S_H$ subunit form a hexagon. The hexagon geometry requires that each pair of "bonding sets" of the $2.5S_H$ polypeptide be related by an angle of 120°. An asymmetric distortion of pairs of sub-

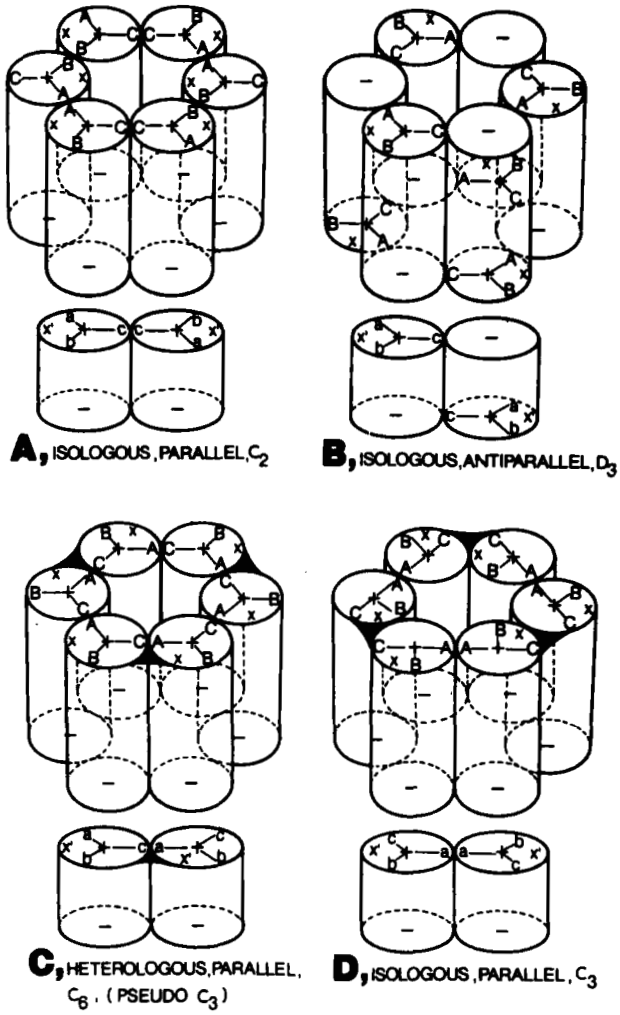


FIGURE 34. Schematic representation of four models for the $12S_H$ and $5S_E$ subunits of transcarboxylase. The carboxyl carrier proteins which bind the dimeric $5S_E$ subunit to the hexameric $12S_H$ subunit are not shown, but the sites for binding to the respective subunits are indicated by X′ and X. The arrangements of the constituent $2.5S_H$ subunits of the $12S_H$ subunit differ in each model as well as the bonding domains (A,A and A,C etc.). The + sign indicates a parallel arrangement and the – sign an antiparallel arrangement. The $5S_E$ subunits have been constructed so as to retain symmetry with the $12S_H$ subunit. The darkened areas indicate asymmetric distortion. A major distortion is indicated in Model D for the "binding set" C,C by the 180° rather than 120° d angle. None of the models fits all the facts unless some rationalization, such as distortion is made Model D seems to be the most suitable. See text for details.

units is assumed in Models C and D (described below). We will consider these models with respect to the three criteria listed above in the evaluation.

a. Isologous, Parallel Model with C_2 Symmetry

Model A is used to illustrate the effect of "open bonding sites" and why such models are not feasible.[8,7] This model has three sets of isologous bonding regions in the $12S_H$ subunit (A,A; B,B; and C,C); it is parallel as indicated by the + and – signs, and it has C_2 symmetry since it must be rotated 180° to transpose identical positions of the $12S_H$ subunit. It is seen that there are "open

bonding sites" A, B, and C on the $12S_H$ subunit. Therefore, the $12S_H$ subunits would combine with each other at these exposed domains and aggregate. Thus, this model does not fulfill the first criterion (i.e., stable $12S_H$ subunits).

It is noted that the $5S_E$ would have three types of bonding (c,c; b,b; and a,a) if it retained symmetry with the $12S_H$ subunit. Consequently, all types would have exposed bonding sites, and the $5S_E$ would not be stable. This model, therefore, does not meet the first criterion with respect to the $5S_E$ subunit.

b. Isologous, Antiparallel Model with D_3 Symmetry

Model B has two sets of isologous binding sets (A,A and C,C,), is antiparallel, and has dihedral symmetry[87] (D_3)-since there is a twofold axis of symmetry is perpendicular to the threefold axis of symmetry. This model satisfies the first criterion with respect to the $12S_H$ subunit (no A or C bonding areas are exposed, thus aggregation would not be expected). The $6S_H$ dimer would occur since the A,A bonding area may be stronger than the C,C bonding area or vice versa. The model does not meet the third criterion with respect to the $12S_H$ subunit, i.e., that six binding sites for peripheral subunits should be provided on each of the two faces of the $12S_H$ subunit and that they should differ. It is seen that both faces are identical, and only three binding sites are provided per face.

Furthermore, the $5S_E$ subunit would not meet the second criterion since the "bonding sets" (X') for the carboxyl carrier proteins are on opposite faces of the $5S_E$ subunit. Thus, only one set is oriented so that the $1.3S_E$ subunit could interact directly with both the $5S_E$ and $12S_H$ subunits.

This model could be brought into partial accord with the third criterion if there was negative cooperativity in the dissociation of the peripheral subunits, i.e., if the loss of one subunit from either face of the $12S_H$ subunit caused the preferential loss of other peripheral subunits from that same face. Even so, it would not account for six binding sites on each face of the $12S_H$ subunit, unless there is a duplicating homology in the sequence of the constituent polypeptides, as proposed by Green et al.[9] and discussed below. Model B is not considered attractive because of its failure to meet the second criterion.

c. Heterologous, Parallel Model with C_6 Symmetry (Pseudo-C_3 Symmetry)

Model C has heterologous bonding (C,A), a parallel arrangement of the polypeptides, and C_6 symmetry. This model is similar to that proposed by Green et al.,[9] except they did not discuss the symmetry of the $5S_E$ subunit. However, their complete model shown in Figure 3 (Section II.B.3) pictures the $1.3S_E$ subunits at each end of the $5S_E$ subunit. This arrangement implies that the $5S_E$ subunit has twofold symmetry, i.e., isologous bonding which is in contrast to the heterologous bonding in the $12S_H$ subunit.

Model C, as shown, fails to meet both the first and second criteria. The $5S_E$ subunit would not be stable because it has exposed binding sites a and c and therefore would aggregate. The $12S_H$ subunit would not give rise to $6S_H$ dimers because all the bonding is the same (C,A); therefore, it would be expected to dissociate to monomers.

In order to account for dimer formation from the $12S_H$ subunit, Green et al.[9] proposed that there is an asymmetric distortion following dimer formation which leads to difference in the bonding strength of the dimers and the bonds which are formed when the dimers are assembled into the $12S_H$ form. Thus, the $12S_H$ subunit would have pseudo-C_3 symmetry. We have indicated this difference in bonding sets by darkening an area to show that distortion occurred during formation of the dimer.* We have also indicated in Model C that there is distortion during dimer formation of the $5S_E$ subunit. Such distortion or some other rationalization would be necessary to prevent self-aggregation of the $5S_E$ subunit. In addition, Green et al.[9] proposed, as we have described earlier, that there may have been gene duplication

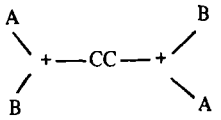
*Insulin was cited by Green et al.[9] as an example of this type of bond formation since it is a hexamer which dissociates to dimers. However, when the insulin hexamer and its dimers are examined closely, it becomes clear that the distortion of the monomers is not responsible for dimer formation. Dimer formation is a result of the isologous antiparallel interaction of the B chains of each monomer between residues number 23 through 28 (Adams et al[78]), which accounts for the formation of dimers as described by Monod et al.[88] and by Cornish-Bowden and Koshland.[89] Thus, insulin does not provide a suitable example of dimer formation as a result of mutual distortion. Although the formation of dimers from structures with heterologous binding as a result of distortion is possible, to our knowledge, there are no known examples.

and fusion with formation of a duplicating amino acid sequence in the $2.5S_H$ polypeptide (Figure 32, Section VIII.B). This rationalization is necessary to account for the binding of peripheral subunits on both faces of the $12S_H$ subunit. We have seen in Section VIII.B.1.b that no evidence was obtained by peptide mapping (Figure 33) for such a duplicating sequence, but this procedure does not exclude the possibility of a duplicating homology.

It is evident from the above discussion that at least three rationalizations are required for Model C.

1. Distortion during formation of the $6S_H$ dimer to provide for its stability.
2. A duplicating homology of the sequence of the $2.5S_H$ polypeptide to accommodate differential binding of the peripheral subunits on both faces of the $12S_H$ subunit.
3. A difference in the symmetry of the $12S_H$ and $5S_E$ subunits or distortion of the $5S_E$ subunit to remove its "open bonding sites" and thus eliminate self-aggregation.

The question of whether or not retention of symmetry between the $5S_E$ and $12S_H$ subunits is an inflexibile requirement rests heavily on the measurements by Fung et al.[14] It is seen in Model C, if it is assumed for purposes of illustration, that if the propionyl CoA (see Figure 14, Section VI.F.1) is oriented with the propionyl group towards the C,A bonding area and the adenine towards B, the propionyl CoA molecules would all have the same orientation on the $12S_H$ subunit.



On the other hand, if the $5S_E$ subunit were isologous, as in the illustration above, the Co^{++} of the $2.5S_E$ polypeptide was near C, and the methyl group of the pyruvate pointed toward B, then there would be two different orientations of the Co^{++} and pyruvate relative to the propionyl CoA. In such a situation, there would not be a unique set of distances and positions of the propionyl CoA relative to the Co^{++} of the $5S_E$ subunit as is shown in Figure 14. Thus, if the measurements of Fung et al.[14] (which indicate there is a unique orientation of the Co^{++} and the bound pyruvate of the $5S_E$ subunit relative to the propionyl CoA on the $12S_H$ subunit) are correct, retention of symmetry between the $5S_E$ and $12S_H$ subunits would be essential (see Section VI.F.5 for possible sources of error in the distance measurements). It is to be noted that over and beyond the change in distances by reversal of the position of the pyruvate per se relative to that of propionyl CoA, there could be that caused by the location of the pyruvate site on the polypeptide. If the sites are located centrally in the $2.5S_E$ polypeptides with respect to the CoA ester sites on the $12S_H$ subunit, the distances between the sites as a whole would remain the same with or without retention of symmetry. However, if the keto acid sites were at the ends of the $2.5S_E$ polypeptides (which are 45 Å long, $5S_E$ is 90 Å, Figure 3) and these sites were opposite the CoA ester sites, then rotation of one of the $2.5S_E$ polypeptides to a position which did not retain symmetry would alter the distance significantly over and beyond that caused by simple reversal of the relationship of the pyruvate per se relative to the propionyl CoA.

The flexibility of the carboxyl carrier protein might make it possible, however, to accommodate the differences in the distances. The biotinyl group which serves as the carboxyl carrier via the 1′N is on an extended peptide chain of which only a portion (the nonbiotinyl peptide) is involved in binding together the $5S_E$ and $12S_H$ subunits. Some 45 residues are between this portion and the biocytin (see Figure 30, Section VIII.A.4). Thus, there is ample opportunity for flexibility, and it is quite possible that there could be oscillation of the biotinyl ring, permitting orientation of the 1′N of the biotin so that it would adjust to the variability in orientation of the substrates. In spite of this possibility, it is clear that a model which provides for retention of symmetry between the $5S_E$ and $12S_H$ subunits is the most attractive.

d. Isologous, Parallel Model with C_3 Symmetry

A model which has isologous, parallel bonding cannot be formulated using cylinders which yield a hexagon unless there is considerable asymmetric distortion of the constituent polypeptides. There are examples of polymeric enzymes which depart from strict symmetry, including chymotrypsin[90,91] and hexokinase.[92,93] For example, there are two monomeric proteins in chymo-

trypsin with identical sequences in which distortion occurs over a very sizable portion of each former monomer.[90,91] Likewise, in hexokinase there are two monomers with identical sequences, and in the enzyme they are related by a 160° axis of rotation and a screw-translation of 13 Å along the rotation axis.[92,93] Thus, there is precedent for assuming that distortion could occur.

In Model D we have formulated isologous bonding (A,A and C,C), but the C,C bondings involve distortion which, for purposes of illustration, is indicated by the 180° angle between the A and C bonding sites instead of the 120° angle; in addition, the asymmetric bonding is indicated by the shaded areas between the C,C bonds. The arrangement of the $2.5S_H$ subunits is parallel, and the $12S_H$ subunit has C_3 symmetry.* Model D meets criterion 1, since the $12S_H$ and $5S_E$ subunits do not have any "open bonding sets" and, therefore, would be stable. Dissociation of the $12S_H$ subunit to $6S_H$ dimers is accounted for since the A,A bonding strength could be stronger than the C,C bonding strength. Criterion 2 is met since the X,X′ sites are oriented so that $1.3S_E$ carboxyl carrier proteins could combine with both the $12S_H$ and $5S_E$ subunits. The third criterion, i.e., bonding of the peripheral subunits on both faces of the $12S_H$ subunit with a difference in the dissociative behavior of the subunits from two faces, is not accounted for by the model, but could be accounted for if there is duplication in the homology or conformation of the $2.5S_H$ polypeptide to provide for the second set of binding sites on the $12S_H$ subunit.

Model D is attractive because the $12S_H$ and $5S_E$ subunits have the same symmetry, i.e., isologous bonding. Therefore, the orientation of the propionyl CoA on the $12S_H$ subunit would be the same in all cases, relative to the Co^{++} and pyruvate of the $5S_E$ subunit. Of the four models presented and others we have considered, Model D appears to be the most suitable.

4. *Number of Substrate Binding Sites*

The number of binding sites for either the keto acids or CoA esters has not been accurately determined. It has usually been assumed that there are two keto acid sites per $5S_E$ subunit. This view was based on the assumption that the metals are part of the keto acid site and that there are two metals per $5S_E$ subunit. However, with the finding that there may be 12 metals (Section II.B.4), this argument may have lost some of its weight (see Section II.B.4). However, in the absence of evidence to the contrary, it is reasonable to assume that there are two keto acid sites per $5S_E$ subunit or six per 18S form of the enzyme, which is equivalent to the number of biotins. This relationship would provide one carboxyl carrier per keto acid site.

The situation with the CoA esters is much more complicated. Fung et al.[39] attempted to determine the number of sites for propionyl CoA using the 18S form of the enzyme. They found three to six sites (Section VI.F.4), but the measurement, as indicated, was not very reliable. There are 12 biotins and six peripheral subunits in the 26S form of transcarboxylase, and all 12 biotins can be carboxylated using [3-^{14}C] methylmalonyl CoA (Section VII.C.2). These results show that all 12 biotins of the carboxyl carrier proteins, six on the one face of the $12S_H$ subunit and six on the opposite face, have access to methylmalonyl CoA sites. The following location and number of binding sites appear to be possibilities.

1. Twelve binding sites, six near the upper face and six near the lower face of the $12S_H$ subunit (two per $2.5S_H$ polypeptide).
2. Six binding sites, three near the upper face and three near the lower face of the $12S_H$ subunit (one per $2.5S_H$ polypeptide).
3. Six binding sites located midway between the upper and lower faces of the $12S_H$ subunit (one binding site per $2.5S_H$ polypeptide).

The electron micrographs of the isolated $12S_H$ subunit (Figure 1E, Section II.A) show a stain-penetrating cleft in the edgewise view of the $12S_H$ subunit (see also the models in Figure 3). This fact suggests there is a bilobal character in the $2.5S_H$ polypeptide, and Green et al.[9] have suggested that these lobes might result from two similar but not identical domains of the tertiary structure. The possibility of two separate domains for binding $6S_E$ subunits and for separate CoA ester sites is

*A pseudoisologous parallel arrangement of the $2.5S_H$ polypeptides analogous to the B 11 dimers of hexokinase[92,93] is also compatible with Model D. In this case, the "binding sets" of the two monomers are nonequivalent and the interaction is heterologous. Because of the specific angles of the interaction, "open binding sets" are not available and infinite polymerization would not be observed. The opposite sides of the two monomers which are not involved in the dimer interaction are distorted and somewhat nonequivalent in quaternary structure.

attractive. The peptide mapping provided no support for this view (Section VIII.B.1.b), but these experiments do not exclude this possibility. If 12 CoA ester sites are demonstrated, it would provide an argument in favor of the duplicating homology in the $2.5S_H$ polypeptide and separate domains of tertiary structure. If, on the other hand, six binding sites are found, three at the upper face and three at the lower face of the $12S_H$ subunit, it would indicate that the 12 biotinyl groups are competing for substrate sites.

The possibility that there are six CoA esters located midway between the two faces of the $12S_H$ subunit to which the carboxyl carrier has access from both faces is difficult to evaluate. Viewed in terms of the model shown in Figure 3 (Section II.B.1), it would appear that the $5S_E$ subunit would be quite far from the CoA ester sites, if the sites were located midway between the two faces of the $12S_H$ subunit. Such an extended distance would definitely not be in accord with the reported measurement of Fung et al.[39] as shown in Figure 14 (Section VI.F.1). However, the peripheral subunits have a flexible linkage to the $12S_H$ subunit, and it is quite possible that the electron micrographs do not give a true picture of the location of the $6S_E$ subunits relative to the $12S_H$ subunit and that the peripheral subunits may not be at the extreme ends of the faces of the $12S_H$ subunit. Therefore, it remains possible that the midway location of the CoA ester site is feasible.

Our efforts to measure the number of sites for the CoA esters have encountered difficulties. Transcarboxylase catalyzes a slow deacylation of propionyl CoA which makes measurements with this compound difficult. Methylmalonyl CoA is not deacylated, but there is transfer of the [C-3] carboxyl to the biotinyl groups of the enzyme, thus producing propionyl CoA. The carboxybiotin spontaneously decarboxylates, which adds to the problem. Efforts to make measurements with the isolated $12S_H$ subunit have not been successful. Perhaps the CoA esters are not bound tightly in the absence of the peripheral subunits. Other means, such as photoaffinity labeling, are being explored as a means of solving the problem.

IX. IMMUNOCHEMISTRY OF TRANSCARBOXYLASE

It is apparent that the $12S_H$ and $5S_E$ subunits of transcarboxylase have many features in common. They both combine with a portion of the $1.3S_E$ carboxyl carrier protein and catalyze the reversible carboxylation of the biotin (Section VI.B), they both combine with the nonbiotinyl portion of the $1.3S_E$ subunit (Section V.A), and they both are composed of polypeptides of molecular weight 60,000 which associate to form dimers. Thus, it seemed possible that there might be structural homology between these two types of subunits. Berger and Wood[101] investigated this possibility using antisera prepared by injecting the isolated $12S_H$ and $5S_E$ subunits into rabbits. Both types of antisera were found to be capable of inhibiting and precipitating intact transcarboxylase. In competitive binding experiments utilizing enzyme inhibition to detect antibody binding, it was found that only the $5S_E$ subunit was capable of preventing the anti-$5S_E$ sera from inhibiting transcarboxylase. In contrast, both the $5S_E$ and $12S_H$ subunits were capable of preventing the anti-$12S_H$ sera from inhibiting the enzyme, and they were equally effective on a weight basis.

Experiments were also done to test the antisera for their capacity to inhibit the two partial reactions of transcarboxylase, each of which is catalyzed specifically by the subunits (Section VI.B). It was found that the anti-$12S_H$ serum, but not the anti-$5S_E$ serum, inhibited the CoA ester partial reaction, which is catalyzed by the $12S_H$ subunit. Pretreatment of the anti-$12S_H$ serum with the $5S_E$ subunit did not interfere with this inhibition. This result confirmed formation of specific antibody against the $12S_H$ subunit. Similar studies demonstrated that not only the anti-$5S_E$ serum inhibited the keto acid partial reaction which is catalyzed specifically by the $5S_E$ subunit, but also the anti-$12S_H$ antiserum, the latter apparently demonstrating cross reactivity between the $12S_H$ and $5S_E$ subunits.

The above observations led to the conclusion that the inhibition of the overall enzymatic reaction by the anti-$12S_H$ serum is due to the presence of a subpopulation of antibodies that react with determinants common to both subunits; under the assay conditions using the intact enzyme, however, only the determinants on the $5S_E$ subunits are accessible to the antibodies. This postulate accounts for the fact that the $5S_E$ subunit neutralizes the anti-$12S_H$ serum for inhibition of the overall reaction but not for inhibition of the partial reaction catalyzed by the $12S_H$ subunit. It

is considered that the equal activity of the $5S_E$ and $12S_H$ subunits in relieving the inhibition of the overall reaction by the anti-$12S_H$ serum indicates that both subunits react equally well with the restricted subpopulation of antibodies being detected by the inhibition of the enzyme. With the anti-$5S_E$ serum it proposed that only those antibodies that bind to unique determinants on the $5S_E$ subunit have sufficiently high affinity to react with and inhibit transcarboxylase under the conditions of this assay; therefore, the $12S_H$ subunit is ineffective in relief of enzyme inhibition by the anti-$5S_E$ serum.

Using the technique of passive hemagglutination-hemagglutination inhibition, it was found that the anti-$5S_E$ serum or anti-$12S_H$ serum agglutinated red blood cells coated with intact transcarboxylase, the $5S_E$ subunit, or the $12S_H$ subunit. The agglutination of each of these types of cells was inhibited by treatment of either serum with either $5S_E$ or $12S_H$ subunits. These results confirm the cross reactivity of the anti-$12S_H$ serum and also indicate that there are antibodies in the anti-$5S_E$ serum that bind to determinants present in both the $12S_H$ and $5S_E$ subunits.

The possibility of cross contamination of the $12S_H$ subunit by the $5S_E$ subunit was investigated using ^{60}Co-labeled $5S_E$ subunit which had been isolated from transcarboxylase purified from propionibacteria grown on media containing the radioactive isotope.[13] Both the anti-$5S_E$ and anti-$12S_H$ sera were capable of precipitating the ^{60}Co-$5S_E$ subunit, and pretreatment of either antiserum with unlabeled $5S_E$ subunit or $12S_H$ subunit neutralized the ability of the antiserum to precipitate the ^{60}Co-$5S_E$ subunit. Comparison of the amount of each subunit necessary to reduce the precipitation of ^{60}Co-$5S_E$ subunit by 50% showed that 25 to 35 times more $12S_H$ subunit was required than $5S_E$ subunit. Since polyacrylamide gel electrophoresis indicated that the contamination of the $12S_H$ preparation with the $5S_E$ subunit was less than 0.5%, it would be expected that at least 200 times more $12S_H$ subunit than $5S_E$ subunit would be necessary to block the precipitation. The observation that the $12S_H$ was effective at lower concentrations is considered to be due to its cross reactivity with antibodies to the $5S_E$ subunit.

Although the subunits were apparently cross contaminated to a minor extent, the contamination was considered insufficient to account for the cross reactivity observed by the precipitation and hemagglutination techniques or to account for the equal effectiveness of the subunits in preventing the antisera from reacting with the intact enzyme. Therefore, it is concluded from the immunological results[101] that the $12S_H$ and $5S_E$ subunits of transcarboxylase contain regions of structural homology leading to cross reactivity of the respective antibodies. It is of interest that in the tryptic peptide mapping, as described in Section VIII.B.1.b, three peptides from the $2.5S_E$ and $2.5S_H$ polypeptides were found to have identical electrophoretic and solvent chromatographic mobilities (i.e., superimposable spots).[20] Since the average length of these peptides was 10 residues, this result is strongly suggestive that there may be at least 30 residues of identical sequence.

Antisera reactive with the $1.3S_E$ biotin carboxyl carrier protein of transcarboxylase were also prepared by immunizing rabbits with the 46- and 65-residue biotinyl peptides described in Sections IV.A and VIII.A.2. To increase the immunogenicity of these small peptides, they were complexed with avidin, and this noncovalent conjugate was used as the immunogen. The resulting antiserum, likewise, inhibited and precipitated intact transcarboxylase. Proof was obtained that the anti-biotinyl peptide serum combined with the peptide chain of the $1.3S_E$ subunit per se by use of Sephadex G-100 to separate the products resulting from combination of the antibody with $1.3S_E$ subunit labeled with [^{3}H] biotin. Two radioactive peaks were observed, one representing the antibody-$1.3S_E$ subunit complex and the other the uncomplexed $1.3S_E$ subunit. Dialysis of the antiserum against 4 m*M* biotin prior to combination with the $1.3S_E$ subunit caused no reduction in the binding of the $1.3S_E$ subunit, indicating that antibodies to the biotinyl group per se had not been formed. When similar studies were carried out using a 10,267 mol wt fragment of the biotin carboxyl carrier protein of acetyl-CoA carboxylase from *E. coli*,[16] the antiserum to the peptides from transcarboxylase failed to bind the *E. coli* protein. This lack of cross reactivity is rather surprising in view of the similar sequences shared by these two proteins (Figure 31). However, further studies by more sensitive methods may show cross reactivity.

Interesting results were also obtained when transcarboxylase was incubated with antibodies reactive with (+)-biotin itself. These antibodies

were produced following the immunization of rabbits with bovine serum albumin to which biotin had been covalently linked.[102] The antibodies inhibited transcarboxylase but failed to precipitate the enzyme even when incubated for long periods of time and at high enzyme (biotin-) concentrations. It was demonstrated, when ^{3}H-biotin or ^{60}Co-labeled transcarboxylase was incubated with the antiserum and the resulting mixtures were analyzed by glycerol density gradient centrifugation or chromatography on Bio-Gel A 1.5 *m*, that radioactive complexes were formed with apparent molecular weights higher than transcarboxylase, but with markedly reduced enzymatic activity. Preliminary studies of these complexes[103] by electron microscopy have revealed some profiles of IgG molecules bound to two $6S_E$ subunits of the same transcarboxylase molecule (much as does avidin, Figure 1C). Thus, it seems possible that the lack of precipitation of transcarboxylase by this antiserum may be due to saturation of many of the IgG molecules by binding to two biotinyl groups of a single transcarboxylase molecule. Such complexes might leave insufficient IgG molecules to form the multiple intertranscarboxylase bridges which are required for formation of the large insoluble complexes of a precipitate.

X. CONCLUDING REMARKS

The developments since the previous review[6] of transcarboxylase in 1972 have added much to our knowledge of this interesting enzyme. The predictions which were made at that time concerning the quaternary structure of transcarboxylase, based on electron micrographs and subunit composition, have been put on a much firmer basis. It was proposed that the biotinyl carboxyl carrier protein serves to bind the large central subunit to the peripheral subunits which contain the Co^{++} and Zn^{++}; flexibility of this polypeptide chain allowed the enzyme to assume the various profiles seen by electron microscopy. The carboxyl carrier protein has now almost been completely sequenced, and the portion of this polypeptide which is involved in the linking of the central and peripheral subunits has been identified (Section V.A).

It was known that transcarboxylation occurs by two partial reactions, and the kinetic studies had indicated that these partial reactions might occur on separate subunits. This prediction has been confirmed; the partial reaction involving the CoA esters is catalyzed by the central subunit and that involving the keto acids is catalyzed by the peripheral subunit (Section VI.B). Thus, the carboxyl transfer is between the central and peripheral subunits. The amino acid sequence of biotinyl peptides which function as carboxyl carriers in the partial reactions has been identified. Biotin or biocytin is ineffective, but the minimum amino acid sequence required for activity in the partial reactions is yet to be determined (Section VI.C).

The biotinyl peptides are only weakly effective in promoting the overall reaction when added to a mixture of $5S_E$ and $12S_H$ subunits or even to trypsinized transcarboxylase in which the $5S_E$ and $12S_H$ subunits still remain attached to each other via the nonbiotinyl peptide portion of the carboxyl carrier protein. Apparently, the nonbiotinyl and biotinyl peptides must be covalently linked, as in the intact carboxyl carrier protein, to promote rapid intersubunit carboxyl transfer between the substrate sites on the $5S_E$ and $12S_H$ subunits (Section VI.D).

The discovery of a 26S form of the enzyme with a molecular weight of 1,200,000 has opened a whole new vista for transcarboxylase. The reversible association-dissociation of the different forms of transcarboxylase, which arise from the 26S form during its conversion to the more stable 18S form of the enzyme, involves loss of three peripheral subunits from one face of the central subunit (Figure 35). The equilibria of the different steps of this association-dissociation are influenced by pH, concentration of the divalent anions, and the concentration of the 18S enzyme and the peripheral subunits (Section VII.C.3.c). The type of transcarboxylase that will exist in the cell evidently depends on these factors and others, and this may play an important part in the regulation of transcarboxylase and metabolism of the propionic acid bacteria.

Many questions arise concerning the quaternary structure of transcarboxylase. What is the structure of the central subunit (Figure 35) which permits binding of a set of three peripheral subunits on opposite faces of the hexameric $12S_H$ subunit by 12 biotinyl $1.3S_E$ subunits? Is there a repeating homology in the sequence of the six $2.5S_H$ polypeptides which provides for such binding, and is the homology sufficiently different so that it causes the dissociation to differ on the two faces? Are there 6 or 12 CoA ester sites on the $12S_H$ subunit which catalyze the carboxylation of

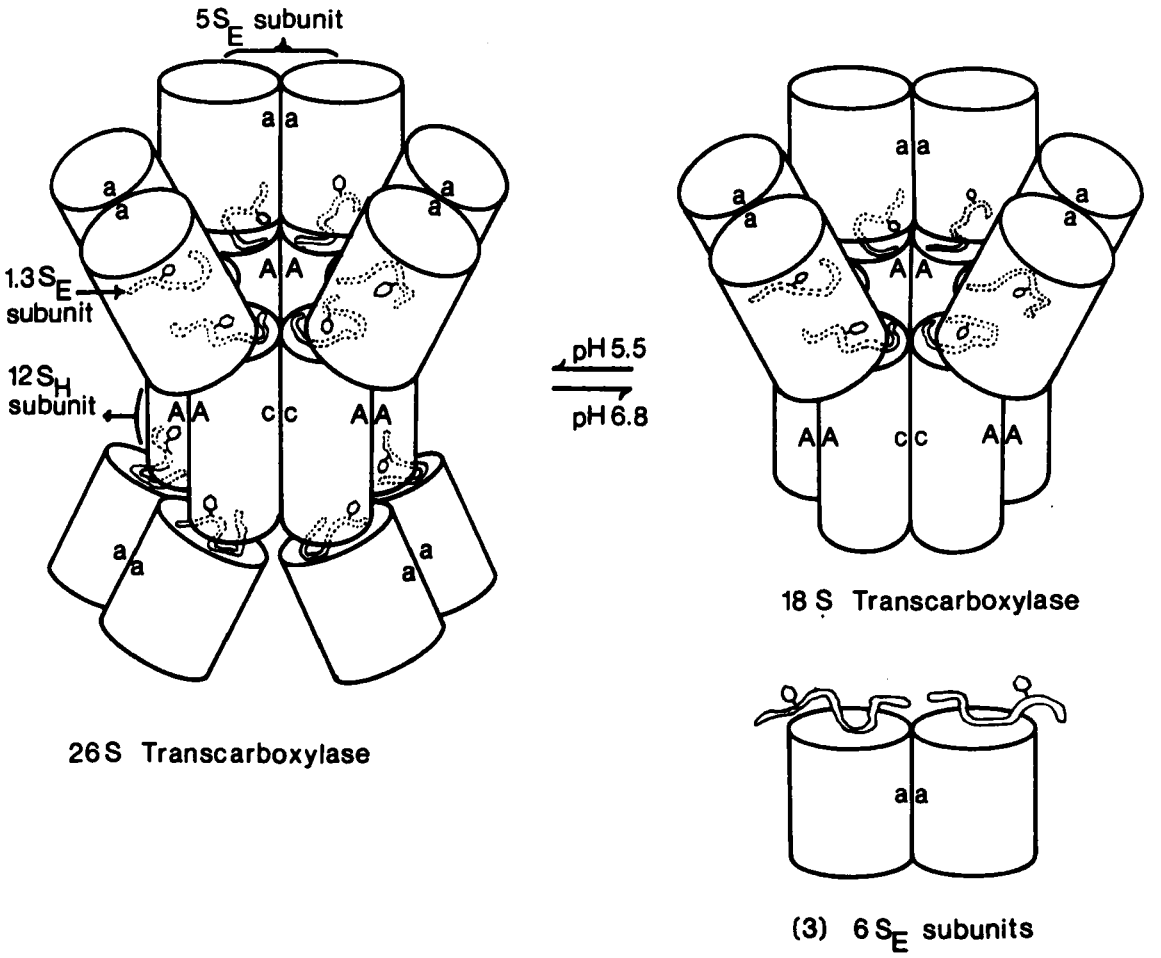


FIGURE 35. A schematic model, corresponding to D of Figure 35, representing the 26S form of transcarboxylase and its dissociation to the 18S form of transcarboxylase and three $6S_E$ subunits. The $1.3S_E$ biotinyl carboxyl carrier proteins are represented by the coiled tubes with hexagons; the hexagons represent the biotin ring of the biotin. One portion of each $1.3S_E$ subunit is attached to the $12S_H$ subunit and another to the $5S_E$ subunit. A,A and C,C indicate different types of binding of the $2.5S_H$ polypeptides, and a,a indicates binding of the $2.5S_E$ polypeptides.

the 12 biotins of the 26S form of transcarboxylase? If there are 12, is this provided for by a repeating sequence homology in the $2.5S_H$ polypeptide? In the transcarboxylation, the carboxyl is transferred by a biotinyl peptide binding alternately near a CoA ester site on the $12S_H$ subunit and a keto acid site on the $5S_E$ subunit. Are the amino acid sequences similar at the binding sites on the $12S_H$ and $5S_E$ subunits? Why should such a complicated enzyme be required to catalyze such a simple reaction? At this moment, more questions are opened than answered (Section VIII.B.3).

The discovery by Rose and colleagues that there is hydrogen transport from one substrate to another which accompanies the carboxyl transfer is intriguing. The enol form of biotin appears to be the best candidate for this transfer. The demonstration by Walsh and co-workers that both partial reactions occur with retention of configuration, likewise, helps define the chemical mechanism (Section VI.G).

The measurements of distances by Mildvan and colleagues between the Co^{++}, pyruvate, and propionyl CoA when bound to transcarboxylase are very interesting. If these measurements are accurate, they are an important step in establishing the quaternary structure of transcarboxylase. These measurements are of the distances between the central subunit and peripheral subunits; if the present values are correct, they indicate that there must be symmetry between the central subunit and the peripheral subunits (Section VIII.B.3.c).

The use of antisera against the $12S_H$ central subunit and the $5S_E$ peripheral subunit has revealed cross reactivity of the antisera. These results point to the existence of homology between the two different types of subunits (Section IX). It would be very interesting to identify the sequences of the central and peripheral subunits which are responsible for this cross reactivity. Since the biotinyl peptide binds alternately at the $12S_H$ central subunit and the peripheral $5S_E$ subunit during the transcarboxylation, it is quite possible that these binding sites are the determinants which lead to cross reactivity of the antisera.

Anti-biotinyl peptide sera, likewise, have been produced, and they precipitate and inhibit transcarboxylase. Surprisingly, they did not bind a biotinyl portion of the carboxyl carrier protein of acetyl-CoA carboxylsase of *E. coli*, even though the two carboxyl carrier proteins do show close homology in the amino acid sequence surrounding the biotin (Section VIII.A.4).

Clearly, much must be done before a more complete understanding is attained of the structure of this enzyme, its mechanism of catalysis, and particularly the mechanism of its regulation in the metabolism by the propionic acid bacteria.

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OBJECTIVES

In the last quarter century Biochemistry has made significant contributions to the advancement of a wide range of disciplines, medicine, molecular biology, genetics, immunology, developmental biology, biophysics, etc. Investigators in these disciplines increasingly require an appreciation of the significance of current biochemical advances. Likewise, biochemists seeking understanding of advances in areas remote from their own specialties are confronted with the same need.

Reviews that organize, evaluate, and present the current status of a particular biochemical area, at a desirable level of detail, in understandable language, are an effective means of meeting these needs – hence, *CRC Critical Reviews in Biochemistry*. The Journal, which will appear quarterly, will include in each issue three or more critical surveys of specific biochemical topics of current interest. Topics will be selected on the advice of an Advisory Board of outstanding biochemists who will also suggest authors of special competence. Topics will be chosen broadly enough to interest most readers, narrow enough to be within the competence of a single author. Authors will be chosen for their perceptiveness, imagination, and ability to communicate. Unsolicited manuscripts will not be considered but the Editor will welcome suggestions of topics and of qualified authors.

A truly critical review is an outstanding device for the integration and meaningful compression of a large, often contradictory, literature. It also provides its author an unparalleled opportunity for creative scholarship by synthesizing out of known facts fruitful hypotheses and new concepts. It is to these roles of the critical review and to a better understanding of the trends of modern biochemistry that this publication is dedicated.

GDF