Isolation of the Subunits of Transcarboxylase and Reconstitution of the Active Enzyme from the Subunits

(Received for publication, June 24, 1974)

HARLAND G. WOOD, FAZAL AHMAD,‡ BIRGIT JACOBSON, MARGARET CHUANG, AND WILLIAM BRATTIN§

From the Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106

SUMMARY

The separation of the 12 SH central subunit, the 5 SE peripheral metallo subunit, and the 1.3 SE biotinyl carboxyl carrier protein which are formed on the dissociation of transcarboxylase has been accomplished by molecular sieving on Bio-Gel. The 12 SH and 5 SE subunits have been obtained in nearly homogeneous form as judged by the sedimentation velocity profiles and by acrylamide gel electrophoresis. The 1.3 SE carboxyl carrier protein has given less consistent results; sometimes a single band of molecular weight of approximately 12,000 is obtained on gel electrophoresis in sodium dodecyl sulfate but sometimes there is an additional band of lower molecular weight of approximately 11,000. This lower molecular weight component may result from limited proteolytic degradation in spite of efforts to prevent it. Two or more bands are obtained in the absence of dodecyl sulfate. This heterogeneity may result from aggregation.

Active enzyme is readily reconstituted from the isolated subunits. The most effective reconstitution is accomplished by a two-step process. First, the 1.3 SE carboxyl carrier protein and 5 SE metallo subunit are combined to form the 6 SE complex; then this product is combined with the 12 SH subunit to yield active enzyme. With a limiting amount of the 6 SE complex and an excess of the 12 SH subunit the resulting enzymatic activity is proportional to the concentration of 6 SE complex. Likewise, with an excess of the 6 SE complex and the 12 SH subunit limiting, the enzymatic activity is proportional to the concentration of the 12 SH subunit. The maximum specific activities of the reconstituted 6 SE complex and the 12 SH subunit were approximately 60% and 50%, respectively of the value estimated for their specific activities in the native 18 S form of the enzyme with three peripheral 6 SE subunits. Because the assay of the 6 SE complex is done using an excess of the 12 SH subunit, this may yield enzyme with a single peripheral subunit. The 6 SE subunit may be less effective in this form. In the case of the 12 SH subunit, the activity is most likely low because the reconstituted 6 SE complex does not contain the full complement of 1.3 SE biotinyl carboxyl carrier proteins.

The carboxyl carrier protein provides the groups which link together the 5 SE peripheral subunits with the central subunit. Evidence is presented that the 1.3 SE biotinyl carboxyl carrier must first combine with the 5 SE subunit to assume a form which effectively provides the combining groups for association with the 12 SH subunit.

Transcarboxylase (methylmalonyl-CoA pyruvate carboxyltransferase (EC 2.1.3.1) has thus far been found only in propionic acid bacteria. It catalyzes the following reaction:

\[ \text{CH}_3\text{CH}(\text{COO}^-)\text{COSCoA} + \text{CH}_2\text{COCOO}^- \rightarrow \]

\[ \text{CH}_3\text{CH}_2\text{COSCoA} + \text{"OOCCH}_2\text{COCO}^-" \]

Transcarboxylase dissociates to subunits which can be reconstituted forming an active enzyme as diagrammed in Fig. 1. The accumulated evidence (1-8) including electron microscopy (1) shows that three peripheral subunits (designated 6 SE) are attached loosely to one face of a cylindrical shaped central subunit (designated 12 SH). The enzyme has a molecular weight of 790,000 and \( \delta_{w} = 18 \) S and dissociates at pH 8 and low ionic strength with sequential loss of the peripheral 6 SE subunits (1, 6). The isolated enzyme frequently contains a mixture of the 18 S form with three peripheral subunits together with a 16 S form of the enzyme with two peripheral subunits (1, 6).

The 6 SE subunit of molecular weight 144,000 dissociates slowly at pH 8 and rapidly at pH 9 to a dimeric metallo subunit of molecular weight 120,000 (designated 5 SE) (1, 6) and to two biotinyl subunits of molecular weight of approximately 12,000 (designated 1.3 SE) (2, 3). In the presence of denaturing agents, the dimeric 5 SE subunit dissociates to its constituent peptides (designated 2.5 SE) (1). The 12 SH subunit likewise dissociates...
zyme can be made from either the 16 S or 18 S form of the enzyme. Recently, Fung et al. (9) have reported that the 12 Sn subunit in 0.25 M acetate at pH 5.0 to 5.2 or in 0.75 M phosphate at pH 6.5 to 6.8 to form active enzyme (1). The present work has been to isolate both the 12 Sn and 6 Sn subunits without their further dissociation to the 6 Sn, 5 Sn, and 1.3 Sn subunits. But unlike many enzymes, the subunits of transcarboxylase readily reassociate; thus, under conditions which stabilize these subunits (e.g., at neutral pH in the presence of polyvalent ions), recombination to the intact enzyme occurs. Only when the 1.3 Sn subunit is removed from the dissociated mixture (17) is it possible to isolate the 12 Sn and 5 Sn subunits in substantially quantitative yields from the native enzyme. The 12 Sn and 5 Sn subunits can be stabilized under these conditions and no longer combine because the 1.3 Sn subunit is required for their combination (8). An example is shown in Fig. 2 of the events encountered when an attempt is made to isolate the 12 Sn and 6 Sn subunits per se from dissociated transcarboxylase. The original enzyme consisted of a combination of the 18 S and 16 S forms of the enzyme (Fig. 2A) i.e., enzyme with three and two peripheral subunits attached (1). The enzyme (480 mg, specific activity 32) was dissociated by dialysis for 23 hours against 6 liters of 0.05 M Tris-HCl (pH 8.0) in 20% glycerol containing 10⁻⁴ M phenylmethylsulfonylfluoride. It then had a specific activity of 1.5 and Fig. 2B shows that it consisted of some ~16 S material and ~12 S material but the major peak was ~6 S material. This dissociated enzyme was placed on a Bio-Gel A-1.5m column (4.5 x 172 cm) which had been equilibrated with 0.05 M phosphate buffer (pH 7.0) and was eluted with the same buffer over a period of about 40 hours at 4°C. Three protein peaks which were not completely resolved were obtained and were separately compared favorably with the spectrophotometric results within 10% of those obtained by the spectrophotometric method of Warburg and Christian (described in Ref. 13). We have compared the spectrophotometric method with other methods frequently used for quantitative determination of proteins: (a) biuret reaction, (b) reaction of the hydrolyzed protein with ninhydrin, and (c) dry weight of the purified enzyme. All of the methods were found to compare favorably (±10%) with the spectrophotometric method. Furthermore, by amino acid analysis on a known amount of the dried enzyme, the total residues accounted for about 95% of the dry weight.

**RESULTS**

**Isolation of Subunits**

Isolation of Subunits from Native Transcarboxylase—Our goal has been to isolate both the 12 Sn and 6 Sn subunits without their further dissociation to the 6 Sn, 5 Sn, and 1.3 Sn subunits. Protein was determined by absorbance at 280 and 260 nm (13). 1 It was reported previously (14) that protein determinations of transcarboxylase by reductive indices gave results within 10% of that obtained by the spectrophotometric method of Warburg and Christian (described in Ref. 13). We have compared the spectrophotometric method with other methods frequently used for quantitative determination of proteins: (a) biuret reaction, (b) reaction of the hydrolyzed protein with ninhydrin, and (c) dry weight of the purified enzyme. All of the methods were found to compare favorably (±10%) with the spectrophotometric method. Furthermore, by amino acid analysis on a known amount of the dried enzyme, the total residues accounted for about 95% of the dry weight. 2

1 We have previously reported that transcarboxylase contains 7 to 8 g atoms of cobalt plus zinc per mol of transcarboxylase of molecular weight 790,000 and provisionally concluded that there are 6 g atoms per mol. Recently, Fung et al. (9) have reported that transcarboxylase contains copper as well as cobalt and zinc and that there are 12 g atoms of metal per mol.

2 Recent evidence (10) indicates that the 24 S form of the enzyme can be made from either the 18 S or 16 S form of the enzyme by treatment with an excess 6 Sn subunit at pH 5.2.
pooled. The protein of the first peak (144 mg) had a sedimentation coefficient of ~17 S and a specific enzymatic activity of ~30 (Fig. 2C). The second peak (110 mg) included considerable overlap from the first and third protein peaks and contained ~18 S, ~16 S, ~12 S, and ~6 S material (Fig. 2D, top). The third protein peak (192 mg) gave a nearly symmetrical peak on sedimentation with an $s_{20,w}$ of 5.8 S (Fig. 2D, bottom). Polyacrylamide gel electrophoresis of this latter protein in 8 M urea gave only one band. Because the 2.5 S$_n$ and 2.5 S$_c$ peptides are separated by this technique (7), this material contained little or no 6 S$_c$ subunit. It was, however, a mixture of 6 S$_n$ and 5 S$_c$ subunits; the latter being formed by partial dissociation of the 6 S$_c$ subunit. That this had occurred was evident because there was a small peak of radioactivity in the eluate from the Bio-Gel column where the 1.3 S$_c$ subunit occurs. It is evident that there was considerable reconstitution to active enzyme during the chromatography on the Bio-Gel column because the dissociated enzyme (480 mg) had a specific enzymatic activity of 1.5, equivalent to 816 units whereas there were ~4320 units in the 1 liter each at 12, 17, and 23 hours against 0.05 M Tris-HCl (pH 9.0) containing 10^{-4} m phenylmethylsulfonylfluoride. This treatment dissociates from the 5 S$_c$ subunit any residual portion of 1.3 S$_c$ subunit that might remain attached to the 5 S$_c$ subunit. The material was then chromatographed on a Bio-Gel A-1.5m column (4.7 x 171 cm) using 0.05 M phosphate buffer (pH 7.0) containing 10^{-4} m phenylmethylsulfonylfluoride for the elution. A single symmetrical protein peak was obtained from the column. The pooled protein fractions were precipitated by 80% saturation with ammonium sulfate and then subjected to ultracentrifugation. The protein had an $s_{20,w}$ = 5.8 S (Fig. 3D). Polyacrylamide gel electrophoresis of this protein and that from the first peak (Fig. 3C, top) in gels containing 8 M urea and sodium dodecyl sulfate [7] gave single bands showing that there was little cross contamination of the 5 S$_c$ subunit by the 12 S$_n$ or 6 S$_c$ subunits or of the 12 S$_n$ subunit by the 5 S$_c$ subunit. These two preparations of subunits in combination with the normal 1.3 S$_c$ subunit were found to be effective in reconstituting active transcarboxylase (see "Reconstitution of Transcarboxylase from Subunits").

*Fig. 2 (left). Sedimentation velocity profiles of transcarboxylase, the dissociated enzyme, and fractions after chromatography of the dissociated enzyme on a Bio-Gel A-1.5m column. A, the original transcarboxylase, 18 S and 16 S; B, the dissociated transcarboxylase, 15.0 S, 10.7 S, and 5.4 S; C, the first protein peak eluted from the Bio-Gel column, 17 S; D (top), second protein peak, 19 S, 16.2 S, 12.6 S and 5.8 S; D (bottom), third protein peak, 5.8 S. Sedimentation was at 52,000 rpm at approximately 4° for 48 min and from right to left. A 30-mm double sector cell was used for the experiments of A, B, and C and a double sector wedge cell for D. The protein concentrations were: A, 2.6 mg per ml; B, 2.0 mg per ml; C, 1.2 mg per ml; D (top), 1.9 mg per ml; and D (bottom), 2.3 mg per ml.*
This trypsinized transcarboxylase may be used for isolation of 12 Sn and 5 Se subunits. However, the yields have not been as good as with dead transcarboxylase because there is some recombination of the dissociated subunits even though a portion of the 1.3 Se subunit is removed by the trypsin treatment. These results are described in detail by Ahmad et al. (8).

A method for isolation of 5 Se and 12 Sn subunits from transcarboxylase by complexing native enzyme with avidin-Sepharose has been developed by Berger and Wood (17). The complex is dissociated at pH 8 to liberate the 12 Sn subunit and then at pH 9 to obtain the 5 Se subunit. The 1.3 Se subunit remains in a complex with the avidin, thus the problem of reconstitution is eliminated and the eluted subunits may be collected under conditions which prevent their dissociation. This procedure is useful in obtaining the 5 Se and 12 Sn subunit but is of no use in obtaining the 6 Sn or 1.3 Se subunits.

**Isolation of 1.3 Sn Biotinyl Carbonyl Carrier Protein**—We have not had consistent results in isolating a homogeneous form of this subunit. Isolation of the 1.3 Sn subunit as described previously involved dissociation of transcarboxylase at 4° in 0.05 M Tris-HCl (pH 8.8) for 72 hours followed by a stepwise elution with an increasing concentration of KCl from a DEAE-Sephadex A-50 column equilibrated with 0.05 M Tris-HCl (pH 8.8). The 1.3 Sn subunit was obtained in a small breakthrough peak as well as in the 0.1 M KCl eluate and gave a single band on polyacrylamide gel electrophoresis at pH 9 and had a molecular weight of approximately 12,000. Subsequently, this and other procedures have not given consistent results because more than one band is often observed on polyacrylamide gel electrophoresis.

One method which we have adopted involves denaturation of transcarboxylase at 100° in 6 M urea plus 10-4 M dithiothreitol and then chromatography on Bio-Gel A-1.5m in 6 M urea plus 10-4 M dithiothreitol. As shown in Fig. 4, the resulting 2.5 Sn and 2.5 Sp peptides in Fractions 23 to 28 are easily observed by ultraviolet absorption at 280 nm and are well separated from the radioactive 1.3 Sn subunit in Fractions 47 to 57. The 1.3 Sn subunit, by virtue of its low content of aromatic amino acids, has a low ultraviolet absorption and is observed only by the presence of the 4H label. The results of analytical polyacrylamide gel electrophoresis of the 2.5 Sn and 2.5 Sp preparation in urea and of the 1.3 Sn preparation in dodecyl sulfate are shown in the insets of Fig. 4. Two major bands were observed in the fractions containing the 2.5 Sn and 2.5 Sp peptides and these had no radioactivity. Fractions 47 to 56 containing the 1.3 Sn subunit gave one major band which was highly radioactive and contained about 85% of the total recovered radioactivity. There was a nearby smaller band with about 15% of the radioactivity. Some preparations obtained by this method give two bands in the radioactivity. Fractions 47 to 56 were pooled separately and each was dialyzed against 2000 ml of 0.02 M sodium phosphate buffer (pH 7) with three changes of buffer. Pool 47 to 56 was concentrated by rotary evaporation to 7.2 ml (~55 mg per ml) and Pool 47 to 56 was pooled separately and each was dialyzed against 2000 ml of 0.02 M sodium phosphate buffer (pH 7) with three changes of buffer. Pool 23 to 28 was concentrated by rotary evaporation to 7.2 ml (~55 mg per ml) and Pool 47 to 56 was pooled separately and each was dialyzed against 2000 ml of 0.02 M sodium phosphate buffer (pH 7) with three changes of buffer. Pool 23 to 28 was concentrated by rotary evaporation to 7.2 ml (~55 mg per ml) and Pool 47 to 56 was pooled separately and each was dialyzed against 2000 ml of 0.02 M sodium phosphate buffer (pH 7) with three changes of buffer. Pool 23 to 28 was concentrated by rotary evaporation to 7.2 ml (~55 mg per ml) and Pool 47 to 56 was pooled separately and each was dialyzed against 2000 ml of 0.02 M sodium phosphate buffer (pH 7) with three changes of buffer. Pool 23 to 28 was concentrated by rotary evaporation to 7.2 ml (~55 mg per ml) and Pool 47 to 56 was pooled separately and each was dialyzed against 2000 ml of 0.02 M sodium phosphate buffer (pH 7) with three changes of buffer.

The protein in Fractions 60 to 76 was precipitated with ammonium sulfate and the protein in Fractions 80 to 102 was concentrated by lyophilization. Gel electrophoresis in the absence of denaturing agents of the preparation containing 6 Sn and 5 Sn subunits gave major bands and gel electrophoresis in sodium dodecyl sulfate of the 1.3 Sn preparation gave a single band aside from a small band near the origin which may be an aggregate. Some preparations of 1.3 Sn have given two radioactive bands by this procedure.

A similar procedure has been used to obtain the 1.3 Sn and 5 Sn subunits from the 6 Sn subunit shown in Fig. 20, bottom. Gel electrophoresis of preparations of the 1.3 Sn subunit in the absence of dodecyl sulfate often yields multiple bands. This observation is considered under “Discussion.”

The concentration of the 1.3 Sn subunit has been calculated from its biotin content on the basis that it contains 1 mol of biotin per mol of molecular weight of approximately 12,000 (2). For this purpose, the specific radioactivity (counts per min per mmol of biotin) of a given batch of transcarboxylase is determined from its total radioactivity and biotin content. Then knowing the total radioactivity of the 1.3 Sn preparation, its biotin content is calculated from the specific radioactivity of the biotin.

**Reconstitution of Transcarboxylase from Purified 12 Sn, 5 Sn, and 1.3 Sn Subunits**—The reconstitution of transcarboxylase has been accomplished previously with the unresolved subunits of dissociated transcarboxylase either by adjustment to pH 5 with acetic acid buffer or by addition of a high concentration of phosphate buffer, pH 6.5 to 6.8 (3, 7). Similarly, a combination of the
linear. Thus, in a limited range of concentrations, and with an 1.3 SE subunit which has very little absorbance at 280 nm.

Assay for the "6 SE" subunit or for the 1.3 SE and 5 SE subunits. Equivalent to 11.0 nmol of 1.3 SE biotinyl subunit bound to reconstitution. The observed AA could likewise have been with the 280 nm absorbance is due to the [3H]biotinyl 1.3 SE excess of the 12 Sn subunit, the procedure could be adapted as an enzyme with the 12 Sn subunit if it forms a complex with the phosphate at pH 6.5 or acetate at pH 5.2 in the experiments of Figs. 6 and 7 that the specific activity of Two peaks of radioactivity were obtained; the first coinciding with the other. Three-aliquot portions of the resulting mixtures were enzyme by the usual spectrophotometric assay which measures concentration of the 12 Sn subunit up to 3.2 pg.

The results of two experiments are shown in Fig. 6 in which the determined by making the one subunit limiting and the other subunit (urea preparation) and was held in ice for 19 hours. Aliquot portions of 0.005 ml (0.029 nmol of 5 SE subunit, 3.5 µg), 0.01 ml (0.058 nmol, 7.0 µg), and 0.02 ml (0.116 nmol, 14.0 µg) from each were then combined with 0.205 nmol (74 µg) of 12 Sn subunit (from trypsinized transcarboxylase) in 0.20 ml of 0.75 M phosphate buffer (pH 6.5) containing 1.0% bovine serum albumin. The mixture was held at 0° and 0.01-ml or 0.005 ml portions were assayed for transcarboxylase activity (11). Controls were set up on the reconstituted "6 SE" subunit and on the 12 Sn subunit separately. The sum of these control values (0.006 ΔA per min or less) was subtracted from the values of the complete mixture.

A similar experiment in which the 12 Sn subunit was limiting and the reconstituted "6 SE" subunit was in excess is shown in Fig. 7. Four concentrations of 12 Sn subunit were used with an excess of the reconstituted "6 SE" complex which was prepared in acetate buffer at pH 5. The reconstitution was done in 0.75 M phosphate buffer (pH 6.8) and the enzymatic activity was assayed in 0.01-ml portions. The results of Fig. 7 show that at 27 hours, the enzymatic activity increased linearly with the concentration of the 12 Sn subunit up to 3.2 µg.

**Determination of Specific Activity of Subunits**—It is evident from the experiments of Figs. 6 and 7 that the specific activity of a given subunit in forming active transcarboxylase could be determined by making the one subunit limiting and the other subunit in excess, much as is usually done in assaying an enzyme. However, the 5 SE subunit can only be effective in forming active enzyme with the 12 Sn subunit if it forms a complex with the 1.3 SE subunit. A comparison between the activities of the reconstituted subunit with that of the native enzyme, therefore, requires an estimation of the number of 1.3 SE subunits bound to each 5 SE subunit. For this purpose, the [3H]biotinyl 1.3 SE subunit and 5 SE subunit were reconstituted and then the unbound 1.3 SE subunit was separated by glycerol gradient centrifugation from the 5 SE subunits containing bound 1.3 SE subunits. The results of such an experiment are shown in Fig. 8. Two peaks of radioactivity were obtained; the first coinciding with the 280 nm absorbance is due to the [3H]biotinyl 1.3 SE subunit bound to the 5 SE subunit and the second to the unbound 1.3 SE subunit which has very little absorbance at 280 nm. Fractions 21 to 28 were combined and contained 13.5 × 10^4 cpm equivalent to 11.0 nmol of 1.3 SE biotinyl subunit bound to the 5 SE subunit. About 58% of the sites of the added 5 SE

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**Fig. 5.** Separation on Bio-Gel A-1.5m of 6 Sn and 5 Sb subunits from the 1.3 Sb subunit by chromatography of transcarboxylase dissociated at pH 9. Transcarboxylase (specific activity = 31) was dissociated by dialysis for 23 hours at room temperature against 4 liters of 0.1 M Tris-HCl, pH 9, containing 10% glycerol, 10% m phenylmethylsulfonyl fluoride, and 0.02% NaN3. The dissociated transcarboxylase (151 µg in 4.6 ml) was placed on a Bio-Gel A-1.5m (100 to 200 mesh) column (4 × 170 cm). The column had been equilibrated with 0.05 M Tris-HCl, pH 9, containing 3% glycerol, 10% m phenylmethylsulfonyl fluoride, and 0.02% sodium azide, and the protein was eluted with the same mixture. Volume of each fraction was 14 ml. Fractions 65 to 76 were pooled and the protein precipitated with ammonium sulfate at 80% saturation. Fractions 89 to 103 (195 ml) were pooled and the protein precipitated with ammonium sulfate subunit (urea preparation) and was held in ice for 24 hours. Aliquot portions of 0.005 ml (0.029 nmol of 5 Sb subunit, 3.5 µg), 0.01 ml (0.058 nmol, 7.0 µg), and 0.02 ml (0.116 nmol, 14.0 µg) from each were then combined with 0.205 nmol (74 µg) of 12 Sn subunit (from trypsinized transcarboxylase) in 0.20 ml of 0.75 M phosphate buffer (pH 6.5) containing 1.0% bovine serum albumin. The mixture was held at 0° and 0.01-ml or 0.005-ml portions were assayed for transcarboxylase activity (11). Controls were set up on the reconstituted "6 SE" subunit and on the 12 Sn subunit separately. The sum of these control values (0.006 ΔA per min or less) was subtracted from the values of the complete mixture.

**Fig. 6.** Assay of the 5 Sb subunit by reconstitution with the 1.3 Sb subunit in phosphate buffer or acetate buffer and then conversion with an excess of 12 Sn subunit to active enzyme. Two reconstitutions with the 5 Sb and 1.3 Sb subunits were done, one in 0.75 M potassium phosphate buffer (pH 6.8) and the other in 0.25 M sodium acetate (pH 5.2). Each contained in 0.2 ml of 1% bovine serum albumin, 1.75 nmol of 5 Sb subunit, and 2.92 nmol of 1.3 Sb subunit (urea preparation) and was held in ice for 19 hours. Aliquot portions of 0.005 ml (0.029 nmol of 5 Sb subunit, 3.5 µg), 0.01 ml (0.058 nmol, 7.0 µg), and 0.02 ml (0.116 nmol, 14.0 µg) from each were then combined with 0.205 nmol (74 µg) of 12 Sn subunit (from trypsinized transcarboxylase) in 0.20 ml of 0.75 M phosphate buffer (pH 6.5) containing 1.0% bovine serum albumin. The mixture was held at 0° and 0.01-ml or 0.005-ml portions were assayed for transcarboxylase activity (11). Controls were set up on the reconstituted "6 SE" subunit and on the 12 Sn subunit separately. The sum of these control values (0.006 ΔA per min or less) was subtracted from the values of the complete mixture.
subunits formed complexes with 1.3 SE subunits.

Pools 21 to 28 from the glycerol gradient were used directly for reconstitution with the 12 SH subunit in 0.75 M phosphate buffer (pH 6.5). The results are shown in Fig. 9. The proportion of 12 SH subunits to "6 SE" subunits was varied so as to obtain ratios of sites which varied from 17 to 0.13 assuming a 12 SH subunit has six sites for binding a 1.3 SE subunit and that one 1.3 SE subunit bound to one 5 SE subunit yields one "6 SE" site. The specific activity of the "6 SE" subunit was calculated from the observed enzymatic activity of the reconstituted mixture and it was assumed that 1 nmol of 1.3 SE subunit bound to a 5 SE subunit is equivalent to 0.072 mg of "6 SE" protein (this follows because 1 nmol of SE subunit containing 2 nmol of 1.3 SE subunits is equal to 0.144 mg). If the enzymatic activity is the same for the 1.3 SE subunit, whether one or two are bound to the 5 SE subunit, then this calculation is correct.

Fig. 9A shows that with 17- or 8-fold excess of sites of the 12 SH subunits per bound 1.3 SE subunit, the specific activities of the "6 SE" subunits were 48 and 51, respectively. A specific activity of the "6 SE" subunit of 50 is equivalent to a specific

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**Fig. 7.** Assay of the 12 SH subunit by reconstitution to active enzyme in the presence of an excess of the reconstituted "6 SE" subunit. The reconstitution with 5 SE and 1.3 SE subunits to "6 SE" subunits was done in 0.5 ml of 0.28 M sodium acetate buffer (pH 5.2) containing 9.9 nmol of 1.3 SH subunit (urea preparation) and 2.1 nmol (0.25 mg) of 5 SE subunit (from dead transcarboxylase). The solution was held at 4°C for 24 hours. Then, four reconstitutions with 12 SH subunit from dead transcarboxylase were set up using 0.02 ml of the above solution of reconstituted "6 SE" (0.14 nmol of 5 SE subunit) and 0.8 μg (0.0022 nmol), 1.6 μg (0.0044 nmol), 3.2 μg (0.0088 nmol), and 6.4 μg (0.0176 nmol) of the 12 SH subunit in 0.2 ml of 0.75 M phosphate buffer, pH 6.8. The mixtures were kept in ice and 0.01-ml portions were assayed for transcarboxylase activity. Controls with the reconstituted "6 SE" subunit or the 12 SH subunit alone were inactive.

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**Fig. 8.** Glycerol gradient separation of the free 3H-biotinyl 1.3 SE subunit from the 1.3 SE subunit bound to the 5 SE subunit. The reconstitution was for 65 hours at 0°C in 0.8 ml of 0.25 M sodium acetate containing 59 nmol of 1.3 SE subunit with 1.22 × 10^6 cpm per nmol in the biotinyl group and 21 nmol (2.56 mg) of 5 SE subunit. The 1.3 SE subunit was isolated from transcarboxylase dissociated at pH 9 and the 5 SE subunit was isolated by avidin-Sepharose chromatography (17). A slight turbidity appeared during the reconstitution which was removed by centrifugation and the protein in the solution was determined to be 2.58 mg per ml and the radioactivity 53,400 cpm per ml. On each of three 10 to 30% glycerol gradients (total volume 5.2 ml), 0.2 ml was layered. Centrifugation was at 55,000 for 13 hours at 3°C in a Beckman SW 65 rotor. The gradients were fractionated from the bottom, the corresponding fractions from each gradient being combined giving a total of 0.4 ml for each combined fraction. The fractions were monitored for absorption at 280 nm, and for radioactivity, O--O. Fractions 21 to 28 were pooled and considered to be representative of the 1.3 SE subunit bound to 5 SE subunit. This pool (3.43 ml) contained 1.396 μg of protein and 15,500 cpm (85.7% and 42%, respectively, of that applied to the gradient). The bound 1.3 SE subunit was 11 nmol (1.35 × 10^5 cpm/1.22 × 10^6 cpm per nmol) or 3.22 nmol per ml. Pool 21 to 28 was used with further purification for the reconstitutions of the experiment of Fig. 9.

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**Fig. 9.** Determination of the specific activities of the subunits of transcarboxylase. Five reconstitutions were set up in 0.12 ml of 0.75 M phosphate buffer (pH 6.5) using the reconstituted "6 SE" subunit of Pools 21 to 28 (Fig. 8) and 12 SH subunit isolated by avidin-Sepharose chromatography of transcarboxylase (17). The five reconstitutions contained the following amounts of the 12 SH subunits to "6 SE" subunits: (1) 12 SH, 0.0213, "6 SE", 0.161; (2) 12 SH, 0.0123, "6 SE", 0.0644; (3) 12 SH, 0.0426, "6 SE", 0.0322; (4) 12 SH, 0.266, "6 SE", 0.0322; (5) 12 SH, 0.533, "6 SE", 0.0322. The ratios of 12 SH subunits to "6 SE" subunit sites thus varied from 0.132 to 16.6. The mixtures were held in ice and assayed for enzymatic activity at intervals. Corrections were made for very small control values obtained with the reconstituted "6 SE" subunit alone and the 12 SH subunit alone. Values are plotted in A for the specific activities of the "6 SE" subunit on the basis that 2 nmol of bound 1.3 SE biotinyl subunit equals 0.144 mg and in B for the specific activities of the 12 SH subunit (six sites per nmol and 1 nmol = 0.360 mg).
activity of 27 for the 18 S form of the enzyme with a full complement of 3 complete 6 SE subunits (50 × 3 × 1.44 × 107/7.90 × 105 where 1.44 × 105 is the molecular weight of the 6 SE subunit and 7.90 × 105 of the 18 S form of the enzyme). The observed specific activity for the 18 S form of the enzyme is approximately 45, thus, the “6 SE” subunit had about 60% of the activity in the reconstituted enzyme as the same amount of this subunit has in the native enzyme.

Fig. 9B gives the specific activities based on the content of the 12 SN subunit. A maximum specific activity of approximately 40 was reached when the ratios of the “6 SE” subunit sites to 12 SN subunit sites were 7.6 and 3.0. A specific activity of 40 for the 12 SN subunit is equivalent to an activity of 18.2 for the 18 S form of transcarboxylase (40 × 3.6 × 107/7.9 × 105 where 3.6 × 105 is the molecular weight of the 12 SN subunit and 7.9 × 105 of the 18 S transcarboxylase). Thus, the 12 SN subunit had about 40% of the activity in the reconstituted enzyme as the same amount of this subunit has in the native enzyme. In other experiments this value has been as high as 50%.

The results of these experiments are presented in Table I, which are expressed as enzymatic activity of the reconstituted mixture on the basis of the specific activity of the 12 SN subunit. It is seen that neither the 1.3 SE subunit nor the nonbiotinyl peptide had a significant influence on the amount of enzyme.

The ratio of the activities was maintained approximately 60:40 for the “6 SE” subunit and 12 SN subunit. A maximum specific activity of approximately 40 was reached when the ratios of the “6 SE” subunit sites to 12 SN subunit sites were 7.6 and 3.0. A specific activity of 40 for the 12 SN subunit is equivalent to an activity of 18.2 for the 18 S form of transcarboxylase (40 × 3.6 × 107/7.9 × 105 where 3.6 × 105 is the molecular weight of the 12 SN subunit and 7.9 × 105 of the 18 S transcarboxylase). Thus, the 12 SN subunit had about 40% of the activity in the reconstituted enzyme as the same amount of this subunit has in the native enzyme. In other experiments this value has been as high as 50%.

### Table I

| Reconstitution mixture | Enzymatic activity<sup>a</sup> | Difference<sup>a</sup> |
|------------------------|-----------------------------|---------------------|
| Control                |                            |                     |
| Plus 1.3 SE subunit    |                            |                     |
| Plus non-biotinyl peptide |                            |                     |
| Plus 5 SE subunit      |                            |                     |

<sup>a</sup> Enzymatic activity was assayed after 48 or more hours and is expressed as specific activity on the basis of the amount of 12 SN subunit, i.e. micromoles of oxalacetate formed per mg of 12 SN subunit per min.

Transcarboxylase is a monofunctional enzyme catalyzing only a single metabolic reaction but in many respects it resembles the multi-enzyme complexes with components capable of catalyzing two or more metabolic reactions. Classical examples of the multiple enzyme complexes are the α-keto acid dehydrogenases (18) and the fatty acid synthetases (19–21) in which the lipoyl moiety serves as an arm to link the various enzymes of the alpha-keto acid dehydrogenases and the d-phosphopantetheine to link the enzymes of the fatty acid synthetases. Transcarboxylase is similar because it involves two partial reactions (shown below) which in combination make up the over-all reaction.

\[
\begin{align*}
\text{CH}_{2}\text{CH}(&\text{COO}^-)\text{COSCoA} + 1.3\text{SE biotin} &\rightarrow 12\text{SN subunit} \\
1.3\text{SE-biotin-COO}^- + \text{CH}_{2}\text{CH}(&\text{COO}^-)\text{COSCoA} &\rightarrow 5\text{SE subunit} \\
1.3\text{SE biotin} + \text{-OCCH}(&\text{COO}^-) &\rightarrow \\
\end{align*}
\]

Chuang et al. (22) have shown that the first partial reaction is catalyzed by the 12 SN subunit and the second partial reaction by the 5 SE subunit. The 1.3 SE biotinyl carboxyl carrier protein serves to link the partial reactions of the individual 12 SN and 5 SE subunits and in this respect it resembles the multi-enzyme complex in which the lipoyl or the phosphopantetheine group serves as the link.

There are numerous biotinyl enzymes in which the biotinyl group serves as a carboxyl carrier (see review by Moss and Lane (23)) but transcarboxylase is the only one which has been isolated as an intact complex, dissociated to its subunits, the subunits isolated, the partial reactions studied with the subunits, and the active enzyme complex reassembled from the isolated subunits.
Similar studies have been done with acetyl-CoA carboxylase of E. coli (24) but in this case the complex has not been isolated nor have the constituent subunits been reassembled into a complex. In this regard, acetyl-CoA carboxylase of E. coli resembles the fatty acid synthetase from E. coli, which has not as yet been obtained as a multi-enzyme complex (24, 25).

Thus far, we have devoted most of our efforts to isolating the 12 S subunit (3), 5 SE and 1.3 S subunits by chromatography on Bio-Gel; separation of the 6 S and 5 SE subunits by chromatography on Bio-Gel; separation of the 6 S and 5 SE subunits by electrophoresis. The dissociation was done by addition of the enzyme to boiling 6 M urea plus 0.3 M phenylmethylsulfonyl fluoride (32). The cells were broken in the presence of these inhibitors and they also were included at reassembly.

We have attempted to prevent proteolysis by use of benzamidine-HCl as employed by Fujikawa et al. (31) and also of phenylmethylsulfonyl fluoride (32). The cells were broken in the presence of these inhibitors and they also were included at each stage of purification of the enzyme. The dissociation was done by addition of the enzyme to boiling 6 M urea plus 10^{-3} M dithiothreitol. Gel electrophoresis in the presence of dodecyl sulfate gave a major single band of the 1.3 S subunit. Our preparations have molecular weights of 11,000 to 13,000.

The present study is the first that the authors are aware of in which it has been possible to determine the activity of a subunit in forming the active enzyme by making the given subunit limiting and adding the other subunits to it in excess. The comparison of the activity of the given subunit with its activity in the intact enzyme is made somewhat uncertain, however, because various preparations of transcarboxylase, albeit pure as judged by their sedimentation behavior, possess varying specific activities. There are two factors which may influence the specific activity. The first factor is the number of peripheral biotinyl subunits that are attached to the central subunit. The 18 S form of the enzyme has three peripheral subunits and has a specific activity of approximately 45 but values in the 50s have been observed occasionally. The 16 S form has only two peripheral subunits and has a correspondingly lower activity. In addition, there is a ~24 S form of the enzyme (1, 6) which may have six peripheral subunits attached to the central 12 S subunit. This form has not been isolated and its specific activity is not known but may be greater than that of the 18 S form. The second factor influencing specific activity is manifest by a loss of enzymatic activity (sometimes quite rapidly) which is not accompanied by a change in the sedimentation coefficient or the dissociation to subunits (6, 12). Sometimes this loss in activity can be restored by incubation in 1.5 M (NH_4)_2SO_4 at 25° (6).

The above factors make it difficult to evaluate the efficiency of reconstitution of active enzyme from the isolated subunits. We have chosen as our standard of comparison a specific activity of approximately 45 for the 18 S form of the enzyme and have compared the observed specific activity of the subunits with this value. Thus, the theoretical maximum specific activity for the 12 S and 5 SE subunits is 98.8 (45 X 7.9 X 10^5/3.6 X 10^5) and for the 6 S subunit is 82.3 (45 X 7.9 X 10^5/3.6 X 10^5) where 7.9 X 10^5 is the molecular weight of the 18 S form of the enzyme, 3.6 X 10^5 of the 12 S subunit and 1.44 X 10^5 of the 6 S subunit (1). On this basis, when the 12 S subunit was made limiting and the reconstituted "6 SE" subunit was added in excess, the specific activity of the 12 S subunit had about 50% of its potential activity. There are several factors which may cause the specific activity to be lower than that calculated from the standard. One is the uncertainty of the values to be used for the standard as explained above. A more important factor is the fact that the "6 SE" subunit formed by reconstitution from the 5 SE and 1.3 SE subunits only carried about 50% of its activity. The full complement of 1.3 SE subunits which is two per 5 SE subunit. Thus, when the 12 S subunit is made limiting, three peripheral "6 SE" subunits which are deficient in the 1.3 SE subunits may form a complex with the 12 S subunit. In this case, some of the 12 S sites would be ineffective in the transcarboxylation reaction because they would lack the biotinyl carboxyl carrier protein which is essential for the activity of that site to become evident. It is not clear why we have been unsuccessful in obtaining a complete conversion of the 5 SE subunit to a 6 SE subunit with its full complement of 1.3 SE subunits. Perhaps a larger excess of the 1.3 SE subunit is required than was used in the reconstitution of the "6 SE" subunit.

The situation with regard to the specific activity of the "6 SE" subunit is somewhat different. Here, the calculation of its specific activity has been done on the basis of each nanomole of complexed 1.3 SE subunit being equivalent to 0.072 mg of "6 SE" subunit (0.144 mg = 1 nmol of 6 SE with 2 nmol of 1.3 SE).

* F. Ahmad, unpublished observations.
Thus, if there is only one 1.3 SE subunit in combination with a 5 SE subunit, only one-half of the weight of 6 SE is considered in calculating the specific activity and as noted above, a theoretical maximum specific activity of 82 would be anticipated for the 6 SE subunit. The observed value was about 50 (Fig. 9) when the 12 SH subunit was in large excess. Under these conditions, the reconstituted enzyme may consist of forms with only one peripheral "6 SE" subunit combined with a 12 SH subunit. Possibly there is cooperativity and a single "6 SE" subunit bound to a 12 SH subunit is less effective than when two or more are bound on the same 12 SH subunit. A more detailed study is required to obtain information about this possibility.

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