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Quantitative Appraisals of Possible Catalytic Intermediates in the Succinyl Coenzyme A Synthetase Reaction*

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ABSTRACT: Previous data have suggested participation of an enzyme-bound form of coenzyme A in the succinyl coenzyme A synthetase reaction. The proposed mechanism requires participation of an oxygen atom from the enzyme or the coenzyme A.

Quantitative ^{18}O studies demonstrate a lack of participation of enzyme or of coenzyme A oxygens and a retention of substrate-^{18}O during catalysis of the succinate $\rightleftarrows$ succinyl coenzyme A, phosphate $\rightleftarrows$ phosphoryl enzyme and phosphate $\rightleftarrows$ adenosine triphosphate exchanges. These results together with a lack of detection of covalently bound coenzyme A and other findings provide compelling evidence against participation of an enzyme-bound coenzyme A form in the catalysis.

An enzyme-bound form of CoA has been suggested as possible catalytic intermediate in the succinyl-CoA synthetase reaction with the enzyme from *Escherichia coli* (Upper, 1964; Moyer and Smith, 1966; Moyer *et al.*, 1967) and from heart tissue (Cha *et al.*, 1965, 1967). The bound CoA was presumably released upon reaction with P_i with concomitant formation of the phosphorylated enzyme, E-P, or was converted into succinyl-CoA by reaction with succinate. Such reactions would require participation of an oxygen atom of either the enzyme or the CoA.

In related studies, Hersh and Jencks (1967) have presented convincing evidence that an enzyme-bound form of CoA participates in the succinyl-CoA–acetoacetate CoA transferase reaction. Further, Benson and Boyer (1969) established that with this transferase ^{18}O from substrates passes through a group on the enzyme in the catalytic cycle. In contrast, however, Moyer *et al.* (1967) did not detect any ^{18}O-labeling of succinyl-CoA synthetase during catalysis with ^{18}O-labeled

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substrates. These various findings made desirable further examination of the possible participation of an enzyme-CoA form with succinyl-CoA synthetase. The results given herein show that participation of an enzyme-CoA form in the synthetase reaction is unlikely.

Materials

Succinyl-CoA Synthetase. Succinyl-CoA synthetase was purified from succinate-grown Crooks strain *E. coli* as described by Ramaley *et al.* (1967); cells purchased from Miles Laboratories were used for the most recent preparations. Enzyme activity and protein concentration were determined as described by Ramaley *et al.* (1967). For experiments using phosphoryl-enzyme, about 14 mg of enzyme was phosphorylated by incubation in a 1-ml total volume with a tenfold excess of ATP in 0.1 M Tris-Cl (pH 7.4), 0.1 M KCl, and 10 mM $MgCl_2$ for 15 min at 25°. To form E-^{32}P, CoA (0.5 mM), and a trace of $^{32}P_i$ were added and the mixture was incubated 30 min at 37°. Phosphoryl-enzyme was then isolated by Sephadex G-50 filtration on a 1 × 50 cm column, equilibrated and eluted with 0.1 M Tris-Cl (pH 7.4) containing 0.1 M KCl. For experiments with dephosphoenzyme, the enzyme was dephosphorylated by incubation in a solution containing 0.1 M Tris-Cl (pH 7.4), 0.1 M KCl, 10 mM $MgCl_2$, 10 mM glucose, 0.1 mM ATP, and 0.05 mg of hexokinase per ml for 15 min at 25°. The protein was then isolated as described above. For some experiments, the E-^{32}P or E was freed of CoA, ATP, and ADP by passage through AG-1 (Cl^-) resin as described under the section on isolation of CoA.

Nucleotides and Coenzymes. ATP and CoA were purchased from P-L Biochemicals. Succinyl-CoA was synthesized from succinic anhydride and CoA (Simon and Shemin, 1953) and purified by chromatography on DEAE-cellulose, formate form, as described by Cha and Parks (1964). Succinyl-CoA concentrations were determined using the absorption coefficients at 230 mμ of 7.7×10^3 M^{-1} cm^{-1} for succinyl-CoA and 3.2×10^3 M^{-1} cm^{-1} for CoA (Stadtman, 1957).

Isotopes. $^{32}P_i$ (Tracerlab) was purified as described by DeLuca *et al.* (1963). After purification greater than 99% of the radioactivity could be extracted into isobutyl alcohol–benzene as the phosphomolybdate complex (Berenblum and Chain, 1938). Succinate-2,3-^{14}C (New England Nuclear Corp.) was used without purification. Greater than 99% of the radioactivity could be converted to succinyl-CoA by succinyl-CoA synthetase. P_i-^{18}O was prepared as described by Cohn and Drysdale (1955) and succinate-^{18}O as described by Falcone and Boyer (1959).

Materials for Chromatography and Gel Filtration. DEAE-cellulose (Sigma Chemical Co.) was purified by recycling three times with 1 N HCl and 1 N KOH before conversion to the desired form. DEAE-Sephadex A-50 and Sephadex G-50 were purchased from Pharmacia Fine Chemicals and treated before use as described by the manufacturer. AG-1 (Cl^-), purchased from Bio-Rad Laboratories, was used without further purification.

Methods

Isolation of CoA. In the absence of other nucleotides, CoA could be recovered from reaction mixtures by use of AG-1 (Cl^-). The reaction mixture (1–2 ml) was applied to an 0.5 × 5 cm AG-1 (Cl^-) column equilibrated with 0.1 M Tris-Cl–0.1 M KCl (pH 7.4). Protein and phosphate were eluted with 10 ml of 0.1 M Tris-Cl–0.1 M KCl (pH 7.4). The CoA was then eluted with about 10 ml of 0.1 M Tris-Cl–1.1 M KCl (pH 7.4). Fractions of 1 ml were collected. The CoA was located by its absorbance at 260 mμ. CoA isolated in this manner retained full activity in the succinyl-CoA synthetase reaction.

Measurement of Free CoA. Determination of CoA at the millimicromole level in the presence of synthetase was made by a modification of method of Garland *et al.* (1965); this depends on fluorescence of DPNH generated by α-ketoglutarate dehydrogenase. Each sample was diluted to 1.00 ml with glass-distilled water and added to 1.00 ml of an assay mix containing 100 mM Tris-maleate (pH 7.0), 25 mM of EDTA, 3 mM α-ketoglutarate, and 0.1 mM DPN in a fluorometer cuvet. The fluorescence of this mixture (excitation at 350 mμ, emission at 465 mμ) was recorded with an Aminco-Bowman fluorometer until a stable base line was obtained. Then 10 units of α-ketoglutarate dehydrogenase (prepared according to Sanadi *et al.*, 1952) were added. When reaction was complete (usually within 5 min) the increment in fluorescence was recorded. The subsequent addition of 1.0 mμmoles of CoA and the resultant increment of fluorescence provided an internal standard for each sample analyzed. The fluorometric assay gave a linear response with 0.1–2.5 mμmoles of CoA and was independent of the presence of succinyl-CoA synthetase.

Isolation and Analysis of ^{18}O-Labeled Compounds. P_i was isolated and its ^{18}O content was determined as described by Boyer and Bryan (1967). For analysis of ^{18}O in the phosphoryl group of E-P, the sample was hydrolyzed in 0.3 M perchloric acid at 100° for 10 min and an appropriate amount of carrier P_i was added. Succinic acid (2–10 μmoles) was isolated and its ^{18}O content determined as described by Benson and Boyer (1969). Succinyl-CoA, if present, was first hydrolyzed at pH 11–12 for 30 min at 37°.

Detection of Radioactivity. Radioactive samples were either dried on planchets and counted with a gas-flow detector (Nuclear-Chicago) or dissolved in Bray's solution (Bray, 1960) and counted with a multichannel scintillation counter (Nuclear-Chicago 720 or Packard Tri-Carb). Quench corrections were made by comparing a known standard in the same milieu or by comparing channel ratios or an automatic external standard to a standard curve. In double-labeling experiments, ^{32}P and ^{14}C were counted in the presence of each other by measurements with the planchets uncovered or covered by aluminum foil; 85% of the ^{32}P counts and only 4% of the ^{14}C counts were passed through the foil.

Phosphorylated-enzyme, E-^{32}P, was measured by a phenol extraction procedure (Ramaley *et al.*, 1967). Succinylated-enzyme was determined as phenol-extracted succinate-^{14}C counts in analogous fashion to the E-^{32}P determination above except that all operations were performed at 5° and were complete within 30 min.

Results

Lack of Participation of Synthetase Oxygens. Earlier data of Moyer *et al.* (1967) indicated that no detectable enzyme oxygens participated in the succinyl-CoA synthetase reaction. Subsequent demonstration of the participation of an enzyme oxygen in a CoA transferase reaction (Benson and Boyer, 1969) necessitated further examination of the findings of

TABLE I: Lack of Participation of Succinyl-CoA Synthetase Oxygens in the E-P $\rightleftarrows$ P_i Exchange.[a]

Sample	E-^{32}P (moles/mole of enzyme)	^{18}O in Total Isolated P_i (atom % excess)
Control, E-^{32}P-^{18}O as isolated from the column	0.78	0.149[b]
Sample, E-^{32}P-^{18}O incubated with CoA and excess P_i	0.09	0.148[c]

[a] Succinyl-CoA synthetase (43 μM) was incubated with 0.1 mM ATP, 0.1 mM CoA, 1.25 mM $^{32}P_i$-^{18}O (53.4 atom % excess), 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 M Tris-Cl (pH 7.4) in 2.02 ml for 60 min at 37°. The E-^{32}P was separated on a 1 × 100 cm column of Sephadex G-50. The fractions containing the enzyme were pooled and divided into two portions. The control sample was made 0.3 M in perchloric acid before additions as for the incubated sample. The incubated sample contained in 5.5-ml total volume 25 μM E-^{32}P, 0.1 mM CoA, 0.73 mM P_i, and KCl, $MgCl_2$, and Tris-Cl as above. After 60 min at 37°, the incubated sample was made 0.3 M in perchloric acid, and P_i was isolated from the control and incubated sample for ^{18}O determination as described in Methods. [b] Expected for 3 ^{18}O from original P_i-^{18}O in the phosphoryl group and dilution by P_i after hydrolysis = 0.157 atom % excess. [c] Expected for equilibration of P_i present with 3 ^{18}O from the original P_i-^{18}O in the phosphoryl group and 1 ^{18}O from the enzyme = 0.209 atom % excess.

TABLE II: Lack of Participation of CoA Oxygens in the E-P $\rightleftarrows$ P_i Exchange.[a]

Sample	^{18}O in P_i (atom % excess)
Control, CoA as isolated plus carrier P_i	0.007
Incubated, isolated CoA equilibrated with E-P and carrier P_i	0.008[b]

[a] Phosphorylated succinyl-CoA synthetase (0.17 mM) was incubated with 0.17 mM CoA, 8.6 mM $^{32}P_i$-^{18}O (53.4 atom % excess), 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 M Tris-Cl (pH 7.4) in a volume of 0.58 ml for 60 min at 37°. CoA was then isolated as described in the Methods section, and divided into two portions. One portion was made 0.3 M in perchloric acid before additions as for the incubated sample. The incubated sample contained in a 2.0 ml volume 17 μM E-P, 17 μM of the isolated CoA, 2.5 mM $^{32}P_i$, and KCl, $MgCl_2$, and Tris-Cl as above. After 60 min, the incubated sample was made 0.3 M in perchloric acid, and P_i was isolated from the control and incubated sample for ^{18}O determination as described in Methods. [b] Compared with an expected value of 0.060 if one CoA oxygen had become labeled from the original P_i.

Moyer *et al.* Experiments were conducted to give a more sensitive test for possible enzyme oxygen participation. Enzyme and ATP were incubated with $^{32}P_i$ highly-labeled with ^{18}O under conditions such that all the oxygens in the phosphoryl group of the E-P formed as well as any other participating oxygens of the enzyme would be equilibrated with the ^{18}O of the P_i-^{18}O. The E-P was then freed of substrates by separation on a Sephadex column, and one portion taken for determination of ^{18}O present in P_i released by acid hydrolysis. This should give P_i containing ^{18}O only from the enzyme-phosphoryl group and not from any other possible ^{18}O-labeled group on the enzyme. The second portion of the enzyme was incubated with CoA and an excess of unlabeled P_i. This should transfer ^{18}O from the enzyme-phosphoryl groups as well as from any participating oxygen group of the enzyme into the relatively large P_i pool. To help ensure valid comparison of the amount of ^{18}O obtained in the P_i under the two conditions, both the control and incubated sample were of identical final composition for P_i-^{18}O isolation.

The results presented in Table I show that the same amount of ^{18}O was found in both the control and the incubated sample. Clearly no extra ^{18}O was released from the enzyme during the incubation. The conditions were such that release of one extra ^{18}O from the enzyme per phosphosphoryl group present should have given a 33% increase in ^{18}O in the incubated sample, a readily detectable amount. The experiment thus demonstrates that the interchange of enzyme phosphoryl with medium P_i in the presence of CoA does not involve participation of enzyme oxygens.

Lack of Participation of CoA Oxygens. The results of Moyer *et al.* (1967) showed that oxygens from CoA of the reaction medium did not readily participate in the synthetase reaction. Sensitivity of these tests, however, did not eliminate the possibility that CoA, which remained firmly bound to the enzyme for an extended period, could participate as an oxygen donor in some type of cyclic fashion and thus escape detection. Because of the lack of participation of enzyme oxygens, experiments were conducted to check on possible participation of CoA oxygens at levels stoichiometric with the enzyme. The sensitive fluorometric assay was used to measure the amount of CoA that remained bound to the enzyme as isolated from the Sephadex column for the experiments reported in Table I. The E-P isolated contained no detectable CoA (less than 1 CoA/100 enzyme molecules). It was thus feasible to incubate a relatively large amount of enzyme with an equivalent amount of CoA in the presence of P_i highly labeled with ^{18}O, isolate the CoA on AG-1 (Cl^-), and test it for the possible presence of ^{18}O by reincubating the CoA with enzyme and nonlabeled P_i.

The results of such an experiment are given in Table II. Measurement of the ^{32}P distribution (not shown in the table) demonstrated that the expected isotopic equilibrium between $^{32}P_i$ and E-P was attained. Because the E-P $\rightleftarrows$ P_i exchange is dependent on CoA, most or all of the CoA (present approximately equimolar with enzyme) should have participated in any oxygen interchange accompanying the catalysis. The re-

TABLE III: Retention of Phosphate Oxygen during the E-P ⇄ P_i and P_i ⇄ ATP Exchanges.[f]

Expt	Incubn Time (min)	Added P_i (μM)	^{32}P with E-P (%)	^{18}O in Total P (μatoms)
1	0	16	0	19.2
	15	16	49[a]	19.1[b]
	30	1630[c]	1	19.2
2	0	61	0	22.0
	15	61	28[d]	22.2[e]
	30	5460[c]	1	22.0

[a] Expected for isotopic equilibrium, 50%. [b] Expected if one enzyme or CoA oxygen participated, 17.5. [c] Carrier P_i added at 15 min. [d] Expected for isotopic equilibrium, 28%. [e] Expected for one oxygen from enzyme or CoA, 20.4. [f] In expt 1, phosphorylated succinyl-CoA synthetase (16 μM) was incubated at 37° in 0.1 mM CoA, 0.1 M KCl, 10 mM $MgCl_2$, and 0.10 M Tris-Cl (pH 7.4) as indicated below. The final volume was 5.0 ml. $^{32}P_i$-^{18}O added initially contained 59.7 atom % excess ^{18}O. Reactions were stopped by addition of perchloric acid to 0.3 M. Carrier P_i was then added such that all samples contained 8.16 μmoles of P_i. In expt 2, succinyl-CoA synthetase (35 μM) was incubated at 37° in 68 μM ATP, 0.1 mM CoA, 50 mM Tris-Cl (pH 7.4), 50 mM KCl, and 10 mM $MgCl_2$. The final volume was 1.48 ml. $^{32}P_i$-^{18}O added initially contained 58.3 atom % excess ^{18}O. Reactions were stopped by the addition of perchloric acid to 0.3 M. Carrier phosphate was then added such that all samples contained 8.29 μmoles. P_i was isolated and ^{18}O was determined as described in Methods. The data are averages of duplicate ^{18}O determinations.

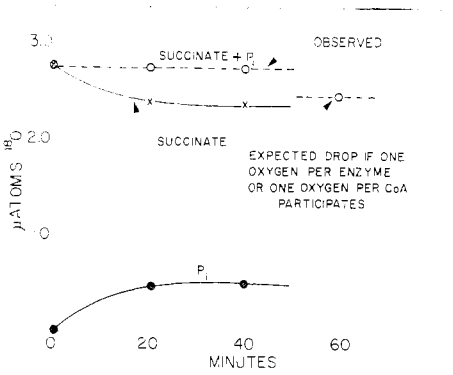

FIGURE 1: Retention of ^{18}O during the succinate ⇄ succinyl-CoA exchange. Succinyl-CoA synthetase (5 μM) was incubated with 5 μM succinate-^{18}O (65 atom % excess), 5 μM succinyl-CoA, 1.8 μM P_i, 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 M Tris-Cl (pH 7.4) in 2.04 ml at 37° for the time indicated. The reaction was stopped by the addition of NaOH to the thymol blue end point. Carrier P_i and succinate (2 μmoles each) were then added, succinyl-CoA was hydrolyzed, and the succinate and P_i were isolated and their ^{18}O content was determined as described in the Methods section. The data are averages of duplicate incubations.

sults show that no detectable ^{18}O was present in the CoA previously incubated with an equivalent amount of E-P and P_i-^{18}O. The experiment was sufficiently sensitive to detect participation of one oxygen for each ten molecules of CoA or enzyme present in the first incubation.

Retention of ^{18}O during the Succinate ⇄ Succinyl-CoA Exchange. The exchange of succinate with succinyl-CoA catalyzed by the synthetase could quite logically involve participation of an enzyme–CoA intermediate. If this were the case, an oxygen from enzyme or CoA must participate in the reaction, and ^{18}O present initially as succinate should, in the presence of sufficiently large amounts of enzyme, be diluted by a detectable amount of interchange with oxygens of the enzyme or the CoA. Such dilution would be in addition to that expected for randomization of the oxygens with the acyl and carboxyl oxygens of succinyl-CoA. An experiment designed to detect such possible dilution is shown in Figure 1.

An apparent loss of ^{18}O from the succinate-succinyl pool was observed. However, the possibility existed that the ^{18}O might have been transferred to a trace contaminant of P_i. This was checked by addition of carrier P_i, and subsequent P_i isolation and ^{18}O analyses. Presence of ^{18}O in the P_i was noted, and the amount of total P_i present during the incubation as estimated from the ^{18}O content was 1.7 μM. As noted in Figure 1, the sum of the ^{18}O in the three reactants (succinate plus succinyl plus P_i) remained constant. The experiment was sufficiently sensitive to detect the participation of one oxygen per every seven enzyme molecules. The results thus allow the conclusion that oxygens of CoA or of the enzyme do not participate in the succinate ⇄ succinyl-CoA exchange in the presence of P_i.

Retention of ^{18}O during the E-P ⇄ P_i and P_i ⇄ ATP Exchanges. The requirement of CoA for the E-P ⇄ P_i and for the P_i ⇄ ATP exchanges (Upper, 1964; Ramaley *et al.*, 1967; Moyer *et al.*, 1967; Cha *et al.*, 1967) suggested the participation of an enzyme-CoA form in these exchanges. This was checked by measurement of the retention of ^{18}O in the P_i plus phosphoryl pool during the exchanges in the presence of relatively large amounts of enzyme. If oxygens from enzyme or CoA participated in these reactions the phosphate ^{18}O level should be diluted by a detectable amount. ^{18}O retention was demonstrated under two conditions. After 15-min incubation the enzymic reaction was stopped with perchloric acid, carrier P_i added, and E-P or E-P plus ATP hydrolyzed to give P_i for comparison of total ^{18}O present with a similar zero-time control. With another sample, the carrier P_i was added at 15 min and the enzymic reaction was stopped at 30 min by perchloric acid addition. Almost all ^{18}O from any participating substances would then be expected to be in the large P_i pool. The distribution of ^{32}P added as $^{32}P_i$ showed that the expected isotopic equilibrations had occurred. The results in Table III demonstrate that in both exchanges all the ^{18}O originally added as ^{18}O-P_i is present in the P_i and enzyme-phosphoryl group (for the P_i ⇄ E-P exchange) or in the P_i, enzyme-phosphoryl group, and terminal phosphoryl group of ATP (for the P_i ⇄ ATP exchange). Thus no oxygen atoms from CoA or enzyme participate in these reactions. The experiments were sufficiently sensitive to detect participation of an additional oxygen for about every eight enzyme molecules.

Lack of Detection of Covalently Bound CoA. Results as reported in the preceding sections, demonstrating lack of participation of CoA or enzyme oxygens in the catalysis, prompted

TABLE IV: Substrates Present with Synthetase Exposed to Succinyl-CoA and Separated on Sephadex.[a]

Synthetase Sample		Assay		
No.	Conditions	^{32}P as E^{32}-P (moles/mole of enzyme)	CoA as Free CoA (moles/mole of enzyme)	Succinyl-^{14}C as Total Succinyl (moles/mole of enzyme)
1	Before column separation	0.14		
2	After column separation	0.14	0.02	0.44[b]
3	As expt 2 but, incubated 15 min, 30°, with 10 mM Mg^{2+} and 0.25 mM $^{32}P_i$	0.55	0.37	
4	As expt 2 but, incubated at pH 13, 10 min, 25°		0.34	
5	As expt 2 but, incubated with 0.3 M NH_2OH, 10 min, 25°		0.27	
6	As expt 2 but, incubated at pH 1, 10 min, 25°		0.07	

[a] Succinyl-CoA synthetase (12 μM, specific activity 16) was incubated with 0.18 mM succinyl-^{14}C-CoA, 0.10 mM each of succinate-^{14}C and CoA (from hydrolysis of the succinyl-^{14}C-CoA preparation before use), 0.019 mM $^{32}P_i$, 0.1 M Tris-Cl (pH 7.3), and 10 mM $MgCl_2$ in a 5-ml total volume for 5 min at 37°. The solution was chilled to near 0°, EDTA (pH 7) was added to give a 100 mM concentration, and the enzyme was separated near 4° on a 1 × 100 cm column of Sephadex G-50, using an eluting solution containing 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, and 0.1 mM EDTA. Assays were made as described in Methods. [b] Of this succinyl, 0.06 mole/mole of enzyme were covalently bound to protein (see Table V).

further examination of the nature and extent of CoA binding to the enzyme. In particular, explanation appeared desirable for the results of Upper (1964), of Cha *et al.* (1965), and of Moyer *et al.* (1967) indicative of bound CoA forms participating in the catalysis. For example, Moyer *et al.* (1967) reported that after incubation of enzyme with succinyl-CoA or of E-P with CoA, a form of the enzyme could be obtained by Sephadex separation that would give E-^{32}P upon incubation with $^{32}P_i$. On the basis of the absence of succinate-^{14}C originally present and from difference spectra indicative of bound nucleotide with the enzyme, the incubation with succinyl-CoA was assumed to give an E-CoA form, capable of reacting with P_i to give E-P. As a further probe, the sensitive fluorometric assay for CoA (see Methods) was used in attempts to demonstrate presence of enzyme-bound CoA under similar and with various modifications of conditions reported by Moyer *et al.* (1967). Results of a representative experiment (see Robinson, 1968) are given in Table IV. The enzyme showed capacity to form E-^{32}P upon incubation with $^{32}P_i$ and Mg^{2+} with concomitant liberation of free CoA. However, the enzyme after the column separation contained bound succinyl sufficient to account for the CoA being present as succinyl-CoA. Support for this possibility came from observation that the CoA was liberated by base or hydroxylamine but not by acid. The results are thus consistent with presence of a firmly bound succinyl-CoA that accounts for most or all of the capacity to form E-P. The sensitivity of the tests were such that no more than about 10% of the enzyme was converted into a form with bound CoA capable of being replaced by reaction with P_i to give E-P.

Several factors appear to account for the previously reported results. The enzyme shows a strong tendency for noncovalent binding of succinyl-CoA. In the experiment reported by Moyer *et al.* (1967), equilibration of succinate-^{14}C with succinyl-CoA may not have occurred thus rendering invalid their test for possible bound succinyl-CoA. Perhaps more important, certain characteristics of the enzyme present pitfalls in the assay for possible presence of E-CoA based on E-^{32}P formation. Only a trace contamination of P_i is necessary for E-P formation in presence of succinyl-CoA. Measurement of capacity for E-^{32}P formation after isolation may reflect exchange of $^{32}P_i$ with E-P already present and not reaction of E-CoA with $^{32}P_i$. In addition, commercial CoA preparations contain traces of ATP (or an ATP-like material) as well as P_i (Robinson, 1968). Because the equilibrium with ATP considerably favors E-P formation (Ramaley *et al.*, 1967), addition of an excess of a CoA preparation to enzyme will give some E-P from the ATP-like contaminant. In the presence of $^{32}P_i$, E-^{32}P will be formed by exchange.

Lack of Borohydride Inactivation. A salient characteristic of succinyl-CoA–acetoacetate CoA transferase reported by Hersh and Jencks (1967) was the ability of borohydride to inactivate the E-CoA form. When ^{3}H-labeled borohydride was used, radioactivity appeared with the inactive protein. A similar E-CoA form if present with succinyl-CoA synthetase might be expected to show borohydride inactivation. The ability of borohydride to inactivate the CoA transferase was confirmed. However, under similar conditions, succinyl-CoA synthetase, treated so as to favor existence of an enzyme-CoA form, showed no inactivation (see Robinson, 1968). Such a result gives additional evidence against the appreciable formation of any enzyme-CoA form with succinyl-CoA synthetase.

The Irreversible Binding of Succinyl to the Enzyme. In the course of experiments on the possible formation of E-CoA forms, a binding of ^{14}C from succinyl-^{14}C CoA to the enzyme was noted. The extent of such binding, as shown in Table V, increases slowly with time and exceeds the capacity to form

TABLE V: Extent of Irreversible Succinylation of Synthetase.[a]

Incubn Period (min)	Succinyl (moles/mole of enzyme)
0	0.0
20	1.8
35	2.3
45	2.6
60	2.9

[a] For each sample, enzyme (2.7 μM, specific activity 25) was incubated in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, 10 mM $MgCl_2$, 10 mM ATP, 1 mM CoA, and 1 mM succinate-^{14}C (final volume 0.13 ml) for the indicated times at 37°. The reactions were stopped by the addition of 5 ml of phenol-saturated water. The protein-bound succinate was extracted into phenol and ^{14}C was determined as described in Methods.

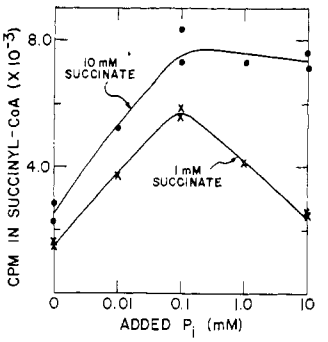

FIGURE 2: Effect of P_i on the succinate ⇄ succinyl-CoA exchange. For each sample, enzyme (0.56 μM, specific activity 16) in 0.1 M Tris-Cl (pH 7.2), 0.1 M KCl, 10 mM $MgCl_2$, 1 mM succinyl-CoA, P_i as indicated, and either 1 mM (x) or 10 mM succinate-^{14}C (●) (30×10^3 cpm/sample) in a final volume of 0.11 ml was incubated for 10 min at 21°. Reaction was stopped by adding 1 ml of 0.1 M formic acid–0.1 M sodium formate. Succinyl-^{14}C CoA was isolated as described by Moyer *et al.* (1967), and 1 ml of the 5-ml eluent containing succinyl-CoA was counted.

phosphoryl-enzyme. In experiments not reported here (see Robinson, 1968), the bound succinate was shown not to decrease the catalytic activity. In addition, the bound succinate was not removed from the enzyme during net reaction with excess unlabeled succinate. The succinylation could thus represent an ability of succinyl-CoA to acylate nonessential groups on the enzyme. It does not appear to be related to the enzymic catalysis.

The Relation of P_i to the Succinate ⇄ Succinyl-CoA Exchange. The occurrence of the succinate ⇄ succinyl-CoA exchange in the absence of added P_i is consistent with the participation of an E-CoA form. The exchange would be expected to be inhibited by P_i, as observed by Moyer *et al.* (1967). Because of the lack of participation of an E-CoA form in the catalysis as presented herein, the effect of reaction conditions on the relation of P_i to the succinate ⇄ succinyl-CoA exchange was further examined. The inhibitory effect of P_i was found to be related to the succinate concentration. As shown by the data in Figure 2, with 10 mM succinate present, increase of P_i above 10^{-4} M did not give the inhibition noted with 1 mM succinate present. Note that the P_i scale is logarithmic, and that added P_i covers a 1000-fold range. Effort was made to reduce P_i contamination of reactants in this experiment, but an exchange in the absence of added P_i was still noted. It is of interest, however, that addition of 10^{-5} M P_i accelerated the exchange with either 1 or 10 mM succinate present.

Discussion

The results confirm and extend the previous demonstration by Moyer *et al.* (1967) that succinyl-CoA synthetase oxygens show no detectable participation in the catalytic reaction. Thus the synthetase behavior is in clear contrast to the succinyl-CoA–acetoacetate CoA transferase, in which oxygen transfer through a group on the enzyme is demonstrable (Benson and Boyer, 1969). In addition, our results show no detectable participation of CoA oxygens at a level stoichiometric with the enzyme. These findings give no support for the participation of an anhydroenzyme-CoA form in the catalysis. They cannot, however, conclusively rule out the participation of such a form. The possibility exists that the enzyme or the CoA could exchange the critical oxygen with solvent water during the isolation procedures required for testing. For example, all that would be required for the enzyme would be a "turnover" about every 10 min or so, many orders of magnitude less than the catalytic rate. It was these uncertainties that stimulated design of the quantitative oxygen balance experiments presented in Table III and Figure 1. These experiments, simple in concept but requiring careful ^{18}O analyses, demonstrate conclusively that no oxygens from either enzyme or CoA participate in the catalyses tested. We must conclude, therefore, that even though under some circumstances small amounts of some enzyme-CoA forms might exist, any such forms are not participants in the catalyses tested.

The P_i ⇄ E-P, the P_i ⇄ ATP, and the succinate ⇄ succinyl-CoA exchanges were chosen for the quantitative oxygen balance studies because each of these reactions can logically be formulated with an enzyme-CoA intermediate. Oxygen balance studies were not attempted with all reactants present because this would necessitate an undue enlargement of the total oxygen pool and thus a smaller potential dilution by any enzyme or CoA oxygens that might participate. The absence of such participation in the three partial reactions that could involve an enzyme-CoA intermediate make it highly unlikely that an enzyme-CoA form participates in the over-all catalysis. This probability receives further support by the lack of detection of any bound CoA, other than succinyl-CoA, that could be liberated upon reaction with P_i to give E-P (Table IV) under conditions previously suggested to give an enzyme-CoA form. The characteristics of the enzyme and other factors mentioned previously as likely accounting for the reported participation of an E-CoA form by Moyer *et al.* (1967) with the *E. coli* enzyme are likely also responsible for the earlier similar report of Upper (964) and for the reports of Cha *et al.* (1965, 1967) of an enzyme-CoA form participating in catalysis by heart succinyl-CoA synthetase. The CoA dependency of the P_i ⇄ E-P and P_i ⇄ ATP exchanges (Upper, 1964; Ramaley *et al.*, 1967; Moyer *et al.*, 1967) cannot be taken as evidence for the formation of an E-CoA in view of the lack of partici-

pation of CoA or enzyme oxygens (Tables II and III) and the lack of binding of CoA (Table IV) reported herein.

Cha *et al.* (1967) appropriately discussed several considerations that necessitated caution in acceptance of an E-CoA form as a catalytic intermediate. Among these was the strong binding of succinyl-CoA and the lack of detection of independent binding of the CoA moiety observed with the pig heart enzyme. Our results with the *E. coli* enzyme are in harmony with these earlier data.

The mechanisms of the succinate $\rightleftarrows$ succinyl-CoA and of the CoA-dependent $P_i \rightleftarrows$ E-P and $P_i \rightleftarrows$ ATP exchanges remain obscure. As noted by the data of Figure 2, the possibility remains that P_i present as a trace contaminant may be a requirement for the exchange. Such a possibility receives support by the data of Figure 1 that show participation of a low level of contaminating P_i in oxygen interchange with added succinate-^{18}O. It is unlikely that contaminating succinate is required for the CoA-dependent E-P $\rightleftarrows P_i$ exchange. Enzyme freed of added succinate-^{14}C by Sephadex separation and ion exchange resin treatment (to at least <1 succinate/100 enzyme molecules) will still catalyze the $P_i \rightleftarrows$ E-P exchange (Benson, 1968). The possibility must thus be entertained that an oxygen from the entering P_i is transferred directly to the departing phosphoryl group, perhaps in a four-centered reaction. Neither the succinate $\rightleftarrows$ succinyl-CoA nor the $P_i \rightleftarrows$ E-P partial reactions are required participants in the over-all catalysis proceeding by a concerted formation of E-P or with a bound succinyl phosphate as an intermediate. Additional considerations of these possibilities are given in the accompanying paper (Robinson *et al.*, 1969).

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