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Stereochemistry of Propionyl-Coenzyme A and Pyruvate Carboxylations Catalyzed by Transcarboxylase†

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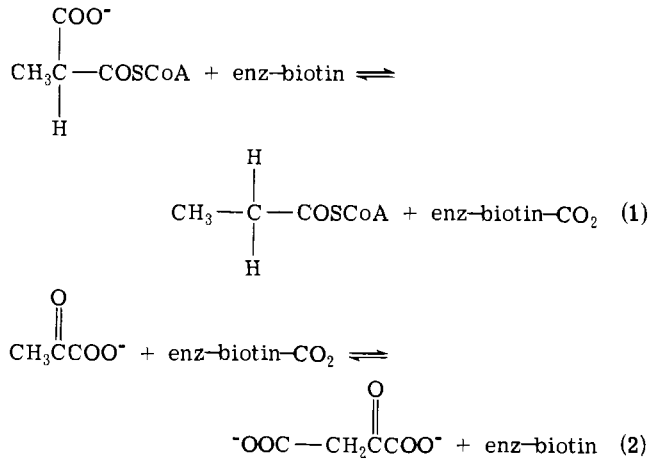
ABSTRACT: The stereochemistry of the two half-reactions catalyzed by the biotin-containing enzyme, transcarboxylase from *Propionobacteria shermanii,* has been determined. The *pro-R* hydrogen at C-2 of propionyl-coenzyme A is replaced by CO_2 in formation of the *S* isomer of methylmalonyl-CoA, defining the process as retention of configuration. This C-2 hydrogen is abstracted at a rate identical with product formation. For the other half-reaction, pyruvate to oxalacetate, the chiral methyl group methodology of Rose (I. A. Rose (1970), *J. Biol. Chem. 245,* 6052) was employed. First, it was determined with [3-$^2H_3$]pyruvate that a kinetic deuterium isotope effect of 2.1 occurs at V_{max} in this carboxyl transfer, indicating that the necessary requirement for discrimination against heavy isotopes of hydrogen existed. Then, 3(*S*)-[3-^{2}H,^{3}H]pyruvate, generated from 3(*S*)-[3-^{2}H,^{3}H]phosphoglycerate, was carboxylated and the oxalacetate trapped as [3-^{3}H]malate using malate dehydrogenase. Exhaustive incubation of the tritiated malate ($^3H/^{14}C$ = 1.95) with fumarase to labilize the *pro-R* hydrogen at C-3 resulted in release of 65% of the tritium into water. Reisolation of the malate after fumarase action yielded a $^3H/^{14}C$ ratio of 0.67, indicating 34% retention as expected. The theoretical enantiotopic distribution for the observed k_{1H}/k_{2H} of 2.1 is 68:32. Selective enrichment of tritium in the *pro-R* position at C-3 of malate indicates enzymatic carboxylation of pyruvate with retention of configuration in this half-reaction also.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase, EC 2.1.3.1) is a metalloenzyme, containing covalently bound biotin, which has been purified from *Propionobacteria shermanii* (Wood et al., 1969). This multimeric enzyme catalyzes the carboxyl transfers of eq 1 and 2. This sequence has been supported by kinetic studies (Northrop, 1969), by partial exchange reactions (Northrop and Wood, 1969), and by the isolation and subsequent reutilization of the carboxybiotinyl-enzyme intermediate (Wood et al., 1963). As with other biotin carboxylases [e.g., bacterial acetyl-CoA carboxylase (Polakis et al., 1974)], it is reasoned that acyl-CoA substrates bind to regions of the active site distinct from regions where keto acid substrates bind (quite probably on distinct subunits) with the biotinyl carrier protein serving as a swinging arm mechanism (Northrop, 1969).



On the other hand, transcarboxylase is an unusual biotin enzyme in at least one sense: ATP and bicarbonate are not substrates, there is no carboxyl activation step, and no energy input is required. In a mechanistic sense it is as though transcarboxylase were composed, in addition to the biotinyl carrier subunit, of the substrate specific subunits of two other well-known ATP-dependent biotin carboxylases, pyruvate carboxylase and propionyl-CoA carboxylase.

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In this regard, it is known that both pig heart propionyl-CoA carboxylase (Retey and Lynen, 1965) and transcarboxylase (Allen et al., 1963; Kellenmeyer et al., 1964) convert propionyl-CoA to (*S*)-methylmalonyl-CoA. It has been determined that the pig heart enzyme does so with specific abstraction of the *pro-R* hydrogen at C-2 of propionyl-CoA (Arigoni et al., 1966; Prescott and Rabinowitz, 1968), thus defining the stereochemical course of carboxylation as retention of configuration. For pyruvate carboxylase, Rose has taken advantage of the isotope effect in pyruvate carboxylation coupled with the use of pyruvate samples containing chiral methyl groups to determine that oxalacetate is likewise formed with retention (Rose, 1970).

In this paper we report the determination of the stereochemistry of both carboxyl transfer steps catalyzed by transcarboxylase and compare these results to those in the ATP-dependent biotin carboxylases.

Experimental Section

Materials. Coenzyme A (lithium salt), *cis*-oxalacetic acid, L-homoserine, pyridoxal 5′-phosphate, sodium pyruvate, and NADH were purchased from Sigma Chemical Company, and (*R,S*)-methylmalonyl-CoA was from PL Biochemicals. All other chemicals and solvents were of commercial reagent grade.

Pig heart propionyl-CoA carboxylase (EC 6.4.1.3) was purified according to the procedures of Kaziro et al. (1961), omitting the alumina gel treatment. The enzyme had a specific activity of 0.7 unit/mg at 20°, and was free of ATPase and methylmalonyl-CoA isomerase activities. Transcarboxylase containing 0.3 mol of Co, 6.4 mol of Zn, 0.7 mol of Cu, and 4.8 mol of biotin per 790,000 mol wt multimer was prepared by Wood's procedure (Fung et al., 1974) as described earlier (Wood et al., 1969) to a specific activity of 27.4 units/mg at 25°, and at the time of the experiments its specific activity was 26 U/mg, when assayed for the production of oxalacetate with malic dehydrogenase. Rat liver γ-cystathionase (specific activity, about 5 μmol per min per mg) was a generous gift from W. Washtien. Lactic dehydrogenase and phosphoglycerate mutase were purchased from Sigma Chemical Company. Malate dehydrogenase, pyruvate kinase, fumarase, and enolase were from Boehringer Mannheim Biochemicals.

The sodium salt of [2-^{14}C]propionic acid (14 Ci/mol) and tritiated water (1 Ci/ml) were purchased from New England Nuclear. The sodium salt of [2-^{14}C]pyruvic acid (6.7 Ci/mol) was also from New England Nuclear; 50 μCi of this was purified by adding to it 1.3 mg of carrier sodium pyruvate, loading onto a small column of Dowex 1 (Cl^- form) column, and elution with 20 m*M* HCl. 3(*S*)-[3-^{3}H,^{2}H]Phosphoglyceric acid (specific radioactivity ca. 3.3 × 10^6 dpm/μmol) was a generous gift from I. A. Rose and H. P. Meloche.

(*R,S*)-[2-^{3}H]Propionic acid was prepared from methylmalonic acid by a previous reported method for the synthesis of (*R,S*)-[2-^{2}H]propionic acid (Murray and Williams, 1958), where 15 mCi of ^3H_2O in a total volume of 1.5 ml was used instead of ^2H_2O.

(*S*)-[2-^{3}H]Propionic acid was prepared by the oxidative decarboxylation of (*S*)-[3-^{3}H]-2-ketobutyric acid, which was in turn synthesized enzymatically by the γ elimination of L-homoserine catalyzed by γ-cystathionase (Krongelb et al., 1968). The incubation mixture contained (in μmoles) sodium phosphate buffer (75, pH 7.5), EDTA (6), 2-mercaptoethanol (6), L-homoserine (300, pH 7.5), 250 mCi of ^3H_2O, pyridoxal phosphate (7.5), and 6.5 units of γ-cystathionase for 1 hr, at the end of which 0.2 ml of 30% H_2O_2 was added to convert the ketobutyrate to propionate. After standing at room temperature for 1 hr, excess H_2O_2 was destroyed by catalase. Excess ^3H_2O was removed by repeated lyophilization. The residue was taken up in 5 ml of water, acidified to pH 1, and the (*S*)-[2-^{3}H]propionic acid continuously extracted into ether. An aliquot of the ether extract was analyzed by thin-layer chromatography (TLC) (silica gel plate, developed in ethanol–NH_4OH, 95:5). Only one radioactive spot was recovered, corresponding exactly to the spot of authentic [2-^{14}C]propionate (R_f 0.45, whereas for α-ketobutyrate, R_f 0.59). The amount of (*S*)-[2-^{3}H]propionate recovered was determined by titration on a pH meter with 0.100 *N* NaOH from a microburet, generating a sharp inflection in the titration curve; 200 μmol of (*S*)-[2-^{3}H]propionate were thus obtained.

(*R*)-[2-^{3}H]Propionic acid was similarly prepared from (*R*)-[3-^{3}H]-α-ketobutyric acid which was enzymatically synthesized from the elimination reaction (Walsh et al., 1973) of D-[α-^{3}H]-*erythro*-β-chloro-α-aminobutyric acid, catalyzed by D-amino acid oxidase.[1]

The acyl-CoA derivatives were prepared from the corresponding ^{3}H-labeled propionic acids by the mixed anhydride method of Arigoni et al. (1966), and subsequently purified by the paper chromatograph system of Bresseler and Wakil (1961), then stored frozen as 1 m*M* HCl solutions. Omission of the paper chromatography step resulted in irreproducibility of subsequent enzymatic experiments.

[3-^{3}H]Pyruvic acid was prepared according to the procedure of Rose (1960). [3-$^2H_3$]Pyruvic acid was similarly prepared, except that 99.8% ^2H_2O was used as the solvent instead. ^2H_2O was also used throughout the whole chromatographic work-up to avoid isotopic dilution via exchange processes. The purified [3-$^2H_3$]pyruvic acid was determined to be at least 95% isotopically pure by nuclear magnetic resonance (NMR) analysis.

(*S*)-[3-^{3}H,^{2}H]Pyruvic acid was prepared from 3(*S*)-[3-^{3}H,^{2}H]-3-phosphoglyceric acid by a modified procedure of H. P. Meloche (private communication). The incubation mixture contained (in μmoles) imidazole buffer (25, pH 6.5), KCl (25), $MgCl_2$ (25), ADP (5, pH 6.5), 4.5 nmol of 2,3-diphosphoglyceric acid, EDTA (0.5), 3(*S*)-[3-^{3}H,^{2}H]phosphoglyceric acid (2, including carrier), 1.6 unit of enolase, and 2 units of pyruvate kinase in a total volume of 0.25 ml. The reaction was started by the addition of 10 units of phosphoglycerate mutase and allowed to proceed at room temperature for 20 min, at the end of which an aliquot was withdrawn, and the pyruvate content determined by assaying with lactic dehydrogenase; 2 μmol (quantitative conversion) of (*S*)-[3-^{3}H,^{2}H]pyruvate was afforded. The whole incubation solution was immediately frozen. The (*S*)-[3-^{3}H,^{2}H]pyruvate therein could be carboxylated to oxalacetate by transcarboxylase without prior isolation.

(*R,S*)-[3-^{3}H]Malate was prepared enzymatically from [3-^{3}H]pyruvate and (*R,S*)-methylmalonyl-CoA, using transcarboxylase, coupled to the immediate NADH-linked reduction of the (*R,S*)-[3-^{3}H]oxalacetate to (*R,S*)-[3-^{3}H]malate by malic dehydrogenase. The incubation mixture contained (in μmoles) Tris-HCl buffer (100, pH 7.4), NADH (0.3), $[NH_4]_2SO_4$ (25), (*R,S*)-methylmalonyl-CoA (0.4), [3-^{3}H]pyruvate (0.75), and 30 units of malate dehydrogenase in a total volume of 1 ml in a Beckman standard

[1] Y. F. Cheung and C. Walsh, unpublished observation.

Table I: Stereochemistry of the Transcarboxylase-Catalyzed Carboxylation of Pyruvate.[a]

Substrate	Malate Isolated: Total ^3H-Content (cpm)	Malate Isolated: ^3H_2O Released by Fumarase (cpm)	Malate Isolated: % Release of ^3H	Malate Recovered from Paper Chromatograph: $^3H/^{14}C$ Ratio before Fumarase Treatment	Malate Recovered from Paper Chromatograph: $^3H/^{14}C$ Ratio after Fumarase Treatment	Malate Recovered from Paper Chromatograph: % Retention of ^3H in Malate
Achiral [3-^{3}H] pyruvate	11,100	5,900	53	1.76	0.87	49
(*S*)-[3-^{2}H,^{3}H] Pyruvate	22,300	14,400	65	1.95	0.67	34

[a] (*R,S*)-[3-^{3}H]Malate and purportedly chiral [3-^{3}H]malate were prepared and purified, from achiral [3-^{3}H]pyruvate and (*S*)-[3-^{2}H,H,^{3}H]-pyruvate, respectively, as described in the Experimental Section. Independently prepared [2-^{14}C]malate was added to achieve appropriate $^3H/^{14}C$ ratios. The fumarase incubation contained 50 μmol of NaP_i buffer (pH 7.2), an aliquot of the [2-^{14}C,3-^{3}H]malate, and 20 units of fumarase in a total volume of 1.0 ml. The reaction was allowed to proceed at room temperature for 1 hr when isotopic equilibrium had clearly been reached, at the end of which the solution was frozen and lyophilized. The ^3H_2O was collected and counted both for ^3H and volatile ^{14}C. No ^{14}C contamination was observed. The residue was dissolved in a minimum amount of water and streaked onto Whatman No. 1 paper. The [2-^{14}C,3-^{3}H] malate was recovered by the aforementioned paper chromatographic system. Fumaric acid (R_f 0.73) moved ahead of malic acid (R_f 0.38). The $^3H/^{14}C$ ratio of the recovered malate sample was determined.

silica cell. The reaction was started by the addition of 2.6 units of transcarboxylase, and allowed to proceed to 30°. The rate of production of malate was followed by the decrease of A_{340} on a Gilford spectrophotometer. Additional aliquots of NADH, (*R,S*)-methylmalonyl-CoA, and [3-^{3}H]pyruvate were added at intervals to yield 1.25 μmol of (*R,S*)-[3-^{3}H]malate. To the incubation solution was added 2.5 μmol of carrier L-malate, and diluted to a total volume of 25 ml. This was passed through a Dowex 1 (Cl^- form] column (0.7 × 5 cm). After washing with 10 ml of water, the column was developed with 25 m*M* HCl, malic acid eluting before pyruvic acid. The radioactive fractions corresponding to (*R,S*)-[3-^{3}H]malic acid were pooled and titrated to neutrality, and could be used without further concentration. Paper chromatography in 1-pentanol saturated with 5 *M* formic acid (Johnson and Hatch, 1969) showed that the radioactive malate was free of contaminating [^{3}H]pyruvic acid.

[2-^{14}C]Malate and (*R*)-[3-^{3}H]-L-malate (whose chirality was subsequently determined as reported in the results section) were prepared in the same manner respectively from [2-^{14}C]pyruvate and the aforementioned enzymatically synthesized (*S*)-[3-^{3}H,^{2}H]pyruvate.

The radiochemical purity of [^{3}H]malate samples was further checked by mixing with independently prepared [^{14}C]malate and ensuring that $^3H/^{14}C$ ratios remained constant in the malate after paper chromatography, confirming that all the tritium was in the malate and not in some contaminant. The radiochemical purity of the (*R,S*)-[3-^{3}H]malate was confirmed by the data of Table I.

Methods. Radioactivity was determined by scintillation counting in 10 ml of Beckman Cocktail D, consisting of 100 g of naphthalene and 5 g of diphenyloxazole per liter of solution in dioxane. The scintillation samples in all cases were adjusted such that 1 ml of water was present, in order to correct for the quenching effect. In the cases of doubly labeled samples, the net ^3H radioactivity was obtained by correcting for the ^{14}C-cross-over, which was separately standardized.

The concentration of purified propionyl-CoA samples was determined by the ATP-dependent carboxylation with propionyl-CoA carboxylase. The ADP thus produced was assayed spectrophotometrically by the coupled enzyme system of pyruvate kinase and lactic dehydrogenase as previously reported (Tietz and Ochoa, 1962). The reaction was run at 30°, monitored by the decrease in A_{340}, until the rate was negligible. The amount of intact propionyl-CoA was estimated from the total decrease in A_{340}. Values of the specific radioactivity for the various [2-^{3}H]propionyl-CoA samples agreed with the ones calculated for their precursor propionic acids. This, together with the fact that exactly 50% of the ^3H label of (*R,S*)-[^{3}H]propionyl-CoA samples was released as ^3H_2O by propionyl-CoA carboxylase, consistent with the previous observation by Prescott and Rabinowitz (1968), supported both the validity of this methodology and the fact that the various [^{3}H]propionyl-CoA's were radiochemically pure.

The chirality of the [3-^{3}H]malate formed by carboxylation of 3(*S*)-[^{3}H,^{2}H]pyruvate was determined by treating a sample with sufficient fumarase, which was known to release only the *pro-R* proton at 3-C in the course of the elimination (Anet, 1960; Gawron et al., 1961), until isotopic equilibrium with the solvent was established (Rose, 1970).

Results and Discussion

Stereochemistry of Propionyl-CoA Carboxylation. It is known from the work of Wood and his colleagues that in the carboxylation of propionyl-CoA from oxalacetate catalyzed by transcarboxylase, the methylmalonyl-CoA formed is specifically the *S* isomer (Allen et al., 1963; Kellenmeyer et al., 1964). This is the same isomer as produced in the ATP and HCO_3^- dependent carboxylation of propionyl-CoA carried out by pig heart propionyl-CoA carboxylase. With this latter enzyme the stereochemical path of methylmalonyl-CoA formation was determined by unambiguous synthesis of (*S*)-[2-^{3}H]propionyl-CoA (Arigoni et al., 1966), (*R*)- and (*S*)-[2-^{2}H]propionyl-CoA (Prescott and Rabinowitz, 1968), incubation with propionyl-CoA carboxylase, followed by analysis of either the tritium or deuterium content in derivatives of the methylmalonyl-CoA formed. These data suggested removal of hydrogen from the *pro-R* position at C-2 of propionyl-CoA and defined the stereochemical course as retention of configuration. An additional observation of note was conducted with [2-^{3}H]propionyl-CoA that had been prepared from the decarboxylation of methylmalonyl CoA in ^3H_2O. Reincubations of the chiral tritiated acyl-CoA with ATP, bicarbonate, and enzyme indicated that tritium washout into water occurred at exactly the rate of methylmalonyl-CoA formation (Prescott and Rabinowitz, 1968), ruling out either a rate-determining abstraction of the *pro-R* C-2 hydrogen or a detectable preequilibrium enolization in the carboxylation mechanism. We have used and confirmed these observations with propionyl-

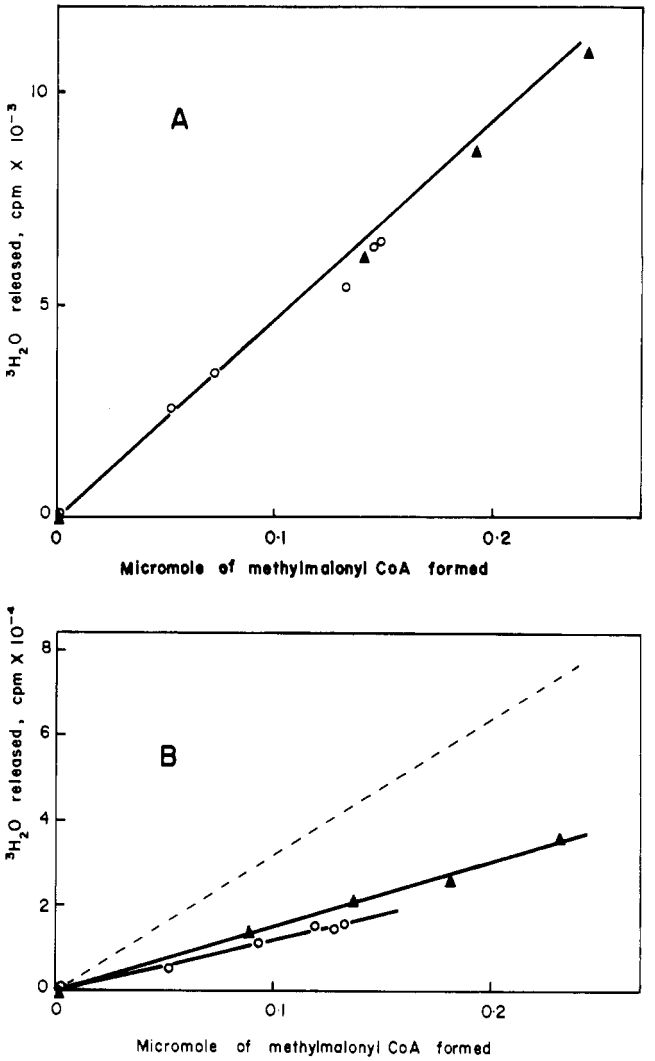

FIGURE 1: ^3H_2O release by propionyl-CoA carboxylase (▲), and transcarboxylase (O) from: (A) (*R,S*)-[2-^{3}H]propionyl-CoA; (—) the theoretical 50% ^3H_2O release (for chiral proton abstraction) based on the specific radioactivity of 93,000 cpm/μmol, determined as described in the Experimental Section, and (B) (*S*)-[2-^{3}H]propionyl-CoA; (- - - -) the theoretical 50% ^3H_2O release based on the specific radioactivity of 640,000 cpm/μmol. The propionyl-CoA carboxylase incubations contained (in μmoles) Tris buffer (150, pH 8.0), $[NH_4]_2SO_4$ (37.5), NADH (0.6), $KHCO_3$ (150), ATP (2.5), phosphoenolpyruvate (1), Mg_2SO_4 (5), dithiothreitol (1.2), 50 units of lactic dehydrogenase, 2 units of pyruvate kinase, and 0.25 μmol of either (*R,S*)-[2-^{3}H]propionyl-CoA or (*S*)-[2-^{3}H]propionyl-CoA in a total volume of 1.6 ml. The reaction was started by the addition of 0.16 mg of propionyl-CoA carboxylase. The transcarboxylase incubations contained (in μmoles) Tris buffer (150, pH 8.0), $[NH_4]_2SO_4$ (37.5), NADH (0.6), oxalacetate (0.3), 50 units of lactic dehydrogenase, and 0.25 μmol of either (*R,S*)-[2-^{3}H]-propionyl-CoA or (*S*)-[2-^{3}H]propionyl-CoA in a total volume of 1.6 ml. The reaction was started by the addition of 44 μg of transcarboxylase. All experiments were conducted at 30°, and the rate was monitored by the decrease in A_{340} on a Gilford spectrophotometer; 0.2-ml aliquots were withdrawn at intervals and immediately delivered into a prechilled round-bottom flask and frozen in liquid nitrogen; 0.8 ml of H_2O was added to the same flask and again frozen. The ^3H_2O was collected by lyophilization of the sample. The amount of methylmalonyl-CoA formed was estimated by the corresponding decrease in A_{340}.

CoA carboxylase as a calibration for the transcarboxylase experiments.

(R,S)-[2-^{3}H]Propionyl-CoA. The first point with transcarboxylase was to determine in similar fashion at what rate one of the C-2 hydrogens of propionyl-CoA was removed during carboxyl transfer from oxalacetate. The data of Figure 1A indicate experiments with (*R,S*)-[2-^{3}H]propionyl-CoA prepared as described in the Experimental Section. The graph indicates the rate of tritium washout, measured as tritiated water production, compared to the rate of methylmalonyl-CoA formation produced by propionyl-CoA carboxylase action. Given a specific activity of 93,000 cpm/μmol we expect production of ^3H_2O at 50% that specific activity since only ^{3}H at the C-2 *pro-R* position is removed by the enzyme. The straight line represents the theoretically predicted washout rate and the experimental points are in good agreement. This control experiment confirms the radiopurity of the (*R,S*)-[2-^{3}H]propionyl-CoA and indicates that the methodology is reliable in our hands. The apparent lack of nonenzymatic exchange of tritium out of the (*S*)-[2-^{3}H]methylmalonyl-CoA was consistent with the model studies of Overath et al. (1962), in which (*S*)-methylmalonyl-*N*-succinylcysteamine was reported to have a half-time of 2500 min at pH 8.1 with respect to the exchange of the methine hydrogen with solvent protons.

The action of transcarboxylase on this racemic tritiated propionyl-CoA is also presented in Figure 1A and the data are indistinguishable from that provided by propionyl-CoA carboxylase. This immediately establishes two points: (1) transcarboxylase removes only one proton at C-2 during propionyl-CoA carboxylation; (2) proton removal is neither the slow step nor is there a preequilibrium enolization of the thiol ester.

(S)-[2-^{3}H]Propionyl-CoA. At this point identification of the C-2 hydrogen removed from propionyl-CoA as the *pro-R* or *pro-S* will complete the stereochemical definition as retention or inversion of configuration, respectively. We chose to synthesize a chiral [2-^{3}H]propionyl-CoA by a combination of enzymatic and chemical syntheses. The work of Krongelb et al. (1968) indicates that in D_2O rat liver γ-cystathionase catalyzes γ elimination of water from L-homoserine to enzyme-bound aminocrotonate. This is then ketonized to 2-iminobutyrate with solvent deuterium entering only at the 3*S* position. This was proved by converting the free [3-^{2}H]-2-ketobutyrate product to [2-^{2}H]propionate with H_2O_2. The optical rotatory dispersion (ORD) spectrum of the enzymatic product was identical with authentic 2(*S*)-[^{2}H]propionate. By repeating the γ-cystathionase reaction in ^3H_2O, we isolated (*S*)-[2-^{3}H]propionic acid which after purification was converted chemically to (*S*)-[2-^{3}H]propionyl-CoA with a final specific activity of 640,000 cpm/μmol.

To check the chiral purity of the 2*S*-tritiated acyl-CoA we submitted it to carboxylation by propionyl-CoA carboxylase and analyzed the rate of tritium washout into water as shown in Figure 1B. A pure (*S*)-[2-^{3}H] sample should not release any tritium into water, since this carboxylase is specific for removal of the *pro-R* proton at C-2. The experimental points define a line with a slope of 23%, suggesting the propionyl-CoA sample has 77% of the tritium at C-2 in the *pro-S* position and 23% in the *pro-R* position. This 23% deviation from chiral purity may stem from some nonenzymatic randomization of tritium at the ketobutyrate stage during the 60-min γ-cystathionase incubation; the 77% content suffices to confirm that propionyl-CoA carboxylase removes the *pro-R* hydrogen.

When transcarboxylase was assayed with the *S*-[2-^{3}H] sample, again a small amount of tritium was released, with the experimental points defining a line of 17% slope. This result shows that transcarboxylase also removes the *pro-R*

hydrogen at C-2 of propionyl-CoA; by this assay the sample is 83% *S* compared to 77% *S* with propionyl-CoA carboxylase.

(*R*)-[2-^{3}]*Propionyl-CoA.* As a corollary to these experiments, it seemed useful to check whether transcarboxylase would in fact quantitatively release radioactivity from a preparation of (*R*)-[2-^{3}H]propionyl-CoA. We had on hand a sample of [2-^{3}H]propionyl-CoA which by propionyl-CoA carboxylase assay releases 80% of the total tritium in assays similar to those of Figure 1A and B, indicating an 80% *R* content. As will be detailed elsewhere,[1] this sample derived from a [3-^{3}H]ketobutyrate sample which in turn was generated in a reaction of D-amino acid oxidase with [2-^{3}H]-2-amino-3-chlorobutyrate. When a sample of this *R*-enriched-[2-^{3}H]propionyl-CoA was submitted to transcarboxylase action, 80% of the total tritium washed out into water at a rate equal to methylmalonyl-CoA formation.

These results with the chiral samples of [2-^{3}H]propionyl-CoA delineate the stereochemistry of acyl-CoA half-reaction catalyzed by transcarboxylase as proceeding with retention of configuration.

Stereochemistry of Pyruvate Carboxylation. In the second half-reaction catalyzed by transcarboxylase the methyl group of pyruvate is converted into the methylene of oxalacetate. The question arises as to whether the carboxyl transfer from the *N*-carboxybiotinyl enzyme intermediate occurs with retention or inversion at C-3 of pyruvate. This question can be answered in principle as a result of the incisive and elegant experiments of Rose (Rose, 1970, and references therein) on the preparation and stereochemical assignment of the isotopically enantiomeric forms of pyruvate. Indeed, Rose has determined that the pyruvate conversion to oxalacetate by the ATP and bicarbonate dependent pyruvate carboxylase occurs with retention.

The following conditions are necessary for the stereochemical determination of this transcarboxylase half-reaction: a known chiral species of pyruvate, a discrimination against the heavier isotopes of hydrogen in the carboxyl transfer to pyruvate, and a methodology for assaying selective retention or loss of tritium in the oxalacetate product. The first condition was met by a gift of 3(*S*)-[3-^{2}H,^{3}H]phosphoglycerate from I. A. Rose and H. P. Meloche (see Rose, 1970). It has been established by Rose that this chiral 3-phosphoglycerate sample can be rapidly and quantitatively converted to 3(*S*)-[3-^{2}H,^{3}H]pyruvate in one tube by reaction of phosphoglycerate mutase, enolase, and pyruvate kinase. The third condition has also been established by Rose in his studies on pyruvate carboxylase. The [3-^{2}H,^{3}H]oxalacetate formed by pyruvate carboxylase was trapped by malate dehydrogenase and NADH to yield the more stable [3-^{2}H,^{3}H]malate. The determination of distribution of tritium at the C(3-*S*) and C(3-*R*) positions of the L-malate can then be determined by incubation with fumarase in H_2O until isotopic equilibrium has been achieved. The stereospecificity of fumarase allows assignment of the tritium recovered as ^3H_2O to the tritium initially present in the *pro-R* position at C-3 of malate.

Kinetic Isotope Selection with Pyruvate. Thus, the second condition of the above paragraph was tested. Does transcarboxylase show a kinetic isotope effect against the heavier isotopes of hydrogen at C-3 of pyruvate? Without such an isotope selection pyruvate molecules with chiral methyl groups will not yield [3-^{3}H]malate with stereoselective enrichment related to the stereochemistry of proton displacement by the incoming carboxyl group.

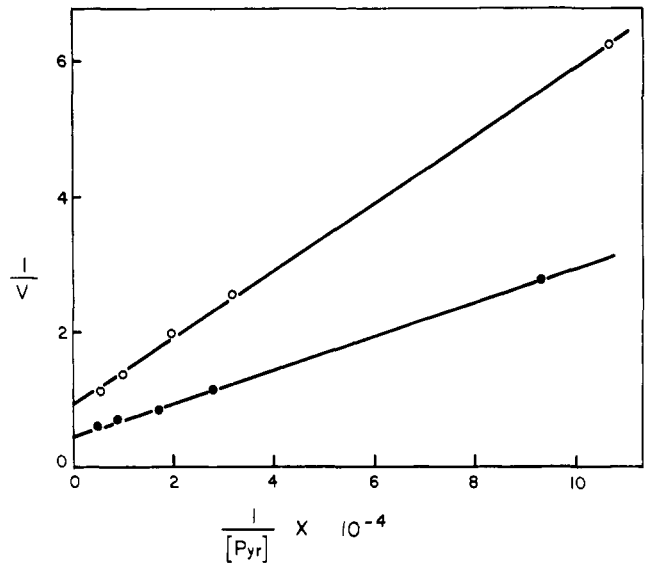

FIGURE 2: Double reciprocal plots of initial velocity of transcarboxylation, expressed in ΔOD_{340}/min vs. concentrations of [3-$^1H_3$]pyruvate (●), and [3-$^2H_3$]puruvate (○). The reaction mixtures contained (in μmoles) Tris buffer (75, pH 7.4), $[NH_4]_2SO_4$ (9.4), NADH (0.3), 0.34 μmol of (*R,S*)-methylmalonyl-CoA, 30 units of malic dehydrogenase, and various amounts of protio or deuteriopyruvate. The reaction was started by the addition of 14 μg of transcarboxylase at 30°, and the rate was monitored by the decrease in A_{340} on a Gilford spectrophotometer. Total volume was 0.85 ml.

We initially attempted to determine the kinetic isotope selection with achiral [^{3}H]pyruvate. Thus [^{3}H]pyruvate and methylmalonyl-CoA were incubated with transcarboxylase, and the oxalacetate formed monitored by malate dehydrogenase. Tritium release during the reaction was measured as ^3H_2O recovered. Due to high backgrounds from nonenzymatic enolization of the [^{3}H]pyruvate, probably during isolation and storage of the compound, a precise number was difficult to obtain, but the selection appeared greater than threefold. In order to confirm this observation we proceeded to determine the deuterium kinetic isotope effect with [$^2H_3$]pyruvate. [$^2H_3$]Pyruvate was kept as a solution in D_2O, and was added second to last to the incubation mixture, immediately followed by the addition of the enzyme transcarboxylase to start the reaction. And since only the initial velocities were required, the problem with nonenzymatic enolization was essentially eliminated. The data of Figure 2 show double reciprocal plots for the protio and trideuteriopyruvates, extrapolated to infinite substrate concentrations. The V_{max} for [3-$^1H_3$]pyruvate is 26 μmol per min per mg while that for [3-^{2}H]pyruvate is 12 μmol per min per mg, so k_{1H}/k_{2H} = 2.1 for this transcarboxylase half-reaction. Extrapolation of both lines of Figure 2 yields an intersection on the abcissa, indicating, as expected, an identical k_m value of 0.55 m*M*.

Stereochemistry with 3(S)-[3-^{2}H,^{2}H]Pyruvate. The k_{1H}/k_{2H} of 2.1 suggested that with a chiral [3-^{2}H,^{3}H]pyruvate sample the maximum stereoselection expected would be a 68:32 partition for ^{3}H in the two enantiotopic positions at C-3 of malate, given absolute chiral purity of the starting pyruvate. Thus, if transcarboxylase carboxylates 3(*S*)-[3-^{2}H,^{3}H]pyruvate with retention of configuration, we expect 68% of the ^{3}H at C-3 of malate to be washed out into water after fumarase treatment, indicating *pro-R* placement, and 32% to be retained indicating a *pro-S* placement.

First we wished to determine that in our hands we could

get reliable numbers from the assay system and decided to use the achiral [3-^{3}H]pyruvate which should give a 50:50 distribution in the C-3 enantiotopic positions of the L-malate. We decided to add [^{14}C]malate to the tritiated sample so that the double label ratio (^{3}H/^{14}C) of the L-malate generated could be checked before and after fumarase treatment. Line 1 of Table I indicates that by analysis of distillable radioactivity after fumarase treatment, 53% of the total tritium in the malate sample had washed out as compared with the 50% expected. When the ^{3}H/^{14}C ratio of the malate, isolated by thin-layer chromatography free from fumarate, was examined after fumarase treatment it was 0.49 the value of the initial ^{3}H/^{14}C ratio, compared to the theoretical 0.50 value expected. This experiment validated the analytical system in our hands.

3(*S*)-[3-^{2}H,^{3}H]Pyruvate was generated from the chiral (*S*)-3-phosphoglycerate by the enzymatic sequence alluded to above (see Experimental Section) and the keto acid immediately carboxylated in situ with transcarboxylase and methylmalonyl-CoA, continuously trapping the oxalacetate as the stable 3-tritiomalate which was then purified and some authentic [^{14}C]malate added to a ^{3}H/^{14}C ratio of 1.95. Fumarase treatment yielded the data of line 2 in Table I; 64% of the initial tritium in malate was released as ^3H_2O. The ^{3}H/^{14}C ratio of the malate recovered after fumarase action was 0.67, a value of 0.34 compared to the initial value. For the k_{1H}/k_{2H} effect of 2.1, the theoretical distribution for retention would have been 68:32, *pro-R*:*pro-S*, so the experimental agreement is excellent and suggests the (*S*)-3-phosphoglycerate and the 3(*S*)-pyruvate generated therefrom were of high chiral purity. Obviously, tritium from 3(*S*)-[3-^{2}H,^{3}H]pyruvate is preferentially accumulated in the 3*R* position of the L-malate and hence in the *pro-R* position at C-3 of the oxalacetate. This result implies retention of configuration (see Scheme II, Rose, 1970).

One might like to see the reciprocal experiment performed with 3(*R*)-[^{2}H,^{3}H]pyruvate to look for the inverse distribution. However, the chiral 3(*R*)-3-phosphoglycerate sample was not available, and two additional factors mitigated against its utility. The synthesis of this isomer reported by Rose yielded material with 200-fold lower specific radioactivity and it appeared to be of lower chiral purity. For instance if the 3(*R*)-pyruvate had a chiral purity of 85%, the calculated transcarboxylase distribution would be 57:43 which is probably close to the limit of experimental differentiation from a 50:50 partition.

The stereochemical path for the *P. shermanii* biotin-containing transcarboxylase then is retention of configuration in both half-reactions. This is the identical stereochemical outcome as catalyzed by the ATP-dependent biotin enzymes propionyl-CoA carboxylase and pyruvate carboxylase. This suggests mechanistic similarity between the transcarboxylation subunits of all three enzymes. More generally it may augur that all biotin dependent enzymes will carboxylate their specific acyl-CoA and keto acid substrates with retention of configuration. In the acyl-CoA carboxylation steps of both propionyl-CoA carboxylase and transcarboxylase, the C-2 *pro-R* hydrogen is abstracted at a rate identical with product formation. Conversely, in the keto acid carboxylation half-reactions, enolization is at least partially rate determining for both transcarboxylase (k_{1H}/k_{2H} = 2.1) and the pyruvate carboxylase (k_{1H}/k_{2H} = 4.2, Rose, 1970).

It has yet to be determined whether biotin enzyme carboxylations are concerted or nonconcerted reactions (Polakis et al., 1974). As Rose (1970) indicated, the concerted mechanism proposed by Mildvan and Scrutton (1967) requires retention. If the enzymatic carboxylation process is discrete carbanion formation followed by carboxylation, then addition of CO_2 is restricted to front-side attack by the stereochemical results.

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