Massey, L. K., Conrad, R. S., and Sokatch, J. R. (1974), *J. Bacteriol. 118,* 112-120.
Moss, J., and Lane, M. D. (1971), *Adv. Enzymol. 35,* 321-442.
Rilling, H. C., and Coon, M. J. (1960), *J. Biol. Chem. 235,* 3087-3092.
Schiele, U., Niedermeier, R., Stürzer, M., and Lynen, F. (1975), *Eur. J. Biochem. 60,* 259-266.
Seubert, W. (1960), *J. Bacteriol. 79,* 426-434.
Seubert, W., Fass, E., and Remberger, U. (1963), *Biochem. Z. 338,* 265-275.
Seubert, W., and Remberger, U. (1963), *Biochem. Z. 338,* 245-264.
Shapiro, B. (1953), *Biochem. J. 53,* 663-666.
Simon, E. J., and Shemin, D. (1953), *J. Am. Chem. Soc. 75,* 2520.
Sokatch, J. R., Sanders, L. E., and Marshall, V. P. (1968), *J. Biol. Chem. 253,* 2500-2506.
Stadtman, E. R. (1956), *Methods Enzymol. 3,* 931-941.
Sumper, M., and Riepertinger, C. (1972), *Eur. J. Biochem. 29,* 237-248.
Tanabe, T., Wada, K., Okazaki, T., and Numa, S. (1975), *Eur. J. Biochem. 57,* 15-24.
Taylor, B. L., Barden, R. E., and Utter, M. F. (1972), *J. Biol. Chem. 247,* 7383-7390.
Utter, M. F., Barden, R. E., and Taylor, B. L. (1975), *Adv. Enzymol. 42,* 1-72.

Evidence against an Acyl-Enzyme Intermediate in the Reaction Catalyzed by Clostridial Phosphotransacetylase†

Jack Henkin‡ and Robert H. Abeles*

ABSTRACT: Clostridial phosphotransacetylase catalyzes acyl group transfer between coenzyme A (CoA) and inorganic phosphate and also the arsenolysis of acetyl-coenzyme A (AcCoA) to yield acetate and CoA-SH. The enzyme mobility on sodium dodecyl sulfate electrophoresis corresponds to a molecular weight of 70 000. Kinetics of both forward and reverse reactions are of the ternary type as previously reported and product inhibition data are consistent with a random binding scheme. One essential sulfhydryl group per 70 000 daltons was inactivated in a pseudo-first-order process by either *N*-ethylmaleimide or 5,5′-dithiobis(nitrobenzoic acid). Reduction of the rate of this inactivation by 50% in the presence of AcCoA or acetyl phosphate concentrations near their kinetic $\bar{K}$ values demonstrates binding of these acyl donors in simple enzyme-substrate complexes. Moreover, pulse-chase experiments show these binary complexes to be functional and also show that they do not dissociate rapidly compared with their rates of catalytic turnover. Incubation of the enzyme with ^{14}C-labeled acyl donors failed to produce labeled protein after passage through Sephadex. This was true despite efforts to mimic "substrate synergism" with desulfo-CoA or to compensate for unfavorable equilibria by means of CoA traps. Very slow isotope exchange reactions of $^{32}P_i$ into acetyl phosphate and [^{3}H]CoA into AcCoA were at first observed. As in the cases of several other enzymes recently reexamined, these were shown on careful inspection to be artifacts of contamination by second substrates. Attempts to detect exchange reactions between acetyl phosphate and P_i, even in the presence of the CoA analogue, desulfo-CoA, were also unsuccessful. Therefore, no evidence for an acyl-enzyme could be detected. Furthermore, our data allow us to develop arguments which, we believe, indicate that an acyl-enzyme intermediate is extremely improbable in the reaction catalyzed by phosphotransacetylase.

It is now well established that the reactions catalyzed by many group transfer enzymes involve covalent intermediates (Spector, 1973). The existence of such covalent intermediates is not surprising, and, as a matter of fact, is expected, for enzymes which show ping-pong kinetics. For enzymes where the kinetic pattern gives intersecting lines, it had been assumed that covalent intermediates are unlikely. This view was seriously altered when it was reported that hexokinase, an enzyme which does not display ping-pong kinetics, catalyzed ATP-ADP[1] exchange and glucose-glucose-6-P exchange (Walsh and Spector, 1971a). The existence of these exchange reactions led to suggestions that this reaction involves a phospho-enzyme intermediate. Subsequently, the occurrence of exchange reactions was reported for phosphoribosyl pyrophosphate synthetase (Switzer, 1970) and glycerate kinase (Walsh and Spector, 1971b). Neither of these enzymes shows ping-pong kinetics. These discoveries led to suggestions that all transfer reactions may involve covalent intermediates, and that proof of their nonexistence is the task of the researcher (Spector, 1973).

In this context, it was decided to reexamine some properties of phosphotransacetylase (EC 2.3.1.8) from *Clostridium*

† This is publication No. 1092 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received March 16, 1976.* This research was supported in part by grants from the National Science Foundation (GB 31952) to R.H.A. and the National Institutes of Health Training Grant GM 212 (J.H.).

‡ Present address: Chemistry Department, Harvard University, Cambridge, Massachusetts 02138.

[1] Abbreviations used: CoA, coenzyme A; AcCoA, acetyl-coenzyme A; E, enzyme; S, substrate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; glucose-6-P, glucose 6-phosphate; P_i, inorganic phosphate; NAC, *N*-acetylcysteamine; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide; NAD, nicotinamide adeninedinucleotide; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

kluyveri. This enzyme carries out reversible acyl group transfer between CoA and orthophosphate and also, the analogous abortive reaction of arsenate and AcCoA to give CoA and acetate (Stadtman and Barker, 1950). Hibbert et al. (1971) reported its kinetics to be of the ternary type and consistent with a rapid equilibrium random order binding scheme (Dixon and Webb, 1964). They have also stated that there is no essential thiol at the active site. The enzyme is fairly nonspecific with respect to acyl phosphates (Marcus and Elliot, 1959), but highly specific for CoA (Shimizu et al., 1970). The molecular weight derived from gel-filtration has been given as 60,000 Daltons (Shimizu et al., 1969).

In our investigations of the mechanism of action of phosphotransacetylase, particular emphasis was placed on the possible occurrence of an acyl-enzyme in the catalytic process. The experiments reported here led to the conclusion that an acyl-enzyme does not occur. After our investigation of the mechanism of action of phosphotransacetylase was completed, new evidence became available which casts considerable doubt on the involvement of covalent intermediates in the reaction catalyzed by phosphoribosylpyrophosphate synthetase, glycerate kinase, and hexokinase.[2]

Experimental Procedure

Enzymes and Materials. Phosphotransacetylase, acetate kinase, malic dehydrogenase, and citrate synthase were obtained from Boeringher. The transacetylase was 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) and typically had a specific activity of 1100 μmol min^{-1} (mg of enzyme)$^{-1}$ under standard assay conditions. CoA, AcCoA, *N*-ethylmaleimide, and adenine nucleotides were purchased from P-L Biochemicals. Dithiothreitol, l-malic acid, oxaloacetic acid, and Ellman's reagent were from Calbiochem. [1-^{14}C]Acetic anhydride, sodium [1-^{14}C]acetate, [^{14}C]AcCoA, and carrier-free $^{32}P_i$ were products of New England Nuclear. Oxy-CoA was a gift of Dr. C. J. Stewart. Desulfo-CoA was prepared from CoA-SH and Raney nickel by the method of Chase et al. (1966), and freed of nickel ion with Chelex 100 from Bio-Rad. Cysteamine was a product of Sigma. This material was N-acetylated with [^{14}C]acetic anhydride in bicarbonate buffer (pH 8.0), followed by cleavage of N,S-diacetylated material with hydroxylamine.

Isotopically labeled acetyl phosphate was prepared from [^{14}C]acetic anhydride by reaction with orthophosphate in pyridine according to Avison (Avison, 1955). [^{3}H]CoA, randomly labeled, was prepared by exposing oxidized CoA to 3 Ci of tritium gas (NEN) for 10 days at 0 °C. The desired product was purified by paper chromatography in an ascending system (isobutyric acid–water–28% NH_3, 66:33:1 (v/v/v)). It was re-chromatographed after enzymatic conversion to AcCoA, and [^{3}H]CoA was then regenerated by enzymatic arsenolysis. All other chemicals were reagent grade and not further purified. In experiments where the final concentration of alkali metal salts would exceed 20 mM, the alkali metal salts were replaced by Tris salts by passage through Dowex-50 (Tris form) and deionized, glass-distilled water was used in all experiments.

Methods. Sodium dodecyl sulfate gel electrophoresis was carried out in one-half cross-linked gels according to the method of Weber and Osborn (1969). Transacetylase was assayed at 25 °C in 0.1 M Tris buffer (pH 7.5) by a modification of the method of Whiteley and Pelroy (1972). This is a coupled assay in which the product, AcCoA is enzymatically condensed with oxaloacetate to give citrate. The oxaloacetate consumed is then replaced by enzymatic oxidation of malate producing 1 equiv of NADH which is observed spectrophotometrically. The assay contained 1 mM CoA, 5 mM acetyl phosphate, 1 mM dithiothreitol, l-malate, NAD, and excess citrate synthase and malic dehydrogenase. Reaction of AcCoA with arsenate or phosphate was measured by disappearance of thiol ester absorption at 232 nm. The effects of thiol reagents on the enzyme were measured by preincubation of enzyme and reagent in 1 mM Tris buffer, both in the presence and absence of substrate, followed by 100–200-fold dilution of an aliquot into a standard assay mixture. Absorbance changes at 340 nm were monitored on a Beckman DU spectrophotometer with a Gilford 2000 recording instrument.

Mono- and diacylcysteamine were separated by paper chromatography using chloroform. Thiol ester was visualized with hydroxylamine and ferric chloride sprays.

Assay of [^{14}C]Citrate and [^{14}C]Acetate. In some experiments the arsenolysis of [^{14}C]acetyl phosphate was carried out in the presence of CoA, citrate synthetase, and oxaloacetate. The products of the reaction were acetate and citrate. These were determined as follows: For the determination of citrate, 50-μl aliquots were removed from the 1.0 ml reaction mixture and added to 0.5 ml of 50% glacial acetic acid containing 2% HCl and 1 mg of citric acid. This was boiled for 5 min to hydrolyze all of the acetyl phosphate to acetate. The reaction mixture was brought to dryness, which removes acetate. Acetic acid was again added and the solution again brought to dryness. This process was repeated once more. The residue which contained only [^{14}C]citrate was then counted. From the number of counts present the amount of citrate formed was determined. The remainder of the reaction mixture was used to determine the amount of acetate formed. The reaction mixture was made 1 M in NH_2OH and allowed to react for 10 min. Under these conditions acetyl phosphate is converted to hydroxamate; citrate and acetate remain unchanged. Glacial acetic acid (0.5 ml) was then added and the reaction mixture was distilled. The distillate contained acetic acid, the only volatile component of the reaction mixture. Aliquots of the distillate were titrated with NaOH and another aliquot was added to Bray's solution to determine [^{14}C]acetic acid. The total amount of ^{14}C in the reaction could then be determined.

Determination of AcCoA Trapping Efficiency. In some experiments acetyl-CoA was produced by the action of phosphotransacetylase on acetyl phosphate and CoA was trapped by the addition of oxaloacetate and citrate synthetase. It was necessary to establish that the trap was sufficiently effective to trap all acetyl-CoA released from the enzyme. To estimate the efficiency of free AcCoA capture by the oxaloacetate + citrate synthase trap employed to compete with transacetylase catalyzed AcCoA arsenolysis, the following control was carried out: [^{14}C]acetyl-CoA in threefold molar excess over the largest amount which could be expected in the reaction was infused steadily, using a motor driven syringe over a 10-min period, into the rapidly stirring reaction. Acetate and citrate were assayed as described above. In no case did the amount of label in acetate exceed 2% of that infused, the remainder being

[2] In case of hexokinase the occurrence of both pairs of exchange reaction and ATPase activity was taken as evidence for phospho-enzyme intermediate (Walsh and Spector, 1971). However, the rate of the exchange reaction was overestimated by a factor of 10^2 (Colowick, 1973). Evidence has been presented that the ATPase activity is not related to the hexokinase activity (Solomon and Rose, 1971). These findings, therefore, make the involvement of a phospho-enzyme intermediate in that reaction very dubious.

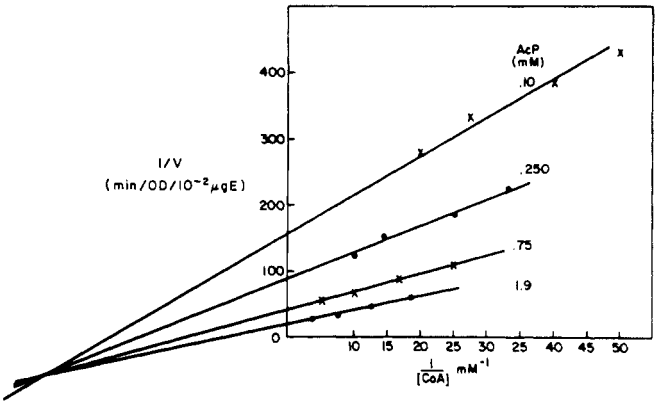


FIGURE 1: Kinetics of AcCoA formation as a function of CoA concentration. Four tubes containing 90 mM Tris-Cl, pH 7.5, 20 mM LiCl, and 20 mM ammonium sulfate were prepared. Each tube also contained 1 mM Tris-L-malate, 1 mM NAD^+, 5 IU of citrate synthase, 2 IU of malic dehydrogenase, and 1 mM dithithreitol. Concentrated Li^+K^+ acetyl phosphate was added to each tube to final [AcP_i] of 0.105, 0.265, 0.70, and 2.0 mM. Each tube was brought to 25 °C in a thermostated water bath and 0.95 ml transferred to a 1-ml, 1.0-cm cuvette. CoA was added to each cuvette to give final [CoA] from 0.02 to 0.30 mM (final volume, 1.0 ml). Finally 5–20 μl of 1 μg/ml enzyme was added and ΔOD measured at 340 nm on a water jacketed Beckman DU spectrophotometer with a Gilford 2000 recording instrument. Rates were expressed as ΔOD/min taken between 0.02 and 0.1 increase in OD, and data plotted in double-reciprocal form lines being fitted by an unweighted, two-parameter, least-squares program.

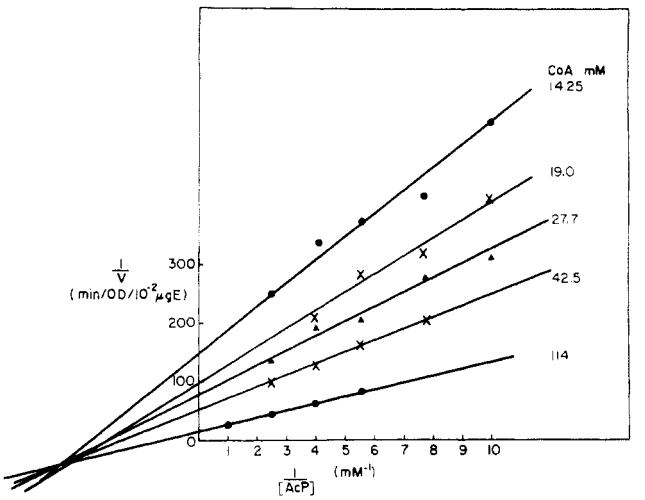


FIGURE 2: Kinetics of Ac-CoA formation as a function of acetyl phosphate concentration. Same as Figure 1 except that tubes were made up to fixed CoA concentration and [AcP] varied in each cuvette. Note: These plots were derived by an iterative procedure. First, a square kinetic matrix was obtained and K_m values were estimated at each fixed value of the second substrate. Then the kinetics were repeated such that each varied substrate ranged approximately from 0.5 to 3 times its estimated K_m.

trapped as citrate.

Pulse-Chase Experiments. Typically 20 μl of enzyme solution containing [^{14}C]acetyl phosphate in buffer was force injected into 2 ml of buffer containing 50 mM cold carrier acetyl phosphate and varying [CoA] stirred at 1000 rpm. A control was carried out at each [CoA] where enzyme alone was injected into [^{14}C]acetyl phosphate solution at the same final specific activity. Reactions were quenched with acetic acid after either 2 or 4 min. Label present in AcCoA was then measured in one of two ways: (A) AcCoA was adsorbed onto Norit A and then released into solution for counting after washing by boiling NaOH; (B) AcCoA was converted to citrate enzymatically. Remaining [^{14}C]acetyl phosphate was converted to acetate by boiling and citrate was counted after several repeated evaporations of cold carrier acetic acid.

Isotope Exchanges. [$^{32}P_i$]acetyl phosphate exchange was measured by incubating $^{32}P_i$ and acetyl phosphate with enzyme in 0.1 M Tris buffer at 25 °C. After 10–60 min, excess ADP and acetate kinase were added to convert acyl-$^{32}P_i$ into [^{32}P]ATP. A small aliquot was then streaked onto Brinkman PEI plates and chromatographed with 0.5 M sodium phosphate, pH 3.5. The ATP spots were visualized under uv light, cut out, and counted in Bray's solution. Controls with no enzyme and with a small amount of CoA present were included to establish values for background and for complete isotopic equilibration.

CoA–AcCoA exchange was measured by incubating [^{3}H]CoA and AcCoA with enzyme in Tris buffer. After 30 min, mixtures were treated with potassium ferricyanide to oxidize CoA to the disulfide which facilitates separation of CoA_{ox} from AcCoA. For the separation of CoA_{ox} and AcCoA aliquots were streaked onto Whatman No. 3 sheets and subjected to ascending chromatography in the isobutyric acid–NH_3–water system previously described. AcCoA spots were counted after elution from paper into Bray's solution. Control experiments were carried out with boiled enzyme and with enzyme + phosphate. Exchange velocity was calculated according to the equation given by Boyer (1959).

$$V_i = \frac{-\ln (1 - f)ab}{(a + b)t} \quad (1)$$

where f is the fraction of maximal counts exchanged, t is the time of incubation, and a and b are the concentrations of CoA and AcCoA.

Micro Assays of P_i and CoA. Inorganic phosphate was measured according to the highly sensitive method of Itaya and Ui (1966) in which the extinction coefficient of phosphomolybdate is enhanced by complexation with malachite green. Low concentrations of CoA (<0.1 nM) were assayed by a modification of the method of Allred and Guy (1969).

Results

Molecular Weight. Phosphotransacetylase treated at pH 7.5 with excess mercaptoethanol exhibited a mobility on sodium dodecyl sulfate electrophoresis (Weber and Osborn, 1969) corresponding to a molecular weight of $70 \pm 2 \times 10^3$. The mobility of unreduced enzyme corresponds to a molecular weight of 60 000 as previously reported by Shimizu et al. (1970). Colorimetric titration of the unreduced enzyme with Ellman's reagent (Ellman, 1959) showed 0.97 ± 0.05 mol of thiol per 70 000 g of protein, confirming the molecular weight of 70 000.

Reaction Kinetics. Double-reciprocal plots of the kinetic analysis of the forward reaction

$$\text{acetyl phosphate} + \text{CoA} \rightarrow \text{AcCoA} + P_i \quad (2)$$

are shown in Figures 1 and 2. The kinetics are clearly of the ternary type in qualitative agreement with reports by Hibbert and co-workers (Hibbert et al., 1971). Ternary kinetics were also obtained for the reverse reaction using either orthophosphate or arsenate as the acyl acceptor. No evidence of ping-pong kinetics was observed over a wide (50-fold) range of substrate concentrations. Kinetic constants were derived from double-reciprocal plots according to methods described by Cleland (1970), and these values are summarized in Table I. The V_{max} values at pH 7.5 and 25 °C correspond to a turnover number of 1280/s in the forward direction and 50/s in the

TABLE I: Summary of Kinetic Constants.[a]

Substrate	K (mM) ($[S_2] \rightarrow \infty$)	$\bar{K}$ (mM) ($[S_2] \rightarrow 0$)	$K/\bar{K}$	V_{max}/E (s^{-1})
Acetyl-P_i	0.70 ± 0.10	0.20 ± 0.04	3.5 ± 1.0	1280 ± 50
CoA-SH	0.12 ± 0.02	0.029 ± 0.004	4.2 ± 1.0	1280 ± 50
Acetyl-CoA	0.20 ± 0.05	0.20 ± 0.05	1	50 ± 5
Phosphate	2.6 ± 0.4			50 ± 5
Arsenate	1.9 ± 0.3			50 ± 5

[a] Kinetic constants were obtained from secondary plots according to Cleland (1970) using slopes and intercepts from Figures 1 and 2. K values were further confirmed at 10× higher concentrations of substrate than the highest used in these figures.

TABLE II: Type of Competition between Various Inhibitors and Substrates.[a]

Inhibitor	Varied Substrate	Reaction[b]	Type of Competition[c]
P_i	Acyl-P_i	F	C
P_i	CoA	F	NC
High [P_i]	CoA	F	M
PP_i	Acyl-P_i	F	C
Arsenate	Acyl-P_i	F	M
AcCoA	Acyl-P_i	F	C
Desulfo-CoA	CoA	F	C
CoA	AcCoA	A	C
PP_i	AcCoA	A	NC

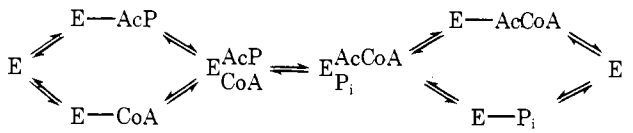
[a] Inhibition type was determined by inspection of Lineweaver-Burke plots for initial velocity kinetics both with and without several concentrations of inhibitor. [b] F, AcP + CoA → AcCoA + P_i; A, AcCoA + AsO_4^{3-} → CoA + AcOH. [c] C, competitive; NC, noncompetitive; M, mixed.

reverse direction. P_i and AsO_4^{3-} react at the same V_{max}. The apparent Michaelis constants of both CoA and acetyl phosphate increased approximately fourfold as the concentration of the corresponding second substrate was increased. The K_m value for CoASH is near the value given by Hibbert et al. (1971). The K_m for acetyl phosphate reported by these authors is, however, ten times greater than obtained here. Their kinetic studies were carried out under very similar conditions and the reason for this discrepancy remains unclear.

Pyrophosphate is a competitive inhibitor with respect to both P_i and acetyl phosphate and desulfo-CoA is a competitive inhibitor with CoA. *N*-Acetylcysteamine (NAC), the functional terminus of CoA-SH, was not inhibitory even in 1000-fold molar excess over CoA-SH. NAC was not detectably acylated by acetyl phosphate under conditions where a turnover number of 0.1/s would have been observable.

The competitive behavior of products and analogues was measured by standard techniques. The results of these kinetic analyses are summarized in Table II. All substrate-product pairs sharing a common chemical group (acyl, phosphate, CoA) are competitive in qualitative agreement with Hibbert et al. (1971). This is consistent with all substrates being able to bind to free enzyme according to the random scheme shown (Scheme I).

Scheme I



Arsenolysis vs. E·AcCoA Dissociation. In the presence of arsenate the enzyme catalyzes the additional reaction:

$$AcP_i \xrightarrow[CoA]{AsO_4^{3-}} \text{acetate} + P_i \qquad (3)$$

Presumably, this is a two-step process involving intermediate formation of AcCoA. An experiment was carried out to establish whether AsO_4^{3-} reacts with free AcCoA or enzyme bound AcCoA. In this experiment the arsenolysis of acetyl phosphate was carried out in the presence of excess citrate synthase and oxaloacetate which serves as a trap for AcCoA which has dissociated from transacetylase. If the trap does not prevent arsenolysis of AcP_i, all of the acetate must be formed via undissociated AcCoA. This would indicate that a compulsory order of product release occurs corresponding to the upper right hand branch of Scheme I if P_i and AsO_4^{3-} obey the same order of binding. If all arsenolysis is abolished by the trap, AcCoA must dissociate prior to the replacement of enzyme bound P_i by AsO_4^{3-}. This will be the case if only the lower right-hand branch of Scheme I is operative. In the event that both P_i and AcCoA may dissociate randomly, and that arsenolysis is not slow compared with AcCoA dissociation from $E_{AcCoA}^{AsO_4}$, it would be expected that the AcCoA trap would reduce the acetyl phosphate arsenolysis to some finite level at saturating [AsO_4^{3-}], the rest of the substrate being trapped as citrate.

The results of the above study starting with [^{14}C]acetyl phosphate and increasing concentrations of AsO_4^{3-} are shown in Figure 3. Details of the experiment are described under Methods along with tests carried out to prove the ability of the enzymatic trap to capture virtually all (>98%) dissociated AcCoA. The graph shows that acetate is formed even in the presence of the AcCoA trap and that citrate formation is not completely repressed by [AsO_4^{3-}]. The ratio of citrate to acetate formed levels off to a constant value of about 2 as AsO_4^{3-} becomes saturating, consistent with a random order of product dissociation as discussed above. While this result does not preclude the predominance of either the upper or lower pathway, it does rule out a compulsory order of product release in agreement with the previous product inhibition studies.

Dissociation Constants from Inactivation Experiments. Phosphotransacetylase is very rapidly and stoichiometrically inactivated by Ellman's reagent. Reaction of a single thiol group per 70 000 daltons proceeds at a pseudo-first-order rate with either the above reagent or with *N*-ethylmaleimide. The inactivation rate is ten times faster at pH 8.5 than at pH 7.5. Saturating concentrations of the substrates, acetyl phosphate or AcCoA, or the inhibitor, PP_i, completely protected the enzyme (>95% reduction in k_{inact}) consistent with the -SH group being at the active site. A 40% reduction in k_{inact} was obtained with saturating orthophosphate at pH 7.5 and a 70% reduction at pH 8.0.

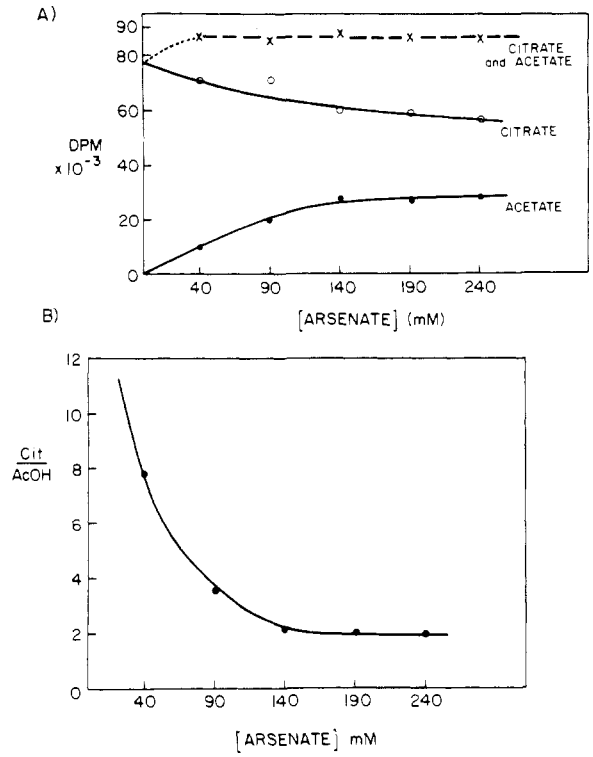


FIGURE 3: Partitioning of E·AcCoA between arsenolysis and dissociation. Six reaction mixtures, each containing 15 mM [^{14}C]acyl-P_i (specific activity, 0.07 μCi/μmol), 0.05 mM CoA, and 1 mM dithiothreitol, were prepared in a total volume of 0.25 ml. These contained from 0 to 240 mM Tris-arsenate, pH 7.5, and ionic strength was maintained in all cases at 740 mM by addition of the appropriate amount of 0.8 M Tris-Cl, pH 7.5. Also present were approximately 5 IU of citrate synthase and 25 mM oxaloacetate. At t = 0, 0.5 μg of transacetylase was added and the reactions were allowed to proceed for 20 min at 25 °C at which point acetate and citrate were assayed as described in Methods. The reaction never proceeded further than one-third to completion. The lower plot is a replot of the data in the upper one.

TABLE III: Comparison of $\bar{K}$ Values with K_D Values Obtained from Protection Experiments.[a]

Substrate or Inhibitor	$\bar{K}$ (mM) ([S_2] → 0)	K_D (from SH Protection)
Acetyl-CoA	0.20 ± 0.05	0.20 ± 0.05
Phosphate	2.3 ± 0.3[b]	2.6 ± 0.4
Acetyl-P_i	0.20 ± 0.04	0.26 ± 0.04
Acetyl-P_i with 40 μM desulfo-CoA	0.20 ± 0.04	0.04 ± 0.01
Desulfo-CoA	0.003 ± 0.001[b]	No protection at 0.08 mM

[a] Values of $\bar{K}$ were obtained from the projection onto the x axis of the common intercept of $1/V$ vs. $1/S$ lines on Figures 1 and 2. Values of K_D were taken from [S] or [I] giving ½ maximal protection. These were identical for NEM and 5,5′-dithiobis(2-nitrobenzoic acid). K_i for competitive inhibitors was estimated from double-reciprocal plots using the equation (Dixon and Webb, 1964): $X_{int} = 1/[K + (1 + (I.K_i))]$. [b] Signifies K_i rather than $\bar{K}$.

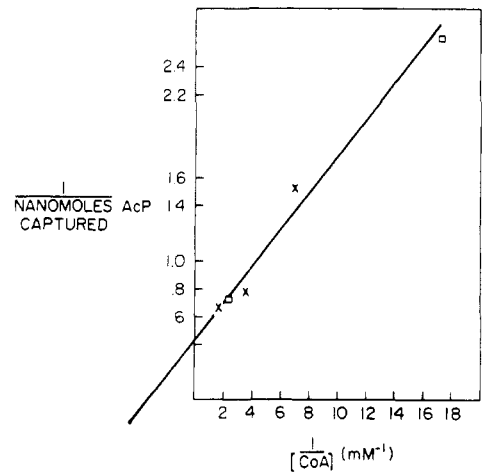


FIGURE 4: Trapping of E·acetyl phosphate by CoA-SH in a single turnover. Enzyme (0.44 ± 0.04 nmol) in 0.15 M Tris buffer in a volume of 20 μl containing 1.0 mM [^{14}C]acetyl phosphate (specific activity, 4200 dpm/nmol) was injected into 50 mM Tris-acetyl phosphate (volume, 2 ml) as described in Methods. At each [CoA] used the excess counts in AcCoA (X) or in citrate (□) from AcCoA, above control, was converted into nanomoles of [^{14}C]acetyl phosphate trapped in the first turnover using the above specific activity. This is plotted in double-reciprocal form (Rose et al., 1974), after fitting a two-parameter, least-squares program. The y intercept corresponds to approximately 60% capture of E·acetyl phosphate.

Substrate protection against thiol inactivation affords a means to directly measure dissociation constants, K_D, of binary enzyme–substrate complexes. This is, in general, equal to the concentration of S which reduces the rate of inactivation by 50%. Such an analysis has been described by Mildvan and Leigh (1964). In Table III K_D obtained from substrate protection experiments is compared with kinetically obtained values of limiting Michaelis constants. The two sets of constants are in good agreement. An interesting result is that desulfo-CoA, a powerful competitive inhibitor of CoA did not itself protect the enzyme against thiol inactivation. However, in the presence of 40 μM desulfo-CoA the acetyl phosphate concentration providing half-maximal protection against NEM was reduced by sixfold. This indicates that acetyl phosphate binds more tightly to enzyme in the presence of desulfo-CoA.

Pulse-Chase Experiments. A proof that complexes formed between enzyme and a single substrate are functional may be obtained by a pulse-chase technique described by Rose et al. (1974), provided that E·S dissociation is not very fast compared with catalytic turnover. In such an experiment, as described in Methods, enzyme premixed with 1 mM [^{14}C]acetyl phosphate in 20 μl of Tris buffer was force injected into 2.0 ml of rapidly stirring 50 mM acetyl phosphate and various CoA concentrations. Controls were carried out where acetyl phosphate was premixed with the cold carrier rather than with enzyme. After rapid mixing and quenching, excess counts in AcCoA compared with controls represent E·[^{14}C]acetyl phosphate captured in a single turnover. The results are shown in Figure 4. At high [CoA], a maximum of 60% of E·acetyl phosphate is converted to AcCoA prior to E·acetyl phosphate dissociation. Substantial (>80%) trapping of AcCoA in a single turnover was also obtained in similar pulse-chase experiments in the reverse reaction. These results indicate that the binary complexes, E·AcP_i and E·AcCoA, are normal catalytic intermediates and that they do not dissociate rapidly compared with their rates of catalytic turnover, as has been inferred from kinetic studies by other workers (Hibbert et al., 1971). Pulse-chase trapping, as found here, has, in the past, been forwarded as evidence for E–X intermediates. For reasons discussed (Rose, 1974), however, such trapping may be explained, as above, without covalent catalysis.

Attempts to Trap an Acyl-Enzyme. The most direct evi-

TABLE IV: Experimental Conditions for Attempted Isolation of Acyl-Enzyme.[a]

% Enzyme Labeled with ^{14}C		Reason for Addition
<1	(1) [^{14}C]Acyl-P_i (1 mM) (2×10^6 cpm/μmol)	
	(2) [^{14}C]Acyl-P_i + 0.05 mM desulfo-CoA	To mimic CoA
	(3) [^{14}C]Acyl-P_i + 0.1 mM 3′-P-ADP	To mimic CoA
	(4) [^{14}C]Acyl-P_i + 0.06 mM oxy-CoA	To mimic CoA
<4	(5) [^{14}C]Acetyl-CoA (1 mM) (8×10^7 cpm/μmol)	
	(6) [^{14}C]Acetyl-CoA + 50 mM Tris-PP_i	To mimic P_i
	(7) [^{14}C]Acetyl-CoA + 1 mM DTNB	To trap free CoA
	(8) [^{14}C]Acetyl-CoA + 50 mM PP_i + 1 mM DTNB	See 6 and 7
	(9) [^{14}C]Acetyl-CoA + 5 mM potassium ferricyanide after 5 min	To trap free CoA
	(10) [^{14}C]Acetyl-CoA + 5 mM potassium ferricyanide at pH 6.0	To reduce hydrolysis
	(11) [^{14}C]Acetyl-CoA + 5 mM potassium ferricyanide + 1 mM desulfo-CoA	To displace and trap CoA
	(12) [^{14}C]Acetyl-CoA + 3 mM P_i	Actual turnover
<1	(13) [^{14}C]Acyl-P_i (1 mM) after 2 min with 50 μM CoA-SH	Turnover with limiting acyl acceptor

[a] After 10 to 20 min in Tris buffer at 25 °C these incubation mixtures were applied to a 0.5 × 20 cm Sephadex G-25 column at 4 °C equilibrated with 50 mM Tris buffer, pH 7.5. Fractions of 0.4–0.5 ml were collected. Flow rate was approximately 0.5 ml/min. Fractions were assayed for enzyme activity as described in Methods and the protein peak tubes pooled and counted in Bray's solution.

dence for covalent catalysis is isolation of an acyl-enzyme. Therefore, attempts were made to label the enzyme using either [^{14}C]acetyl phosphate or [^{14}C]AcCoA under a variety of conditions including the presence of chemical traps for CoA and the presence of second substrate analogues. The various incubation conditions are listed in Table IV. No significant amount of ^{14}C was associated with protein passed through Sephadex G-25 at 4 °C after incubation with acyl donors at 25 °C for 15–20 min. We attribute the small amount of label in the case of AcCoA to occlusion of this molecule by denatured enzyme.[3] Since simple incubation was unsuccessful second substrate analogues were added to seek evidence of substrate synergism. Neither the addition of desulfo-CoA to enzyme and [^{14}C]acetyl phosphate nor of pyrophosphate and [^{14}C]AcCoA yielded labeled protein after Sephadex treatment. Thiol reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) and ferricyanide were also included in some incubation since they would be expected to overcome an unfavorable equilibrium that might prevent accumulation of any acyl-enzyme. This was also without effect. The possibility was even considered that, like citrate lyase (Buckel et al., 1971), transacetylase might initially exist as an acyl-enzyme, and, therefore, require "discharging" with an acyl acceptor prior to reacting with [^{14}C]acyl donors. This was ruled out in experiments 12 and 13 of Table IV where enzyme was preincubated with small amounts of CoA and P_i.

It may be proposed that an acyl-enzyme was actually formed but hydrolyzed during gel filtration. This can be dismissed because enzyme isolation was carried out within 15–20 min at 4 °C while we have measured an upper limit to the rate of AcCoA hydrolysis by the enzyme at 25 °C to be 0.1 mol mol^{-1} enzyme h^{-1}.

Isotope Exchange Experiments. The hypothetical half reactions for an acyl-enzyme mechanism are minimally:

$$E + AcP_i \rightleftharpoons \text{acyl-E} + P_i \quad (4)$$

and

$$E + AcCoA \rightleftharpoons \text{acyl-E} + CoA \quad (5)$$

These virtual reactions may be observable by means of isotope exchange between acyl donor–acceptor pairs in the absence of the second substrate.

When 1 mM acetyl phosphate and 10 mM ^{32}P were incubated with enzyme in the presence of 1 mM dithiothreitol, ^{32}P incorporation into acetyl phosphate was observed without addition of CoA. This apparent isotope exchange absolutely requires added thiol and occurs at a rate 1000 times slower than that observed with added CoA. That the exchange was not due to a contaminating enzyme was established by demonstrating that time courses of inactivation for both "exchange" and normal activity were parallel when inactivation was carried out with NEM, heat, or trypsin. It was noted, however, that the velocity of ^{32}P incorporation increased as the square of the enzyme concentration, suggesting that CoA might be present as a contaminant. That this was, indeed, the case was proven by exhaustive dialysis of enzyme against buffer containing 10 mM dithiothreitol. Enzyme thus treated lost all ability to catalyze $^{32}P_i$ incorporation into acyl-P_i, even in the presence of oxy-CoA or desulfo-CoA. Sensitive CoA analysis (Allred and Guy, 1969) then showed that one CoA molecule is bound as a disulfide to 25–30 enzyme molecules. This amount is sufficient to account for the observed $^{32}P_i$ incorporation into acetyl phosphate. It is, therefore, concluded that no exchange between acetyl phosphate and P_i occurs. The detection limit in these experiments was such that an exchange rate equal to 10^{-6} the rate of the overall reaction could have been detected.

The other potential isotope exchange, CoA–AcCoA was also, at first, observed when 1 mM [^3H]CoA and 5 mM AcCoA were incubated with enzyme and without added orthophosphate. The isotope equilibration under these conditions occurred at a rate approximately 1% of that found with added P_i. A contaminating protein was again ruled out by parallel courses of heat, NEM, and trypsin inactivation for normal and isotopic equilibration activities of the enzyme. When, however, both [AcCoA] and [CoA] were increased, but kept at a constant ratio of 10:1, the observed rate of isotope incorporation

[3] In a control experiment boiled enzyme was incubated with [^{14}C]-acetyl-CoA for 15 min. The enzyme was then dialyzed against Tris-Cl (pH 7.5) for 8 h and finally 8 h against 1 mM HCl. At this point 0.71 mol of [^{14}C]acetyl-CoA were associated with each mole of enzyme. Dialysis against 6 M guanidine hydrochloride at pH 3.0 released all ^{14}C.

into AcCoA appeared to increase without limit. This suggested the presence of a trace amount of P_i as contaminant. Total [P_i] in AcCoA + CoA was assayed by the method of Itaya and Ui (1966) after separation from CoA on a 0.7 × 35 cm Sephadex G-10 column using carrier-free $^{32}P_i$ as a phosphate elution marker. CoA contained approximately 0.2 mol % inorganic phosphate. When this relative amount of P_i was added to an [^{3}H]CoA + AcCoA + enzyme mixture, the rate of AcCoA labeling was very nearly twice as rapid as in the absence of added P_i. This indicates that the observed [^{3}H]CoA → AcCoA incorporation was entirely an artifact of P_i contamination. Thus, both apparent isotope exchanges were due to contaminating second substrate. No detectable isotope exchange in the total absence of second substrate could be found.

Discussion

Phosphotransacetylase from *Clostridium kluyveri* was found by sodium dodecyl sulfate electrophoresis to have a molecular weight of 70 000. The previous report of 60 000 (Shimizu et al., 1969) may be due to the compressing effect of a disulfide since enzyme untreated with mercaptoethanol also migrated farther on sodium dodecyl sulfate gels. One essential -SH group was found per 70 000 molecular weight.

The reaction kinetics, in agreement with previous reports (Hibbert et al., 1971), are of the ternary type and are inconsistent with any simple ping-pong mechanism. Product inhibition studies demonstrated competitive behavior among all product-substrate pairs, except CoA-P_i, in agreement with a random binding scheme. In addition, the random binding order on the product side was directly proved by the observation that high [AsO_4^{3-}] prevented some, but not all, AcCoA dissociation from the enzyme.

The ability of acyl-donor substrates to bind to free enzyme at concentrations near their $\bar{K}$ was directly demonstrated by protection experiments with thiol reagents. This method also revealed a sixfold greater binding of acyl-P_i in the presence of desulfo-CoA. It may be speculated that this tight fit in the complex $E_{acyl\text{-}P}^{desulfo\text{-}CoA}$ reflects the enzyme's tendency to compress CoA and P_i together in the normal ternary complex, the absence of sulfur in the analogue making compression less strained (Jencks, 1969).

The complete protection of the thiol by acyl donors, only partial protection by P_i, and the lack of protection by desulfo-CoA suggest that the thiol is nearest the acyl group. One might speculate that the SH group is situated between the entering P_i and leaving CoA-SH and serves as a proton shuttle between attacking and leaving groups.

In the pulse-chase experiments, the binary complex E·acetyl phosphate in the presence of high [CoA] was converted to products in a single turnover in about two out of three times, prior to its dissociation. This proves the functional ability of the binary complex and also indicates that rapid equilibrium binding, as postulated by Hibbert et al. (1971), cannot be the case, because, if it were, no E·acetyl phosphate would be captured as product prior to its dissociation.

All attempts to isolate a covalent enzyme-substrate adduct failed. Very slow apparent isotope exchange reactions were, at first, observed. These were shown, however, to be artifacts of contamination by traces of second substrates. Similar explanations of initially reported slow partial reactions have now been recognized in the cases of phosphoribosylpyrophosphate synthetase (Switzer and Simcox, 1974) and phosphoglycerate kinase (Johnson et al., 1975) demonstrating the caution which must be applied when very slow partial reactions are observed. Although the acyl donors can bind to free enzyme, no evidence for an acyl-enzyme could be isolated despite attempts to mimic substrate synergism with a variety of CoA or phosphate analogues. It has been claimed that negative evidence cannot completely disprove the existence of covalent catalysis (Spector, 1973). We think, however, it is worthwhile to examine in detail the significance of the negative evidence and to examine further the reasons why a covalent intermediate should be completely undetectable in the absence of the second substrate. Experiments were carried out in which acetyl-CoA and enzyme were reacted in the presence of a CoA trap. Under these conditions one would expect all of the enzyme to be converted to the acyl-enzyme even if the equilibrium for its formation were unfavorable. The enzyme and acetyl-CoA were allowed to react for 20 min and less than 10% of the enzyme was converted to acyl-enzyme.[4] $t_{1/2}$ for the formation of acyl-enzyme must, therefore, be at least 60 min. If the acyl-enzyme were to participate in the catalytic process, its rate of formation should be commensurate with the rate of conversion of acetyl-CoA to acetyl phosphate; i.e., $t_{1/2}$ should be less than 1.2×10^{-2} s. These experiments, therefore, establish that acyl-enzyme formation in the absence of the second substrate is too slow by a factor of 10^5 to 10^6 to be catalytically competent. There could be several reasons for the failure to detect an acyl-enzyme: (1) formation of the acyl-enzyme does not occur; (2) the rate of formation of the acyl-enzyme or the release of CoA-SH is slow in the absence of the second substrate; (3) the equilibrium for the reaction AcCoA + E ⇌ Ac·E·CoA-SH is highly unfavorable. This seems improbable on energetic grounds. If the second of the above reasons applies, it is possible that in the catalytic reaction the binding of the second substrate (P_i) supplies sufficient energy (7–8 kcal) to overcome the kinetic or thermodynamic barrier to acyl-enzyme formation or CoA release. However, we also carried out experiments in which we incubated the enzyme with acetyl-CoA in the presence of PP_i, a competitive inhibitor of P_i which presumably binds at the same site as P_i. K_D for P_i and K_i for PP_i are very similar. It appears probable that, if the failure to form an acyl-enzyme were due to the lack of binding energy provided by the second substrate, PP_i should provide this energy. Therefore, the failure to form an acyl-enzyme even in the presence of PP_i suggests that an acyl-enzyme does not occur. The argument presented above can be criticized on the ground that PP_i is sufficiently different from P_i and cannot replace it in promoting formation of an acyl-enzyme.

Another set of experiments, which we have carried out, is not subject to this criticism. In these experiments, an attempt was made to detect an exchange reaction between acetyl phosphate and P_i catalyzed by phosphotransacetylase. Such an exchange would be expected if an acyl-enzyme occurs. The detection techniques were such that it could be established that exchange must occur at less than 10^{-6} times the rate of the exchange in the presence of CoA. As before, failure to detect exchange means: (1) the intermediate is not involved; (2) the binding energy of the second substrate, in this case CoA-SH, must be utilized to overcome a kinetic or equilibrium barrier to the formation of the acyl-enzyme or the release of P_i. The exchange reaction also was carried out in the presence of desulfo-CoA-SH, a close analogue of CoA-SH. Since *N*-acetylcysteamine is neither a substrate nor inhibitor, the binding contribution of the -SH of CoA-SH is not substantial. Any binding mode available to CoA-SH must be available to de-

[4] We have chosen a value of 10% because of the uncertainty introduced by nonspecific binding of CoA. The amount of acyl-enzyme actually formed is probably considerably less than that value.

sulfo-CoA. Therefore, desulfo-CoA should be able to supply a significant fraction of any binding energy which CoA-SH might supply toward the reduction of an energy barrier to the formation of an acyl-enzyme. However, even in the presence of that analogue, no exchange could be detected. We therefore conclude that no acyl-enzyme intermediate is involved in the reaction catalyzed by phosphotransacetylase.[5]

In many group transfer reactions, convincing evidence has been obtained for the occurrence of covalent intermediates. It is more difficult to establish that a transfer reaction proceeds with direct transfer, i.e., without covalent intermediates. We believe that the study with phosphotransacetylase discussed here shows that it is possible to conclude from negative evidence, with reasonable certainty, that a reaction proceeds without the participation of a covalent intermediate.

References

Allred, J. B., and Guy, D. G. (1969), *Anal. Biochem. 29,* 293.

Avison, A. W. D. (1955), *J. Chem. Soc. A,* 732.

Boyer, P. D. (1959), *Arch. Biochem. Biophys. 82,* 387.

Buckel, W., Buschmeier, V., and Eggerer, H. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352,* 1195.

Chase, J. F. A., Middleton, B., and Tubbs, P. K. (1966), *Biochem. Biophys. Res. Commun. 23,* 208.

Cleland, W. W. (1970), *Enzymes, 3rd Ed. 2,* 63.

Colowick, S. P. (1973), *Enzymes, 3rd Ed. 9,* Chapter 1.

Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, London, Longmans.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Hibbert, F., Kyrtopoulos, S. A., and Satchell, D. P. N. (1971), *Biochim. Biophys. Acta 242,* 39.

Itaya, K., and Ui, M. (1966), *Clin. Chem. Acta 14,* 361.

Jencks, W. P. (1969), in Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, p 294.

Johnson, P. E., Abbott, S. J., Orr, G. A., Sémériva, M., and Knowles, J. R. (1975), *Biochem. Biophys. Res. Commun. 62,* 382.

Kaji, A., and Colowick, S. P. (1965), *J. Biol. Chem. 240,* 4454.

Marcus, A., and Elliot, W. B. (1959), *J. Biol. Chem. 234,* 1011.

Mildvan, A. S., and Leigh, R. A. (1964), *Biochim. Biophys. Acta 89,* 393.

Rose, I. A., O'Connell, E. L., Bar Tana, J., and Litwin, S. (1974), *J. Biol. Chem. 249,* 5163.

Shimizu, M., Suzuki, T., Hosokawa, Y., Nagase, O., and Abiko, Y. (1970), *Biochim. Biophys. Acta 222,* 307.

Shimizu, M., Suzuki, T., Kin-Ya, K., and Yasushi, A. (1969), *Biochim. Biophys. Acta 191,* 550.

Solomon, F., and Rose, I. A. (1971), *Arch. Biochem. Biophys. 147,* 349.

Spector, L. B. (1973), *Bioorg. Chem. 2,* 311.

Stadtman, E. R., and Barker, H. A. (1950), *J. Biol. Chem. 184,* 769.

Switzer, R. L. (1970), *J. Biol. Chem. 245,* 483.

Switzer, R. L., and Simcox, P. D. (1974), *J. Biol. Chem. 249,* 5304.

Walsh, C. T., and Spector, L. B. (1971a), *Arch. Biochem. Biophys. 145,* 1.

Walsh, C. T., and Spector, L. B. (1971b), *J. Biol. Chem. 246,* 1255.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Whiteley, H. R., and Pelroy, R. A. (1972), *J. Biol. Chem. 247,* 1911.

[5] A possible exception to the argument presented above could be that CoA undergoes chemical reaction (for instance, disulfide interchange) prior to formation of acyl-enzyme. If that were the case, two covalent intermediates would have to participate, acyl-enzyme and CoA-enzyme.