Structure of the Subunits of Transcarboxylase and Their Relationship to the Quaternary Structure of Transcarboxylase†

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ABSTRACT: The structures of the central $12S_H$ subunit and the attached $5S_E$ subunits of transcarboxylase (EC 2.1.3.1) have been investigated by amino-terminal determinations and enzymatic peptide mapping of their constituent peptides. Previously, it had been proposed that the polypeptides of the $12S_H$ subunit may have duplicating sequences as a consequence of gene duplication and fusion. Peptide maps were constructed from tryptic digests of both succinylated and nonsuccinylated subunits and also from digests of the subunits with the AL-1 protease of myxobacter. This latter enzyme cleaves specifically at the amino terminal of lysines. If there has been gene duplication and fusion, subsequent mutations have been so extensive that the identity of the duplicated peptides has been destroyed and the homology cannot be detected by the peptide mapping. It was shown that the six polypeptides of the $12S_H$ subunit are identical as are the two polypeptides of the $5S_E$ subunit. The NH_2 terminal of the $12S_H$ constituent polypeptide is alanine, while that of the $5S_E$ polypeptide appears to be blocked. Comparison of the amino acid composition of the polypeptides of the central $12S_H$ and $5S_E$ subunits indicates there is considerable homology between these peptides. This observation is in accord with the previously observed immunological cross-reactivity of the subunits. The 26S form of transcarboxylase has six $5S_E$ subunits attached by $1.3S_E$ subunits to the $12S_H$ subunit (a $5S_E$ subunit with two $1.3S_E$ subunits still attached to it is designated as a $6S_E$ subunit). In other studies, it has been shown that the 26S form dissociates to an 18S form of the enzyme with the loss of three $6S_E$ subunits exclusively from only one face of the central $12S_H$ subunit leaving three remaining $6S_E$ subunits on the opposite face of the $12S_H$ subunit. The question of whether this selective dissociation occurs because the two faces of the $12S_H$ subunit differ or because of negative cooperativity (loss of one subunit promoting loss of the other subunits from the same face and stabilization of the attached subunits at the other face) has been investigated using ^{60}Co-labeled $6S_E$ subunits. The ^{60}Co-labeled $6S_E$ subunits were exchanged into unlabeled 26S transcarboxylase and the labeled enzyme was isolated. Then the labeled 26S enzyme was dissociated and the resulting transcarboxylase and the dissociated subunits were isolated. The specific radioactivity of the dissociated subunits was much higher than that found for the $6S_E$ subunits which remained attached to the enzyme. These results indicate that there was preferential exchange of subunits on one face of the $12S_H$ subunit and also preferential loss of the same set of attached subunits when the 26S enzyme was dissociated. These findings are considered evidence that the two faces of the central subunit differ in structure and have different properties. Models for the quaternary structure of transcarboxylase have been evaluated in view of these findings and a model is proposed in which the six $2.5S_H$ polypeptides which comprise the $12S_H$ subunit have two types of isologous interactions and are arranged in a parallel manner with C_3 symmetry.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxytransferase (EC 2.1.3.1)) is a biotin containing enzyme found in propionic acid bacteria. It catalyzes the following reaction:

$$CH_3CH(COO^-)COSCoA + CH_3COCOO^- \rightleftharpoons CH_3CH_2COSCoA + {}^-OOCCH_2COCOO^- \quad (1)$$

Two forms of this enzyme have been studied, the 18S form and the 26S form. The latter (see Figure 1 for model of 26S form) has been isolated recently (Wood et al., 1977) and has been shown to consist of a central subunit of mol wt 3.6×10^5 which is designated the $12S_H$ subunit. To this central subunit, six dimeric Co^{2+}- and Zn^{2+}-containing subunits are attached, three at each face of the central subunit (Wrigley et al., 1977). These attached subunits have a mol wt of 1.2×10^5, a sedimentation coefficient of 5.9 S and are designated the $5S_E$ subunit. The $5S_E$ subunits are each attached to the central subunit by two biotinyl carboxyl carrier proteins of mol wt 0.12×10^5 with an $s_{20,w} = 1.3$ S and are designated the $1.3S_E$ subunits.

The 26S form of the enzyme dissociates at neutral pH to the 18S form of the enzyme with loss of three of the $5S_E$ subunits with the two $1.3S_E$ biotinyl carboxyl carrier proteins still attached to each of them. This latter combination is called the $6S_E$ subunit and it has a mol wt of 1.44×10^5. The 18S enzyme is more stable and does not dissociate completely until ~pH 8 with loss of the three remaining $6S_E$ subunits from the central $12S_H$ subunit. Because the 26S enzyme is so labile, it can only be isolated when the pH is maintained near 5.5 (Wood et al., 1977); studies prior to 1976 had been done with the 18S form of the enzyme. At pH 9 the dissociation of the $6S_E$ subunit to the constituent $5S_E$ subunit and the two $1.3S_E$ biotinyl subunits is complete. Also, at pH 9, the hexameric $12S_H$ subunit dissociates to three dimeric subunits of mol wt 1.2×10^5, $s_{20,w} = 6$ S, which are designated the $6S_H$ subunit. These dissociations are summarized below where TC is transcarboxylase:

$$26S\text{-}TC \underset{\text{pH 5.5}}{\overset{\text{pH 7}}{\rightleftharpoons}} 18S\text{-}TC + (3)\ 6S_E \text{ subunits} \quad (2a)$$

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FIGURE 1: A model of the 26S form of transcarboxylase. The six dimeric outside $5S_E$ subunits and the central cylindrical hexameric $12S_H$ subunit are linked to each other by the $1.3S_E$ biotinyl carboxyl carrier proteins which are represented by the small tubing with a hexagon attached to it. The hexagon symbolizes the biotin. The biotin serves as the carboxyl carrier by oscillating between a keto acid site on the $5S_E$ subunit and a CoA ester site on the $12S_H$ subunit. There are 12 biotinyl carboxyl carrier proteins, 2 per $5S_E$ subunit. The 2 carboxyl carrier proteins in combination with the $5S_E$ subunit are designated as the $6S_E$ subunit.

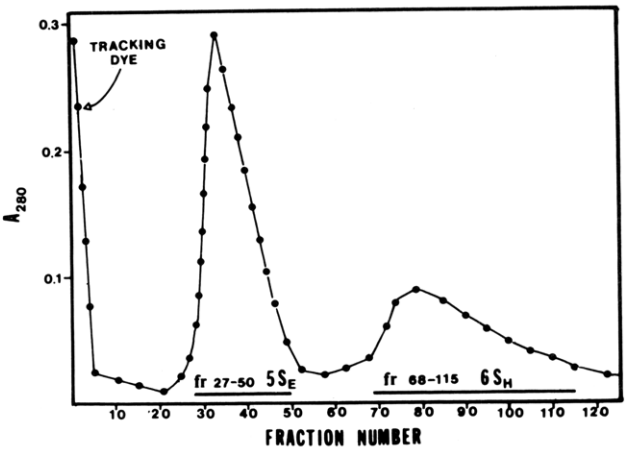

FIGURE 2: Separation of the $5S_E$ and $6S_H$ subunits by preparative disc gel electrophoresis. In this separation, 19.0 mg (1.8 mL) of the $5S_E$ and $6S_H$ subunit mixture in 0.05 M Tris-HCl, pH 9.0, with 10% glycerol was applied to a PD 320/2 column which contained a 2.3-cm gel (7.7 mL) of 7.5% polyacrylamide (Gabriel). The upper electrophoresis buffer was 0.005 M Tris–0.038 M glycine, pH 8.3, and the lower and elution buffers were 0.06 M Tris-HCl, pH 8.1. The elution buffer was pumped across the bottom of the gel, up through the center of the column core, and into the fraction collector at a constant flow rate of 0.70 mL/min. The electrophoresis was performed with a current of 13 mA and a potential of 500 V, and the apparatus was cooled with cold tap water. The elution was monitored by the absorbance at 280 nm and the fraction volume was 2.35 mL. The tracking dye was bromophenol blue. The $5S_E$ and $6S_H$ subunits were pooled according to the bars in the figure and were recovered in 44% (8.40 mg) and 28% (5.34 mg) yield, respectively.

$$18S\text{-}TC \underset{\text{pH 5.5}}{\overset{\text{pH 8}}{\rightleftharpoons}} 12S_H \text{ subunit} + (3)\ 6S_E \text{ subunits} \quad (2b)$$

$$(6)\ 6S_E \text{ subunits} \underset{\text{pH 5.5}}{\overset{\text{pH 9}}{\rightleftharpoons}} (6)\ 5S_E \text{ subunits} + (12)\ 1.3S_E \text{ subunits} \quad (2c)$$

$$12S_H \text{ subunit} \xrightarrow{\text{pH 9}} (3)\ 6S_H \text{ subunits} \quad (2d)$$

$$\text{Sum: } 26S\text{-}TC \rightarrow (3)\ 6S_H \text{ subunits} + (6)\ 5S_E \text{ subunits} + (12)\ 1.3S_E \text{ subunits} \quad (2)$$

The reviews by Wood (1976) and Wood and Zwolinski (1976) should be consulted for references and diagrams which illustrate the quaternary structures of transcarboxylase.

The purpose of the present investigation has been to study the structure of the $12S_H$ subunit in relation to the 18S and 26S forms of the enzyme. Peptide mapping of the $2.5S_H$ polypeptide of the dimeric $6S_H$ subunit has been undertaken in order to test the proposal of Green et al. (1972), Wood et al. (1972), and Green (1972) that there may be a duplicated sequence in the $2.5S_H$ polypeptide. In addition, peptide maps have been made of the $2.5S_E$ polypeptide of the dimeric $5S_E$ subunit. In neither case was evidence obtained for a closely duplicated sequence.

Tests were undertaken with ^{60}Co-labeled $6S_E$ subunit to determine if the two faces of the $12S_H$ subunit differ. The results indicate there is a structural difference.

Experimental Procedure

Materials. The 18S transcarboxylase containing [^{3}H]biotin was purified and assayed as described by Wood et al. (1969) and the 26S transcarboxylase was obtained as described by Wood et al. (1977). Trypsin (treated with 1-tosylamido-2-phenylethyl chloromethyl ketone) was from Worthington Biochemical Corp. The Myxobacter AL-1 Protease II was a generous gift from Dr. R. S. Wolfe, University of Illinois, Urbana.

Isolation and Purification of $6S_H$ and $5S_E$ Subunits. Purified 18S transcarboxylase containing [^{3}H]biotin was dissociated into its subunits by dialysis against 0.05 M Tris[1]-HCl, pH 9.0, which contained 10% glycerol, 10^{-4} M dithiothreitol, 10^{-4} M phenylmethanesulfonyl fluoride, 10^{-5} M EDTA, and 0.02% sodium azide at 4 °C for 48 h. The resulting $6S_H$ and $5S_E$ subunits were separated from the $1.3S_E$ biotin labeled subunit by gel filtration on a Bio-Gel A-1.5m, 100–200 mesh column (4.4 × 165 cm) using 0.05 M Tris-HCl, pH 9.0, which contained 3% glycerol, 10^{-4} M phenylmethanesulfonyl fluoride, 10^{-5} M EDTA, and 10^{-4} M dithiothreitol. The mixture of $6S_H$ and $5S_E$ subunits was located by measuring the absorption at 280 nm and the $1.3S_E$ subunit by its radioactivity (Wood et al., 1975).

The mixture of $6S_H$ and $5S_E$ subunits was concentrated by lyophilization and then dialyzed against 0.05 M Tris-HCl, pH 9.0, containing 10% glycerol, 10^{-4} M phenylmethanesulfonyl fluoride and 0.02% sodium azide. The $6S_H$ and $5S_E$ subunits were separated and purified by preparative disc electrophoresis (Canalco chamber from Canal Industrial Corp., Rockville, Md.). The elution buffer was pumped through the apparatus by a mini pump Model 196-89 produced by the Milton Roy Co., St. Petersburg, Fla. A typical separation is shown in Figure 2. Glycerol was added to the separated subunit pools to a final concentration of 1% and the pools were lyophilized to a volume of ~3 mL. The $6S_H$ subunit tends to denature during lyophilization and the glycerol was added to retard or prevent denaturation. The samples were dialyzed against 0.025 M potassium phosphate buffer, pH 7.0, containing 1% glycerol and were centrifuged to remove any denatured protein. The purity of the separated subunits was determined by analytical polyacrylamide gels (Berger and Wood, 1975) and in each case only a single band was observed.

Amino Acid Analyses. The purified $6S_H$ and $5S_E$ subunits

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

were treated with performic acid (Moore, 1963) and hydrolyzed at 100 °C in vacuo in 6 M HCl for 24 h, and the hydrolysates were analyzed with a Beckman amino acid analyzer equipped with an automatic integrator. The tryptophan content was determined by the method of Hugli and Moore (1972).

Determination of the NH_2-Terminal Amino Acid of the $6S_H$ and $5S_E$ Subunits. The NH_2-terminal amino acids were determined by dansylation according to the procedure of Gros and Labouesse (1969). Extracts of the hydrolyzed dansylated protein were spotted on silica gel thin-layer chromatographic plates and were chromatographed with a series of standard dansyl amino acids. The dansylation products of both subunits were chromatographed in two solvent systems, chloroform–benzyl alcohol–acetic acid (100:30:5 v/v) and 2-butanone–propionic acid–water (15:5:6 v/v), to ensure reliability of the results.

S-Carboxymethylation of the Subunits after Reduction with 2-Mercaptoethanol. Each of the purified subunits (4.0 mg) was dialyzed against 0.1 M ammonium bicarbonate and was lyophilized to dryness, suspended in distilled water, and lyophilized to dryness. The last procedure was performed twice. The proteins were dissolved in 2.0 mL of 0.5 M Tris-HCl at pH 8.0 which contained 0.1 M mercaptoethanol and 7 M guanidine hydrochloride. Sodium iodoacetate (45.8 mg, 0.11 M) was added to the above reduced proteins at room temperature in the dark. The reactions were terminated after 20 min by the addition of 0.14 mL of 2-mercaptoethanol. The $5S_E$ carboxymethylated proteins were dialyzed against water overnight and lyophilized to dryness. The $6S_H$ carboxymethylated protein was dialyzed against water for 6 h and then against 5% acetic acid overnight. The mixture was then lyophilized.

Succinylation of the Carboxymethylated Proteins. The method of succinylation with a few minor changes was that of Waterson and Konigsberg (1974). Each of the proteins (1 mg) was dissolved in 0.3 M sodium phosphate, pH 9.0, containing 6.0 M guanidine hydrochloride and treated with 10 mg of succinic anhydride in portions of 2, 2, 2, and 4 mg. The pH was maintained during the reaction between 8.0 and 9.0 by the addition of the buffer or 0.5 M NaOH. The reaction was allowed to proceed for 1 h. The mixture was dialyzed exhaustively against 0.05 M ammonium bicarbonate, pH 8.5, and then lyophilized. The product was taken up in a minimal amount of water and lyophilized. Lyophilization was repeated three additional times.

Cleavage at Arginine Residues (Trypsin Digest of Succinylated Proteins). The succinylated proteins (1 mg) were taken up in 0.25 mL of 0.05 M ammonium bicarbonate, pH 8.5, and 0.01 mL of trypsin solution (2 mg/mL in 0.001 M HCl) was added, and the digestions were for 20 h at room temperature. The digests were lyophilized to dryness.

Myxobacter Al-1 Protease II Digests. This protease cleaves specifically at the α-amino group of lysine (Wingard et al., 1972). The proteins (1 mg) were taken up in 0.25 mL of 0.05 M ammonium bicarbonate, pH 8.5, and 0.01 mL of the protease solution (1 mg/mL in 0.001 M HCl) was added. The digestions were for 16 h at 30 °C after which the samples were lyophilized to dryness.

Trypsin Digest of Proteins. The proteins (1 mg) were taken up in 0.25 mL of ammonium bicarbonate, pH 8.5, and 0.01 mL of a trypsin solution (2 mg/mL in 0.001 M HCl) was added and the digestion was for 20 h at room temperature. The mixtures were lyophilized to dryness.

Thin-Layer Electrophoresis. The thin-layer chromatography plates (20 × 20 cm) used in preparing the peptide maps were precoated silica gel, 60 F-254 glass plates of 0.25-mm layer thickness from E. Merck, Darmstadt, West Germany. The electrophoresis was performed with a Desaga/Brinkman double chamber, thin-layer electrophoresis apparatus (C. Desaga, Heidelberg, West Germany).

The plates were sprayed with electrophoresis buffer (10% acetic acid titrated with pyridine to pH 3.6) to give a wet matted finish. The digests were taken up in 0.25 mL of the electrophoresis buffer and aliquots between 0.015 and 0.045 mL (60–180 μg of protein) were spotted onto the plates. The electrophoreses were done at 350 V for 5.5 to 7 h and the apparatus was cooled by circulating cold tap water through the cooling plate. After the electrophoresis, the thin-layer plates were dried at room temperature.

Ascending Thin-Layer Chromatography. The chromatography was in 1-butanol–acetic acid–water (3:1:1 v/v) (pH 2.35) and the plates were dried at room temperature in a fume hood. In the mapping of trypsin digests, when the lysine residues were not succinylated, it was found necessary to do a double chromatographic development (Perry et al., 1973) in order to resolve all of the tryptic peptides. The thin-layer plates were dried after the first ascending solvent chromatography and chromatographed again in the same direction in the same solvent system.

Identification of the peptides in the Mapping Experiments. The plates were sprayed with fluorescamine [4-phenylspiro-(furan-2(3*H*),1′-phthalan)-3,3′-dione] solution (2 mg/10 mL acetone) and the peptides were located using long wave UV light (366 nm). In all experiments the peptides were clearly identified and there was no smearing or trailing.

The biotin determinations, glycerol gradients, and ultracentrifugation were done as previously described (Wood et al., 1977).

Isolation of $5S_E$–$6S_E$ Subunits Containing ^{60}Co. Transcarboxylase was isolated from *P. shermanii* grown in ^{60}Co-containing medium as described by Ahmad et al. (1972) under conditions yielding the 18S enzyme. The enzyme preparation (16.2 U/mg, 51 mg of protein, 489 000 cpm in 10 mL) which had been dialyzed vs. 0.25 M sodium acetate buffer, pH 5.5, at 4 °C, containing 10^{-4} M phenylmethanesulfonyl fluoride was applied at 4 °C to an 80-mL column of cellulose phosphate, equilibrated with 0.25 M Tris·SO_4, pH 8, containing 20% glycerol (v/v). This causes dissociation of the $6S_E$ subunits and their elution from the column. Approximately 11 mg of protein, 24 900 cpm/mg, was eluted. The biotin content was 1.4 ± 0.1 nmol per nmol of $6S_E$ subunit. Thus, during the isolation, part of the $6S_E$ subunits had dissociated with loss of $1.3S_E$ subunits. On heavily overloaded gels (Berger and Wood, 1975) there was a dark band of $5S_E$ subunits and only a trace amount of $6S_H$ material. This material in combination with a carrier of unlabeled $5S_E$ subunits and $1.3S_E$ subunits were used in the exchange experiments.

Results

Peptide Mapping. The amino acid compositions of the $2.5S_H$ and $2.5S_E$ polypeptides are presented in Table I. Comparison with the average amino acid composition of 307 proteins of known sequence (Dayhoff, 1972) shows that 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 amount of alanine, methionine, and glutamic acid plus glutamine is high. The $2.5S_E$ polypeptide is characterized by a lower than average content of aromatic residues and a slightly higher than average amount of hydrophobic residues. Individually, the methionine, alanine, and the combined glu-

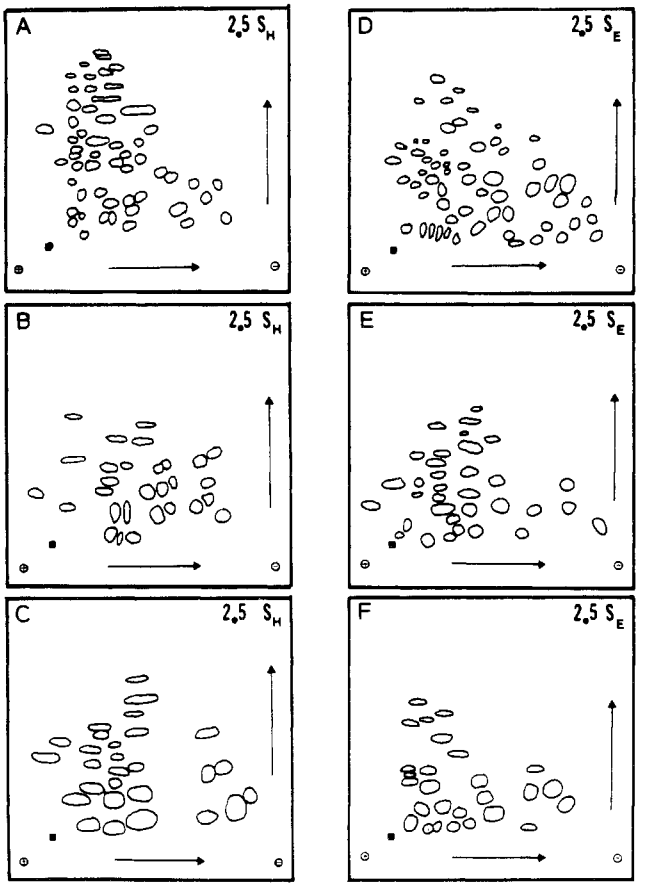

FIGURE 3: Peptide maps of the $2.5S_H$ and $2.5S_E$ polypeptides of transcarboxylase. (A and D) The sample was digested with trypsin and electrophoresed for 5.5 h and then subjected to a double chromatographic development. The peptide map indicates 55 lysyl and arginyl peptides for the $2.5S_H$ polypeptide and 59 for the $2.5S_E$ polypeptide. (B and E) The subunit was digested with AL-1 protease II of myxobacter and electrophoresed for 7 h for B and 5.05 h for E, and then each was chromatographed in the perpendicular direction. The peptide map indicates 29 lysyl peptides for B and 34 for E. (C and F) The ϵ-amino groups of the lysyl residues were succinylated, digested with trypsin, and then electrophoresed for 5.05 h and chromatographed. This peptide map indicates 28 arginyl peptides for C and 27 for F. Electrophoresis was horizontal; chromatography was vertical. The dark square indicates the origin. For experimental methods, see text.

TABLE I: Amino Acid Composition of $2.5S_H$ and $2.5S_E$ Polypeptides of Transcarboxylase.[a]

Amino acid	$2.5S_H$ Molar ratios	$2.5S_H$ Residues per mole	$2.5S_E$ Molar ratios	$2.5S_E$ Residues per mole
Lys	3.44	30	2.82	34
His	1.00	9	1.00	12
Arg	2.82	24	2.15	26
Asp	7.05	61	4.45	53
Thr	3.15	27	2.52	30
Ser	2.85	25	2.03	24
Glu	8.28	71	6.15	73
Pro	3.13	27	2.76	33
Gly	5.80	50	3.45	41
Ala	7.15	62	4.67	56
½-Cystine	0.77	7	0.40	5
Val	4.87	42	3.30	40
Met	2.31	20	2.33	28
Ile	3.15	27	1.55	19
Leu	4.31	37	3.12	37
Tyr	1.23	11	1.30	16
Phe	2.49	22	1.33	16
Trp	0.56	5	0.33	4
Total residues		557		547

[a] The half-cystine and methionine residues were determined on the products of performic acid oxidation as cysteic acid and methionine sulfone, respectively. Tryptophan molar ratios were determined from its molar ratio with histidine by the method of Hugli and Moore (1972). The residues/mole of $2.5S_H$ and $2.5S_E$ subunits were calculated on the basis that both of these subunits have a molecular weight of 60 000 (Green et al., 1972). Since the $6S_H$ and $5S_E$ subunits are composed respectively of two identical subunits, the residues/mole of the $6S_H$ and $5S_E$ are twice the respective values of the $2.5S_H$ and $2.5S_E$ subunits.

tamic acid glutamine residues are present in large quantities.

It has previously been assumed, because single bands are obtained on electrophoresis, that the two $2.5S_H$ polypeptides comprising the $6S_H$ subunit are identical and also that the two $2.5S_E$ polypeptides comprising the $5S_E$ dimer are identical (Green et al., 1972; Wood et al., 1972). This conclusion is now substantiated chemically. Dansylation of the $6S_H$ subunit yields only alanine as the NH_2-terminal amino acid. In addition, Edman degradation of the $6S_H$ subunit (Hartley and Massey, 1956) and the subsequent identification of the phenylthiohydantoin derivative of the NH_2 terminus also gave alanine as the NH_2-terminal residue. The $6S_H$ subunit ($2 \times 2.5S_H$ polypeptides) has 60 lysyl and 48 arginyl residues and, if the two polypeptides were completely different, a tryptic digest should yield 109 peptides. Fifty-five peptides were observed (Figure 3A), which indicates that the two $2.5S_H$ polypeptides comprising the $6S_H$ subunit are identical. The situation is analogous with the $5S_E$ subunit. Dansylation of the $5S_E$ subunit indicated that the NH_2 terminus of the $2.5S_E$ polypeptide is blocked. There are 68 lysyl and 52 arginyl residues in the $5S_E$ subunit (Table I) which would yield 121 peptides after a tryptic digest, if the two $2.5S_E$ polypeptides were different. The peptide maps of Figure 3D show 59 peptides which is, within experimental error, the number expected if the two polypeptides are identical and if there is no duplication in the sequence.

To further confirm that there is no duplication of sequences, a digestion was done with AL-1 protease II from myxobacter, which cleaves specifically at the α-amino group of the lysines (Wingard et al., 1972). Twenty-nine different lysyl peptides were observed for the $2.5S_H$ polypeptide (Figure 3B) out of a possible 30, indicating there is no duplication. In order to perform another specific cleavage, the ϵ-amino groups of lysyl residues were succinylated and the protein digested with trypsin to obtain cleavage at the arginyl groups. Twenty-eight peptides were observed which is three more than the maximum number of 25 expected (Figure 3C). It is likely that a few of the lysine residues may not have been succinylated thereby allowing trypsin to cleave at these unprotected lysine residues. Each of these peptide mapping experiments on the $2.5S_H$ polypeptide indicates that there is no extensive sequence identity in peptides derived from the $2.5S_H$ polypeptide.

Similar experiments were likewise done on the $2.5S_E$ polypeptide. A myxobacter Al-1 protease II digest of the $2.5S_E$ polypeptide should give 35 lysyl peptides if there are no identical sequences and Figure 3E shows 34 lysyl peptides. The tryptic cleavage of the $2.5S_E$ polypeptide in which the lysyl residues had been succinylated gave the expected 27 arginyl peptides (Figure 3F).

Exchange of $[^{60}Co]6S_E$, $5S_E$ *Subunits with Unlabeled Transcarboxylase and Determination of the Distribution by Dissociation of the Resulting 26S Transcarboxylase.* The

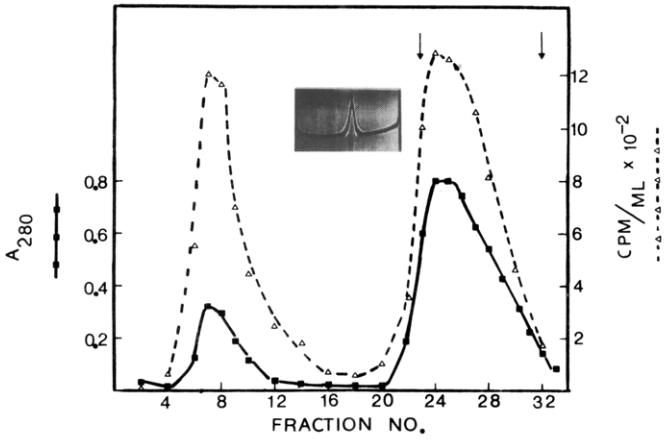

FIGURE 4: Separation of ^{60}Co-labeled 26S enzyme from excess [^{60}Co]-$6S_E$,$5S_E$ subunits by glycerol gradient centrifugation following exchange. The exchange mixture contained in 1 mL: 1.56 mg of the [^{60}Co]$6S_E$ subunit, 2.98 mg of unlabeled $5S_E$ subunit, 0.61 mg of $1.3S_E$ subunit, and 15.7 mg of 26S transcarboxylase (containing 12 biotins per mol). The mixture was dialyzed against 0.1 M phosphate buffer, pH 6.3, at 4 °C for 12 h and then against 0.1 M acetate buffer, pH 5.5, for 3 h. After dialysis, the sample was diluted to 1.8 mL and contained 20.3 mg of protein, 29 440 cpm of ^{60}Co, and 433 units of enzyme. Aliquots (0.3 mL) were layered on six 11-mL glycerol gradients, 10–30% (v/v), buffered with 0.1 M acetate, pH 5.5, and centrifuged at 39 000 rpm at 4 °C for 13 h in a Du Pont Sorvall OTD-65 ultracentrifuge with a Spinco SW 41 head. The gradients were fractionated (0.3 mL per fraction) and the radioactivity and the absorption at 280 nm were measured. Fractions 6–11 were combined for the $6S_E$, $5S_E$ subunit pool and 23–32 for the [^{60}Co]26S transcarboxylase pool as indicated by the arrows. The pool of 26S enzyme was dialyzed vs. 0.1 M acetate, pH 5.5, for 2 h to remove glycerol. The volume was 22 mL of which 4.5 mL was precipitated with $(NH_4)_2SO_4$ (75% saturation) and then dialyzed against 0.1 M acetate buffer, pH 5.5. It was diluted to 2.1 mL and 0.1 mL used for biotin determinations and 2.0 mL for ultracentrifugation (see inset). The remaining 17.5 mL was precipitated with $(NH_4)_2SO_4$ and taken up in 0.7 mL of 0.03 M phosphate, pH 6.8, and treated as described in Figure 5. Inset: The sedimentation velocity profile of the 26S pool. The sample, 1.1 mg in 1.0 mL of 0.1 M acetate buffer, pH 5.5, was centrifuged at 52 000 rpm at 4 °C in a Beckman Model E. The photograph is at 36 min. $s_{20,w}$ = 26.6 S. Sedimentation is from left to right.

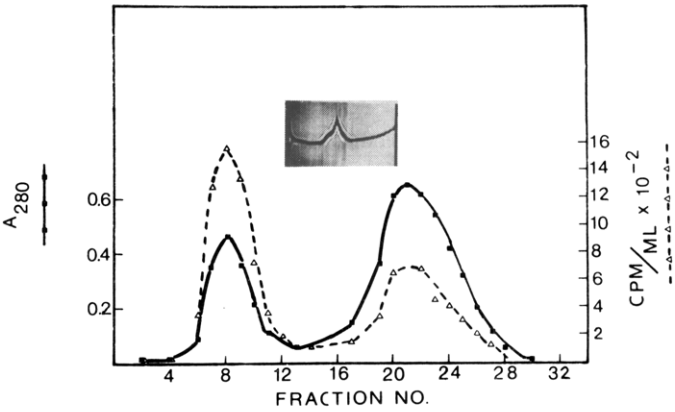

FIGURE 5: Separation of transcarboxylase from $6S_E$, $5S_E$ subunits by glycerol gradient centrifugation following dissociation of the 26S transcarboxylase by dialysis in 0.03 M phosphate buffer, pH 6.8, at 4 °C for 3 h. The mixture, in 0.7 mL, contained 6.0 mg of protein, 7686 cpm and 128 units of enzyme. The glycerol gradient in 0.05 M phosphate buffer, pH 6.8, was done in two tubes as described in Figure 4. Fractions 7–11 were combined for the $6S_E$, $5S_E$ subunits and 17–25 for the transcarboxylase. The combined fractions were each dialyzed vs. 0.1 M acetate, pH 5.5, for 2 h, and the proteins were precipitated with 75% saturated $(NH_4)_2SO_4$. The biotin content and radioactivity were determined and the transcarboxylase was sedimented in the ultracentrifuge after dialysis against 0.1 M acetate buffer, pH 5.5 (see inset). Inset: Conditions as in Figure 4. Photo is at 36 min. $s_{20,w}$ of major peak = 22.1 S and of shoulder, 18.0 S.

purpose of these experiments was to determine whether the exchange and subsequent loss of $6S_E$ subunits were random at the two faces of the $12S_H$ subunit (identical faces) or whether the exchange and loss were preferential (nonidentical faces) (see Figure 1). The experiments consisted of two parts: (1) exchange of [^{60}Co]$6S_E$ plus $5S_E$ subunits with unlabeled 26S transcarboxylase and isolation of the products; (2) dissociation of the isolated [^{60}Co]26S transcarboxylase and then isolation of the resulting $6S_E$ subunits and dissociated form of the transcarboxylase.

1. Exchange of [^{60}Co]$6S_E$, $5S_E$ Subunits with Unlabeled 26S Transcarboxylase and Isolation of the Products. The results from this portion of the experiment are shown in Figure 4 and Table II. The exchange was conducted by dialysis in 0.1 M phosphate buffer at pH 6.3 for about 12 h at 4 °C using [^{60}Co]$6S_E$ plus $5S_E$ subunits and $1.3S_E$ subunit and 26S transcarboxylase in a molar ratio of about 3 to 6 to 1. Complete exchange was not desired since the purpose was to determine whether one type of attached subunit exchanged more rapidly than the other. Following the 12-h dialysis, the mixture was dialyzed for 3 h at 4 °C against 0.1 M acetate buffer at pH 5.5 to stabilize the 26S form of the enzyme. There is no exchange of [^{60}CO]$6S_E$, $5S_E$ subunits into transcarboxylase in this buffer.

The [^{60}Co]transcarboxylase resulting from this exchange was isolated by glycerol gradient centrifugation and had a sedimentation coefficient of 26.6 S (Figure 4). It contained 11.3 ± 0.7 nmol of biotin per nmol of 26S transcarboxylase (Table II). The $6S_E$, $5S_E$ subunit mixture recovered from the exchange contained 0.58 ± 0.05 nmol of biotin per nmol (Table II).

The number of the $6S_E$ subunits that had exchanged with the unlabeled 26S transcarboxylase was estimated from the cpm/nmol of the $6S_E$, $5S_E$ subunits and the cpm/nmol in the 26S transcarboxylase and found to be 3.97 (footnote *c*, Table II). This is probably a maximum value since the specific radioactivity of the $6S_E$, $5S_E$ subunit pool would decrease as the exchange progressed. Since the [^{60}Co]26S transcarboxylase contained (11.3 ± 0.7) biotins, it is clear that most, if not all, of the exchanged subunits contained their full complement of two $1.3S_E$ subunits.

2. Dissociation of the [^{60}Co]26S Transcarboxylase and Isolation of the Resulting $6S_E$ Subunits and Dissociated Transcarboxylase. The results from this portion of the experiment are shown in Figure 5 and Table II. The dissociation (reaction 2a) was done by dialysis at 4 °C against 0.03 M phosphate buffer, pH 6.8, for 3 h. These conditions were selected even though the dissociation was not completely to the 18S form of the enzyme since it was desired to avoid exchange of the $6S_E$ subunits into the 18S enzyme via reaction 2b during this dissociation.

The dissociated transcarboxylase after isolation by glycerol gradient centrifugation gave a major peak on sedimentation (Figure 5) with an $s_{20,w}$ = 22.1 S. From this value it is estimated that this transcarboxylase had about four attached subunits and a mol wt of ~934 000 (Poto and Wood, 1977). Using this value and the determined nmol of biotin per mg of protein, the nmol of biotin per nmol of the transcarboxylase was estimated to be 7.3. In addition, by use of this mol wt, the cpm per nmol of the transcarboxylase was calculated to be 658 and thus the cpm per nmol of the attached $6S_E$ subunits to be 165 (Table II).

The $6S_E$ subunits recovered from the glycerol gradient contained only 0.61 ± 0.12 nmol of biotin per nmol of subunit. Apparently, the $6S_E$ subunit dissociated via reaction 2c to $5S_E$

TABLE II: Exchange of [^{60}Co]6S_E, 5S_E Subunits with 26S Transcarboxylase and Dissociation of the Product to Lower Molecular Weight Transcarboxylase and 6S_E Subunits.

	Biotin content (nmol/mg)	Biotin content (nmol/nmol)	Sedimentation, $s_{20,w}$ (S)	Enzyme act. (U/mg)	Radioactivity (cpm/mg)	Radioactivity (cpm/nmol)	Estimated av. radioact. of attached 6S_E subunit (cpm/nmol)	No. of 6S_E subunits exchanged
Exchange of [^{60}Co] Subunits with 26S Transcarboxylase								
6S_E, 5S_E subunits	4.55 ± 0.25	0.58 ± 0.05[a]	ND[g]		3062	389[b]		
26S transcarboxylase	9.25 ± 0.55	11.3 ± 0.7	26.6	27	1267	1546[c]	258[d]	3.97[c]
Dissociation of [^{60}Co] Transcarboxylase								
6S_E, 5S_E subunits	4.8 ± 1	0.61 ± 0.12[a]	ND[g]		2729	346[b]		
Transcarboxylase	7.8 ± 0.7	7.3 ± 0.7[e]	22.1	29	703	658[e]	165[f]	

[a] If x is the nmol of biotin per nmol of the mixture of 6S_E, 5S_E subunits, x may be calculated from the expression $x/(0.120 + 0.012x)$ = nmol of biotin/mg of the mixture of 6S_E, 5S_E subunits, 0.120 is the weight of a nmol of 5S_E subunit in mg and 0.012 of the 1.3S_E subunit. When the nmol of biotin/mg is 4.55, x = 0.58. The value 0.61 for the mixture of 6S_E, 5S_E subunits from the dissociation of the 26S transcarboxylase was calculated in a similar manner. [b] The average mol wt of a mixture of 6S_E, 5S_E subunits containing 0.58 nmol of biotin is 120 000 + 0.58 × 12 000 = 126 960 where 120 000 is the mol wt of the 5S_E subunit and 12 000 is the mol wt of the 1.3S_E subunit. The cpm per nmol of the 6S_E, 5S_E subunits is 3062 × 0.127 = 389 where 3062 is the cpm/mg and 0.127 the mol wt in mg of a nmol of the 6S_E, 5S_E mixture. The cpm/nmol of the mixture of 6S_E, 5S_E subunits (346) was calculated in a similar manner. [c] The cpm per nmol in the [^{60}Co]26S transcarboxylase has been calculated on the basis of its mol wt (1.22×10^6) and is equal to 1267 × 1.22 = 1546 where 1267 is the cpm/mg in the 26S transcarboxylase. The number of 6S_E subunits that has undergone exchange is estimated to be 1546/389 = 3.97, where 389 is the cpm/nmol in the [^{60}Co]6S_E, 5S_E subunits. [d] The 26S transcarboxylase is considered to contain six attached 6S_E subunits and the ^{60}Co is exclusively in the 5S_E subunit; therefore, the cpm/nmol of 26S transcarboxylase (1546) divided by 6 is the average cpm/nmol in the attached subunits. [e] The major peak on sedimentation was 22.1 S with an 18.0 S shoulder and a very minor shoulder at 25.5 S (Figure 5). It is estimated that the average number of attached subunits of this transcarboxylase was about 4 (Poto and Wood, 1977). Thus, the average mol wt would be 360 000 + 4 × 144 000 = 934 000 where 360 000 is the mol wt of the 12S_H subunit and 144 000 of the 6S_E subunits. Therefore, 7.8 × 0.934 = 7.3 nmol of biotin/nmol of transcarboxylase, where 7.8 is the nmol of biotin per mg of protein and 703 × 0.934 = 658 cpm/nmol of transcarboxylase where 703 is the cpm/mg of the transcarboxylase. [f] The number of attached subunits is considered to be 4. The cpm/nmol in the transcarboxylase was 658 which divided by 4 gives the average cpm/nmol in the attached 6S_E subunits, i.e., 165. [g] ND, not determined.

and 1.3S_E subunits during the dissociation of the 26S transcarboxylase and isolation of the subunits. The average mol wt of the mixture of 6S_E, 5S_E subunits was calculated and the cpm per nmol of the subunit was found to be 346 (Table II). Clearly, this value is significantly larger than that of the subunits which remained attached to the transcarboxylase (165 cpm). Thus, there is little doubt that some subunits of the 26S transcarboxylase exchanged more rapidly and also dissociated more rapidly than others of the 26S transcarboxylase. These results suggest that the two faces of the 12S_H subunit differ structurally. The significance of this observation will be considered in the Discussion.

Discussion

There are some very interesting problems concerning the quaternary structure of transcarboxylase (Figure 1) and the structure of the 12S_H subunit. The hexameric 12S_H subunit apparently provides 12 binding sites for the 12 biotinyl carboxyl carrier proteins of the 6S_E subunits of the 26S enzyme, i.e., two per 2.5S_H polypeptide. All 12 biotins of the 26S enzyme are carboxylated by methylmalonyl-CoA (Wood et al., 1977) and the CoA ester sites are on the 12S_H subunit (Chuang et al., 1975). There are two CoA ester sites per 2.5S_H polypeptide (Poto and Lau, 1977). The dissociation of the 6S_E subunits from the 26S enzyme is selective; three of the six subunits dissociate preferentially from one face of the 12S_H subunit while the three on the opposite face remain attached yielding the 18S form of the enzyme (Wrigley et al., 1977). Thus, there may be structural differences between the two faces of the 12S_H subunit.

Provision for two binding sites per 2.5S_H polypeptide for the 1.3S_E subunits was made by Green et al. (1972), Green (1972), and Wood et al. (1972) by proposing that there is a duplicating sequence in the 2.5S_H polypeptides. This duplication was proposed to arise via gene duplication and fusion. They also proposed a parallel arrangement of the 2.5S_H polypeptides to account for a difference in properties of the two faces of the 12S_H subunit.

Significance of the Peptide Maps. Peptide mapping was undertaken in an attempt to determine whether or not there is a duplicating amino acid sequence in the 2.5S_H polypeptide. The peptide maps showed that all six 2.5S_H polypeptides are identical and by this technique no evidence was obtained of homology within the sequence. Similar results were obtained for the two polypeptides of the 5S_E subunit. Nevertheless, these results do not necessarily exclude homology; they only show that the homology does not involve an identical amino acid sequence in any of the tryptic peptides. That there can be homology without exact duplication of sequence is evident by examination of proteins of known sequence which contain two or three extensive regions of homology and which are considered to have been derived by gene duplication and fusion (Dayhoff, 1972). Wood and Zwolinski (1976) have considered the following proteins: cytochrome c_3 of *Desulfovibrio vulgaris*, the γ heavy chain of immunoglobulin G of rabbit, the protease inhibitor from the lima bean, the β chain of human hemoglobin and the E chain of alcohol dehydrogenase (horse). They found that, if a hypothetical tryptic digestion (cleavage at lysyl and arginyl residues) of these proteins is considered, almost the exact number of peptides would result as expected from the number of lysyl and arginyl residues. Thus, it was necessary in these cases to know the amino acid sequences to demonstrate the homology.

If the 2.5S_H polypeptides originally consisted of two large duplicated sequences, 27 substitutions in the appropriate lysyl and arginyl peptides would give the full number of tryptic peptides expected of a nonduplicated sequence, but a more realistic figure for random substitutions probably is around 84 (15%). This amount of change could still leave a basic homology in a polypeptide of 557 residues (Table I). Such

regions of homology (hydrophobicity and charge type) could be responsible for binding the peripheral subunits at both ends of the $2.5S_H$ subunit. This result would be analogous to the Rossmann and Liljas (1974) finding that there is little sequence homology in the NAD binding portions of several dehydrogenases even though their conformational structural domains are the same.

Peptide mapping has, in some cases, however, successfully demonstrated sequence homology that may have arisen by gene duplication and fusion. The following are examples: human transferrin (Mann et al., 1970), leucyl-tRNA synthetase (Waterson and Konigsberg, 1974), methionyl-tRNA synthetase (Koch et al., 1974; Bruton et al., 1974), and valyl-tRNA synthetase (Koch et al., 1974). With each of these proteins the peptide maps revealed fewer peptides than expected for a nonduplicated sequence.

Exchange Experiments with ^{60}Co-Labeled Subunits. The rationale of the experiments with ^{60}Co-labeled subunits is as follows. (a) If there is a *structural difference* in the two faces of the $12S_H$ subunit and this difference is the cause of the differential dissociation of $6S_E$ subunits from one face of the $12S_H$ subunit, then with the 26S enzyme, an exchange of $6S_E$ subunits with $6S_E$ subunits of the 26S enzyme should be more rapid on one face of the $12S_H$ subunit than on the other face. Likewise, upon dissociation, these *same* subunits which entered by exchange should undergo dissociation most rapidly. Thus, the dissociated subunits would have a higher specific radioactivity than those that remained attached to the enzyme. (b) On the other hand, if the two faces of the $12S_H$ subunit are identical and the selective loss of $6S_E$ subunits from one face of the $12S_H$ subunit of the 26S enzyme is due to cooperativity, then the exchange should be random on the two faces. This situation arises because initially, in the 26S species, there would be an equal chance of loss of a $6S_E$ subunit from either face of the $12S_H$ subunit. Exchange would continue to occur on any face that had already lost a $6S_E$ subunit. Likewise, during the dissociation, the loss of subunits would be most rapid from any face that had lost a subunit. In this case the specific radioactivity of the subunits which remained attached to the enzyme would be the same as those that dissociated.

The results given in Table II show that the cpm per nmol in the dissociated $6S_E$, $5S_E$ subunits was more than twice that of $6S_E$ subunits which remained attached to 18S transcarboxylase (346 compared with 165). These results indicate that the binding of $6S_E$ subunits differs at the two faces of the $12S_H$ subunit and that the structures of the two faces differ. A number of experiments were done which were not as complete as the experiment of Table II (sedimentation profiles not determined). The results (data not shown) were quite similar to those of the experiment of Table II.

Another indication that the structures of the two faces differ has been obtained by dissociation of 26S transcarboxylase which was prepared by addition of $6S_E$ subunits to 18S transcarboxylase which had been treated with trypsin. The 18S transcarboxylase is inactive because the biotin is removed in the form of biotinyl peptides but the $5S_E$ subunits still remain attached to the $12S_H$ subunit (Ahmad et al., 1975). The 26S enzyme is active because of the addition of three $6S_E$ subunits which do contain biotin (Wood et al., 1975). However, on dissociation to the 18S form of the enzyme, the outside subunits are on only one face of the $12S_H$ subunit (Wrigley et al., 1977). If the dissociation was random from the two faces it would be expected that half of the $6S_E$ subunits with biotins would remain attached to the resulting 18S enzyme and that the product would retain half of the enzymatic activity observed with the 26S enzyme. However, when active 26S enzyme was prepared in this manner from trypsinized 18S enzyme and $6S_E$ subunit and then dissociated at pH 6.8, no enzymatic activity remained in the 18S enzyme (unpublished results, J.-P. Chiao and H. G. Wood). This method is not infallible since the effects of trypsin on the 18S enzyme on the face of the $12S_H$ subunit not carrying $6S_E$ subunits are not known and the $6S_E$ subunit might dissociate preferentially from this face.

The test for difference by the exchange procedure, likewise, is not infallible. For example, incorrect conclusions could result if the 26S transcarboxylase used for the exchange did not have a full complement of six $6S_E$ subunits. In this case, if there was cooperativity, those molecules of transcarboxylase which did not have their full complement would undergo exchange more rapidly than those with the full complement. If these same faces remained deficient during the exchange, then during the dissociation to 18S transcarboxylase and $6S_E$ subunits, the release of $6S_E$ subunits would be fastest from these faces. Thus, the attached subunits would have a lower specific radioactivity than those which dissociated and it would appear there was a structural difference in the two faces when none existed. We believe we have overcome this problem by using 26S transcarboxylase which had the full complement of six attached subunits. The enzyme, when assayed, gave 12 ± 0.2 nmol of biotin per 1.2 mg which is within experimental error of 12 per mol of 26S enzyme (mol wt 1.2×10^6). Furthermore, to maintain the 26S form of the enzyme during the exchange, $1.3S_E$ carboxyl carrier subunit was included, thus maintaining the equilibrium so that it was favorable for isolation of 26S transcarboxylase with its full complement of $6S_E$ subunits. This was apparently accomplished. The enzyme following the exchange (fractions 23 to 32 of Figure 3) had a $s_{20,w} = 26.6$ S and contained 11.3 ± 0.7 nmol per 1.2 mg. It should be noted that the specific activity of the enzyme (29 units/mg) is somewhat less than values previously published (Wood et al., 1977). Transcarboxylase often loses activity on storage, but, as far as we can determine, no change in subunit composition parallels this loss. Therefore, we believe that the data are reliable.

A second possible source of error is that $[^{60}Co]6S_E$ subunits might be introduced into the 18S transcarboxylase by exchange during the dissociation of $[^{60}Co]6S_E$ subunits from the $[^{60}Co]26S$ transcarboxylase. If this occurred, the ^{60}Co content of the $6S_E$ subunit and the resulting transcarboxylase might become equal because of this equilibration. This result would tend to make it appear that there was no difference between the two faces of the $12S_H$ subunit. To avoid extensive exchange during the dissociation, a pH of 6.8 was used rather than a more alkaline pH, which might have given more complete dissociation. Furthermore, the period of dissociation was kept relatively short. Since we found a difference in the radioactivity of the dissociated $6S_E$ subunits and those attached to the transcarboxylase, this error could not alter the conclusion. It could only make the observed difference less in magnitude than actually existed after the exchange.

Another possible source of error which is more difficult to assess and control could result if the exchanged subunits in some manner differed from those that did not exchange and the exchanged subunits dissociated more rapidly than the subunits that had not undergone exchange.

The above-described shortcomings of the method are presented for clarification of the procedure but we believe we have conducted the experiments under conditions which minimize these possible sources of error and that the results are quite reliable. To our knowledge, this is the first attempt to demonstrate such a structural difference by a procedure of this type. However, x-ray crystallography probably will be required to provide direct proof of the difference in structure of the two

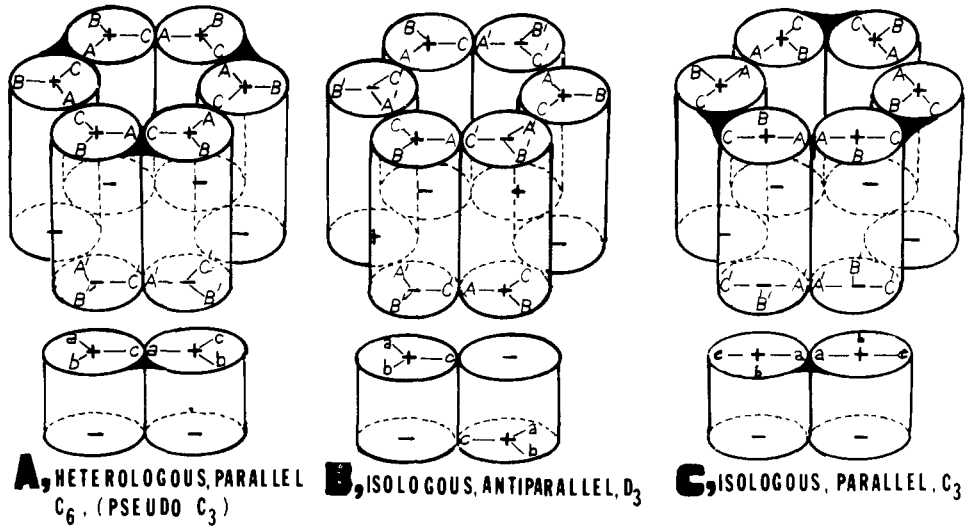

FIGURE 6: Schematic representation of three models for the $12S_H$ and $5S_E$ subunits of transcarboxylase. The polypeptides are represented by cylinders and A, B, C, and A′, B′, C′ and also a, b, c indicate bonding sets as well as subsites, see text for full discussion of conventions adopted. Symmetry of the subsites has been maintained throughout, i.e., the $5S_E$ subunit may be superimposed on the upper face of the $12S_H$ subunit so that b coincides with B or B′ and a, c coincides with A, C, or A′, C′. Also, the symmetry between A, B, C and A′, B′, C′ has been maintained within the $2.5S_H$ polypeptides; i.e., both the top and the bottom letters occur in either a clockwise or counterclockwise order. This fact may not be obvious from the illustration since both sets of letters are presented as if they were viewed from the top. However, if the bottom of the model is viewed from beneath, then the clockwise or counterclockwise order of the letters is seen to be the same (for the bottom sequence, look at it reflected in a mirror). The lettering of these models is somewhat different than that of Wood and Zwolinski (1976). As lettered in their models, the correspondence of a clockwise or counterclockwise symmetry between the top and bottom of the polypeptides of the $12S_H$ subunit was not always present and in addition the subsites on the $5S_E$ subunit were not always superimposable on those of the $12S_H$ subunit. The resulting changes in lettering required modifications of the designations for the bonding domains and substitution of double letters for single letters in models A and B. In models A and B the bonding surface of a cylinder is represented by two letters (A, C′ for example). This combination indicates the polypeptide chain is twisted and both the A surface and C′ surface of the polypeptide participate in the bonding.

faces of the $12S_H$ subunit and verify the reliability of the procedure as applied to transcarboxylase.

Models for Transcarboxylase. The following facts are of particular importance in considering the models of transcarboxylase:

1. The $12S_H$ and $5S_E$ subunits are stable, i.e., they do not polymerize and can be isolated as such.

2. The $1.3S_E$ subunits bind together the $12S_H$ and $5S_E$ subunits (Ahmad et al., 1975).

3. Dissociation of $6S_E$ subunits occurs selectively from one face of the $12S_H$ subunit during the conversion of 26S transcarboxylase to 18S transcarboxylase (Wrigley et al., 1977).

4. There is retention of symmetry between the $12S_H$ and $5S_E$ subunits. This symmetry is required if the measurements of Fung et al. (1974, 1976a,b) are correct.

5. The two faces of the $12S_H$ subunit appear to be structurally different (present results).

6. There appears to be a sequence homology within the constituent polypeptides of the $12S_H$ subunit.

Criterion 6 is included because Poto and Lau (1977) have shown that there are 12 CoA ester sites per 26S transcarboxylase. Since the CoA ester sites are on the hexameric $12S_H$ subunit (Chuang et al., 1975), their results indicate there are two sites per polypeptide. This fact indicates there are two similar parts in the polypeptide even though the difference could not be detected by peptide mapping.

Three models are presented in Figure 6. In these models the $1.3S_E$ subunit is omitted and only the $12S_H$ subunit and one $5S_E$ subunit are considered. The following conventions have been adopted:

1. The polypeptides are represented as cylinders.

2. It is assumed there is homology in the polypeptides of the $12S_H$ subunit and two domains of binding are indicated A, B, C in one portion of the sequence and A′, B′, C′ in the other portion. The primes are used to indicate that the binding forces at these two portions probably are not identical.

3. The $12S_H$ subunit is shown as a hexagon. This geometry requires that each pair of "bonding sets" of the $2.5S_H$ polypeptide be related by an angle of 120°. The darkened areas indicate asymmetric distortions from this 120° angle.

4. Lower case letters a, b, c are used to indicate bonding domains for the $5S_E$ subunit.

5. The letters are also used to indicate subsites; this is useful in visualization of the symmetry between the $2.5S_H$ and $2.5S_E$ polypeptides. For this purpose, B and B′ are assigned as the binding sites for the carboxyl carrier protein on the $2.5S_H$ polypeptide, as is b on the $2.5S_E$ polypeptide. B or B′ binding sites are provided on both faces of the $12S_H$ subunit to account for formation of the 26S form of the enzyme. In addition, A, C and A′, C′ are considered to be the CoA ester subsites on the $12S_H$ subunit and a, c the keto acid subsites on the $5S_E$ subunit.

6. The + and − signs indicate parallel or antiparallel arrangements, respectively, of the polypeptide.

7. Symmetry of the subunits has been maintained throughout (see legend of Figure 6 for a more complete explanation).

Model A is similar to the model proposed by Green et al. (1972) except that these authors did not discuss the symmetry of the $5S_E$ subunit. However, their picture of a model of the $6S_E$ subunit shows a $1.3S_E$ subunit at each end of the $5S_E$ subunit which implies there is twofold symmetry, i.e., isologous bonding rather than the heterologous bonding as shown in Model A of Figure 6. Such bonding, as illustrated in Figure 7, would not maintain symmetry between the $2.5S_H$ polypeptides of the $12S_H$ subunit and the $2.5S_E$ polypeptides of the $5S_E$ subunit. Thus, criterion 4 would not be met.

Whether or not criterion 4 must be met rests heavily on the results of Fung et al. (1974, 1976a,b). By use of NMR and

EPR, they measured the distances of the Co^{2+} of the transcarboxylase from [1-^{13}C], [2-^{13}C], and the protons of bound pyruvate (Fung et al., 1974). The values were 6.3, 5.0, and 6.3 Å, respectively. Fung et al. (1976a) also determined the distances of the protons and phosphorus of propionyl-CoA from the Co^{2+} and Fung et al. (1976b), using a nitroxide analogue of propionyl-CoA determined the distances between the [1-^{13}C], [2-^{13}C], and the methyl protons of bound pyruvate and the bound analogue. The propionyl-CoA was found to have a unique U-shaped orientation around the Co^{2+} with Co^{2+} 6.5 Å from the methylene protons of the propionyl of the CoA ester. The nitroxide radical was found to be 7.9 Å from the methyl group of pyruvate. Since the Co^{2+} (AHMAD et al., 1972) and the keto acid sites (Chuang et al., 1975) are on the $5S_E$ subunit and the CoA ester sites are on the $12S_H$ subunit (Chuang et al., 1975), these results indicate that the $12S_H$ and $5S_E$ subunits must be very close together.

There almost certainly must be retention of symmetry between the $2.5S_H$ polypeptide of the $12S_H$ subunits and the $2.5S_E$ polypeptides of the $5S_E$ subunit if these distances are correct. This conclusion arises from the fact that, if the symmetry were not maintained and there was rotation of the $2.5S_E$ polypeptide relative to that of the $2.5S_H$ polypeptide, the distance of the respective carbons of the pyruvate relative to those of the propionyl-CoA would vary beyond the observed measurements. These relationships are discussed in detail by Wood and Zwolinski (1976) as well as the possibilities of errors in the measurements of the distances.

It is to be noted in Model A that the domains of bonding of the $2.5S_H$ polypeptide are all heterologous but identical in the $12S_H$ subunit (AC′ to CA′ surfaces); therefore, the $12S_H$ subunit would be expected to dissociate to the $2.5S_H$ monomer rather than to stable $6S_H$ dimers. In addition, the $5S_E$ subunit would not be expected to be a stable dimer since it has "open binding sets", a and c (Klotz et al., 1970). Therefore, the $5S_E$ subunit would be predicted to polymerize into an infinite net or to larger closed cyclic structures whereas the $5S_E$ subunit does not polymerize.

In order to account for the formation of $6S_H$ dimers from $12S_H$ subunits, Green et al. (1972) proposed an asymmetric distortion between alternate pairs of the $2.5S_H$ polypeptides (indicated by the shaded areas in Model A of Figure 6) thereby making two different types of heterologous interactions of different strengths. Distortion would also have to occur in the $5S_E$ subunit. Such distortion or some other mechanism would be necessary to prevent self-aggregation via the "open binding sets" of the $5S_E$ subunit.

Model A does account for the difference in dissociation rates of the $6S_E$ subunits from the two faces of the $12S_H$ subunit since the binding sites B for the carboxyl carrier protein on one face of the $12S_H$ subunit differ from those on the other face which are indicated by B′.

Model B presents an example of an antiparallel arrangement of the polypeptides. The bonding is isologous (the A,C′ and C′,A surfaces being identical). Both faces of the $12S_H$ subunit are identical in this Model. Thus, this structure is not in accord with the results of the exchange experiments which indicate the structures are not identical. Model B would account for dissociation of the $12S_H$ subunit to dimers. There are two different types of isologous bonds: A,C′ to C′,A and A′,C to C,A′. Thus, the bonding between pairs of polypeptides differs and dimers would be predicted on dissociation. However, the antiparallel arrangement of the $5S_E$ subunit places the bonding groups b for the carboxyl carrier protein on opposite faces. With this arrangement, it is difficult to account for the fact that each $5S_E$ subunit is bonded to the $12S_H$ subunit via two $1.3S_E$

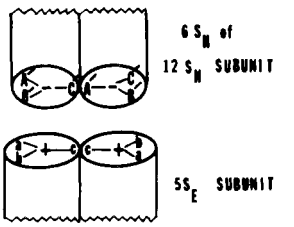

FIGURE 7: Illustration of isologous bonding of the $2.5S_E$ polypeptides of the $5S_E$ subunit in place of the heterologous bonding of $5S_E$ of model A of Figure 5. As a consequence, on the right set of the polypeptides, there is asymmetry between the subsites B′ and b and A′,C′ and a,c.

carboxyl carrier subunits. Furthermore, the keto acid sites a,c are located on opposite faces and, therefore, both sites could not superimpose on a CoA ester site (A′,C′ or A,C) and thus provide equal distances between the sites.

Model C has been proposed by Wood and Zwolinski (1976). The advantage of this model is that it provides two different types of bonding for the polypeptides of the $12S_H$ subunit and thus provides for the formation of dimers. Also, there are no open bonding sets on the $5S_E$ subunit which might cause polymerization. However, the isologous bonding of the C,C′ surface to the C,C′ surface of the $2.5S_H$ polypeptides in the $12S_H$ subunit requires a distortion, which for purposes of illustration is indicated by the shaded areas and the 180° angle between the "binding sets" A and C of each $2.5S_H$ polypeptide. It should be noted that a similar distortion in the constituent $2.5S_E$ polypeptides of the $5S_E$ subunit is required to maintain strict symmetry with the $12S_H$ subunit. Experimental evidence that such a distortion might occur was provided by Wood et al. (1975). They found that a great excess of $1.3S_E$ subunit was ineffective in competing with the $6S_E$ subunit for the $12S_H$ subunit during reconstitution of transcarboxylase. Thus, it appears that a conformational change occurs during combination of the $1.3S_E$ and $5S_E$ subunits which increases the binding capacity of the $1.3S_E$ portion of the $6S_E$ subunit for the $12S_H$ subunit. There is precedent for assuming that distortion could occur since there is a departure from strict symmetry in chymotrypsin (Tulinsky et al., 1973; Vandelin and Tulinsky, 1973) and also in hexokinase (Anderson and Steitz, 1975; Steitz et al., 1976). The latter authors have suggested that in hexokinase there are two monomers with identical sequences, which are related by a 156° axis of rotation and a screw-translation of 13.8 Å along the rotational axis.

Both models A and C meet the requirements of the six facts listed above if the proposed distortions occurred.

Homology between the $2.5S_H$ and $2.5S_E$ Polypeptides. There are a number of features of these two polypeptides which indicate they may have homology. Both the $5S_E$ and $12S_H$ subunits combine with a portion of the carboxyl carrier protein and catalyze the carboxylation of the biotin (Chuang et al., 1975). Both subunits combine with the nonbiotinyl portion of the carboxyl carrier protein (Ahmad et al., 1975). Both polypeptides have molecular weights of 60 000 and associate to form dimers. There is substrate inhibition at high concentrations (Northrop, 1969) indicating the CoA esters bind at the keto acid sites and the keto acids bind at the CoA ester sites. Thus, there may be homology at these subsites. Furthermore, Berger and Wood (1976) prepared antibodies against the $12S_H$ and $5S_E$ subunits and found cross-reactivity of the antibodies toward the subunits. Also, three peptide spots of the tryptic peptide maps of the $2.5S_H$ and $2.5S_E$ polypeptides (Figure 3) appear to superimpose.

Methods have been developed for estimating sequence homology from the amino acid composition of the proteins and

TABLE III: Comparison of the Relatedness of Glutamic Dehydrogenases and the $2.5S_H$ and $2.5S_E$ Subunits.[a]

	Bovine GDH	*Neurospora* GDH	$2.5S_H$ polypeptide	$2.5S_E$ polypeptide
		Deviation ($S\Delta Q$)		
Chicken GDH	5.5	49	40	54
Bovine GDH		67	49	67
Neurospora GDH			37	60
$2.5S_H$ polypeptide				14
		Correlation Coefficient		
Chicken GDH	0.98	0.88	0.91	0.84
Bovine GDH		0.82	0.87	0.80
Neurospora GDH			0.91	0.85
$2.5S_H$ polypeptide				0.97

[a] The values were calculated from the data of Table I and from the data of Blumenthall et al. (1975), Moon and Smith (1973), and Moon et al. (1973). See text. GDH = glutamate dehydrogenase.

we have applied them to the $2.5S_E$ and $2.5S_H$ subunits. Marchalonis and Weltman (1971) have calculated a deviation function, $S\Delta Q$, which is the sum of the squares of the individual differences in the mole percent of each amino acid in two proteins. They found that, in 98% of the pairs of proteins compared, $S\Delta Q$ was greater than 100 for unrelated proteins and in no case was $S\Delta Q$ less than 50 for unrelated proteins. Related proteins such as hemoglobins, immunoglobulins, and cytochromes *c* from various sources gave $S\Delta Q$ values of 80, 30, and 20, respectively (the lower the $S\Delta Q$, the greater homology in the primary structure). However, Dedman et al. (1974) have shown that calculations of this type tend to indicate a spuriously high degree of homology as the proteins being compared increase in size. These authors defined a parameter, the composition coefficient, which essentially represents a correlation coefficient between the amino acid compositions being compared. Less scatter was found using this analysis than those based on deviation functions, but this procedure, likewise, loses reliability with increase in molecular weight. To give some indication of the reliability in the case of larger proteins, the $S\Delta Q$ values and the composition coefficients of the $2.5S_H$ and $2.5S_E$ polypeptides were calculated for comparison with glutamate dehydrogenases (mol wt ~50 000) from three sources (Moon et al., 1973; Moon and Smith, 1973; Blumenthall et al., 1975). As shown in Table III, bovine and chicken glutamate dehydrogenase (95% homology) gave a $S\Delta Q$ of 5.5 and bovine and chicken dehydrogenase when compared with *Neurospora* gave values of 49 and 67 where the homology is ~18%. When the dehydrogenases were compared with either the $2.5S_E$ or $2.5S_H$, $S\Delta Q$ values ranged between 37 and 67, suggesting some degree of homology, whereas none should exist. However, the value of 14 from comparison of $2.5S_E$ and $2.5S_H$ polypeptides is low enough to indicate there may be homology between these subunits.

Bovine and chicken glutamate dehydrogenase gave a composition coefficient of 0.98 whereas comparison with *Neurospora* glutamic dehydrogenase gave values of 0.88 and 0.82. The values when the $2.5S_H$ and $2.5S_E$ subunits were compared with the dehydrogenase ranged from 0.80 to 0.91 again indicating homology which no doubt is erroneous. However, the comparison of $2.5S_H$ with $2.5S_E$ polypeptides gave a value of 0.97 which again indicates there is homology between these subunits. If there is homology it might indicate the subunits had a common origin. However, determination of the sequence of these two polypeptides will be required in order to determine whether or not there is homology between the $2.5S_H$ and $2.5S_E$ polypeptides and also homology within the $2.5S_H$ polypeptide.

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A·I and A·G Polynucleotide Pairing. Controlling Effect of Amino-Group Hydrogen Bonds to Solvent Water†

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ABSTRACT: Poly(2-aminoadenylic acid) (poly($2NH_2A$)) forms a well-defined double helix with poly(I), in contrast to unsubstituted poly(A), but does not form a triple helix under any conditions. This $2NH_2A$·I double helix has the same stability as that of the poly(A)·2poly(I) triple helix. Poly($8NH_2A$) also forms a complex with poly(I), less well-defined than the previous one because of its low stability. In the $2NH_2A$·I complex there is not enough space for a solvent water molecule to be hydrogen bonded to one of the 2-amino protons when I is on the N(1) side of A. Conversely, in the $8NH_2A$·I pair the hydrogen-bonding potential of the 8-NH_2 group could not be satisfied because of steric exclusion of water when pairing occurs at N(7) of A but could be satisfied when pairing occurs at N(1) of A. We conclude that it is the inability of 2-NH to make a hydrogen bond to water in a triple helix which restricts pairing to a 1:1 complex and that the structure of the 2-NH_2A·I complex involves bonding at N(7) of A rather than N(1). The possibility that base pairing is restricted by direct steric interference of the A 2-NH_2 group with poly(I) has been excluded by examining the interaction of poly(2MeA) with poly(I). The latter polymers form only a 1:2 complex, which has the same stability as poly(A)·2poly(I). Since relevant geometric features of A·G pairing are the same as those of the above $2NH_2A$·I systems (the 2-substituents being merely transposed), the same conclusion can be applied to A,G interaction. Base pairing of G to either the N(1) or the N(7) side of A is prevented by steric exclusion of a solvent water molecule, necessary to satisfy the hydrogen-bonding potential of the amino groups. The poly($2NH_2A$)·poly(I) helix is the first purine·purine heteroduplex helix to be described. The I is bonded to N(7) of A, and the two strands are presumably parallel. In A·I complexes of either 1:1 or 1:2 stoichiometry, bonding at N(7) is more stable than bonding at N(1), in striking contrast to A·U systems.

Interactions of A and I residues in polynucleotides are of interest in several contexts. Studies with A and I in polynucleotide model systems help to establish fundamental principles of polynucleotide structure and reactivity, with special reference to purine–purine interactions. There is a biological interest in the A·I pair because of the presence of I as the third base in the anticodon of many tRNA molecules and in the nature of its pairing with A in messenger RNA during protein synthesis. Finally, a possible role of poly(A)[1] and poly(I) as the basis of a primitive prebiotic self-replicating system has been suggested. In this paper, we approach A, I interactions in the first of these contexts and discuss the implications of our results for the other two.

Interaction of A and G would be of major importance if such pairing could be observed, but it has in fact never been demonstrated. Rich (1959) had suggested that guanine could replace hypoxanthine in an A·I pair, since the additional amino group attached to C(2) of the purine ring would not introduce any steric interference. In considering nonstandard base pairs to account for observed degeneracy in the third letter of mRNA codons, Crick (1966) proposed wobble pairings of U to G or I and of I to A. He concluded, however, that a G·A pair to N(1) of A would not occur, "because the NH_2 group of guanine cannot make one of its hydrogen bonds, even to water".

The possibility of G pairing to A at N(7) rather than at N(1)

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[1] Abbreviations used are: poly($2NH_2A$), poly(2-aminoadenylic acid); poly(I), poly(inosinic acid); poly(G), poly(guanylic acid); poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(2MeA), poly(2-methyladenylic acid); poly($2NH_2A$)·poly(I), 1:1 complex of these components; poly(2MeA)·2poly(I), 1:2 complex of these components; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.