The Participation of an Enzyme-bound Oxygen Group in a Coenzyme A Transferase Reaction*

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SUMMARY

A well supported mechanism for succinyl-CoA:3-ketoacid coenzyme A transferase (EC 2.8.3.5) involving an enzyme-CoA intermediate requires the participation of an oxygen atom of the enzyme or of the CoA moiety. Measurements of loss of $^{18}O$ from acetoacetate show that CoA oxygens do not participate. An oxygen-containing group on the enzyme is reversibly labeled from succinate-$^1$O in the presence of acetoacetyl-CoA or succinyl-CoA. The $^{18}$O-labeled enzyme does not contain succinyl or CoA moieties. A binding of acetoacetate-$^{14}$C to the enzyme preparation does not appear to reflect an anhydride precursor to the enzyme-CoA form.

Succinyl-CoA:3-ketoacid coenzyme A transferase (EC 2.8.3.5) (referred to herein as CoA transferase) catalyzes the transfer of CoA from succinyl-CoA to a 3-ketoacid carboxyl group (Equation 1).

\[
\text{Succinyl-CoA} + 3\text{-ketoacid} \rightarrow 3\text{-ketoacyl-CoA} + \text{succinate} \tag{1}
\]

The enzyme, discovered independently by Green et al. (1) and by Stern, Coon, and del Campillo (2), was first isolated in an almost pure state from pig heart by Stern et al. (3). The enzyme is specific for succinyl-CoA but a variety of C₂ to C₆ 3-ketoacids serve as CoA acceptors, the most active being acetoacetate. The enzyme activity is not decreased by presence of iodoacetate (3), indicating that free CoA does not have an appreciable lifetime in the over-all reaction. Reports of unpublished experiments† that CoA transferase catalyzes the exchange of succinate into succinyl-CoA in the absence of acetoacetate and of labeled acetoacetate into acetoacetyl-CoA in the absence of succinate led to the postulate that the over-all reaction proceeds through two half reactions with an enzyme-CoA intermediate (4) (Equations 2 and 3).

\[
\text{Acetoacetyl-CoA} + E \rightleftharpoons E\text{-CoA} + \text{acetoacetate} \tag{2}
\]
\[
E\text{-CoA} + \text{succinate} \rightleftharpoons E + \text{succinyl-CoA} \tag{3}
\]

The observation that $^{18}O$ from the succinate carboxyl group was transferred to the acetoacetate formed from acetoacetyl-CoA (6) led to the suggestion that the reaction proceeded through formation of a mixed anhydride between the carboxylic acid substrates or between the carboxylic acids and enzyme. In order to avoid the thermodynamic barrier to anhydride formation and account for the apparent lack of free CoA formation, Jenck (7) proposed a concerted mechanism proceeding through a four-centered transition state. The more recent data of Hersh and Jencks provide elegant evidence that the reaction proceeds through an enzyme-CoA intermediate (8, 9). Their conclusion is based on a detailed kinetic analysis of the over-all reaction, which followed "ping-pong" kinetics, and the isolation and properties of an E-CoA derivative. The E-CoA forms succinyl-CoA or acetoacetyl-CoA on addition of succinate or acetoacetate, and it has properties indicative of a thiol ester bond between CoA and enzyme.

If the CoA transferase reaction does proceed through an E-CoA intermediate, an indirect transfer of oxygen between the acid substrates is required (Equations 4 and 5).

\[
E + \text{R-C-SCoA} \rightleftharpoons E\text{-SCoA} + \text{R-C-O}^- \tag{4}
\]
\[
E\text{-SCoA} + \text{R-C-O}^- \rightleftharpoons E + \text{R-C-SCoA} \tag{5}
\]

The first reaction consists of formation of the E-CoA intermediate and free acetoacetate from enzyme and acetoacetyl-CoA. If this reaction occurs, the carboxyl oxygen of free acetoacetate must be derived from enzyme or from the CoA portion of the thiol ester. Similarly during the second partial reaction, the carboxyl oxygen of succinate must be donated to enzyme or CoA to discharge E-CoA and form free enzyme and succinyl-CoA.

In this publication we report that an oxygen-containing group on the enzyme participates in the CoA transferase reaction.

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† Supported by Training Grant GM 463 of the United States Public Health Service.
‡ The exchange of labeled succinate into succinyl-CoA was found in unpublished experiments of Gilvarg cited in Reference 4. The exchange of acetoacetate into acetoacetyl-CoA was found in unpublished experiments of Stern and del Campillo cited in Reference 6.
Materials and Methods

Enzymes and Enzyme Assays—CoA transferase was prepared through the second (NH₄)₂SO₄ fractionation as described by Hersch and Jencks (8). The dialyzed enzyme preparation was then chromatographed on a DEAE-Sephadex column (4 x 40 cm) previously equilibrated with 0.005 m potassium phosphate (P₁), pH 7.0. The nonabsorbed protein was removed by washing with 2 liters of the same buffer. The CoA transferase activity was then eluted with 2 liters of 0.005 m P₁-0.05 m KCl, pH 7.0. Fractions of 15 ml were collected. The use of DEAE-Sephadex is essential as DEAE-cellulose supplied by the Whatman Chemical Company inactivated CoA transferase and the CoA transferase activity was not retained on DEAE-cellulose supplied by Sigma. Most experiments could be performed with enzyme at this stage of purity as it was free of detectable deacylase activity.

If a more purified preparation was desired, the protein was chromatographed on calcium phosphate gel prepared by the method of Siegelman, Wieczorik, and Turner (10). The sample was first dissolved in 0.005 m P₁-0.05 m KCl, pH 7.0, and applied to a calcium phosphate column (2 x 30 cm) previously equilibrated with 0.005 m P₁, pH 7.0. The column was developed with a linear gradient consisting of 1 liter each of 0.05 m P₁ and 0.10 m P₁, both at pH 7.4. A summary of one preparation is given in Table I. For unknown reasons the chromatographic methods were not always reproducible. Protein was estimated by the method of Warburg and Christian as described by Layne (11).

The enzyme assay described by Stern et al. (3) cannot be used with preparations containing deacylase or thiolase activity. For this reason an assay system was developed which could be used at all stages of purity. The assay consisted of measuring the succinate-dependent loss of acetoacetyl-CoA, measured by the change in absorbance in 310 μm. The assay medium contained 67 mM Tris-Cl, pH 8.1, 5.33 mM MgCl₂ and 0.05 mM acetoacetyl-CoA in a total volume of 1 ml at 25°C. Enzyme (0.1 to 1 unit) was added and the loss of acetoacetyl-CoA was measured for 2 to 3 min. The loss of acetoacetyl-CoA during this time is due to contaminating deacylase and thiolase enzymes. Succinate was then added to a final concentration of 1 mM and the loss of acetoacetyl-CoA was measured for an additional 3 to 5 min. The succinate-dependent loss of acetoacetyl-CoA used to determine the CoA transferase activity is linear until 50% of the acetoacetyl-CoA is consumed. One unit of enzyme as defined by Stern et al. (3) will form 0.0025 pmole of acetoacetyl-CoA per min at 25°C. In the assay system described, 1 Stern unit is equivalent to a change in absorbance at 230 μm reported by Stadtman (14). Acetoacetyl-CoA was synthesized from diketene (K and K Fine Chemicals) and CoA by the method of Lynen et al. (15). Acetoacetyl-CoA concentrations were determined by using the absorption coefficients of 7.7 x 10⁵ M⁻¹ cm⁻¹ for succinyl-CoA and 3.2 x 10⁵ M⁻¹ cm⁻¹ for CoA at 230 μm reported by Stadtman (14). Acetoacetyl-CoA was synthesized from diketene (K and K Fine Chemicals) and CoA by the method of Lynen et al. (15). Acetoacetyl-CoA concentrations were determined by using the absorption coefficients of 11.9 x 10⁵ M⁻¹ cm⁻¹ at 310 μm as reported by Stadtman (14). The thiol esters were purified by chromatography on DEAE-cellulose, formate form, as described by Parks and Cha (16). The thiol esters were stored as the lyophilized powders at -20°C. Acetoacetate was prepared by hydrolysis of ethyl acetoacetate as described by Selsky (17). ¹⁸O-Labeled acetoacetate was prepared by hydrolysis of ethyl acetoacetate-3-¹⁸O (New England Nuclear Corporation) with an equivalent amount of KOH for 24 hours at 25°C. Unhydrolyzed ester was removed by extraction with an equal volume of ether at 25°C. Ether remaining in the solution was removed by brief exposure to vacuum. The acetoacetate prepared in this manner contained approximately 6% of a radioactive contaminant which could not be converted to acetoacetyl-CoA by CoA transferase. ¹⁸O-Labeled succinate was purified by chromatography on DEAE-Sephadex, formate form, as described by Falcione and Boyer (6), and ¹⁸O-labeled glutamate as described by Boyer, Keppe, and Luchinger (18).

Assays—CoA associated with CoA transferase was determined using the α-ketoglutarate dehydrogenase system of Garland, Shepherd, and Yates (19) as modified by Robinson (20). With the use of this method 0.1 μmole of CoA could be detected. Prior to the assay, any E-CoA present was first hydrolyzed to enzyme and CoA by incubation at pH 7.4 for 5 hours at 0°C.

Acetoacetate was decarboxylated as described by Falcione and Boyer (6). Succinate oxygen was converted to CO₂ by pyrolysis with guanidine hydrochloride. Succinic acid (2 to 10 μmoles) was isolated from reaction mixtures by extraction with isobutyl alcohol-benzene. About 80% of the succinate was extracted into the organic layer. The isobutyl alcohol-benzene extracts were evaporated with a stream of air filtered through a cotton plug. Succinic acid was dissolved in 0.2 ml of water, transferred to a breakseal tube, and lyophilized by brief exposure to vacuum. The acetoacetate prepared in this manner contained approximately 6% of a radioactive contaminant which could not be converted to acetoacetyl-CoA by CoA transferase. ¹⁸O-Labeled succinate was purified by chromatography on DEAE-Sephadex, formate form, as described by Falcione and Boyer (6), and ¹⁸O-labeled glutamate as described by Boyer, Keppe, and Luchinger (18). The thiol esters were purified by chromatography on DEAE-cellulose, formate form, as described by Parks and Cha (16). The thiol esters were stored as the lyophilized powders at -20°C.

Summary of CoA transferase purification from 1 kg of pig heart

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Total units</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial extract</td>
<td>36,400</td>
<td>0.5</td>
<td>18,200</td>
</tr>
<tr>
<td>Heat-acid treatment</td>
<td>5,030</td>
<td>2.9</td>
<td>14,700</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>3,500</td>
<td>3.4</td>
<td>11,500</td>
</tr>
<tr>
<td>Succinate</td>
<td>67</td>
<td>120</td>
<td>8,050</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>6.4</td>
<td>750</td>
<td>4,830</td>
</tr>
</tbody>
</table>

Determinied by using the absorption coefficients of 7.7 x 10⁵ M⁻¹ cm⁻¹ for succinyl-CoA and 3.2 x 10⁵ M⁻¹ cm⁻¹ for CoA at 230 μm reported by Stadtman (14). Acetoacetyl-CoA was synthesized from diketene (K and K Fine Chemicals) and CoA by the method of Lynen et al. (15). Acetoacetyl-CoA concentrations were determined by using the absorption coefficients of 11.9 x 10⁵ M⁻¹ cm⁻¹ at 310 μm as reported by Stadtman (14). The thiol esters were purified by chromatography on DEAE-cellulose, formate form, as described by Parks and Cha (16). The thiol esters were stored as the lyophilized powders at -20°C. Acetoacetate was prepared by hydrolysis of ethyl acetoacetate as described by Selsky (17). ¹⁸O-Labeled acetoacetate was prepared by hydrolysis of the ester in H₂¹⁸O (Yeda Research Corporation). Acetoacetate-3-¹⁸O was prepared by hydrolysis of ethyl acetoacetate-3-¹⁸O (New England Nuclear Corporation) with an equivalent amount of KOH for 24 hours at 25°C. Unhydrolyzed ester was removed by extraction with an equal volume of ether at 25°C. Ether remaining in the solution was removed by brief exposure to vacuum. The acetoacetate prepared in this manner contained approximately 6% of a radioactive contaminant which could not be converted to acetoacetyl-CoA by CoA transferase. ²⁻¹⁸O-Labeled succinate was purified by chromatography on DEAE-Sephadex, formate form, as described by Falcione and Boyer (6), and ²⁻¹⁸O-labeled glutamate as described by Boyer, Keppe, and Luchinger (18).

Promase, B grade, was purchased from Calbiochem. Leucine aminopeptidase was purchased from Worthington. Before use the preparation was dialyzed overnight against 1 liter of 5 mM Tris-Cl-5 mM MgCl₂, pH 8.5.

Substrates—Sucinyl-CoA was synthesized from succinic anhydride and CoA (P-L Biochemicals) by the method of Simon and Shenin (13). Succinyl-CoA concentrations were determined by the method of McDonald (12) with values 17% higher than the spectrophotometric method.

Hersh and Jencks (9) reported that the protein concentration determined by the method of E. coli (12) gives values 17% higher than the spectrophotometric method.
Succinate standards gave the expected \(^{18}O\) content with this per mg) was incubated with 4 mM acetoacetate-\(^{18}O\) (0.22 atom \% excess), 1 mM acetocetyl-CoA, 0.02 \(\mu\)g F, pH 7.4, at 25° in a final volume of 1.0 ml. Reactions were stopped at the times indicated by freezing the sample with liquid nitrogen. The samples were lyophilized to dryness and the acetocetyl was decarboxylated as described by Falcone and Boyer (6). The data presented are averages of duplicate incubations.

procedure did not exchange carboxyl oxygens with water. \(^{18}O\)-Sucinate standards gave the expected \(^{18}O\) content with this procedure of isolation and decarboxylation.

Glutamate and aspartate (1 to 5 \(\mu\)moles each) were isolated from amino acid mixtures on Amberlite CG-4B, Cl\(^{-}\) form (21). To amino acid mixtures obtained from enzymatic digestion of CoA transferase, aspartate-2-\(^{13}C\) (about 10,000 dpm) and glutamate-2-\(^{13}H\) (about 100,000 dpm) were added to aid in identification. The sample was then applied to an Amberlite column (0.5 \(\times\) 5 cm) previously washed with 100 ml of distilled water, followed by 10 ml of distilled water. Glutamic and aspartic acids were then eluted with about 200 ml of 0.003 \(N\) HCl as fractions of 1 to 2 ml. Fractions containing glutamic or aspartic acid were combined and lyophilized to dryness. Conversion of glutamic or aspartic oxygens to CO\(_2\) was accomplished as described for succinic acid. Control experiments showed that glutamate-4\(^{18}O\) isolated in this manner did not exchange oxygens. After aspartic acid was eluted, the column was washed with 15 ml of 0.1 \(M\) HCl, and the eluate containing low molecular weight peptides was lyophilized to dryness. The oxygen atoms in the fraction were converted to CO\(_2\) with Hg(CN)\(_2\) and HgO\(_2\) as described by Anbar and Guttmann (22).

CoA transferase was digested to free amino acids by treatment with Pronase and leucine aminopeptidase (probably contaminated with prolidase). In a typical experiment 5 \(\mu\)g of denatured CoA transferase in 5 mm Tris-Cl, 5 mm MgCl\(_2\), pH 8.5, were incubated with 0.1 \(\mu\)g of Pronase for 24 hours at 37°. The solution was then heated to 100° for 10 min. After cooling, 1 \(\mu\)g of leucine aminopeptidase was added and incubated at 37° for 24 hours. Protein remaining was then precipitated by adding an equal volume of 1% picric acid. The precipitate was removed by centrifugation. Aspartate and glutamate were recovered for \(^{18}O\) analysis as described above.

To determine \(^{14}C\)-acetocetyl bound to CoA transferase, \(^{14}C\)-acetocetyl and \(^{14}C\)-acetocetyl-CoA were removed by washing the precipitated protein with acetone-H\(_2\)O (4: 1, v/v) at 0°. For the experiments reported in this paper, 1 ml of 6 \(M\) urea, pH 7.1, was added to denature the CoA transferase, followed by 100 \(\mu\)moles of unlabeled acetocetyl. After thorough mixing, 5 ml of acetone-H\(_2\)O were added to precipitate the protein and the mixture was filtered through a glass fiber filter (Whatman Chemical Company) on a Hirsch funnel with suction. Denatured protein remains on the filter pad. The protein was then washed with ten 0.5-ml portions of acetone-H\(_2\)O. After the final wash, the filter pad was placed in 0.5 ml of 0.1 \(M\) KOH and \(^{14}C\) was determined. With the use of this method, protein could be separated from substrates in 2 to 3 min.

Levels of \(^{18}O\) in the CO\(_2\) were determined by measurement of the mass ratio (46: 44) on a Consolidated Engineering Corporation mass spectrometer. \(^{14}C\) and \(^{3}H\) were determined in Bray’s solution (23) with a Packard Tri-Carb liquid scintillation spectrometer.

Fig. 1. Retention of substrate oxygen during acetocetyl-CoA \(\Rightarrow\) acetocetyl exchange. CoA transferase (0.24 \(\mu\)g, 750 units per mg) was incubated with 4 mm acetocetyl-\(^{18}O\) (0.22 atom \% excess), 1 mm acetocetyl-CoA, 0.02 \(\mu\)g F, pH 7.4, at 25° in a final volume of 1.0 ml. Reactions were stopped at the times indicated by freezing the sample with liquid nitrogen. The samples were lyophilized to dryness and the acetocetyl was decarboxylated as described by Falcone and Boyer (6). The data presented are averages of duplicate incubations.

RESULTS

Retention of Substrate Oxygen during Acetocetyl-CoA \(\Rightarrow\) Acetocetyl Exchange—To determine if oxygen from the CoA portion of acetocetyl-CoA participate in the CoA transferase reaction, the acetocetyl-CoA \(\Rightarrow\) acetocetyl exchange reaction was conducted with a catalytic amount of CoA transferase. \(^{18}O\) was present originally in the acetocetyl carboxyl group. After the exchange has reached isotopic equilibrium, the \(^{18}O\) should be diluted by an amount equivalent to the carboxyl oxygen in the thiol ester bond. However, if oxygen from the CoA portion of the thiol ester participates in the reaction mechanism, the dilution will be greater than that expected. As can be seen in Fig. 1, the dilution of \(^{18}O\) was only that expected from the carboxyl oxygen. This result eliminates the possibility of a facile equilibration of CoA oxygens with the carboxyl group of substrates. The experiment cannot, however, detect participation of oxygen in amount stoichiometric with the enzyme.

Participation of an Enzyme Oxygen in CoA Transferase Reaction—The experiments shown in Table II establish that an oxygen-containing group on the enzyme participates in the CoA transferase reaction. Substrate amounts of enzyme were incubated with acetocetyl-CoA and succinate-\(^{18}O\). The enzyme was then freed of all substrates by Sephadex G-50 filtration. Re-incubation of this enzyme with unlabeled succinate and succinyl-CoA resulted in the appearance of \(^{14}C\) in succinate in an amount significantly higher than a zero time sample. The lack of \(^{18}O\) in succinate isolated from the zero time sample (alkali added before the \(^{18}O\) labeled enzyme) shows that no succinate \(^{18}O\) was isolated with the enzyme. A succinyl group on a thiol, carboxyl, or imidazole residue of the enzyme would be expected to be cleaved by the alkaline conditions used. Also, in separate experiments under the same conditions, no \(^{14}C\) (<0.02 mole per mole of enzyme) from added succinate-\(^{14}C\) was detected. Similar results were also obtained (Table II) when the initial incubation was conducted with succinyl-CoA and succinate-\(^{18}O\), eliminating the possibility that the \(^{18}O\) was derived from contaminating acetocetyl-\(^{18}O\). The enzyme also contained no detectable CoA (less than 1 CoA per 10 enzyme molecules). Thus the \(^{18}O\) observed after incubation must be derived from an \(^{18}O\)-containing group on the enzyme. This experiment cannot establish the minimum number of oxygens per catalytic site that participate in the reaction because of the uncertainty in the concentration of active sites. However, it is clear from the experimental results that enzyme oxygens do become equilibrated with substrate oxygens during the catalytic sequence.
Possible Nature of $^{18}O$-Containing Residue in CoA Transferase—

Identification of the residue containing $^{18}O$ would be of obvious value and interest, but it is handicapped by the limited amount of enzyme available and the problems attendant to $^{18}O$ analysis. If the residue is on an amino acid, a means of hydrolyzing the protein to amino acids without oxygen exchange is essential. Since both acidic and basic hydrolysis of CoA transferase would exchange the $\gamma$-carboxyl oxygen of glutamate with water (24), an enzymic digestion with Pronase and leucine aminopeptidase was used. With conditions as given under "Materials and Methods," the enzymic digestion released at least 90% as many free amino acids as did conventional acid hydrolysis. This means that prolidase activity was likely present in the commercial leucine aminopeptidase preparation (25). Glutamate-$^{18}O$ incubated under identical conditions followed by re-isolation retained at least 65% of its original $^{18}O$. This result established that if $^{18}O$-labeled glutamate or aspartate is released during digestion at least 90% of it is converted to amino acids without oxygen exchange is essential. If the residue is on an amino acid, a means of hydrolyzing the protein to amino acids without oxygen exchange is essential. Since both acidic and basic hydrolysis of CoA transferase would exchange the $\gamma$-carboxyl oxygen of glutamate with water (24), an enzymic digestion with Pronase and leucine aminopeptidase was used. With conditions as given under "Materials and Methods," the enzymic digestion released at least 90% as many free amino acids as did conventional acid hydrolysis. This means that prolidase activity was likely present in the commercial leucine aminopeptidase preparation (25). Glutamate-$^{18}O$ incubated under identical conditions followed by re-isolation retained at least 65% of its original $^{18}O$. This result established that if $^{18}O$-labeled glutamate or aspartate is released during digestion it can be identified. Since dilution by unlabeled amino acid residues might obliterate a positive result, the purity of the enzyme is also a crucial factor. With use of 1000 units of CoA transferase in a total of 5 mg of protein, and assuming only 1 $^{18}O$ per enzyme molecule and that the protein is 5% glutamate, the final expected atom per cent excess of isolated glutamate would be about 0.08. This value is well within the limits of detectability.

Enzyme (0.018 amole, specific activity 174 units per mg) was labeled with $^{18}O$, isolated by Sephadex G-50 filtration, divided into two equal portions, and incubated with succinate and succinyl-CoA as described in Table II, Experiment 2. The protein in the zero time sample remained labeled with $^{18}O$. The protein in the incubated sample transferred its $^{18}O$ to succinate (about 1 $^{18}O$ per enzyme molecule, see Table II, Experiment 2). The protein samples were isolated by centrifugation and each was digested with Pronase and leucine aminopeptidase as described under "Materials and Methods." Glutamate and aspartate were isolated by chromatography on Amberlite CG-4B (see "Materials and Methods") and tested for $^{18}O$. There was no detectable $^{18}O$ (<0.001 atom % excess) in either glutamate or aspartate. However, the fraction containing low molecular weight peptides did contain measurable excess $^{18}O$ (about 0.008 atom % excess) as compared to a control sample identical except that the enzyme was first incubated $^{14}O$ succinate and succinyl-CoA to remove any $^{18}O$ prior to digestion.

This experiment was repeated using less pure CoA transferase (10 units per mg). After labeling with $^{18}O$ from succinate-$^{14}C$ and succinyl-CoA, the protein was isolated by Sephadex G-50 filtration, digested with Pronase and leucine aminopeptidase, and chromatographed as described previously. The material eluted from the column with 0.1 M HCl was tested for $^{18}O$ after conversion with Hg(CN)$_2$ and HgCl$_2$. It contained measurable $^{18}O$ (about 0.004 atom % excess). After acid hydrolysis (5 M HCl, 110°, 18 hours), amino acid analysis showed the fraction contained only 3 to 4% of the amino acid residues present in the original protein.

Studies on Route of Formation of E-CoA—All evidence suggests that E-CoA participates in the CoA transferase reaction. If E-CoA is a thiol ester as suggested by its chemical properties (9), at least two routes of formation merit consideration.

One is a four-centered reaction (Equation 6).

\[
\begin{align*}
E-C-O^+ & \leftrightarrow E-C + C-R \\
CoAS-C-R & \leftrightarrow CoAS - O
\end{align*}
\]

Another more chemically appealing route is through a mixed anhydride intermediate (Equation 7).

\[
E-C-O^+ + CoAS-C-R \Rightarrow E-C=SCoA + R-C-O^- 
\]

Attempts to measure the formation of an anhydride with enzyme were conducted with concentrations of acetoacetyl-CoA and $^{14}C$-acetocetate well above the reported $K_m$ values of 0.7 mM for acetoacetyl-CoA and 0.2 mM for acetocetate (8). Incubation of CoA transferase at saturating concentrations of acetocetate and acetoacetyl-CoA would be expected to favor maximum accumulation of any intermediate anhydride between free enzyme and the E-CoA form. After denaturation of the enzyme, unbound substrates were removed by washing with acetone-H$_2$O and the $^{14}C$ remaining with the enzyme was determined. To determine if any bound $^{14}C$-acetocetate repre-
An intriguing possibility considered for the CoA transferase reaction and for the succinyl-CoA synthetase reaction is that an oxygen-containing group on the CoA participates in the catalysis. The quantitative \(^{18}O\) balance experiment presented in Fig. 1 provides clear evidence that oxygens of CoA in the reaction medium do not participate in the catalysis. Similar results have been obtained with succinyl-CoA synthetase (27). Further, such balance experiments have shown that with the synthetase, in contrast to the transferase, oxygen-containing groups on the enzyme do not participate in the catalysis. Substrate-oxygen interchange with the synthetase is direct between substrates rather than indirect through the enzyme as with the transferase.\(^{4}\)

Acknowledgments—The able technical assistance of Sue Galyean and Donna Bryan, particularly for \(^{18}O\) analyses, is gratefully acknowledged.

REFERENCES


\(^{4}\) Note Added in Proof—Identification of an enzyme \(\gamma\)-glutamyl coenzyme A intermediate from coenzyme A transferase has recently been reported by Solomon and Jencks (28).
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