

BBA 66349

KINETIC STUDIES WITH PHOSPHOTRANSACETYLASE

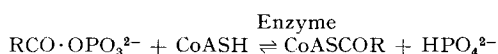
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SUMMARY

1. We have studied the phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalysed acylation of coenzyme A (CoASH) by various acyl phosphates in Tris buffers at 25°.



Under the conditions used, the equilibrium position lies well to the right for all the acyl phosphates studied, and our results refer predominantly to the forward step.

2. We have established the kinetic form of the acylation. Both substrates, CoASH and acyl phosphate, are adsorbed by the enzyme, probably under pre-equilibrium (Michaelis-Menten) conditions. Kinetic analysis leads to the various substrate dissociation constants, K_s , and to the rate constant, k_2 , for the transformation of the enzyme-substrate ternary complex to products.

3. The stability of the acyl phosphate-enzyme adduct depends upon the electron availability on the phosphate and carbonyl oxygen atoms, but rather little upon the steric bulk of the group R in the acyl phosphate. The value of k_2 , however, is very dependent on this steric bulk.

4. The product CoASCOR is not taken up by phosphotransacetylase in the presence of an excess of acyl phosphate. The product HPO_4^{2-} is less strongly absorbed than any of the acyl phosphates studied.

5. Experiments at pH values between 6 and 9 show that the acyl phosphates are taken up by an acidic centre in the enzyme of effective $\text{p}K_a > 9$. The adsorption is assisted by the acid form of a group with $\text{p}K_a = 6.9$ in the free enzyme and $\text{p}K_a = 6.4$ in the enzyme-acyl phosphate complex. The adsorption of CoASH probably involves its thiol group, which becomes attached to a basic group in the enzyme-acyl phosphate complex of $\text{p}K_a < 6$. Two other groups, one in the enzyme ($\text{p}K_a = 8.7$) and the other in the enzyme-CoASH complex ($\text{p}K_a = 7.2$), both active in their acid forms, are involved in binding CoASH.

6. The value of k_2 is also pH-dependent. Two groups in the ternary complex, one ($\text{p}K_a = 7.3$) active in its basic form, the other ($\text{p}K_a = 8.7$) active in its acidic form, are involved.

7. The spontaneous reaction between CoASH and acetyl phosphate in the

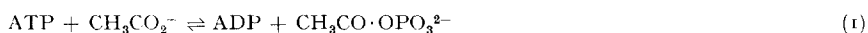
relevant pH region is very slow. The acceleration produced by phosphotransacetylase constitutes a factor of at least $5 \cdot 10^5$.

8. The spontaneous reaction of aliphatic carboxylic anhydrides with CoASH proceeds at a rate at least 10^8 -fold faster than does the spontaneous reaction with acyl phosphates.

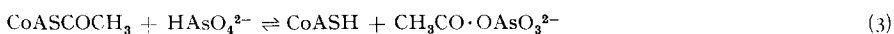
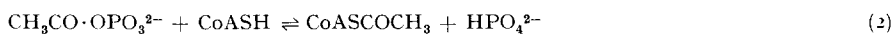
9. The mechanism of the enzymatic reaction is discussed and an outline scheme presented which is compatible with all the evidence. An essential role of the enzyme is to bring into close proximity the two reactants, both of which carry more than one negative charge. Multiple, simultaneous, intramolecular acid and base catalysis is also probably involved.

INTRODUCTION

Phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalyses Reaction (2), the second stage of an important route, (1)–(2), to acetyl coenzyme A. The first stage is catalysed by acetate kinase¹.



Phosphotransacetylase is usually isolated from *Clostridium kluyveri*^{2–5}. Most of the early work with this enzyme is due to STADTMAN and co-workers^{3–7}, who normally studied Reaction (2) in the presence of added arsenate ions. The overall result is then the hydrolysis of acetyl phosphate, and Reactions (2), (3) and (4) were suggested for this process. Reaction (3) was also assumed to be catalysed by phosphotransacetylase.



From various semi-quantitative studies of the overall hydrolysis, monitored by observing the loss of acetyl phosphat, STADTMAN and co-workers concluded (i) that the velocity is proportional to the enzyme and CoASH concentrations, (ii) that the velocity is a maximum at $\text{pH} \simeq 7.6$, (iii) that the enzyme is activated by NH_4^+ and K^+ , and (iv) that the reaction is inhibited by Li^+ and Na^+ , and non-competitively by orthophosphate ions. STADTMAN and co-workers also estimated the equilibrium constant for Reaction (2) as $K = [\text{CoASCOCH}_3][\text{HPO}_4^{2-}]/[\text{CoASH}][\text{CH}_3\text{CO} \cdot \text{OPO}_3^{2-}] \simeq 60$.

Other early work^{3–5,8} showed that propionyl, butyryl and fluoroacetyl phosphates could replace the acetyl derivative, but with less effect. Since 1960 there have been two main developments. First it has been found⁹ that an enzyme capable of catalysing Reaction (2) can be isolated from *Clostridium acidiurici*, although this enzyme requires the presence of a divalent metal, and does not catalyse Reaction (3). Secondly the enzyme from *C. kluyveri* has been crystallised by BERGMAYER *et al.*² who made a brief, but direct, study of Reaction (2) by following the appearance of acetyl coenzyme A. They found (i) a pH-velocity maximum at pH 7.5 (under otherwise unspecified conditions), (ii) $K = 144 \pm 25$ (at 25°), (iii) Michaelis constants for

CoASH and acetyl phosphate at an unspecified pH, (iv) maximum enzyme activity, under otherwise unspecified conditions, when $[\text{NH}_4^+] \geq 7 \cdot 10^{-3} \text{ M}$, with Na^+ , K^+ , and Mg^{2+} unable to replace NH_4^+ , and (v) slight activation when $[\text{HPO}_4^{2-}] > 10^{-3} \text{ M}$.

It will be evident that there exist both points of agreement and of discrepancy between these findings and those of STADTMAN and co-workers. Both sets of workers find that the oxidised (dimeric) form of CoASH is inactive; STADTMAN and co-workers always added sodium thioglycollate to reduce any CoAS-SACo.

We now report a detailed, quantitative, kinetic study of the forward step of the general Reaction (5) (*cf.* Reaction (2)).



MATERIALS AND METHODS

(1) Chemicals

Phosphotransacetylase (*ex C. kluyveri*) was obtained from the Boehringer Corp. as a suspension (1 mg/ml) in 3.6 M $(\text{NH}_4)_2\text{SO}_4$. All reactions were studied at 25°. A given enzyme sample displayed constant activity over a period of several days if stored below 0° and allowed to reach room temperature just prior to use. Different, fresh batches of enzyme all had the same specific activity. In calculating the enzyme molarity its molecular weight was taken² as $4 \cdot 10^4$, and the samples assumed to contain no foreign protein. The calculated molarities, if in error, will be so by a constant factor. Trilithium coenzyme A and acetyl coenzyme A, dilithium acetyl phosphate (purity 95%) and tris(trihydroxymethyl methylamine) buffer were also Boehringer products. The coenzyme A contained little, if any, dimer. Dilithium acetyl phosphate was prepared by STADTMAN AND LIPMANN's¹⁰ method and the corresponding salts of *n*-propionyl, *i*-butyryl, and chloroacetyl phosphates by AVISON's¹¹ method. Dilithium benzoyl phosphate was a gift from Mr. G. F. White. Analyses of the recrystallised products for phosphate and acyl content showed the purities to be acetyl: $98 \pm 1\%$, propionyl: $70 \pm 2\%$, chloroacetyl: $84 \pm 2\%$, *i*-butyryl: $60 \pm 2\%$, and benzoyl: $95 \pm 2\%$. The impurity was largely K_2HPO_4 , and proved very difficult to remove. In calculating the concentrations of the acyl phosphates we have everywhere allowed for the potassium phosphate impurity. Sodium thioglycollate and *p*-chloromercuribenzoate were obtained from British Drug Houses. The remaining chemicals were the purest available commercial grades, AnalaR material being used in most cases. All the experiments involved aqueous solutions only. pH values were measured with a Radiometer N26 pH meter.

(2) Preliminary experiments

(i) *Hydrolysis of acyl phosphates.* To conduct the enzymatic Reactions (5) without significant concurrent loss of acyl phosphate *via* non-enzymatic hydrolysis (6), it is necessary to know this rate of hydrolysis. Reaction (6) was followed by



observing the removal of acyl phosphate using KOSCHLAND's¹² modification of Lipmann and Tuttle's method. In the presence of a large excess of an aqueous Tris buffer the observed loss of acyl phosphate was an accurately first-order process over

TABLE I

SPONTANEOUS HYDROLYSIS OF ACYL DERIVATIVES AT 25°

[Acyl phosphate]_{initial} $\simeq 10^{-2}$ M; ionic strength = 0.10 M; pH 8.32; k_1 = first-order rate constant.

Acyl derivative	$k_1 \times 10^4$ (min ⁻¹)
Acetyl phosphate	6.60 (6.51) ^b
Propionyl phosphate	2.65
Isobutyryl phosphate	1.5 ^a
Chloroacetyl phosphate	3.15
Acetyl coenzyme A	4.37
Chloroacetyl coenzyme A	3.90

^a Calculated from results in ref. 13 for ionic strength = 0.60 M.^b This run contained $5 \cdot 10^{-8}$ M enzyme.

at least three half-lives. The first-order rate constants (k_1) were obtained from the slopes of plots of $\log [\text{acyl phosphate}]$ against time. Values of k_1 were reproducible to within $\pm 3\%$. Our results are in Table I, and are compatible with earlier work¹³.

(ii) *Stability of thiols in the reaction medium.* Thiols undergo oxidative dimerisation, especially in alkaline media¹⁴. Since STADTMAN and co-workers found it necessary to include thioglycollate to prevent oxidation of CoASH, we have tested the stability of CoASH in our systems. At 25°, in a Tris buffer at pH 8.3, and in the absence of other reagents, CoASH (10^{-4} M) is converted to a non-acylatable species (probably dimer) by a zero-order process of $t_{\frac{1}{2}} \simeq 110$ min. At pH 9.3 the reaction has $t_{\frac{1}{2}} < 1$ min. The loss of CoASH was followed by rapidly acylating the unreacted material at suitable intervals with an excess of acetic anhydride, and determining spectrophotometrically the acetyl coenzyme A formed (see (iii) below). The dimerisation of thioglycollate can be followed in the same way, the acetyl derivative of the monomer absorbing strongly at 235 nm. At pH 8.3 and 25°, $t_{\frac{1}{2}} = 27$ min for the zero-order removal of 10^{-4} M thioglycollate. Acetyl thioglycollate has a larger extinction coefficient at 235 nm than has acetyl coenzyme A. The same situation probably obtains for other acyl derivatives of these compounds. This can lead to difficulties if thioglycollate is present during enzymatic acylations of CoASH followed spectrophotometrically, for, with acyl phosphates containing electron withdrawing groups (*e.g.* chloroacetyl) the acyl coenzyme A formed is a powerful enough acylating agent to attack the thioglycollate at a significant rate in a non-enzymatic process, and can thus lead to a spurious optical density change.

When studying Reaction (5) the foregoing facts make it desirable to avoid (a) the presence of thioglycollate, (b) storage of CoASH solutions, and (c) enzyme catalysed reactions with half-lives comparable with that for the spontaneous disappearance of CoASH.

(iii) *Reaction stoichiometry and equilibrium positions.* The equilibrium position of (5) can be readily determined by estimating spectrophotometrically the amount of acyl coenzyme A formed under given conditions. Species CoASCOR have an absorption maximum near 258 nm; the most favourable wavelength for use in analysing their mixtures with CoASH is 235 nm. When R is an unsubstituted, saturated aliphatic group, the thiol ester hydrolyses relatively slowly (Table I). When $R = \text{CH}_3$,

TABLE II

EXTINCTION COEFFICIENTS OF COENZYME A SPECIES

 ϵ = extinction coefficient at 235 nm. Coenzyme A species = CoASH.

R	$\epsilon \times 10^{-4} \text{ (cm}^2 \cdot \text{mole}^{-1}\text{)}$
CH ₃ CO	0.69 ± 0.02
C ₂ H ₅ CO	0.73 ± 0.02
<i>i</i> -C ₃ H ₇ CO	0.72 ± 0.02
ClCH ₂ CO	0.68 ± 0.03
H	0.38 ± 0.02

an accurate value of the extinction coefficient of the ester can therefore be obtained by dissolution of a known amount of the solid ester. The same value is obtained, within experimental error, by acylating a known concentration of CoASH (about 10^{-4} M) non-enzymatically, by addition of an excess (about 10^{-2} M) of acetic anhydride. This acylation with anhydride is therefore apparently quantitative. It proceeds very rapidly ($t_{\frac{1}{2}} < 0.1$ min). The extinction coefficients of CoASCOR where R = C₂H₅, *i*-C₃H₇, and ClCH₂ were also obtained in this way, *via* reaction with the appropriate anhydride. Our results are in Table II. When R = ClCH₂ the ester hydrolyses at a significant rate, and ϵ is obtained by extrapolation of the absorbance measurements to the time of mixing.

Spectroscopic examination of the reaction mixtures for the enzymatic process (5), using the acyl phosphates under a variety of concentration and pH conditions, showed that (5) proceeds well to the right under all the conditions used in the present study. The production of CoASCOR was, in every case, $> 96\%$ of that expected for complete conversion of the CoASH. For R = CH₃ this result is compatible with the available²⁻⁵ estimates of the equilibrium constant for (5). Under certain special circumstances, less than the expected amount of thiol ester is detected. This is (a) when R = ClCH₂ at low enzyme concentrations, and (b) when the enzymatic reaction has $t_{\frac{1}{2}} \lesssim 30$ min. Under Condition (a) a significant amount of the thiol ester hydrolyses before the enzymatic process is complete (see above); under Condition (b) a significant amount of CoASH is lost by oxidation (see (ii) above).

(3) Kinetics of Reaction (5)

We have followed the forward step of (5) by determining (i) the concentration of free SH groups remaining at any stage, using the colourimetric analysis of MAHLER *et al.*¹⁵, (ii) the concentration of inorganic phosphate at any stage by MISSON'S^{16,17} method, and (iii) the concentration of thiol ester at any stage by spectrophotometric measurements at 235 nm (see (2) (iii) above). Methods (i) and (iii) are appropriate when CoASH is in deficit compared with the acyl phosphate. They lead to the same rate constant, within experimental error. Method (ii) is suitable if CoASH is in excess of the acyl phosphate. Since Method (iii) is experimentally the easiest our concentration conditions were, so far as possible, arranged accordingly.

In a typical run, to a freshly prepared stock solution of dilithium acyl phosphate, made up in Tris buffer of the required pH, were added known concentrations of Li⁺, K⁺ and NH₄⁺ (as chlorides). The final solution normally had an ionic strength, $I = 0.18$ M and, for any set of runs, fixed concentrations of K⁺ and NH₄⁺. LiCl,

which has little effect on the reaction velocity (Table IV), was the salt used to adjust the ionic strength. This final acyl phosphate solution was transferred (3 ml) to an ultraviolet spectrophotometer cell (1 cm path, quartz) and the cell placed in a thermostatted housing. After cell and contents had reached 25°, reaction was initiated by the rapid addition of a small (1–10 μ l) volume of a stock solution of enzyme, and a small quantity (about 0.2 mg) of solid CoASH. The cell was quickly shaken and optical measurements begun. This procedure avoids deterioration of enzyme and CoASH in the mixture before the reaction is actually begun. The initial CoASH concentration was normally about $6 \cdot 10^{-5}$ M and the acyl phosphate was usually kept in a 10-fold, or greater, excess of this. The enzyme concentration varied between about 10^{-10} and $8 \cdot 10^{-8}$ M. Under most of the concentration conditions the formation of acyl coenzyme A (or loss of CoASH) was an accurately first-order process for at least three half-lives. The observed first-order rate constants (k_{obs}) were obtained from the slopes of plots of $\log [\text{CoASH}] \equiv \log (A_{\infty} - A_t)$ against time. A_t and A_{∞} represent the absorbances at 235 nm at times t and infinity (about 10 half-lives), respectively. For reactions with $t_{\frac{1}{2}} \simeq 30$ min, wherein significant loss of CoASH *via* oxidation must occur, and for those involving readily hydrolysable reactants or prod-

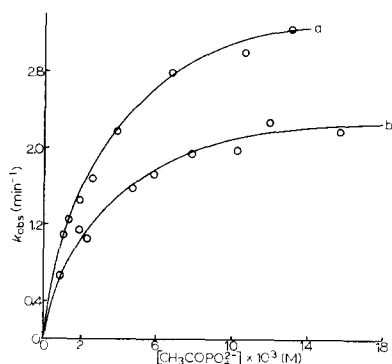


Fig. 1. Michaelis curves for acetyl phosphate. pH 8.32; $[\text{NH}_4^+] = 7 \cdot 10^{-3}$ M; $[\text{CoASH}]_{\text{initial}} \simeq 6 \cdot 10^{-5}$ M. a, $[\text{Enzyme}] = 4.1 \cdot 10^{-8}$ M; b, $[\text{Enzyme}] = 2.4 \cdot 10^{-8}$ M.

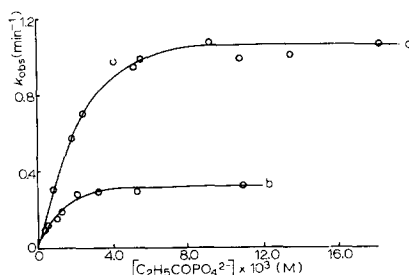


Fig. 2. Michaelis curves for propionyl phosphate. pH 8.32; $[\text{NH}_4^+] = 7 \cdot 10^{-3}$ M; $[\text{CoASH}]_{\text{initial}} \simeq 6 \cdot 10^{-5}$ M. a, $[\text{Enzyme}] = 7.8 \cdot 10^{-8}$ M; b, $[\text{Enzyme}] = 2.5 \cdot 10^{-8}$ M.

ucts, as with chloroacetyl phosphate, the initial slope only of the first-order plot was used to calculate k_{obs} . In these cases a theoretical value of A_{∞} was used. Values of k_{obs} were reproducible to within $\pm 3\%$.

With analytical Methods (i) or (ii) the same general procedure was adopted for making up reaction mixtures and for initiating the reaction, except that, for Method (ii), the relative initial concentrations of CoASH and acyl phosphate were reversed. Reaction mixtures were placed in suitable ground-glass stoppered vessels suspended in a thermostat bath, and samples withdrawn at appropriate intervals for analysis by the relevant colour test. Sampling necessitated the study of slower reactions (where necessary we used less enzyme) and k_{obs} was calculated from the initial slope of the plot of $\log [\text{CoASH}]$ or $\log [\text{HPO}_4^{2-}]$ against time. Values of k_{obs} were reproducible to within $\pm 5\%$.

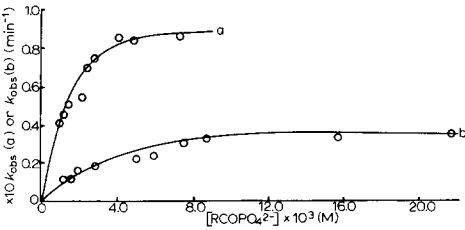


Fig. 3. Michaelis curves for *i*-butyryl and chloroacetyl phosphates. pH 8.32; $[\text{NH}_4^+] = 7 \cdot 10^{-3} \text{ M}$; $[\text{CoASH}]_{\text{initial}} \simeq 6 \cdot 10^{-5} \text{ M}$; $[\text{Enzyme}] = 2.5 \cdot 10^{-8} \text{ M}$. a, *i*-butyryl; b, chloroacetyl.

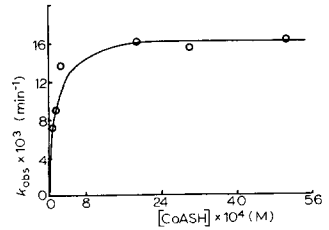


Fig. 4. Michaelis curve for coenzyme A. pH 8.32; $[\text{NH}_4^+] = 7 \cdot 10^{-3} \text{ M}$; $[\text{Enzyme}] = 2.5 \cdot 10^{-8} \text{ M}$; $[\text{CH}_3\text{COPO}_4^{2-}]_{\text{initial}} \simeq 3.7 \cdot 10^{-4} \text{ M}$.

Our kinetic results for a variety of CoASH, enzyme, and acyl phosphate concentrations for the four acyl phosphates are shown in Figs. 1–6. These results all refer to pH 8.32.

(4) *The effect of pH on Reaction (5) when $R = \text{CH}_3$*

Results in Figs. 1 and 4 lead to Michaelis constants for CoASH and acetyl phosphate at pH 8.32. Further experiments, along lines suggested by DIXON^{18,19}, examined the effect of pH on these constants, and upon the velocity of product for-

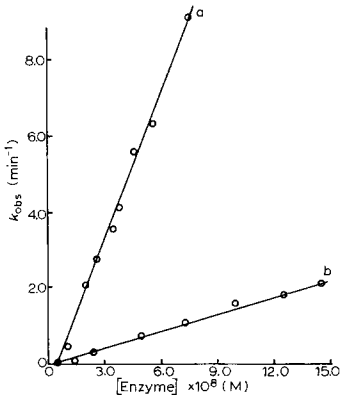


Fig. 5. Reaction order in enzyme. pH 8.32; $[\text{RCOPO}_4^{2-}] = 2.7 \cdot 10^{-2} \text{ M}$; $[\text{CoASH}]_{\text{initial}} \simeq 6 \cdot 10^{-5} \text{ M}$. a, acetyl phosphate; b, propionyl phosphate.

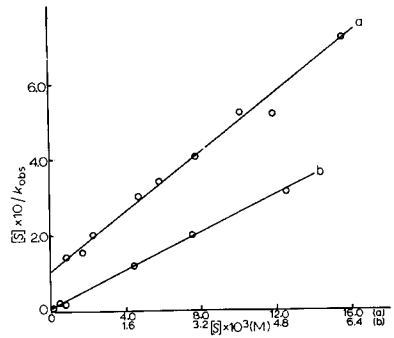


Fig. 6. Reciprocal plots. a, acetyl phosphate; b, coenzyme A.

mation from the enzyme–substrate complex. The pH range 5.9–9.2 was covered using Tris buffers. Our results are in Table III and Figs. 7–9; those referring to $\text{pH} \lesssim 9$ must be treated with reserve owing to the rapid oxidation of CoASH at high pH; initial slopes were used in calculating k_{obs} at high pH.

(5) *Inhibition and activation*

(i) *Effects of products*

(a) *Phosphate ions.* Previous authors^{2–5} disagree about the effect of added

TABLE III

EFFECT OF pH ON THE PHOSPHOTRANSACETYLASE CATALYSED ACYLATION OF COENZYME A WITH ACETYL PHOSPHATE AT 25°
Tris buffers used throughout. Initial and final pH values for all runs agreed within ± 0.02 unit.

(a) Effect on acetyl phosphate binding

[CoASH]_{initial} ≈ 6 · 10⁻⁵ M; ionic strength = 18.2 · 10⁻² M; [K⁺] = 2.0 · 10⁻² M; [NH₄⁺] = 8.0 · 10⁻³ M; [Enzyme] = 2.5 · 10⁻⁸ M. *k*_{obs} = observed first-order rate constant from Equation (14).

[CH ₃ COPO ₄ ²⁻] × 10 ³ (M)	<i>k</i> _{obs} (min ⁻¹)			
	pH 5.68	pH 6.15	pH 7.15	pH 8.34
0.10	0.21			
0.30	0.48	0.15		
0.50		0.19		
0.70	1.03	0.44		
1.00		0.57	0.28	0.46
1.30	1.16			
2.00	1.55	0.72	0.40	0.76
3.00	1.43	0.61	0.57	1.11
4.00			0.66	1.02
5.00			0.85	1.28
6.00	1.67	0.84		1.52
7.00			0.87	
8.00			0.97	1.68
10.0	2.47	1.00		
12.0			1.06	1.80
16.0			1.11	1.92
20.0			1.06	2.16

(b) Effect on coenzyme A binding

[CoASH]_{initial} ≈ 4 · 10⁻⁵ M; [CH₃COPO₄²⁻] = 2.67 · 10⁻² M; [Enzyme] = 2.5 · 10⁻⁸ M; [NH₄⁺] = 7.0 · 10⁻³ M; [K⁺] = [Li⁺] = 2.67 · 10⁻² M. *k*_{obs} = observed first-order rate constant (min⁻¹) from Equation (19).

pH	6.15	6.66	7.03	7.35	7.58	7.90	8.34	8.84	9.04
<i>k</i> _{obs}	0.60	0.82	1.13	1.32	1.60	1.82	1.79	0.85	0.45

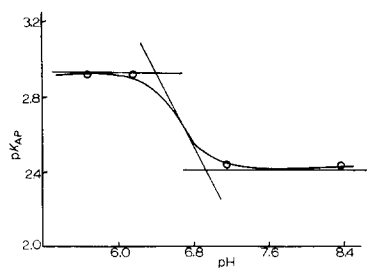


Fig. 7. Effect of pH on *pK*_{AP}.

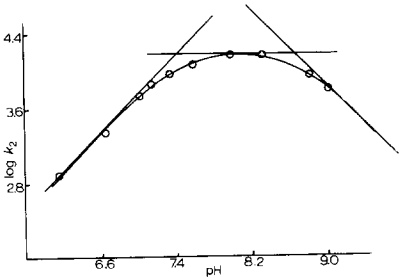


Fig. 8. Effect of pH on *k*₂.

phosphate ions. Our results, to be published in detail elsewhere, show that HPO_4^{2-} is only feebly adsorbed by the enzyme. It has a K_s value much greater than any of the acyl phosphates studied here.

(b) *Acetyl coenzyme A*. Added acetyl coenzyme A had no observable effect on the rate of the enzymatic reaction between acetyl phosphate and CoASH. However, it was not practicable to use high acetyl coenzyme A concentrations (for fear of significantly disturbing Equilibrium (5)) and acetyl phosphate was always present at a concentration appreciably in excess of that of acetyl coenzyme A.

(ii) *Effects of substrates*

In the concentration ranges used there is no evidence for any inhibition by substrates, even when these are present in relatively high concentrations.

(iii) *Effects of other inhibitors*

(a) *Sodium p-chloromercuryl benzoate*. This compound largely inhibits the reaction when its concentration is comparable with, or greater than, that of the CoASH. It apparently attacks CoASH, not the enzyme.

(b) *Iodoacetic acid*. The effect of this compound, known to react with thiols, was tested on the forward step of Reaction (3) as catalysed by phosphotransacetylase. In contrast to the forward step of (2), the substrates contain no free thiol groups. It was found that at concentrations of about 10^{-4} M iodoacetic acid has a decelera-

TABLE IV

EFFECTS OF K^+ AND Li^+ ON THE PHOSPHOTRANSACETYLASE CATALYSED ACYLATION OF COENZYME A BY ACETYL PHOSPHATE AT 25°

$[\text{CoASH}]_{\text{initial}} \simeq 6 \cdot 10^{-5}$ M; $[\text{CH}_3\text{COPO}_4^{2-}] = 2 \cdot 0 \cdot 10^{-3}$ M; $[\text{Enzyme}] = 0 \cdot 8 \cdot 10^{-8}$ M; pH 8.32; $[\text{NH}_4^+] = 1 \cdot 2 \cdot 10^{-3}$ M. k_{obs} = observed first-order rate constant.

(a) *Effect of K^+ ($[\text{Li}] = 2 \cdot 0 \cdot 10^{-3}$ M)*

$[\text{K}^+] \times 10^2$ (M)	0.20	1.50	2.70	4.00	5.30
k_{obs} (min^{-1})	0.33	0.45	0.61	0.68	0.67

(b) *Effect of Li^+ ($[\text{K}^+] = 2 \cdot 0 \cdot 10^{-3}$ M)*

$[\text{Li}^+] \times 10^2$	0.20	1.50	2.70	5.30	7.80
k_{obs} (min^{-1})	0.34	0.32	0.32	0.28	0.24

tory effect of $< 5\%$. It is evident therefore that there is no rapid inhibition of important thiol groups in the enzyme. Since the iodoacetic acid is present in about 10^4 -fold excess of the enzyme it is probable that an accessible thiol group plays no part in the active site—at least for Reaction (3). The result with *p*-chloromercuryl benzoate suggests the same is true for Reaction (2) also.

(iv) *Effects of metal ions*

Table IV shows that Reaction (2) is accelerated by K^+ , but slightly decelerated by Li^+ . These results are similar to those of STADTMAN and co-workers for the overall hydrolysis (Reactions (2)–(4)). It was these findings, and those in refs. 2–5, that led us to maintain constant concentrations of the various ions in most sets of experiments.

(6) *Relevant related reactions*

Experiments showed (i) that benzoyl phosphate is not a satisfactory substrate

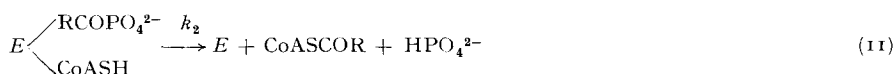
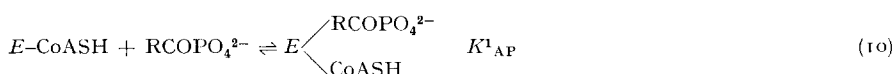
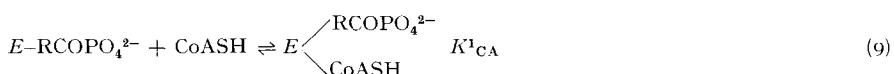
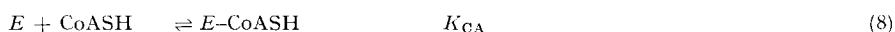
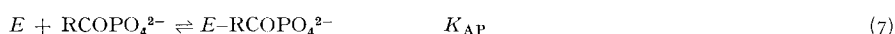
for phosphotransacetylase, no reaction at all being observed, and (ii) that the velocity of the spontaneous (non-enzymatic) reaction between CoASH and acetyl phosphate, at pH 8.3, is negligible compared with that of the enzymatic reaction, and has a second-order rate constant $k_2 \gtrsim 10^{-1} \text{ mole}^{-1} \cdot \text{l} \cdot \text{min}^{-1}$. Added Mg^{2+} ions ($5 \cdot 10^{-2} \text{ M}$), which catalyse the hydrolysis of acetyl phosphate²⁰ and probably²¹ its reactions with thiols, also led to a negligibly slow reaction between CoASH and acetyl phosphate at pH 8.3.

DISCUSSION

(1) Outline reaction mechanism

Under all the concentration conditions used Equilibrium (5) lies far to the right for the aliphatic acyl phosphates studied, and our kinetic results refer to the forward step of (5). The shapes of the curves in Figs. 1–4 suggest that for each acyl phosphate, both this substrate and coenzyme A are taken up by the enzyme. We are dealing therefore with at least a two substrate system. Since a K^+ and/or an NH_4^+ concentration of about 10^{-3} M is needed for the maximum enzyme activity to be displayed, it may be that these ions are also important and essential substrates. Since, however, the majority of our experiments involved constant concentrations ($> 10^{-3} \text{ M}$) of these ions, it is probably legitimate to treat their effects as constant.

It can be shown^{18,19}, for a two-substrate system in which both substrates are adsorbed by the enzyme, and adsorption is followed by a product-forming reaction on the enzyme (Reactions (7)–(11), where E represents enzyme) that, for pre-equilibrium (Michaelis–Menten) conditions, the rate equation can be represented by (12).



In Equation (12) the various equilibrium constants represent the dissociation constants of the adducts, and E_0 is the total enzyme concentration.

If it is assumed, as a first approximation, that the adsorption processes do not affect each other, then $K_{\text{AP}} = K^1_{\text{AP}}$ and $K_{\text{CA}} = K^1_{\text{CA}}$. Under these conditions Equation

$$\begin{aligned} d[\text{Products}]/dt &= -d[\text{CoASH}]/dt = -d[\text{RCOPO}_4^{2-}]/dt = \\ &= \frac{k_2 E_0 [\text{CoASH}] / (K^1_{\text{CA}} + [\text{CoASH}])}{1 + \frac{1}{[\text{RCOPO}_4^{2-}]} \left(\frac{K_{\text{AP}} K^1_{\text{CA}} + K^1_{\text{AP}} [\text{CoASH}]}{K^1_{\text{CA}} + [\text{CoASH}]} \right)} \quad (12) \end{aligned}$$

$$-d[\text{CoASH}]/dt = -d[\text{RCOPO}_4^{2-}]/dt = \frac{k_2 E_0 [\text{CoASH}] [\text{RCOPO}_4^{2-}]}{([\text{RCOPO}_4^{2-}] + K_{\text{AP}}) ([\text{CoASH}] + K_{\text{CA}})} \quad (13)$$

(13) is obeyed. If either $[\text{CoASH}] \ll K_{\text{CA}}$ at constant $[\text{RCOPO}_4^{2-}]$, or $[\text{RCOPO}_4^{2-}] \ll K_{\text{AP}}$ at constant $[\text{CoASH}]$, Equation (13) reduces to either (14) or (15), respectively*. Under such conditions the reaction will become first-order in both enzyme and the low-concentration substrate, and simple Michaelis-Menten behaviour should be found on changing the concentration of the other substrate. At fixed pH our findings show this pattern (Figs. 1-5). Normally CoASH was the species present in deficit,

$$-d[\text{CoASH}]/dt = \{k_2 E_0 [\text{RCOPO}_4^{2-}] / (K_{\text{AP}} + [\text{RCOPO}_4^{2-}]) K_{\text{CA}}\} [\text{CoASH}] = k_{\text{obs}} [\text{CoASH}] \quad (14)$$

$$-d[\text{RCOPO}_4^{2-}]/dt = \{k_2 E_0 [\text{CoASH}] / (K_{\text{CA}} + [\text{CoASH}]) K_{\text{AP}}\} [\text{RCOPO}_4^{2-}] = k_{\text{obs}} [\text{RCOPO}_4^{2-}] \quad (15)$$

and k_{obs} refers to Equation (14). The order in enzyme is unity except at very low enzyme concentrations. The various parameters in Equations (14) and (15) can be calculated using reciprocal plots. Thus from Equations (14) and (15) we have (16) and (17), respectively.

$$\frac{[\text{RCOPO}_4^{2-}]}{k_{\text{obs}}} = \frac{K_{\text{CA}} K_{\text{AP}}}{k_2 E_0} + \frac{K_{\text{CA}} [\text{RCOPO}_4^{2-}]}{k_2 E_0} \quad (16)$$

$$\frac{[\text{CoASH}]}{k_{\text{obs}}} = \frac{K_{\text{CA}} K_{\text{AP}}}{k_2 E_0} + \frac{K_{\text{AP}} [\text{CoASH}]}{k_2 E_0} \quad (17)$$

A plot of $[\text{RCOPO}_4^{2-}]/k_{\text{obs}}$ against $[\text{RCOPO}_4^{2-}]$ leads to K_{CA}/k_2 and K_{AP} . Good straight lines were always obtained (Fig. 6). An even better method of obtaining these parameters is that of WILKINSON²². Our values for K_{CA} , and for K_{AP} and k_2 , for the different acyl phosphates, collected in Table V, were calculated using his method and a programme appropriate for the King's College CDC6600 computer.

Since the observed kinetic behaviour is in keeping with the foregoing equations, it seems reasonable to consider, for this two-substrate system, either that pre-equilibrium conditions obtain, or that steady-state conditions apply with one substrate (that present in excess) being taken up by the enzyme much faster than the other. In what follows we assume pre-equilibrium conditions, but the assumption has little effect on our subsequent arguments. Where they are affected we point this out.

(2) Effect of acyl phosphate structure on K_{AP} and k_2

The results in Table V suggest that the electron availability at one, or all, of the oxygen atoms of the acyl phosphates is important in determining K_{AP} . Thus electron withdrawal by R leads to a large K_{AP} value and electron release to a small

* If it is assumed that the substrate present in low concentration is taken up by the enzyme much more slowly than the other, the same equations result under steady state (Briggs-Haldane) conditions, except that K_{CA} in Equation (14) and K_{AP} in Equation (15) are no longer true dissociation constants but involve k_2 also¹⁹.

TABLE V

COLLECTED MICHAELIS PARAMETERS

(a) *Acetyl phosphate and coenzyme A*

<i>pH</i>	$k_2 \times 10^4 \text{ (min}^{-1}\text{)}^a$	$K_{CA} \times 10^4 \text{ (moles} \cdot \text{l}^{-1}\text{)}^b$	$K_{AP} \times 10^3 \text{ (moles} \cdot \text{l}^{-1}\text{)}$
9.04	0.60	3.35	—
8.84	0.80	2.34	—
8.34	1.20, 1.25 ^c	1.66	3.6 ± 0.3^c
8.32	1.30 ^d	1.54 ^e	2.9 ± 0.4^d
7.90	1.23	1.68	—
7.58	1.12	1.77	—
7.35	0.88	1.66	—
7.15	0.68 ^c	1.41	3.7 ± 0.3^c
7.03	0.53	1.17	—
6.66	0.22	0.66	—
6.15	0.08 ^c	0.32	1.2 ± 0.2^c
5.68	—	—	1.3 ± 0.4^c

(b) *Other acyl phosphates at pH 8.32*

<i>Acyl phosphate</i>	$K_{AP} \times 10^3 \text{ (moles} \cdot \text{l}^{-1}\text{)}$	$k_2 \times 10^{-4} \text{ (min}^{-1}\text{)}^f$
$\text{C}_2\text{H}_5\text{COPO}_4^{2-}$	1.1 ± 0.4	0.23
$i\text{-C}_4\text{H}_7\text{COPO}_4^{2-}$	1.4 ± 0.3	0.0064
$\text{CH}_2\text{ClCOPO}_4^{2-}$	3.9 ± 0.5	0.26

^a From Equation (18) unless otherwise indicated. Reproducibility within $\pm 5\%$.^b From Equations (18) and (19) unless otherwise indicated. See also Table III (b).^c At constant ionic strength (Table III (a)).^d Average value from measurements at two different E_0 values (Fig. 1).^e From results in Fig. 4.^f Calculated using $K_{CA} = 1.54 \cdot 10^{-4}$.

value. The rather small, overall spread of the values is, however, surprising. Steric hindrance is probably of some importance, since the *i*-butyryl derivative forms a slightly less stable complex than does the *n*-propionyl derivative. It is evident, however, that just from the viewpoint of adsorption by the enzyme, a wide variety of acyl phosphates would probably be suitable for phosphotransacetylase.

The results for k_2 , the rate constant for decomposition of the ternary complex in Reaction (11), reveal a very different situation. They suggest that electron availability is now relatively unimportant, but that steric effects are crucial. No doubt this is the reason that benzoyl phosphate leads to negligible reaction. Clearly a particular configuration (or a narrow range of configurations) is required of the acyl phosphate for successful interaction with the adsorbed CoASH.

(3) *Effect of pH when $R = \text{CH}_3$*

(a) *Effect on acetyl phosphate binding.* Free acetyl phosphate, with $\text{p}K_a$ values of 1.2 and 4.9, exists in solution predominantly in its dianionic form at all the pH values studied here. Results in Table III and Fig. 7 suggest therefore that a group in the free enzyme with $\text{p}K_a \simeq 6.9$ (and of $\text{p}K_a \simeq 6.4$ in the enzyme-acetyl phosphate complex) affects the binding between $\text{CH}_3\text{COPO}_4^{2-}$ and the enzyme. This group is effective when protonated, but is not itself a site of firm adsorption since

its ionisation can still be detected in the complex (Fig. 7). It is evident therefore that the principal site of adsorption of the acyl phosphates involves a group, or groups, which do not undergo acid-base ionisation within the pH range 6-9, but whose action is facilitated by a neighbouring, protonated group of $pK_a \approx 6.9$.

(b) *Effect on k_2* . If both $[RCOPO_4^{2-}]$ and $[CoASH]$ are made large compared with their respective dissociation constants, K_{AP} and K_{CA} , then Equation (13) reduces to (18).

$$-d[CoASH]/dt = k_2 E_0 = v_{\max} \quad (18)$$

The kinetics are then zero-order in both substrates, and the relevant plots lead to values of k_2 . The effect of pH on k_2 is shown in Fig. 8. The value obtained at pH 8.34 is in good agreement with the values obtained independently from the Michaelis curves referring to a similar pH (Table V). The ternary complex appears to contain two groups, of $pK_a \approx 7.4$ and $pK_a \approx 8.7$, respectively, which catalyse Reaction (11). The former group is active in its base form, the latter in its acid form.

(c) *Effect on CoASH binding*. If $[RCOPO_4^{2-}] \gg K_{AP}$ and $[CoASH] < K_{CA}$, Equation (13) reduces to (19), which predicts that the reaction will then be first-order in coenzyme A, with $k_{\text{obs}} = k_2 E_0 / K_{CA}$. Hence, from Equation (18), at any fixed pH

$$-d[CoASH]/dt = k_2 E_0 [CoASH] / K_{CA} = k_{\text{obs}} [CoASH] \quad (19)$$

and E_0 values, $K_{CA} = v_{\max} / k_{\text{obs}}$; the effect of pH on K_{CA} can therefore be determined*. It happens that, at low pH, the magnitude of K_{CA} falls, and eventually $K_{CA} \approx [CoASH]_{\text{initial}}$ (see Tables III and V). Under such conditions departures from good first-order kinetic behaviour are to be expected; and, at pH < approx. 6.8, we found such departures. In these cases the quoted values of k_{obs} were obtained from the slopes of the latter parts of the reactions, where the conditions of Equation (19) are more closely satisfied. The corresponding K_{CA} values can, however, only be approximate.

The shape of Fig. 9 suggests that at least two groups are involved in the binding of CoASH. The first, situated in the enzyme-substrate complex and active in its

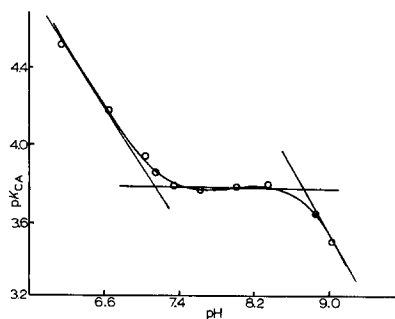


Fig. 9. Effect of pH on pK_{CA} .

* If steady-state conditions obtain, K_{CA} determined this way will be $(k_2 + k_{-9})/k_9$. A combined effect of pH on CoASH binding and on k_2 would therefore be involved. However, the effect on k_2 is known from (b) above, and clearly involves two *different* groups from those now identified for K_{CA} . It is evident that these latter groups can indeed be taken as referring to CoASH binding only. The implication is that steady-state conditions do not, in fact, obtain.

protonated form, has $pK_a \simeq 7.2$. The second, situated either in the free enzyme or in CoASH itself, and also active in the protonated form has $pK_a \simeq 8.7$. The pK_a values of three of the numerous basic centres in CoASH have been determined²³; it is claimed that the thiol group has $pK_a \simeq 9.6$, a secondary phosphate group has $pK_a \simeq 6.4$, and the primary amino group of the adenine system has $pK_a \simeq 4.0$. None of these is sufficiently close to 8.7 to identify this group, and none of the other basic centres in CoASH seems likely to possess a pK_a value of this magnitude. Perhaps therefore the group is in the free enzyme.

It should be noted that the thiol group of CoASH will be largely in the S-H form in the pH range studied.

(4) General conclusions

The foregoing sections lead to the following general picture of the forward step of Reaction (5) between pH 6 and pH 9. The enzyme adsorbs both CoASH and various acyl phosphates, but the product HPO_4^{2-} is much less strongly adsorbed than any of the acyl phosphates. The other product, acyl coenzyme A, is not taken up by the active site, at least not in the presence of an excess of acyl phosphate. This result suggests that the thiol end of the molecule is important to the binding of both coenzyme A species.

The pH profile for CoASH does not, however, reveal a basic group (G_1) in the enzyme which could be receiving the thiol proton; this group, if it exists, must therefore have a pK_a value < 6 . Two other groups (G_2 , G_3) are more certainly involved in binding CoASH; both are active in their protonated forms. That having $pK_a \simeq 8.7$ in the free enzyme is perhaps an ammonium group. The other group, $pK_a \simeq 7.2$, is only detected in the ternary complex, not in $E-RCOPO_4^{2-}$ or CoASH; this suggests that conformational changes occur on adsorption of CoASH. The schematic picture for CoASH binding is Fig. 10.

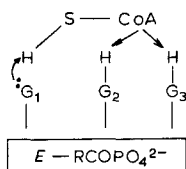


Fig. 10. G_1 , $pK_a < 6$. G_2 , $pK_a \simeq 7.2$. G_3 , $pK_a \simeq 8.7$, $-NH_2$?

The acyl phosphates are taken up by the enzyme at an acid centre, or centres (G_4 , G_5), which do not respond to pH changes in the range 6–9. These could be $-OH$ groups or perhaps a metal ion. Adsorption is assisted by a group (G_6) detectable in both the free enzyme and the enzyme–acyl phosphate complex with pK_a values suggesting imidazole. Since HPO_4^{2-} is less strongly attached to the enzyme than is $RCOPO_4^{2-}$, the involvement of the carbonyl oxygen atom is probable for the latter (G_7), but the adsorption process does not have very stringent steric requirements for R. Fig. 11 is a possible schematic picture.

The rate of reaction between the adsorbed species is very dependent upon the steric bulk of R in $RCOPO_4^{2-}$. Probably a nice juxtaposition is necessary. Apart from the reacting centres of CoASH and $RCOPO_4^{2-}$, at least two other groups (G_8 , G_9),

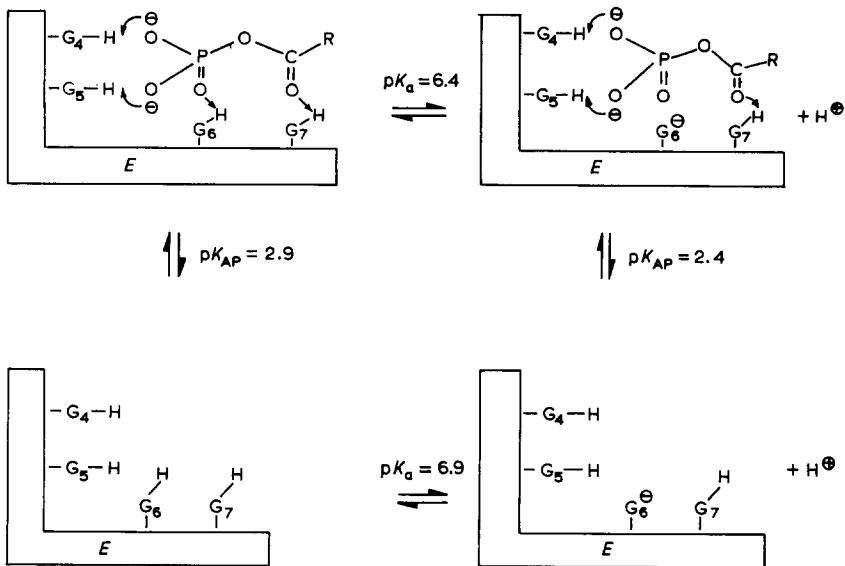


Fig. 11. G_4 , G_5 , $pK_a > 9$. G_6 , $pK_a \approx 6.4$ – 6.9 , imidazole? G_7 , $pK_a > 9$.

not yet accounted for, influence the rate of reaction of the ternary complex. One, $pK_a \approx 7.4$, is active in its basic form, the other, $pK_a \approx 8.7$, in its acidic form. Two reasons why the spontaneous reaction between CoASH and acyl phosphates is very slow are (a) the reagents both carry more than one negative charge and will therefore repel each other, and (b) an $-SH$ group is a poor nucleophile, and $RCOPO_4^{2-}$ is a relatively poor acylating agent owing to the basicity of its leaving group²⁵ (PO_4^{3-})*. Useful roles the enzyme could perform are therefore to render one (or both) reagents effectively neutral, to increase the nucleophilicity of the $-SH$ group by basic catalysis, and to facilitate the departure of the leaving group by acid catalysis²⁵. The composite schematic representation in Fig. 12, compatible with all our experimental facts, is designed with such roles in mind. In addition it is suggested that, on reaction, the sulphur atom is brought closer to the carbonyl carbon atom owing to H-bonding between group G_8 and the $-NH$ (or $-C=O$) group (G_9) in the pantetheine side chain

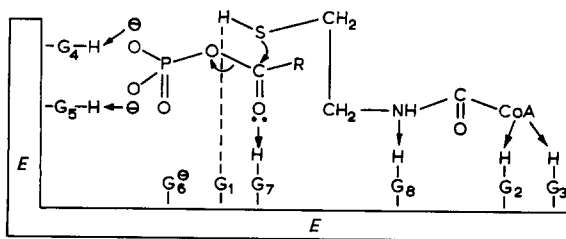


Fig. 12. G_8 , $pK_a \approx 8.9$. G_1 – G_7 as in Figs. 10 and 11.

* The comparatively great speed of the spontaneous reaction between CoASH and carboxylic anhydrides is undoubtedly due to the latter species being neutral and possessing relatively good leaving groups.

of CoASH, to which we assign a $pK_a \simeq 7.4$. Clearly, whatever the exact three-dimensional arrangement, the size of R could be crucial in allowing a sufficiently close approach of the reacting centres.

With reaction complete, the products can probably coexist on the enzyme. Once desorbed, however, under conditions where the acyl phosphate is present in large excess of coenzyme A species, the site G_7 will rapidly become inaccessible to acyl coenzyme A owing to the adsorption of another molecule of acyl phosphate—always provided K_{AcylCA} is not very much smaller than K_{AP} at the pH in question. It is in this way that the absence of inhibition by acyl coenzyme A which we have observed (at pH 8.3) can be understood. Acyl coenzyme A will certainly be expected to undergo adsorption (with a K_{AcylCA} value probably similar to that of K_{CA}), but only if the relative acyl phosphate concentration is small enough.

Our postulated overall mechanism: (a) effectively renders the acyl phosphate a neutral species as acylation occurs, (b) involves intramolecular attack at the carbonyl carbon atom by a locally formed $-S^-$ group, (c) employs a 2-fold, intramolecular acid catalysis of leaving group departure, and (d) involves acid catalysed activation of the carbonyl group towards nucleophilic attack. These effects, often observed separately, or in pairs, in non-enzymatic acylation²⁵⁻²⁷, and here postulated as operative simultaneously, would certainly produce a very large value of k_2 . Our tentative scheme can, of course, be only a crude approximation. It ignores the activation of the enzyme by NH_4^+ or K^+ . We hope next to throw more light on the problem by studying in detail the forward step of Reaction (3).

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