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# Substrate Synergism and Phosphoenzyme Formation in Catalysis by Succinyl Coenzyme A Synthetase*

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ABSTRACT: The phosphorylated form of succinyl coenzyme A synthetase (E-P) is shown by rapid mixing and quenching experiments to participate on the major or exclusive catalytic pathway. The initial rate of appearance of E-P is as least as great as the initial steady-state rate of the over-all reaction in either direction and, in the direction of adenosine triphosphate synthesis, the steady-state level of E-P appears to be reached before the establishment of the steady-state rate of adenosine triphosphate formation. Succinyl coenzyme A synthetase provides an excellent example of a phenomenon termed substrate synergism. This refers to acceleration by a substrate or substrates of a reaction undergone by other substrates of a multisubstrate enzyme. Theoretical considerations show that such synergism can be conclusively detected by appropriate comparison of rates of isotopic exchange between certain substrates in the presence or absence of other substrates. Substrate synergism occurs with succinyl coenzyme A synthetase as measured by the adenosine diphosphate $\rightleftarrows$ adenosine triphosphate, succinate $\rightleftarrows$ succinyl coenzyme A, and E-P $\rightleftarrows$ adenosine triphosphate exchange reactions. Acceleration of the adenosine diphosphate $\rightleftarrows$ adenosine triphosphate exchange by succinyl coenzyme A or by all other substrates likely reflects modifications important in net catalysis because both the exchange and over-all reaction probably involve E-P as an intermediate.

Multisubstrate enzymes frequently catalyze partial reactions that may reflect steps in the over-all catalysis. For example, succinyl-CoA[1] synthetase will catalyze an ADP $\rightleftarrows$ ATP exchange (Kaufman, 1955), and aminoacyl-tRNA synthetases will catalyze a P–$P_i$ $\rightleftarrows$ ATP exchange (DeMoss and Novelli, 1956). With succinyl-CoA synthetase, as with other enzymes, such partial reactions have frequently been observed to be relatively slow, thus raising doubts as to whether the reactions responsible are participants in the catalysis. A plausible explanation often considered is that the presence of other substrates may markedly increase the rate of the step responsible for the partial reaction. Such rate acceleration could logically arise from existence of a synergism between or among substrates for promotion of catalysis. Evidence for such acceleration has been presented with succinyl-CoA synthetase (Ramaley *et al.*, 1967), aminoacyl-tRNA synthetases (Hele, 1964; Ravel *et al.*, 1965; Mitra and Mehler, 1966; Deutscher, 1967; Lee *et al.*, 1967), and argininosuccinate synthetase (Rochovansky and Ratner, 1967). A definitive synergism is shown in the promotion of acylation of glyceraldehyde 3-phosphate dehydrogenase by the binding of DPN (see Malhotra and Bernhard, 1968). With citrate synthase, L-malate, probably by mimicing oxalacetate, promotes exchange of the methyl hydrogens of acetyl-CoA (Eggerer, 1965). This likely represents acceleration of a catalytic step. Closely related to synergism among substrates are observations of McElroy *et al.* (1967) and of Baldwin and Berg (1966), demonstrating promotion of hydrolysis of enzyme-bound intermediates by presence of other substrates.

Convincing experimental and theoretical means for quantitative demonstration of such substrate synergism have been lacking. One purpose of this paper is to present data obtained with succinyl-CoA synthetase, together with theoretical considerations, that clearly establish occurrence of substrate synergism in this enzymic catalysis.

The findings on substrate synergism are intimately

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: succinyl-CoA, succinyl coenzyme A; acetyl-CoA, acetyl coenzyme A.

associated with other experiments reported in this paper which show that the phosphorylated form of succinyl-CoA synthetase (Ramaley *et al.*, 1967) satisfies some kinetic requirements for an intermediate in the over-all catalysis. Possible participation of the phosphorylated form as a catalytic intermediate is of definitive interest because of the unknown catalytic mechanism for this important enzyme (see Hager, 1962; Nishimura and Meister, 1965; Moyer *et al.*, 1967). Phosphoenzyme participation is also pertinent to considerations of substrate synergism because such participation makes it highly likely that the step responsible for the ADP $\rightleftarrows$ ATP exchange as a partial reaction is also a step in the over-all catalysis.

## Materials and Methods

*Enzyme and Substrates.* Succinyl-CoA synthetase was prepared from *Escherichia coli* (Crookes strain) and assayed as described by Ramaley *et al.* (1967). The enzyme used in these studies had specific activities ranging from 7 to 26. Where necessary, solutions of enzyme were freed of traces of CoA by passage through columns (0.5 $\times$ 4 cm) of Dowex 1 ($Cl^-$), equilibrated with 0.1 M Tris-Cl–0.1 M KCl (pH 7.2).

Succinyl-CoA was synthesized from succinic anhydride and CoA, with purification by chromatography on DEAE-cellulose as described by Cha and Parks (1964). For preparation of ADP-$^{14}$C, about 10 $\mu$Ci of ATP-8-$^{14}$C (Schwarz) in 1 ml of 50% ethanol was diluted to 3 ml with water and lyophilized. The powder was dissolved in 0.2 ml of a solution containing 0.05 M Tris-Cl (pH 7.2), 0.05 M KCl, and 0.005 M $MgCl_2$, then 0.1 ml of 0.2 M glucose and 10 $\mu$g of hexokinase (Sigma type C-300) were added. The mixture was incubated at 25° for 10 min, and then placed in a boiling-water bath for 1 min. The solution was placed on a 2 $\times$ 1 cm column of DEAE-cellulose (bicarbonate form). Traces of unidentified $^{14}$C-containing substances were removed by washing the column with 5 ml of water followed by 1 ml of 0.2 M $NH_4HCO_3$. ADP-8-$^{14}$C was largely eluted by a further 2 ml of 0.2 M $NH_4HCO_3$, lyophilized, and taken up in water. About 97% of the radioactivity migrated with added carrier ADP on thin-layer chromatography as described below.

ATP-$\gamma$-$^{32}$P was prepared by $^{32}P_i \rightleftarrows$ ATP exchange catalyzed by succinyl-CoA synthetase as described previously (Ramaley *et al.*, 1967).

*Measurement of Rapid Reactions.* A flow device was constructed according to the general design of Barman and Gutfreund (1964), except that the syringes were driven by springs instead of a motor. Four stretched garage door springs were arranged with a suitable support device for expelling the contents of two syringes through a multijet mixing chamber of 0.32-ml volume (Gibson and Milnes, 1964). A hydraulic jack was used to stretch the springs, and the extended springs were held with a trip device. The flow rate after release of the trip device was varied by adjusting the orifice in the needle valve of the jack. The reactant mixture passed from the mixing chamber into a capillary of known volume, and reactions were stopped by expelling the reaction mixture from the capillary into an appropriate quenching solution. Flow rates as high as 40 ml/sec were readily obtained with the use of 2-ml syringes.

The apparatus was tested in two ways. Adequate uniformity of flow rates was indicated by observations that the time required for discharge of a given volume from the first or last portions of a filled syringe were close to equal. As another experimental evaluation, measurement was made of the rate of hydrolysis of 2,4-dinitrophenyl acetate in 0.80 N NaOH as described by Barman and Gutfreund (1964). The second-order rate constant was found to be 53.3 $M^{-1}$ $sec^{-1}$ at about 24°, which agrees favorably with the value of 49.5 $M^{-1}$ $sec^{-1}$ reported by Barman and Gutfreund (1964) at 25°.

For measurement of the rates of E-P (phosphorylated enzyme) and of product formation, one syringe contained enzyme in buffered solution and the other syringe contained substrates, including ATP-$^{32}$P or $^{32}P_i$, depending upon the reaction direction to be measured. Using flow rates ranging from 9 to 40 ml per sec, the reaction mixture (4.74 ml) was expelled into 4 ml of 8% w/v phenol–1 mM $P_i$ (pH 7.0). Aliquots of the quenched solution were removed for E-P determination by phenol extraction (Boyer and Bieber, 1967) and for ATP-$^{32}$P or -$^{32}P_i$ determination by extraction of the phosphomolybdate complex of the $P_i$ into isobutyl alcohol–benzene (Berenblum and Chain, 1938). Previous studies in this laboratory have shown that close to the same amount of $^{32}$P as phosphorylhistidine is obtained whether the enzymic reaction is stopped by addition of phenol or urea at acid, neutral, or alkaline pH, by addition of perchloric or trichloroacetic acid, or by organic solvent addition (Boyer and Bieber, 1967). The assumption thus appears justified that the method measures the amount of $^{32}$P covalently bound as phosphohistidine prior to addition of the stopping agent.[2]

*ADP $\rightleftarrows$ ATP Exchange.* Samples of reaction mixture (5–10 $\mu$l) containing 1–20 m$\mu$moles each of ADP and ATP were applied to 20 $\times$ 20 cm thin-layer chromatography plates of polyethyleneimine cellulose prepared as described by Randerath and Randerath (1964). The chromatograph was developed with 0.1 M LiCl for 90 sec, followed by 1.0 M LiCl until the solvent front came within 0–3 cm of the top of the plate (about 60 min). The plates were dried and the ADP ($R_F$ 0.26) and ATP ($R_F$ 0.06) spots were located in ultraviolet light. The spots were scraped off with a scalpel and transferred to tubes containing 1 ml of 0.1 N HCl. The cellulose was pulverized with a glass rod, and the suspension was heated at 100° for 30 min, with brief mixing every 10 min. The acidic suspension was neutralized by the addition of 0.1 ml of 1 N NaOH and centrifuged. An aliquot of the supernatant was then removed for scintillation counting in Bray's (1960) solution. When ATP or ADP, labeled with $^{14}$C in the 8 position of the adenine ring, was tested with this procedure,

[2] The procedure used for measurement of E-P does not distinguish between free E-P and E-P complexed with substrates (*e.g.*, E-P·ADP). The total E-P present is likely distributed among a number of substrate complexes.

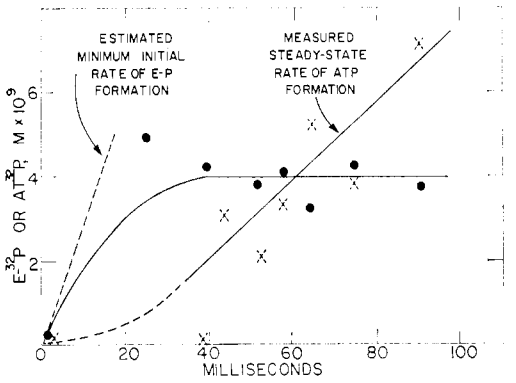



FIGURE 1: Rate of E-$^{32}$P and ATP-$^{32}$P formation from $^{32}P_i$. Measurements were made as described under Materials and Methods at about 24°. One syringe contained 0.05 mM $^{32}P_i$ ($1.2 \times 10^7$ cpm/$\mu$mole), 0.1 mM ADP, 0.2 mM succinyl-CoA, and 0.01 M Tris-Cl (pH 6.6). The other syringe contained enzyme (0.5 mg/ml, sp act. 7) in 0.05 M Tris-Cl (pH 7.4), 0.1 M KCl, 8 mM $MgCl_2$, and 0.1 mM EDTA. The steady-state velocity of ATP formation as indicated on the figure was measured separately in reactions of 2–15 sec duration; observed ATP-$^{32}$P values (×) observed E-$^{32}$P (●).

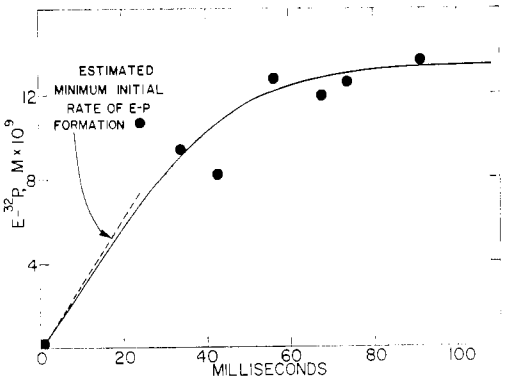



FIGURE 2: Rate of E-$^{32}$P formation from ATP-$^{32}$P. One syringe contained enzyme (0.25 mg/ml, sp act. 7) in 0.05 M Tris-Cl (pH 7.4), 0.1 M KCl, 8 mM $MgCl_2$, and 0.1 mM EDTA. The other syringe contained 0.02 mM CoA, 0.08 mM ATP-$\gamma$-$^{32}$P ($9 \times 10^6$ cpm/$\mu$mole), and 0.05 M sodium succinate in the same buffer as the enzyme solution. The rate of $^{32}P_i$ formation was measured separately in reactions of 2–15-sec duration. Observed E-$^{32}$P (●).

95–100% of the radioactivity was recovered, probably as free adenine.

*Succinate ⇄ Succinyl-CoA Exchange.* Samples (50 $\mu$l) containing approximately 0.04 $\mu$mole each of succinate and succinyl-CoA were pipetted onto 0.5 × 5 cm columns of DEAE-cellulose (formate form). Succinate was completely removed by washing the column with 6 ml of 0.1 M formic acid–0.1 M sodium formate. Succinyl-CoA was eluted with 5 ml of 0.5 M formic acid–0.5 M sodium formate. Aliquots of the eluent were added to Bray's (1960) solution for scintillation counting.

Rates of interchange between given reaction partners A and B were calculated from measured isotope exchanges and the expression (see Boyer, 1959)

$$\text{rate} = \frac{([A][B])}{([A] + [B])t} \ln (1 - F) \qquad (1)$$

where $F$ represents the fraction of isotopic equilibrium attained in time, $t$.

## Results

*Rate of E-P Formation from $P_i$ or ATP.* Two different sets of measurements were made to assess whether the phosphorylated form of the enzyme met kinetic criteria for a catalytic intermediate.[2] One was to compare the initial rate of E-P formation from $^{32}P_i$ with the rate of ATP formation starting with $P_i$, succinyl-CoA, and ADP; the other was to compare the initial rate of E-P formation from ATP-$^{32}$P with the rate of $P_i$ formation, starting with ATP, succinate, and CoA. These experiments were somewhat limited by the amounts of enzyme required, but were sufficient to establish clearly the necessary quantitative relationships.

Figure 1 shows the rate of E-P and ATP formation starting from the $P_i$ side. The concentration of E-P has apparently reached a steady-state within the first 20-msec reaction. From the initial slope, as given in the figure, the estimated initial rate of E-P formation under these conditions is at least 1.1 m$\mu$moles/sec.

In contrast to the fair accuracy of measurement of E-P formation, the measurements of initial appearance of ATP-$^{32}$P were subject to considerable error because of the very small amount formed. Thus it was deemed desirable to establish the steady-state rate of ATP formation by observations made over a longer period. The steady-state rate shown in Figure 1, equivalent to 0.34 m$\mu$mole/sec, is not an approximation from the data given with Figure 1, but corresponds to the rate measured in separate experiments of 2–15-sec duration. This steady-state rate of ATP formation is only about one-third of the minimal initial rate of E-P formation.

The approximations of ATP-$^{32}$P appearance given in Figure 1 do suffice to show a definite lag in ATP-$^{32}$P formation. After the short lag, ATP-$^{32}$P formation continues as anticipated from the separate rate measurements. These observations show that the initial rate of E-P synthesis is at least two to three times faster than the subsequent steady-state rate of ATP formation, and indicate that the E-P concentration reaches a plateau at approximately the time that the steady-state rate of ATP formation reaches a maximum.

The kinetics of E-P formation in the reverse direction from ATP-$\gamma$-$^{32}$P, CoA, and succinate are shown on Figure 2. The initial rate of E-P synthesis as estimated from these results is 1.5 m$\mu$moles/sec. The steady-state rate of $P_i$ formation was estimated as 1.1 m$\mu$moles/sec, again by quenching the reaction 2–15 sec after mixing under identical conditions. The E-P formation is thus also sufficiently rapid in this direction to be considered an intermediate in the catalysis.

It is of interest that in the direction of ATP formation (Figure 1) the ratio of E-P/total potential phosphoryl enzyme = 0.04 in the steady state, whereas under the conditions used for the reverse reaction (Figure 2), this ratio is approximately 0.3. These values are based on the total potential phosphorylation by exposure of enzyme to excess ATP (Ramaley *et al.*, 1967).

TABLE I: Rates of Some Reactions Catalyzed by Succinyl-CoA Synthetase.

| Reaction | Temp (°C) | Moles of Substrate Reacting/Mole of Enzyme per min |
|---|---|---|
| Net initial velocity | 25 | 7000 |
| $RCOO^- \rightleftarrows RC(=O)-CoA$ | 25 | 70 (no $P_i$) |
| | | 24 (10 mM $P_i$) |
| ADP ⇄ ATP | 25 | 167 |
| $P_i$ ⇄ ATP | 25 | <0.02 (0.1 mM CoA) |
| $P_i$ ⇄ ATP | 25 | 0.6 (0.1 mM CoA and 5 μM succinate) |
| $P_i$ ⇄ ATP | 25 | 380 (all substrates)[a] |
| $P_i$ ⇄ E-P | 0 | 0.01 (0.5 mM CoA) |
| ATP ⇄ E-P | 0 | 1.1 (no CoA) |
| | | 3.0 (0.038 mM CoA) |

[a] Concentrations of substrates were as follows: succinyl-CoA, 10 μM; succinate, 52 μM; CoA, 52 μM; ADP, 52 μM; ATP, 520 μM; and $P_i$, 780 μM.

*Rates of Some Partial Reactions.* Table I gives a compilation of some measurements, made in this laboratory at various times during the past 2 years, of the rates of the various partial reactions catalyzed by succinyl-CoA synthetase. It is clear from the rates given that each of the partial reactions measured is much slower than the net initial velocity, and moreover, that the rates of some of the partial reactions are increased in the presence of additional substrates. Although these comparisons are hampered by lack of uniformity of conditions and concentrations, they represent the kind of observations which prompted us to consider the phenomenon of substrate synergism in a study which was properly controlled and on a firm theoretical basis.

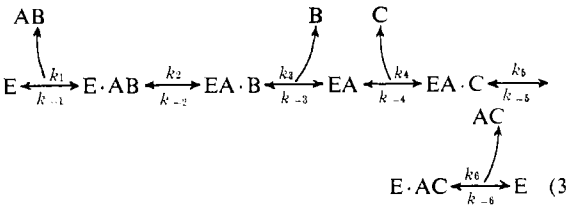
*A Simple Basis for Detection of Substrate Synergism.* If net reaction rates are rapid compared with partial reactions, synergism would appear probable. However, comparison of partial reaction rates with net reaction velocities does not readily allow definitive conclusions as to whether or not substrate synergism occurs. This is because net reactions are usually measured in the absence of a product necessary for the partial reactions. For example, measurement of the rate of succinyl-CoA cleavage requires presence of ADP but not ATP, but ATP must obviously be present for measurement of the ADP ⇄ ATP exchange. Presence of ATP could have inhibitory effects expressed only in the partial and not the net reaction.

Simple relationships between rates in the presence of all substrates and partial reaction velocities follow from consideration of their respective reaction fluxes at equilibrium. The partial reaction is of necessity an equilibrium situation with no net catalysis. Comparisons of exchange rates between pairs of substrates in the presence and absence of some or all other substrates at equilibrium can lead to definitive conclusions regarding the existence of synergistic effects.

Consider the system for transfer of a group A from B to C

$$AB + C \rightleftarrows AC + B \qquad (2)$$

where




(3)

In eq 3 the center dot indicates a Michaelis complex and juxtaposition a covalent band, *e.g.*, E·AB is the Michaelis complex of AB and enzyme, and EA·B designates the Michaelis complex of B with the enzyme to which the group A is covalently bound. Equations for the AC ⇄ C interchange with this system are readily obtained by the approach previously described (Boyer, 1959; Boyer and Silverstein, 1963).[3] The rate is given by

$$R = \frac{k_3k_5k_6}{k_{-4}k_{-5} + k_{-4}k_6 + k_5k_6} \times \frac{(E)_t}{\frac{1}{(C)} + \frac{k_4}{k_{-4}} + \frac{k_4k_5}{k_{-4}k_{-3}} + \frac{k_4k_5k_6}{k_{-4}k_{-5}k_{-6}(AC)} + \frac{(B)}{(C)}\left(\frac{k_{-2}k_{-3}}{k_2k_3} + \frac{k_{-3}}{k_3}\right)} \qquad (4)$$

Examination of eq 4, the rate equation for AC ⇄ C exchange in the presence of all substrates, shows that even at high substrate concentrations the rate remains an inverse function of (B)/(C), as predicted by the fact that both of these substrates are competing for the same enzyme form, EA.

For the partial reaction of AC ⇄ C interchange with no AB or B present, the derivation is as above but the terms (E·AB) and (EA·B) need not be considered

[3] For this derivation, let (E·AC) = $a + b$, where $a$ = (E·AC) derived most recently from AC and $b$ = (E·AC) derived most recently from C; (EA·C) = $c + d$, where $c$ = (EA·C) derived most recently from AC and $d$ = (EA·C) derived most recently from C.

The rate of AC ⇄ C is equal to $k_5b$ (or $k_{-4}c$). An expression for $b$ is readily attained by consideration that at equilibrium $0 = db/dt = k_5d - (k_{-5} + k_6)b$ and $0 = dd/dt = k_4(EA)(C) + k_{-5}b - (k_{-4} + k_5)d$ from which it follows that $b = (EA)(C) - k_4k_5/(k_{-4}k_{-5} + k_{-4}k_6 + k_5k_6)$. (EA) may be formulated from the enzyme conservation equation and the various equilibria involved

$$(EA) = \frac{(E)_t}{1 + (C)\left\{\frac{k_4k_5k_6}{k_{-4}k_{-5}k_{-6}(AC)} + \frac{k_4k_5}{k_{-4}k_{-5}} + \frac{k_4}{k_{-4}}\right\} + (B)\left\{\frac{k_{-2}k_{-3}}{k_2k_3} + \frac{k_{-3}}{k_3}\right\}}$$

and eq 4 follows from simple substitution and rearrangements.

in the enzyme conservation equation. The rate equation thus becomes

$$R = \frac{k_4k_5k_6}{k_{-4}k_{-5} + k_{-4}k_6 + k_5k_6} \times \frac{(E)_t}{\frac{1}{(C)} + \frac{k_4}{k_{-4}} + \frac{k_4k_5}{k_{-4}k_{-5}} + \frac{k_4k_5k_6}{k_{-4}k_{-5}k_{-6}(AC)}} \quad (5)$$

Equation 5 differs from eq 4 only in that the (B)/(C) term is missing from the denominator. This additional denominator term reflects the fact that the velocity must be correspondingly reduced when measuring the exchange with all substrates present, because some of the enzyme will be in complexes (E·AB, EA·B) that do not participate in the AC ⇄ C exchange. Similar considerations hold for comparison of any exchange measured as a partial reaction in the presence or absence of all substrates where the catalytic route for the exchange is unchanged.[4]

From these considerations it is evident that the rate of the partial reaction in the absence of other substrates must exceed the rate of the same exchange reactions as measured at the same reactant concentrations but with all other substrates present if the same catalytic steps and efficiencies are involved. If the opposite situation holds, substrate synergism must exist, and either the catalytic efficiency must be modified by change in the values for one or more rate constants in the presence of other substrates, or a different reaction pathway must be involved.

*Synergism in E-P Formation from ATP.* Figure 3 represents an experiment designed in cognizance of the above theoretical considerations. The rate of ADP ⇄ ATP exchange was measured under identical conditions except for the presence or the absence of all other substrates at equilibrium. The exchange rate is shown to be increased about fivefold in the presence of other substrates, clearly revealing the existence of substrate synergism.

The presence of each additional substrate above that necessary for the partial reaction introduces corresponding terms in the denominator of the rate equation. The presence of various additional substrates individually might reveal a larger synergism if this substrate was responsible for most or all of the synergism effect. The results of such experiments are given in Table II.

TABLE II: Stimulation of the Rate of ADP ⇄ ATP Exchange by Other Substrates.[a]

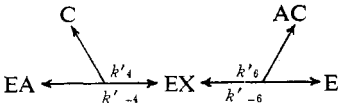
| Additions | | ADP ⇄ ATP |
|---|---|---|
| Substrate | Concn (mM) | (μM/min) |
| None | | 10.3 |
| Succinyl-CoA | 0.17 | 59.3 |
| Succinate | 0.92 | 13.0 |
| CoA | 0.92 | 13.5 |
| CoA | 0.0092 | 12.0 |
| $P_i$ | 1.88 | 11.7 |

[a] Succinyl-CoA synthetase (15.8 μg, sp act. 17.8) was incubated, in a final volume of 0.55 ml, with 0.1 M Tris-Cl (pH 7.2), 0.1 M KCl, 0.01 M $MgCl_2$, 0.185 mM ADP-8-$^{14}$C (sp act. 2.6 × $10^6$ cpm/μmole), 1.85 mM ATP, and the indicated additions. After 2 min at 25°, the reactions were stopped by the addition of 0.25 ml of 0.1 M EDTA (sodium salt, pH 7.2), and 10-μl samples were removed for thin-layer chromatography as described under Methods. The rates given were calculated using eq 1 and are averages of duplicate measurements.

They show that each of the substrates exhibits at least a slight synergistic effect on the ADP ⇄ ATP exchange. By far the largest effect is seen with the addition of succinyl-CoA, suggesting that succinyl-CoA is primarily responsible for the stimulation. However, due to the relatively rapid nonenzymic hydrolysis of succinyl-CoA, finite concentrations of succinate and CoA must have been present during this incubation, and it is therefore not possible to conclude that the effect seen is ascribable only to succinyl-CoA.

A further refinement in assessment of factors influencing the ADP ⇄ ATP exchange is plausible by measurement of the ADP ⇄ E-P exchange. The effect of CoA concentration on this partial reaction is shown in Figure 4. The exchange rate is stimulated by CoA according to simple saturation kinetics. This is consistent with the effect of CoA resulting from binding to noninteracting sites. The concentration of CoA giving half-maximal stimulation (6 μM) is near the $K_m$ measured for CoA as a substrate in the net over-all reaction (3.6 μM), implying that the stimulatory effect of CoA on the exchange is related to its role as a substrate. The stimulation observed in this experiment is considerably greater than that seen in Table II where the ADP

[4] When steps involving changes in bound substrates or enzyme, such as steps governed by $k_2$ and $k_{-2}$ and by $k_5$ and $k_{-5}$ of eq 3, are omitted from consideration, equations for exchange among labeled substrates can be conveniently expressed in terms of kinetic instead of rate constants (Cleland, 1967). Thus if the AC ⇄ C exchange is considered to be governed only by




(6)

the equation for exchange in terms of rate constants is

$$R = \frac{k'_4k'_6}{k'_{-6} + k'_4} \frac{(E)_t}{\frac{1}{(AC)} + \frac{k'_6}{k'_{-6}} + \frac{k'_4k'_6}{k'_{-4}k'_{-6}(C)}} \quad (7)$$

and in terms of kinetic constants (Cleland, 1967) is

$$R = \frac{V_1(K_{iac}/K_a)(C)(AC)}{K_{ic}(AC) + K_{iac}(C) + (AC)(C)} \quad (8)$$

We have preferred to use equations in terms of rate constants because of the actual enzyme phosphorylation and dephosphorylation steps (*e.g.*, such as steps governed by $k_{-2}$ and $k_2$ and by $k_{-5}$ and $k_5$ in eq 3) are included in the rate expressions.

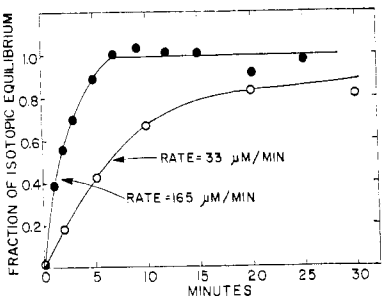



FIGURE 3: Substrate synergism in the ADP ⇄ ATP exchange reaction. One sample contained, in 0.57 ml, 0.016 mg of succinyl-CoA synthetase (sp act. 26.0), 0.1 M Tris-Cl (pH 7.2), 0.1 M KCl, 10 mM $MgCl_2$, 0.18 mM ADP, and 1.8 mM ATP. A second sample contained, in addition, 0.88 mM succinate, 0.17 mM succinyl-CoA, 0.088 mM CoA, and 1.18 mM $P_i$. The solutions were incubated for 10 min at 25° to assure attainment of chemical equilibrium, then 10 μl of 0.4 mM ADP-8-$^{14}$C (sp act. 7 × $10^7$ cpm/μmole) was added to each. Aliquots (50 μl) were removed at intervals and added to 25 μl of 0.1 M EDTA (sodium salt, pH 7.2) to stop the reaction. Portions (10 μl) of the resulting mixtures were analyzed for ATP-$^{14}$C by thin-layer chromatography as described under Materials and Methods. The ADP ⇄ ATP reaction rates were calculated according to eq 1 for each point before equilibrium and averaged. Exchange with only ADP and ATP present (○—○); exchange with all substrates present (●—●).

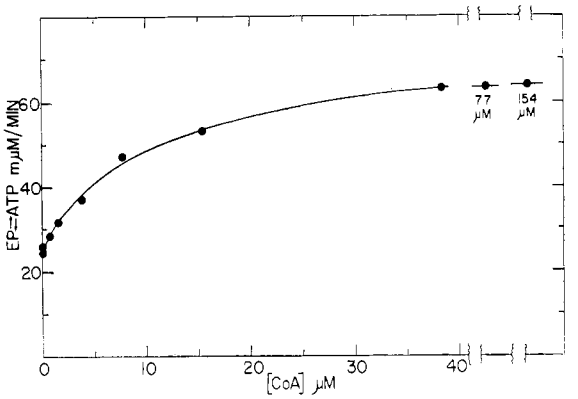



FIGURE 4: The effect of CoA on the rate of EP ⇄ ATP exchange. Succinyl-CoA synthetase (1.6 mg, sp act. of 26.0) was converted into the phosphorylated form by incubation with 5 mM ATP for 10 min at 0°. The E-P was isolated by passage through a 0.5 × 4 cm column of Dowex ($Cl^-$) equilibrated with 0.1 M Tris-Cl (pH 7.2). Portions of the E-$^{32}$P (sufficient for a final concentration of 0.5 μM) were incubated with 8.5 μM ADP, 14.6 μM ATP-γ-$^{32}$P (6.9 × $10^7$ cpm/μmole), 0.077 M Tris-Cl (pH 7.2), 0.077 M KCl, 7.7 mM $MgCl_2$, and CoA at the indicated concentrations. After 5 min at 0°, the reactions were stopped by the addition of 1.5 ml of liquified phenol, and E-$^{32}$P was determined after exhaustive extraction of the phenol layer (Boyer and Beiber, 1967).

and ATP concentrations used were much higher, suggesting that one effect of CoA may be on the binding constants for ADP and/or ATP.

*Synergism as Demonstrated by Measurement of the Succinate ⇄ Succinyl-CoA Exchange.* Exchange between succinyl-CoA and succinate has been observed and could reflect formation of a form of succinyl-CoA synthetase containing firmly bound CoA (Upper (1964), Moyer *et al.* (1967), and Cha *et al.* (1967)). Even though such a form may exist, evidence is lacking to definitively support its participation as an intermediate on the major catalytic path. Nevertheless, this exchange exhibits substrate synergism (Figure 5) in a manner analogous to the ADP ⇄ ATP exchange but with more pronounced effects. The rate in the presence of all substrates is about 60 times that at the same concentrations of succinate and succinyl-CoA but in the absence of substrates.

When each of the substrates is tested individually under the same conditions (Table III), only ATP provides a marked stimulation of the rate of succinate ⇄ succinyl-CoA exchange. The effect could, however, reflect cleavage of ATP to ADP and $P_i$ in the presence of small amounts of CoA arising from hydrolysis of succinyl-CoA. All substrates would thus be present, and dynamic reversal of the over-all reaction could be responsible for the exchange.

## Discussion

Our observations show that E-P formation occurs at rates equal to or greater than the corresponding net reactions starting from either the $P_i$ or ATP side of the succinyl-CoA synthetase reaction. These results are clearly consistent with participation of the phosphoenzyme in the major or the only catalytic route. Two additional criteria need to be met before a conclusion may be reached that the phosphoenzyme is indeed a catalytic intermediate. One is the phosphoenzyme must, in addition to rapid formation, give rise to product at a rate equal to or greater than the net catalytic rate. That is, will E-P formed from ATP react to give $P_i$ at rate equal to or greater than the net catalytic rate. A second criterion is whether the E-P formed from $P_i$ or from ATP is identical. Present information suggests that both criteria will be met and that a single type of E-P serves as an intermediate, but reservations in this conclusion must be recognized.

That E-P disappears as well as forms rapidly is definitely suggested although not proved by the low steady-state levels of E-P reported in Figures 1 and 2. The equilibrium between ATP and E considerably favors enzyme phosphorylation (Ramaley *et al.*, 1967). Yet only about one-third of the enzyme was phosphorylated during the steady state while net reaction was ensuing starting with ATP. When starting with $P_i$ and succinyl-CoA, the steady-state level of E-P was only about $^1/_{20}$ of the potential level. The equilibrium for E-P formation from the $P_i$ side has not been measured (for unknown reasons, with higher succinyl-CoA concentrations E-P formation is depressed (Ramaley *et al.*, 1967)). However, concentrations of $P_i$ and succinyl-CoA used for data of Figure 1 would, in the absence of ADP for net reaction, give a considerably higher portion of E-P than the observed steady-state level. Although the presence of other substrates could modify equilibria involved, the rapid attainment of low steady-state levels of E-P point strongly to rapid disappearance as well as rapid formation of E-P during net catalysis.

With respect to the possible identity of E-P formed

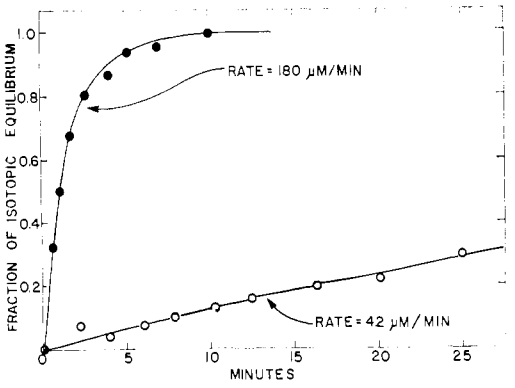



FIGURE 5: Substrate synergism in the succinate ⇄ succinyl-CoA exchange. One sample contained in 0.75 ml of 0.080 M Tris-Cl (pH 7.2), 0.080 M KCl, 8.0 mM $MgCl_2$, 0.79 mM succinyl-CoA, 0.49 mM succinate-2,3-$^{14}$C (sp act. 5 × $10^6$ cpm/μmole), and 48 μg of succinyl-CoA synthetase (sp act. 10). A second sample contained, in addition, 0.69 mM CoA, 1.17 mM $P_i$, 0.18 mM ADP, and 1.76 mM ATP, but only 36 μg of the enzyme preparation. Aliquots (50 μl) were removed at intervals and added to 25 μl of 0.1 M EDTA (sodium salt, pH 7.2) to stop the reaction, then 50-μl portions of the resulting mixture were removed for measurement of succinyl-CoA-$^{14}$C as described under Materials and Methods. Slight corrections were applied to account for the nonenzymic hydrolysis of succinyl-CoA ($k_{HOH}$ = 0.0103 $min^{-1}$). Succinate ⇄ succinyl-CoA exchange rates were calculated as described in the text. Exchange with succinate and succinyl-CoA present (○—○); exchange with all substrates present (●—●). Because in the experiment with all substrates only three-fourths as much enzyme was present, the ratio of rates calculated for equal enzyme concentrations is about 60.

from either $P_i$ or ATP, present evidence is consistent with such identity (Ramaley *et al.*, 1967). However, the ability to form more E-P starting with succinyl-CoA and $P_i$ than with ATP (Ramaley *et al.*, 1967) suggests caution in interpretation. Studies now underway in this laboratory on phosphorylated and nonphosphorylated subunits may provide a definitive answer to these and other relationships.

The number of enzymes known to form phosphoryl derivatives is quite limited. Among enzymes catalyzing intermolecular transfer of a phosphoryl group, evidence has been presented that catalysis by nucleoside diphosphokinase (Norman *et al.*, 1965; Mourad and Parks, 1966) and by isocitrate lyase (Inoue *et al.*, 1968) may involve phosphoenzyme intermediates. Kinetic measurements of the rate of phosphoryl enzyme formation and disappearance have not been presented. Catalytic participation is in contrast to the regulating role ascribed to the phosphorylation of phosphorylase (Fischer and Krebs, 1966) and to the adenylation of glutamine synthetase (Shapiro *et al.*, 1967). Knowledge of such possible alternate function of an enzyme phosphorylation was one of the factors that stimulated the present studies.

Results presented in this paper demonstrate clearly the phenomenon of substrate synergism for the ADP ⇄ ATP and succinate ⇄ succinyl-CoA partial reactions. Both reactions are slow when measured at the same concentrations of exchanging substances in the absence as compared with the presence of some or all other substrates. Of particular value is the synergism demonstrated in ADP ⇄ ATP exchange because this partial reaction reflects E-P formation and is known from the studies reported herein to constitute the only or a major catalytic pathway. In contrast, the succinate ⇄ succinyl-CoA exchange could be the result of a nonphosphorylated high-energy enzyme form (Moyer *et al.*, 1967), could reflect E-P formation in the presence of very low levels of contaminating $P_i$, or could result from some side reaction not on the catalytic pathway.

TABLE III: Effect of Addition of Other Substrates on Rate of Succinate ⇄ Succinyl-CoA Exchange.[a]

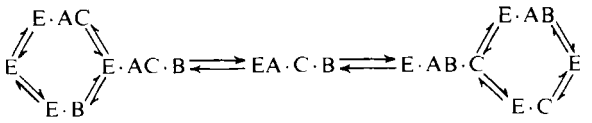
| Addition | | Succinate ⇄ Succinyl-CoA |
|---|---|---|
| Substrate | Concn (mM) | (mM/min) |
| None | | 4 |
| $P_i$ | 1.17 | 6 |
| CoA | 0.69 | 6 |
| ADP | 0.18 | 6 |
| ATP | 1.76 | 120 |

[a] The experiment was done under the same conditions as described with Figure 5, but with samples removed only 2 min after addition of enzyme. Each sample contained 0.49 mM succinate, 0.79 mM succinyl-CoA, and 0.22 mM CoA (present due to hydrolysis of succinyl-CoA). Rates were calculated using eq 1 and are averages of duplicate determinations.

Both intuitively, and, more precisely, from the theoretical considerations given herein, important conclusions follow from the observed substrate synergisms. Either the presence of additional substrates in some manner increases the rate of one or more steps involved in the partial reaction, or opens up an alternate catalytic route for the partial reaction. The synergistic effects could be the result of increased binding affinity as well as increase in reactivity of the bound substrate. Further insight into these possibilities may be attained by assessment of synergistic effects over a range of reactant concentrations.

Two discrete explanations merit consideration for an increase in binding or in reactivity of bound substrates. One is that binding of substrates at sites other than at the catalytic site promotes catalysis by changing protein conformation or subunit interaction. This would be analogous to what is frequently termed an allosteric effect. Conversely, the presence of one substrate at the catalytic site could promote reactivity or binding of another substrate. An illustration of how an increase in reactivity might operate may be helpful. For example, the AC ⇄ C exchange in the presence of only AC and C could occur *via* the sequence E + AC ⇄ E·AC ⇄ EA·C ⇄ EA + C, but might proceed more rapidly in the presence of all substrates by a route involving the

participation of ternary complexes such as




Modifications in reactivity or binding could result from a change in protein conformation or position of groups at the catalytic site. Such a change is inherent in the "induced-fit" concept developed by Koshland (1964). Alternatively, two or more substrates bound closely together at one catalytic site could mutually promote catalysis by steric interactions of their own groups. In addition, bound substrates could mutually influence reactivity through charge interaction, by effective change in dielectric constant, or by themselves furnishing catalytic or binding groups at the site. All these possibilities for catalytic synergism as a consequence of binding at a specific site can conveniently be grouped as catalytic site packing effects. Detailed and sophisticated studies as well as considerable knowledge of the structure of the active site will be necessary to give a satisfactory understanding of such packing effects.[5]

The limited studies on the relationship between CoA concentration and acceleration of the E-P $\rightleftarrows$ ATP exchange (Figure 4) suggest that the CoA effect results from binding at the catalytic site. The CoA concentration *vs.* activity plot shows simple saturation behavior as expected for a single site, and with CoA concentration for half-maximal activation close to the apparent $K_m$ for CoA in the net initial reaction.

The theoretical basis for detection of substrate synergism as described herein offers definite advantages over the previous situation in which an adequate basis for such comparisons was lacking. The present approach does have some shortcomings that must be recognized. Although the approach will allow, under some circumstances, the definite conclusion that substrate synergism in catalysis exists, it does not allow accurate evaluation of the magnitude of the synergistic effect. This magnitude could be assessed by an independent measurement of specific rates. For example, by use of the rapid mixing and quenching techniques, the rate of E-P formation from ATP in the presence and absence of other substrates could be measured and a quantitative evaluation of the synergistic action could be obtained.

Because of the above-mentioned limitation, the present approach could fail to detect synergism under one set of circumstances. This would be if the magnitude of the synergistic promotion of rate produced by an additional substrate or substrates were weak and less than decreases in the rate of the partial reaction produced by converson of some of the total enzyme into forms inactive in the partial reaction. The net effect would be an observed decrease in the rate of the partial reaction in the presence of the additional substrates. The approach thus suffices to detect instances where strong promotion of catalysis occurs Fortunately, these are likely the most interesting cases for study.

[5] Uncertainty about the mechanism of the catalytic promotion as described herein is a principal reason for designation of the phenomenon by the operational description as substrate synergism. The term homosterism has been suggested by McElroy *et al.* (1967) for instances where one substrate bound at the catalytic site may promote reaction of another substrate because of changes in protein conformation. Homosterism would thus be a special case of substrate synergism where the mechanism is known.

Substrate synergism is distinguished from the cooperative effects of a substrate in that the latter is usually taken to imply promotion of reaction of one bound substrate molecule by binding of a second molecule of the same substrate. Synergism implies two or more different substrates are involved.

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# The Interaction of Trypsin with Neutral Substrates and Modifiers*

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ABSTRACT: In an attempt to assess the role of the positive charge in trypsin specificity, the hydrolysis constants of neutral substrates were compared with their charged analogs. Replacement of the side chain of arginine by that of citrulline and of lysine by that of heptyline resulted in decreases of $10^2$–$10^6$ in $k_0/K_0$. Enzyme treated to remove chymotrypsin impurities did not lose activity toward these substrates. Trypsin inactivated with diisopropylphosphofluoridate or 1-chloro-3-tosylamido-7-amino-2-heptanone failed to hydrolyze the neutral substrates. Ternary complex formation involving the enzyme, a positively charged molecule, and a neutral molecule was indicated by the following data. (1) The rate of hydrolysis of a given substrate was affected in a qualitatively different manner by modifiers of different charge types at equivalent $[M]/K_i$ ratios: phenol was a competitive inhibitor of benzoylcitrulline methyl ester hydrolysis while phenylguanidinium chloride was a noncompetitive inhibitor. (2) A given modifier affected the hydrolysis of different substrate charge types in a qualitatively different manner: phenol was a competitive inhibitor of benzoylcitrulline methyl ester hydrolysis and a noncompetitive inhibitor of benzoylarginine methyl ester hydrolysis. An auxiliary binding site to which neutral molecules preferentially bind is thus implicated.

This paper seeks to confirm and extend earlier observations on the relationship between the hydrolysis of neutral substrates by trypsin and evidence for a second or auxiliary binding site for this enzyme (Sanborn and Hein, 1967). Early work on enzymatic cleavage catalyzed by trypsin established its high specificity for bonds in which L-lysine or L-arginine contributed the carbonyl function (Bergmann *et al.*, 1939). These original conclusions on specificity were supported by subsequent studies on ester and amide derivatives (Neurath and Schwert, 1950). More recently, however, reports of neutral substrates hydrolyzed by trypsin have appeared (Inagami and Sturtevant, 1960; Inagami and Mitsuda, 1964; Cohen and Petra, 1967).

That trypsin specificity is highly dependent upon the length of the side chain containing the positive charge has been shown by Elmore and coworkers using lysine and arginine homologs (Baines *et al.*, 1964; Baird *et al.*, 1965; Elmore *et al.*, 1967). Inhibitory capacity is also dependent upon the nature of the side chain and its orientation with respect to the positive charge (Mares-Guia and Shaw, 1965, 1967; Inagami, 1964; Geratz, 1966, 1967).

Evidence for an auxiliary binding site which can accommodate both positively charged (Trowbridge *et al.*, 1963) and neutral (Howard and Mehl, 1965) molecules and result in increased catalysis has been reported. The present study seeks to further clarify the role of the positive charge in trypsin specificity by investigating combinations of charged substrates (arginine (Ia) and lysine (IIa) derivatives) and neutral analogs (citrulline (Ib) and heptyline (IIb) derivatives). In the process of studying the behavior of positively charged and neutral modifiers toward these substrates, information has been gained about the nature of an auxiliary binding site.

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