Identification of the catalytic groups in β-ketoacyl CoA thiolase by covalent modification of the protein

by

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ABSTRACT

FACULTY OF SCIENCE, PHYSIOLOGY AND BIOCHEMISTRY

Master of Philosophy

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β-Ketoacyl CoA thiolase is known to possess a sulfhydryl group at the active site that is essential for its catalytic activity. This work was concerned with identifying any other groups at the active site that participated in the catalytic process. For this purpose various anhydrides were used which covalently modify the protein by specifically reacting with any amino groups potentially present at the active site. Since these reagents may also react with thiol groups at the enzyme active site, the enzyme amino was modified with these reagents after masking the thiols present by an alkyl alkane thio sulfonate type reagent (methyl methanethiosulfonate) that specifically reduces the thiol generating the inactive thiomethylated enzyme. When this procedure was followed, the enzyme could be undoubtedly modified at its amino by the anhydride used leading to a doubly modified protein. The thiomethyl group could now be removed by reduction with dithiothreitol yielding an enzyme modified solely on the amino residues. The amino group could be unblocked in turn by exposure to acidic pH's.

The principle results from this work were as follows. Thiolase was inactivated by methyl methane thiosulphonate and this was protected by acetoacetyl CoA and acetyl CoA. The inactivated enzyme was reactivated by exposure to dithiothreitol or mercaptoethanol. Succinic anhydride, phthallic anhydride and citraconic anhydride also inactivated thiolase, however only acetoacetyl CoA provided any protection against inactivation. When thiomethyl-citraconyl thiolase was reduced with dithiothritol the enzyme remained inactive, but when the doubly modified enzyme was exposed to pH5 then the reduction led to formation of an active enzyme.These results are interpreted as demonstrating a role for an amino group at the enzyme active site. A catalytic mechanism is proposed for the enzyme which incorporates this group.

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INTRODUCTION

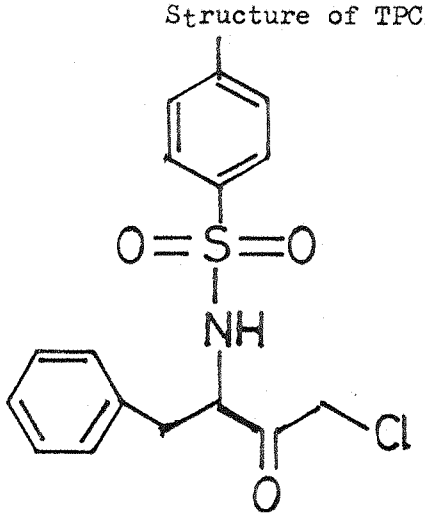
The main aim of this thesis is to identify the functional groups at the active sites of β-ketoacyl-CoA thiolase (acetyl-CoA-acetyl-CoA-C-acetyltransferase EC 2.3.1.9). This is approached by using reagents that covalently modify different groups on the enzyme, thus giving valuable information about the structure of the active site.

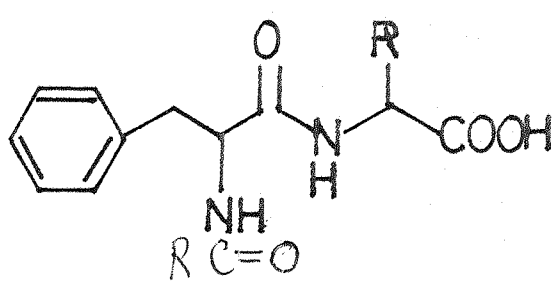
Functional groups essential for catalysis are usually identified by two main methods.

(a) Use of reagents capable of modifying different types of functional groups in an enzyme molecule to see whether these groups are essential for enzyme activity. A classical example is when ribonuclease is treated with iodoacetate at pH 5.5 (Gundlach et al, 1959; Crestfield et al, 1963). The alkylation leads to two inactive forms of enzyme, in one the imidazole ring of histidine residue 119 is alkylated, in the other histidine 12 is alkylated. These histidine residues of ribonuclease are essential for catalytic activity since no other functional groups are alkylated.

(b) The second method for mapping active sites is affinity labelling where the enzyme is allowed to react with a molecule resembling its true substrate but contains in addition a functional group capable of rapid covalent reaction with a specific group of the enzyme near the active site. An example of this approach is the use of N-tosyl-L-phenylalanylchloro methyl-ketone (TPCK) (Shaw, 1970), which is an affinity lable for chymotrypsin made to resemble the normal substrate. Instead of the usual susceptible structure of the substrate (-CO-NH-), this contains potent alkylating group (-CO-CH_2Cl) which binds to the active site of the enzyme alkylating at Histidine 57.

Structure of TPCK vs normal substrate





CHAPTER I

General Features of Enzyme-Catalysed Reactions

(1.1) Function of enzymes

Enzymes are biological catalysts that accelerate chemical reactions without being consumed during the reaction. In doing so, they do not alter the "overall" thermodynamic characteristics such as the equilibrium constant (Keq), the Gibbs free energy (ΔG°), the entropy (ΔS) or the enthalpy (ΔH) of the reaction that it catalyses. The equilibrium position is defined by the standard free energy change (ΔG°) of the reaction which is a function of its thermodynamic equilibrium constant at a certain temperature as related by:

$$\Delta G^{\circ} = -RT \ln Keq$$

For the reaction $A \rightleftharpoons B$ if ΔG° is negative, then the equilibrium lies to the right side of the equation liberating energy (exothermic). If ΔG° is positive, then Keq < 1, the reaction proceeds but the Keq lies to the left side of the equation and energy is consumed (endothermic).

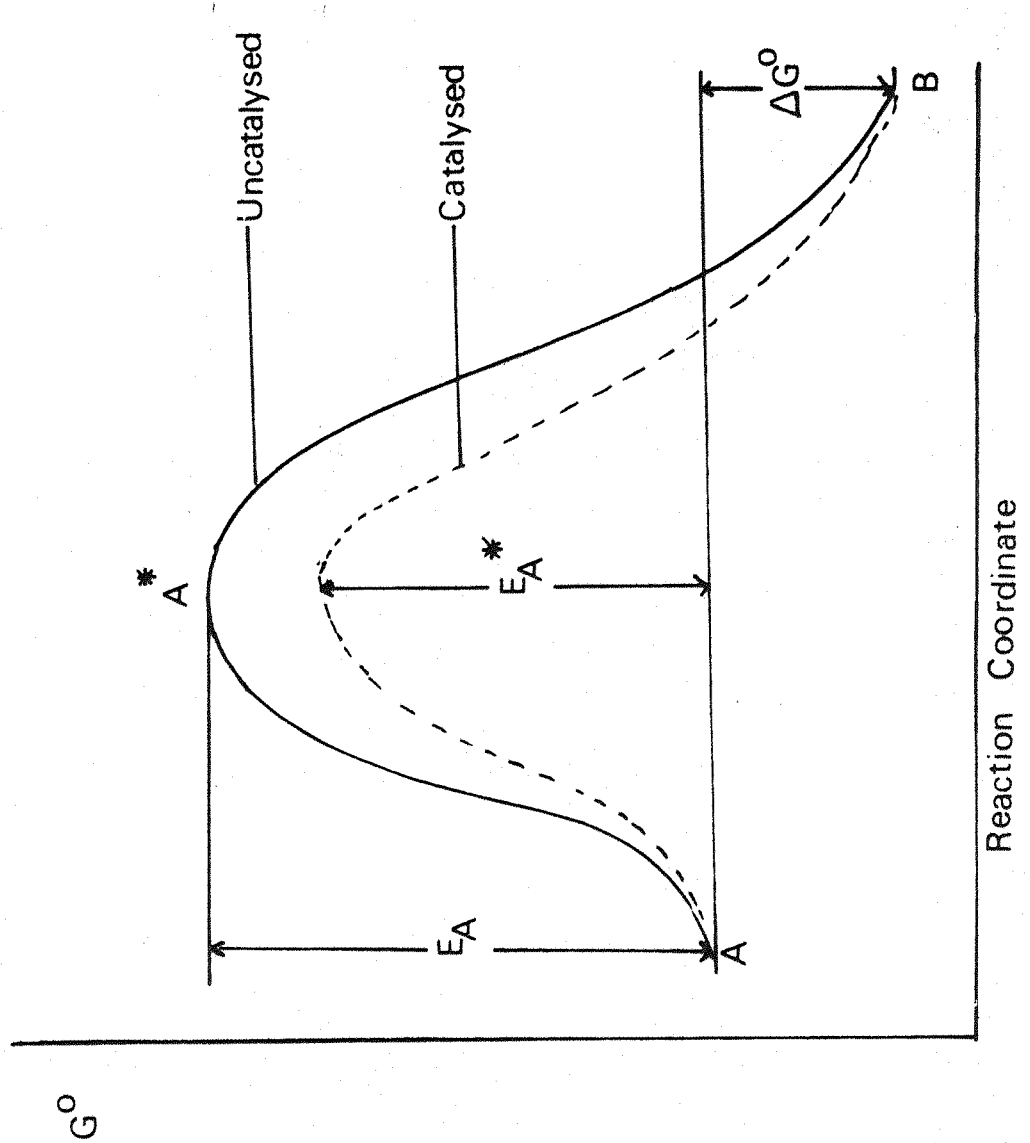
According to the transition state theory shown in Fig. (1), the conversion of $A \rightleftharpoons B$ is accomplished by the intermediate formation of a transition state complex A* whose potential energy is greater than that of the reactants by a quantity that is the activation energy (E_A) of the reaction. It is proposed that the pathway of an enzyme catalysed reaction involves a lower E_A than that of a non-catalysed reaction, by modifying the reactants so that E_A is decreased. The change in Gibbs free energy (ΔG°) remains the same for a catalysed or non-catalysed reaction.

The magnitude of the energy of activation determines, to a large extent, the rate of the reaction. The Arrhenius equation provides a means whereby E_A can be determined for a reaction whose rate constant is k.

$$k = Ae^{-\frac{E_A}{RT}} \quad \text{becomes} \quad \ln k = \ln A - \frac{E_A}{RT}$$

Using this equation it can be estimated that if k increases by a factor of 10, then E_A decreases by around 1.38k cals/mole at 27°. Since many enzymes increase the uncatalysed reaction rate by between 10^6 - 10^{10} times, this means that they must lower the activation energy for the reaction by 8.3 to 13k cals/mole

Figure 1


Uncatalysed
Catalysed
ΔG^o
B
$^*A$
E_A^*
E_A
A
Reaction Coordinate
G^o

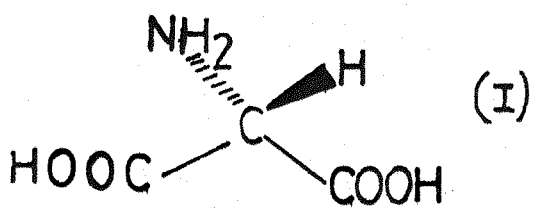
(1.2) Stereochemical specificity

By lowering the activation energy, enzymes exert a specificity for substrates to catalyse defined chemical reactions. This property is related to binding of substrates to the active site of the protein in an appropriate conformation.

The first stereochemical specificity of enzyme action was reported by Pasteur (1858) who found a form of yeast which fermented D-tartaric acid and not L-tartaric acid. This showed that enzymes can stereospecifically metabolize one enantiomer of a certain substrate. This was followed by the theory of the tetrahedral structure of four-valent carbon by Van't Hoff (1874) which accounts for the optical activity observed in many organic compounds. This achievement made possible the representation of the three dimensional structure of organic molecules.

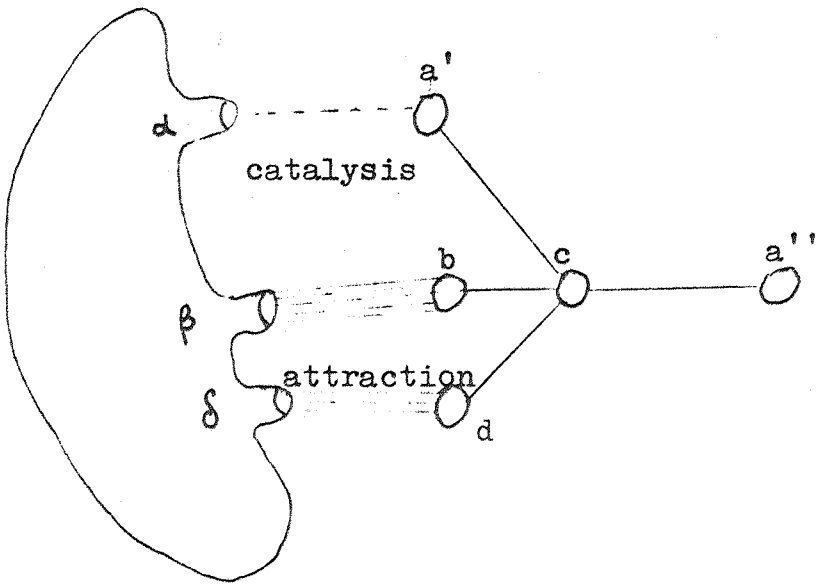
Along with the growth of the knowledge of the stereochemistry of chemical reactions, it was important to investigate the assymmetrical nature of the enzyme catalysed reactions. Before the knowledge of protein structure existed, enzymes were considered as assymmetrical reagents capable of distinguishing between two identical ligands in a structure of the Ca'a"bd type. Ogston (1948) postulated a model in which a Ca'a"bd type molecule is bound to a hypothetical enzyme area in which there are two binding sites β and δ, specific for groups b and d, and one catalytic site, α, specific for groups a' or a" (Fig.2).

There is only one orientation of this molecule on the enzyme which permits the approach of one, and only one, of the two identical groups to the catalytic site (a' in Fig.2). Ogston illustrated this by showing how the two carboxyl groups of aminomalonic acid (I) could be differentiated by its attachment to citrate synthase at three points.

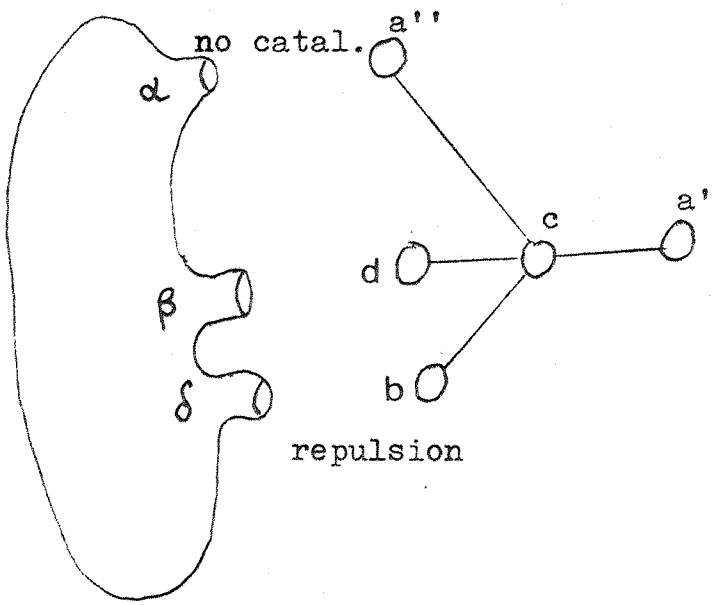


(I)

Figure 2.



2(a)

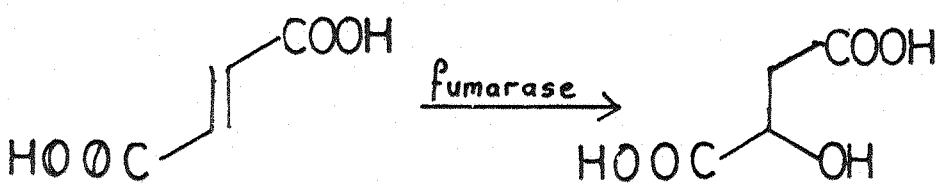


2(b)

The other important discovery concerned with the stereospecificity of enzyme reactions was by Levy et al (1962) who pointed out in their review of enzyme reactions involving prochiral carbon atoms that Van't Hoff's tetrahedral model of the chiral $C(R_1R_2R_3R_4)$ structure illustrated that there was no spontaneous shift in the relative positions of the four different ligands around the chiral centre and that this same principle must apply to identical ligands around chiral or prochiral centres also.

The next important discovery was made by Vennesland and Westheimer (Fisher et al, 1953; Loewus et al, 1953) and their colleagues relating to oxidoreductases (dehydrogenases), which were shown to transfer hydrogen atoms between substrates and diphospho- or triphosphopyridine nucleotide coenzymes in a stereospecific manner.

The stereospecificity of enzymic reactions applies also to unsaturated compounds showing cis or trans geometric isomerism such as the reaction catalysed by fumarase which involves the hydration of the trans and not the cis isomer of fumaric acid.



(1.3) Types of enzyme mechanisms

The ability of enzymes to catalyse reactions in a specific manner indicate their efficiency in enhancing reaction rates. The general theories to explain how this is achieved are as follows:

(a) Orientation of substrate in a specific manner allowing it to be in close proximity with the functional groups of the enzyme molecule so that the transition state is easily formed.

(b) Covalent bond formation of an enzyme-substrate intermediate that will go on to form the product.

(c) Enzymes provide functional groups that will act as proton donors or proton acceptors which will bring about general acid-base

catalysis.

(d) Enzymes induce strain - or distortion in the substrate forcing its conformation towards the transition state (Jenks , 1975).

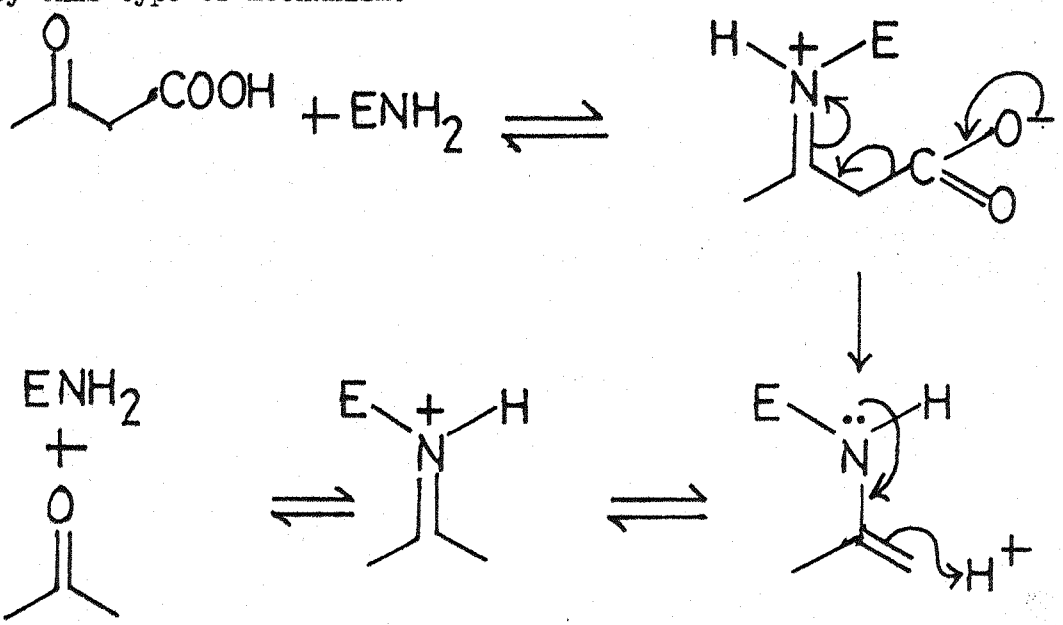
(1.4) Covalent catalysis

This is characterised by the formation of a covalently bound enzyme-substrate intermediate, the overall reaction rate being determined by the formation or decomposition of this intermediate. These reactions mainly involve nucleophilic type of catalysis. The reaction can be accelerated through two steps involving low energy barriers instead of one high barrier.

There are several types of covalent intermediates that are exemplified by this type of catalysis mainly:

(i) Schiff's base intermediate (Lysine class):

Acetoacetate decarboxylase catalyses the decarboxylation of β-ketoacids by this type of mechanism:



This type of reaction is important for reactions requiring activation of the carbon atom α to the carbonyl group where the observed catalysis results from the action of the protonated Schiff base serving as an electron sink.

(ii) Acyl-enzyme intermediate (Serine class):

These include acyl group transfer enzymes like chymotrypsin which transfers the acyl groups from a number of donors such as esters, amides and acid to a number of acceptors such as water, alcohols, and amines. The chymotrypsin catalysed reaction proceeds with the intermediate formation of an acyl enzyme. This was shown by the use of quasi-substrates such as p-nitrophenylacetate and diisopropyl-fluorophosphate which covalently labelled chymotrypsin. Chemical degradation of several acylchymotrypsins revealed that the acyl group was attached to a hydroxyl functional group of serine 195.

(iii) Phosphoenzyme intermediate (Histidine class):

Succinyl CoA synthetase catalyses the formation of Succinyl CoA which involves a phosphoenzyme intermediate as suggested by Kaufmann (1955) on the basis of an ADP-ATP isotopic exchange reaction. Further evidence that such an intermediate was involved was obtained when a phosphorylated protein was obtained when the enzyme was incubated with succinyl CoA and phosphate, or with ATP(Mitchell, 1964). The phosphate group was bound to the enzyme at a histidine residue (Remaley, 1967).

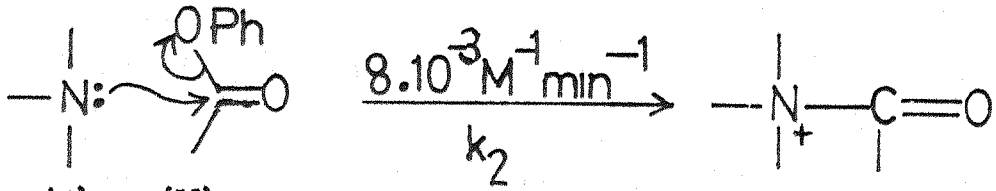
(1.5) Proximity, orientation, and orbital steering.

The mode of enzyme catalysis may depend on intermediates formed before a particular reaction is completed. On forming the original enzyme - substrate complex, conformational change may occur which may involve the catalytic groups at the active site yielding a second enzyme-substrate intermediate before products are formed. This complex is important in determining how enzymes work in that it can induce a proximity effect in positioning all nucleophiles and acidic and basic groups in a correct stereochemical fashion for entrance to the transition state.

One concept in explaining the proximity effect is that catalysis occurs by virtue of the substrate binding to the active site of the enzyme which results in a substantial increase in the concentration of substrates in a highly localized zone. Since chemical reactions generally proceed at a rate proportional to reactant concentrations, then the increase in concentration at the active site might explain the enhancement of catalysis.

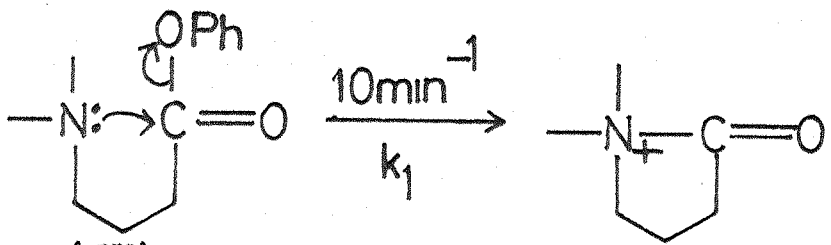
Reactions of monophenyl esters as studied by Bruice (1970) showed that there are other factors concerned in the enhancement of this type of catalysis. The reactions are:

(i) Intermolecular:



(I) (II)

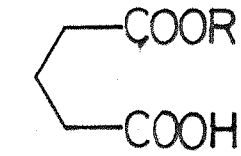
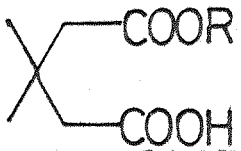
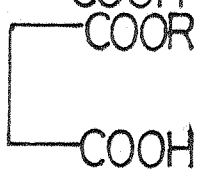
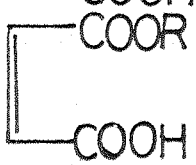
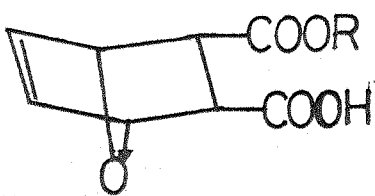
(ii) Intramolecular:



(III)

$$k_1/k_2 = 1250M$$

This means that if (I) and (III) are present at equal concentration, then for the bimolecular reaction to proceed at the same rate as the unimolecular reaction then(II) must be present at 1250M. This is impossible to achieve. Hence the rate increase must be much more than a concentration effect which lead Bruice to assume that there is an orientation factor involved in the enhancement of reaction rates and the greater the proximity of the groups involved in the reaction, the faster the rate. He showed that in the intramolecular catalysis of the hydrolysis of monophenyl esters of dicarboxylic acids in which the free carboxylate group functions as a catalyst, the greater the proximity of the catalytic carboxylate group to the susceptible ester bond, the greater the reaction rate. Enhancement of the rate was increased as much as 53,000 in the most extreme case:

Ester	Relative rates of esterhydrolysis
	1
	20
	230
	10,000
	53,000

(1.6) Strain or distortion in catalysis

A theory on the importance of strain and conformational modification of substrates that might enhance enzymic reactions was proposed recently by Jenks (1975). He suggested that when a substrate binds to the enzyme, by definition the reaction is exothermic. The energy released is not solely used in binding the substrate to the enzyme, but part of it induced a strain or distortion factor which enables the substrates to reach the transition state for them to be catalysed. How this is achieved is complex to explain in detail.

(1.7) Acid-Base catalysis

There are two general types of acid-base catalysis

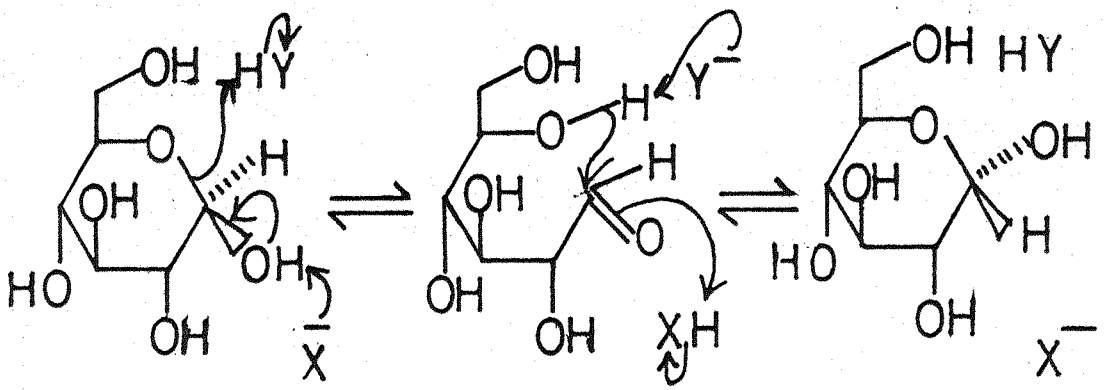
(i) Specific acid-base catalysis:

The reactions rates depend on concentration of H^+ or OH^- ions, but do not vary with the concentration of other acidic or basic species in solution.

(ii) General acid base catalysis:

The reaction rates are a function of the concentration of all

acids and bases is solution. Enzymes contain many functional groups which may participate in general acid-base catalysis, e.g. amino, imidazole, carboxyl, sulphydryl. An example of concerted general acid-base catalysis is the mutarotation of glucose:



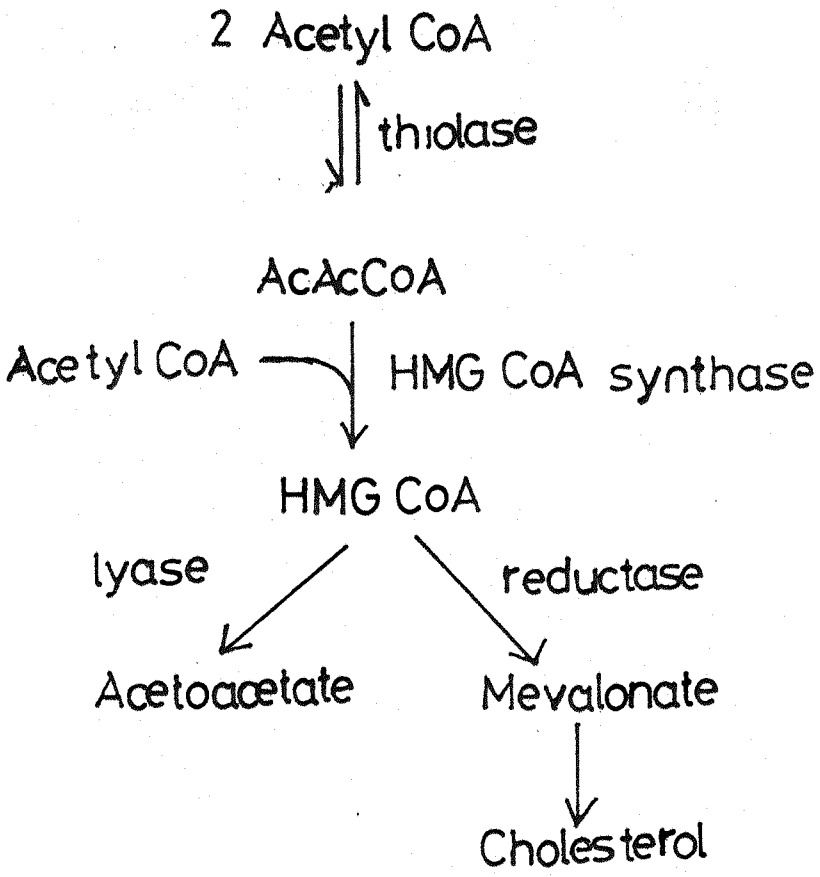
General acid base catalysis performs an important function in enzyme catalysed reactions. The side chains of many amino acids have appreciable acidic or basic character and may function as general acid base catalysts, and the pH rate profiles of many enzymic reactions indicates the importance of the ionization state of various side chain amino acids in enzyme catalysed reactions.

CHAPTER 2

The Properties of β-ketoacyl CoA-thiolase

(2.1) Physiological role

β-Ketoacyl CoA-thiolase exist both in the mitochondria and cytoplasm (Clinkenbeard et al, 1972). Intracellular fractionation studies in chicken and rat liver revealed that thiolase and β-hydroxy-β-methylglutaryl-CoA(HMG-CoA) lyase were found in the mitochondrial fraction and 44% and 21% respectively were present in the cytoplasm. Apart from β-oxidation, thiolase is involved in cholesterol biosynthesis and ketogenesis which are diverse functions of the liver that require independent regulation, yet the first two steps of these pathways are chemically identical (Scheme 1):



The cytoplasm of liver contains all the enzymes required for the conversion of HMG-CoA to cholesterol (Bucher, 1956) while only mitochondria appear to possess the lyase needed to convert HMG-CoA to acetoacetate and acetyl-CoA.

The distinction between the cytoplasmic and mitochondrial HMG-CoA pools is guaranteed by the impermeability of the inner mitochondrial membrane to the CoA derivatives (McGarry, 1969). Consequently, HMG-CoA generated in the cytoplasm serves as a precursor for cholesterol, while mitochondrial HMG-CoA is precursor for acetoacetate. Cytoplasmic thiolase has been shown to possess different molecular properties compared to the mitochondrial forms which participate in ketogenesis and β-oxidation (Clinkenbeard, 1973).

(2.2) Role in β-oxidation

In the β-oxidation scheme, a saturated acyl CoA is degraded by four reactions. The first is the formation of the trans isomer of the unsaturated acyl CoA by acyl CoA dehydrogenase, then the hydration of this trans isomer by enoyl CoA hydrase to form the L(+)β-hydroxyacyl-CoA. This is oxidized by β-OH-hydroxyacyl CoA dehydrogenase to form the β-ketoacyl CoA which undergoes thiolysis according to the following reaction:

$$R.CO.CH_2.CO.SCoA + CoASH \rightleftharpoons R.CO.SCoA + CH_3.CO.SCoA$$

Thiolase carries out this cleavage which leads to the shortening of the acyl CoA by 2 carbon atoms. This is a highly favourable reaction having a ΔG° = -6.65 Kcal/mole. The acetyl CoA produced in the thiolysis reaction may be subsequently oxidized to CO_2 and H_2O by means of the citric acid cycle. The shortened acyl CoA (n-2) undergoes another cycle of oxidation starting with the reaction catalysed by acyl CoA dehydrogenase. In each reaction cycle, an acyl CoA is shortened by 2-carbon atoms and one $FADH_2$, one NADH and one acetyl CoA are formed:

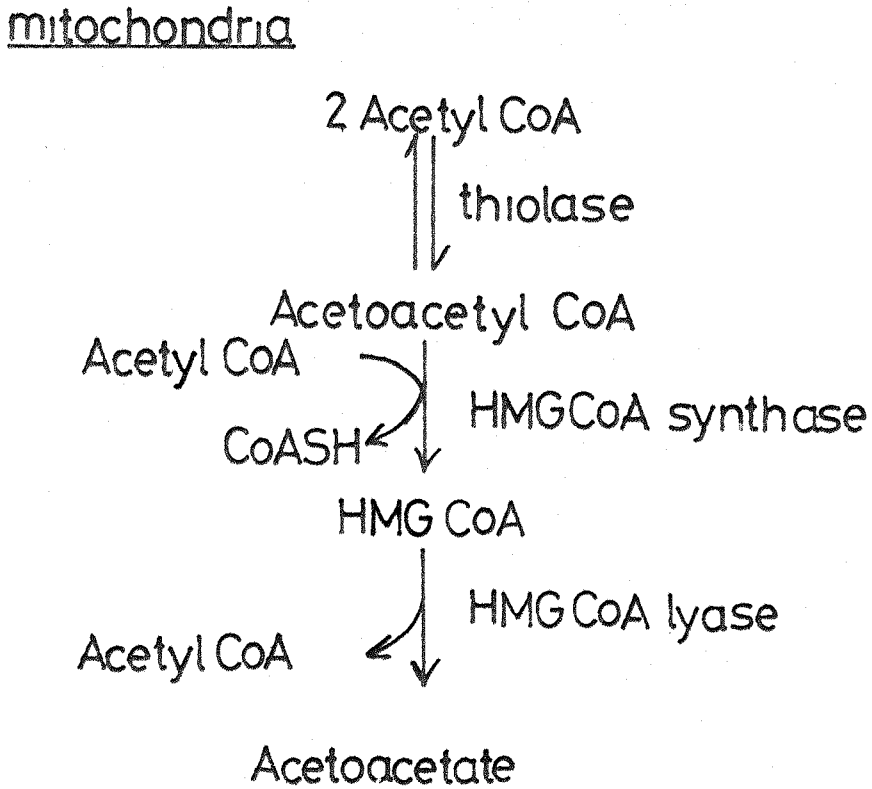
$$C_n \text{ acyl CoA} + FAD + NAD^+ + H_2O + CoA \longrightarrow C_{n-2} \text{ - acyl CoA} + FADH_2 + NADH + \text{acetyl CoA} + H^+$$

(2.3) Role in ketogenesis

Acetyl CoA enters the citric acid cycle by reacting with

oxaloacetate to form citrate. This depends on the availability of oxaloacetate. Acetyl CoA undergoes a different fate if oxaloacetate is present at low concentrations. In diabetes, oxaloacetate is used to form glucose and is thus unavailable for condensation with acetyl CoA. So, acetyl CoA is directed to the formation of acetoacetate and D-3-hydroxybutyrate. These with acetone are the ketone bodies.

Acetoacetate formation is solely a mitochondrial process (Bucher et al, 1960). A general scheme for its formation is as follows:



Formation of acetoacetate:

(1) The first step is the reversal of the thiolysis step in the oxidation of fatty acid where thiolase condenses two molecules of acetyl CoA:

$$2\ CH_3.CO.SCoA \xrightleftharpoons{\text{thiolase}} CH_3.CO.CH_2.CO.SCoA + CoASH \qquad (1)$$

This has a positive $\Delta G^{o'}$ which is accompanied by an unfavourable equilibrium, but is compensated by the favourable equilibrium of the following reaction (2) due to the hydrolysis of ester linkage :

$$CH_3.CO.CH_2.CO.SCoA + CH_3.CO.SCoA \xrightleftharpoons{\text{HMG - SCoA synthase}} CH_3.COH.(CH_2.COOH).CH_2CO.SCoA. \quad (2)$$
(II)

This results in the formation of HMG-CoA (II) catalysed by HMG-CoA synthase.

(3) Then the HMG-CoA is cleaved to acetyl CoA and acetoacetate which can undergo enzyme catalysed decarboxylation to acetone, or more importantly can be reduced to 3-hydroxybutyrate in the mitochondrial matrix. The ratio of hydroxybutyrate to acetoacetate depends on the $NADH/NAD^+$ ratio inside the mitochondria. The acetoacetate and 3-hydroxybutyrate diffuse from liver mitochondria into the blood to the peripheral tissues. There they are fuels of respiration providing an energy source.

(2.4) Role in cholesterol biosynthesis

β-ketothiolase has been isolated in the cytosol as well as the mitochondria from many sources such as chicken, bovine liver, and rat liver (Middleton, 1971;1973). It is proposed that cholesterol biosynthesis occurs exclusively in the cytoplasm (Clinkenbeard et al, 1975)

The first few steps leading to formation of HMG-CoA are identical to acetoacetate formation, but here HMG-CoA is a precursor for the formation of mevalonate by HMG-CoA reductase, which in turn passes through many intermediates leading to the formation of cholesterol in the liver.

(2.5) The Thiolase reaction

The physiological role of thiolase and how it regulates many reactions in the mitochondria and cytoplasm leads to the discussion

of the nature of the fundamental thiolase reaction, its equilibrium characteristics and the true substrates for the enzyme.

Thiolase catalyses the general reaction:

$$R.CH_2.CO.CH_2.CO.SCoA + CoASH \rightleftharpoons R.CH_2CO.SCoA + CH_3.CO.SCoA$$

(2.6) Equilibrium of the reaction

The equilibrium constant for the reaction in the direction of condensation of 2 molecules of acetyl CoA is:

$$2 \text{ Ac CoA} \rightleftharpoons \text{AcAc CoA} + \text{CoA}$$

$$\text{Keq} = \frac{[\text{AcAc CoA}]\,[\text{CoA}]}{[\text{Ac CoA}]^2}$$

This was reported by Goldman (1954) as 5.8×10^{-5} at pH 8.5 and 9.4×10^{-5} at pH 8.8, Stern et al (1953) reported 2×10^{-5} at pH 8.2. Therefore the equilibrium strongly favours the thiolytic cleavage.

(2.7) Isolation and Occurance

Thiolase is widely distributed in nature as a result of the occurance of the β-oxidation cycle. In yeast, both cytosolic and mitochondrial forms exist (Kornblatt, 1971) with isoelectric points of pH 5.3 and 7.8 respectively. In mammals, it is found in liver, heart, kidney, and occurs in the mitochondria of all tissues that have been examined with different levels of activity (Hartmann and Lynen, 1961). Coldman (1954) purified the enzyme from beef liver mitochondria by ammonium sulphate and ethanol fractionation techniques that yielded a partially purified enzyme.

The cytoplasmic form from rat liver was highly purfied (Middleton, 1974) by means of column chromatography techniques and had a specific activity of 37 units/mg. The purity was shown by polyacrylamide gel electrophoresis (Neville, 1971) which showed one band corresponding to the enzyme. Thiolase has also been purified from rat brain(Middleton, 1971) and avian liver (Clinkenbeard, 1972). The purest preparations were obtained from pig heart muscle (Stern, 1955) with a specific activity 33.8 units/mg. It is unstable during the purification procedure, but the final pure enzyme is stable between pH 5.3 - 9.5 compared to other isoenzymes which denature below pH6.

Huth et al (1975) isolated two mitochondrial forms of thiolase from rat liver. Upon assaying the thiolytic reaction in the condensation reaction (acetoacetyl CoA formation), the affinity of enzyme A for acetyl CoA was affected by acetoacetyl CoA, while enzyme B showed simple Michaelis-Menten kinetics with acetyl-CoA that were not altered by a acetoacetyl CoA. This indicates that one of the isoenzymes is probably specific for acetoacetyl CoA and participates in ketone body formation whereas the other isoenzyme uses the longer chain length substrates produced in β-oxidation.

(2.8) Enzyme Substrates

The importance of thiolase in the β-oxidation of fatty acids and in the metabolism of ketone bodies is reflected in its specificity

to β-ketoacyl substrates. These substrates include coenzyme A and pantetheine derivatives of fatty acids the specificity of which depends on their chain length. The beef liver enzyme is active on β-ketoacyl substrates with a chain length of 4-16 carbon atoms (Seubert, 1968); the maximum activity was observed with β-ketocaproyl CoA with acetoacetyl CoA reacting at a slower rate. This chain length specificity is different with pure thiolase from pig heart which is highly specific for acetoacetyl CoA (Gehring, 1968). Middleton (1973) showed that cytoplasmic thiolase from rat liver had a higher activity with acetoacetyl CoA than with 3-oxohexanoyl CoA, while the mitochondrial activity, however, differs significantly, having a higher relative activity with the longer chain substrate.

The true substrate for the enzyme purified from rat liver (Middleton, 1973) or mitochondrial pig heart (Gehring, 1968) is the keto form of acetoacetyl CoA which was verified by Middleton (1973) who studied the effect of Mg^{++} ions on the enzyme activity. He showed that Mg^{++} had no effect on the rate of the condensation reaction (acetoacetyl CoA formation), but inhibited the cleavage reaction. This is due to the interaction of the Mg^{++} cations with the enol form of acetoacetyl CoA to form a chelate, and the inability of Mg^{++} to interact with acetyl CoA in the direction of acetoacetyl CoA formation. This had originally been suggested by Stern (1956) who showed that at pH values greater than 7.5, Mg^{++} interacted with the enol form of acetoacetyl CoA to form the chelate thus decreasing the concentration of the free keto form present. By using the constants controlling this process as calculated by Stern(1956), Middleton (1974) calculated the concentrations of the free keto, enol and chelate forms of acetoacetyl CoA at 0 - 5 mM Mg^{++} concentrations. As the Mg^{++} concentration was increased, the keto form decreased and the chelate and enol form increased which led him to conclude that the true substrates for the thiolase reaction is the keto form of acetoacetyl CoA, and that the inhibitory effect of Mg^{++} was due to the depletion of this form to give the enol-chelate.

Acyl acceptors such as Coenzyme A and pantetheine are other substrates for the enzyme. Thiolase from different sources has similar affinities for Coenzyme A with a Michaelis constant of around 5×10^{-5} M (Gehring, 1968a; Mazzei et al, 1970). Pantetheine also serves as an active acyl acceptor but the rate of the reaction is decreased by about one third, and the K_m is increased by a factor of 2 - 3 as compared to Coenzyme A (Gehring et al, 1968b).

(2.9) Structure

Thiolase from pig heart has a molecular weight of 170,000 (Gehring et al, 1968). In the presence of 5M-guanidine-HCl it is dissociated to a molecular weight of 39,000 - 44,000. Quantitative determination of the N-terminal amino acid residues by reaction with dinitroflurobenzene yielded 4 valine residues per mole of protein (Gehring and Harris, 1970). From these N-terminal sequence studies, combined with peptide mapping and gel electrophoresis, it was concluded that thiolase consisted of 4 very similar protein chains each containing approximately 400 amino acid residues corresponding to a molecular weight of 44,000. The units are linked by non-covalent bonds and each individual chain contains an active cysteine which catalyses the thiolytic reaction.

These results have been further verified by inhibition studies. Thiolase from pig heart is specifically inhibited by iodoacetamide. This is due to the reaction of at least one out of a total of 20 cysteine residues per mole (170,000g) of enzyme protein (Gehring et al, 1968b). The chemical nature of the enzyme inhibitor product was studied by Gehring and Riepertinger (1968). When the enzyme was allowed to react with iodo-[^{14}C]acetamide, digested with trypsin, three radioactive peptides were found. When these were isolated in pure form amino acid studies revealed that they were derived from a unique sequence around one particular cysteine residue in the primary structure of the enzyme. The region of the identical sequence around the active cysteine was extended to 26 residues which supports the findings of Gehring and Harris (1970) that thiolase is composed of 4 similar and probably identical polypeptide chains.

The 26 amino acids that each of these peptid es consist of is:

Gln - Ala - Val - Leu - Gly - Ala - Gly - Leu - Pro
- (Cys, Asn, Thr_3, Ser, Pro, Ile_2) - Lys - Val - Cys*
- Ala - Ser - Gly - Met - Lys

* The cysteine marked with an asterisk was present as the amido-carboxymethyl derivative.

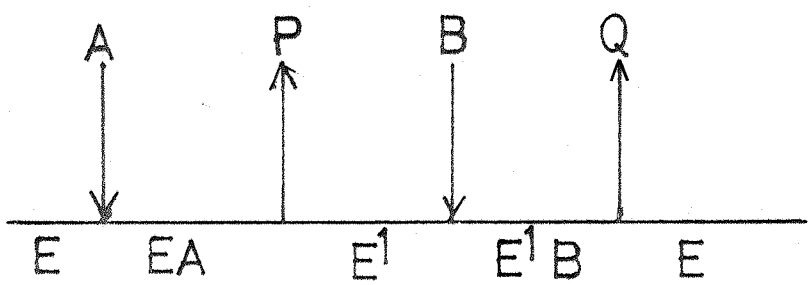
(2.10) Enzyme Mechanism

A stepwise mechanism was proposed by Gehring et al (1972a) involving an acetyl-enzyme intermediate

$$R.CH_2.CO.CH_2CO.SCoA + Enz\ S^- \rightleftharpoons CH_3.CO.SCoA + R.CH_2.CO.SEnz \quad (1)$$

$$R.CH_2.CO.SEnz + CoASH \rightleftharpoons R.CH_2.CO.SCoA + EnzS^- \quad (2)$$

The kinetic behaviour is consistent with a ping-pong type mechanism (Cleland 1970) in which the first substrate reacts with the enzyme, then the first product is released before the second substrate binds. In such a mechanism the enzyme oscillates between two or more stable forms, each substrate reacting with a different form of the enzyme. Both substrates are not required on the enzyme active site at the same time. A scheme for this is:



A = First substrate
B = Second substrate
P = First product released
Q = Second product released

E = Original form of enzyme
E^1 = Modified form of enzyme

Huth et al (1975) purified two forms of mitochondrial thiolase (A & B) from ox liver. In the direction of acetoacetyl CoA cleavage, enzyme A showed a double competitive substrate inhibition when acetoacetyl CoA was varied at different fixed CoA concentrations, while with enzyme B, a parallel kinetic pattern was obtained under the same conditions. In the condensation reactions (acetoacetyl CoA synthesis) both enzymes showed linear reciprocal plots of initial velocities against acetyl-CoA concentrations in the absence of CoA. These initial velocity kinetics in the cleavage and condensation reactions agree with a ping-pong type mechanism with the formation of an acetyl-Enzyme intermediate.

In the first partial reaction (1), the active thiol (ES^-) exerts a nucleophilic attack on the carbonyl carbon of the β-ketcacyl CoA substrate forming an acetyl-enzyme intermediate and cleaving off acetyl CoA. The second reaction (2) involves the transfer of the newly formed acyl group to Coenzyme A. The overall reaction being

$$R.CH_2.CO.CH_2.CO.SCoA + CoASH \rightleftharpoons R.CH_2.CO.SCoA + CH_3.CO.SCoA.$$

Experiments in favour of this general ping pong mechanism are as follows.

(a) <u>The enzyme possesses an essential thiol</u>

There have been many studies to prove that the enzyme is inactivated in a specific manner by thiol (SH) blocking reagents. The first reagent reported by Lynen (1953) was iodoacetate. Gehring et al (1968b) reported that reagents like iodoacetamide inactivate the enzyme with concentrations as low as 5×10^{-5} M by 50% in 5 - 8 minutes in the following manner:

$$ES^{\ominus} + I - CH_2 - \overset{O}{\overset{\|}{C}} - NH_2 \longrightarrow ES.CH_2.CO.NH_2 + I^-$$

The enzyme was also inactivated by N-ethylmaleimide and p-chloromercuribenzoate (Mazzei et al, 1970). Chase and Tubbs (1966) found out that substrate analogues like bromoacetyl CoA were also active site directed inhibitors.

All these reagents, being effective inhibitors of thiolase, proved that the enzyme contains thiol (SH) groups essential for enzyme activity by taking the following points into consideration:
(i) When the enzyme was preincubated with its substrate, it was protected against inactivation (Gehring et al, 1968b) showing that the substrate and the inhibitor compete for the active thiol in the reactive cysteine residue of the enzyme.
(ii) By using [^{14}C]- labelled iodoacetamide, it was shown by Gehring et al, (1968c) that the loss of enzyme activity of mitochondrial pig heart thiolase was directly proportional to the initial rate of incorporation of radioactivity, and by isolating a [^{14}C]-carboxymethylcysteine, they proved that the inhibitor had in fact reacted with one SH group.

(b) Acetyl CoA covalently labels thiolase to form an acetyl-enzyme complex

The thiol blocking reagents confirmed the stepwise mechanism of thiolase discussed previously. Incubation of the enzyme with acetyl CoA resulted in binding of acetyl groups to the protein as shown by Gehring et al (1968d). They found that the enzyme contains at least three (almost certainly 4) reactive cysteine residues per mole of enzyme. The following experiments were carried out to prove that these cysteines serve as acyl acceptors during the acyl transfer reaction:
(i) On incubating thiolase with [^{14}C] - acetyl CoA, acetyl groups bound to the enzyme. They were located in the same cysteine residue as that which had been modified by iodoacetamide. When pantetheine was included in the reaction mixture then an exchange reaction was demonstrated as shown below. This exchange requires the participation of an acetyl-enzyme intermediate.

$$[^{14}C]\text{- acetyl CoA + Enzyme} \rightleftharpoons [^{14}C]\text{ - acetyl - enzyme + CoA}$$

$$[^{14}C]\text{- acetyl - enzyme + pantetheine} \rightleftharpoons [^{14}C]\text{ - acetyl - pantetheine + enzyme}$$

(ii) Gehring and Harris (1970) incubated [^{14}C] acetyl CoA with the enzyme and isolated a unique heptopeptide containing [1-^{14}C] acetyl cysteine derived from tryptic digests of [1-^{14}C] acetyl-enzyme formed by the reaction with [1-^{14}C] acetyl CoA. Consequently they concluded that [1-^{14}C]-acetyl CoA was bound in thio ester linkage to cysteine residues in the enzyme, and that these reactive cysteines occurred in a unique sequence in the primary structure of the enzyme protein. In all these studies, the incorporation of radioactivity into protein was dependent on the specific activity of the enzyme.

CHAPTER 3

EXPERIMENTAL SECTION

I - Preparation of acyl CoA esters and thiols

(3.1) Preparation of Acetoacetyl CoA

5 mg of CoASH was dissolved in 1 ml of 0.1 M - $KHCO_3$. N_2 gas was bubbled through the solution. Test with nitroprusside for direct thiol reaction (Toennies and Kolb, 1951). 10µl of diketene was then added. The formation of the thiolester was confirmed by the nitroprusside test. If the reaction was not complete, a further 10µl of diketene was added and the pH was made alkaline with $KHCO_3$. After completion, the pH was lowered to 1 with 1M-HCl to stop the reaction. Excess diketene was extracted with 4 x 2 ml of diethyl ether. Contaminating diethyl eter in the aquoues phase was removed by passing through a N_2 gas stream. The aqueous phase was adjusted to pH 5 - 6 with 0.05 ml 1M-$KHCO_3$. This method gave at least 60% yield (2.7 µmoles acetoacetyl CoA).Stored at $0^{\circ}C$, the acetoacetyl CoA was stable for a few weeks.

(3.2) Preparation of Acetyl CoA

This was prepared by the same method as acetoacetyl CoA except that 10µl of acetic anhydride was used instead of diketene.

(3.3) Preparation of Acetoacetyl pantetheine

This was prepared by the same method as acetoacetyl CoA except for the use of pantetheine instead of CoASH as the thiol acceptor.

(3.4) Preparation of purified thiolesters

The crude acyl CoA ester was initially prepared on a four fold increased scale. This was applied to a DEAE-52 cellulose column (column size 25 x 1.5 cm) which had been equilibrated with 0.02 M-LiCl

+ 2mM - HCl. Fractions (2ml) were collected using an LKB fraction collector. The acyl CoA esters were eluted with a LiCl gradient. The fractions that showed an optical density change at 260 nm (the wavelength at which the thioester absorbs), were pooled, freeze dried and dissolved in 5 ml of water. This sample was then applied to a sephadex G-10 (75 x 1.5 cm) column which had been equilibrated with H_2O, and the acyl CoA was eluted with water and the fractions collected, freeze dried and resuspended in 1 ml of H_2O ready for use. This procedure gave 56% yield overall and was reasonably free of contaminating salts.

II - Preparation of reagents for the determination of thiolesters

(3.5) Hydroxylamine reagent

28% (w/v) Hydroxylamine hydrochloride was nearly neutralized by the addition of an equal volume of 14% (w/v) NaOH. The mixture had a pH of 6.4. This solution was diluted 1:1 with water for reagent purposes.

(3.6) Nitroprusside reagent

Sodium nitroprusside (1.5g) was dissolved in 5ml of 1M-H_2SO_4. Methanol (95ml) was added along with 10ml of concentrated NH_4OH (28%, v/v). The precipitate was filtered off and the deep red reagent was stored at 0°C.

(3.7) 5% Methanolic KOH

5g of KOH dissolved in 100ml of methanol.

III - Determination of thiolesters

(3.8) Quantitive Method

Thiolesters reacted quantitatively with neutral hydroxylamine reagent to form hydroxamic acids which were estimated by the method of Lipmann and Tuttle (1945).

(3.9) Qualitative method

(a) Test for a free thiol (-SH)

Place a drop of test solution (thiol in 0.1M $KHCO_3$) on a narrow strip of Whatman No.3 filter paper and dip in nitroprusside reagent, immediate appearance of a red spot indicates the presence of free-SH groups (Toennis and Kolb 1951).

(b) Test for acylthiolester

After reaction with the acid anhydride, a drop of the test solution was spotted on paper, dipped in nitroprusside reagent and then diethyl ether. No colour should appear. Then it was dipped in 5% (w/v) methanolic KOH followed by diethyl ether, a pink colour appeared indicating the formation of the acyl thiolester. Diethyl ether intensified the pink colour corresponding to the acyl thiolester formation.

III - Preparation of thiols

(3.10) Preparation of pantetheine

Pantetheine was prepared by the reduction of pantethine by sodium borohydride:

$$\underset{\text{pantethine}}{R\text{-}S\text{-}S\text{-}R} \xrightarrow{NaBH_4} \underset{\text{pantetheine}}{2RSH}$$

To 1ml of 20mM-panthethine (11mg/ml) 4mg of $NaBH_4$ was added. This was left for 40 minutes at 0°C. When the reduction was complete, the solution gave a strong nitroprusside colour (positive test). This was adjusted to pH2 by addition of 1M-HCl (H_2 evolved) to destroy any excess $NaBH_4$. Then adjust to pH 8 with 1M-$KHCO_3$.

In the preparation of acetoacetyl pantetheine, 0.2ml of freshly prepared pantetheine was mixed with 0.8ml of 0.1M - $KHCO_3$ and reacted with diketene (10µl).

IV - Techniques used in enzyme preparations

(3.11) Determination of protein concentration by Buiret method, (Layne, 1957)

The Biuret reagent was prepared by dissolving 1.5 g of $CuSo_4.5H_2O$ and 6g of sodium potassium tartarate. $4H_2O$ in 500ml of H_2O. Then 300ml of 10% (w/v) NaOH was added with continuous stirring. The solution was then diluted to 1 litre of H_2O.

To determine the protein concentration, 1ml of test protein was mixed with 4ml of Biuret reagent. This was allowed to stand for 30 minutes at room temperature, and the optical density was then measured spectrophotometrically at 540 nm, using a blank containing 1ml of H_2O and 4ml of reagent. A standard curve was plotted using bovine serum albumin of concentration 1-10mg./ml. 1mg protein gave an optical density change of 0.063.

(3.12) Determination of protein concentration by Lowry method, (Lowry, 1951)

Reagents needed:

(A) 2% (w/v) $Na_2 CO_3$ in 0.1 M-NaOH

(B) 1% (w/v) $Cu SO_4.5H_2O$

(C) 2% (w/v) sodium potassium tartarate

(D) Folin reagent - undiluted stock

0.1ml of reagent (B) was mixed with 0.1ml reagent (C) and the mixture was added to 0.2ml of the protein solution (5-100µg). The mixed solution was allowed to stand for 10 minutes, then 0.1ml of reagent (D) was added and left for 30 minutes at room temperature. This was measured spectrophotometrically against a blank which was prepared as above but with the protein replaced by a suitable buffer. The protein concentration was estimated with respect to a standard curve between 0-100µg bovine serum albumin.

(3.13) Modification of the Lowry method for proteins in the presence of thiol groups such as β-mercaptoethanol and dithiothreitol (Geiger and Ressman, 1972)

To a sample containing sulphydryl compound and up to 100mg of protein, water was added to make 0.8ml. Then add 1ml of alkaline copper reagent and mix well. This was followed by 0.2ml of 3% (w/v) H_2O_2 solution and again mixed well. The alkaline copper solution was heated

for 10 minutes at 50°C - 60°C and stood for half an hour. 2ml of working phenol reagent was added with vigorous mixing. The mixture was heated again at 60°C and stood for half an hour before reading the absorbance at 500-750 mμ depending upon the sensitivity required.

(3.14) Polyacrylimide gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gels were run according to the gel procedure of Neville (1971).

(3.15) Calcium phosphate column chromatography

Calcium phosphate for column chromatography was prepared was follows: (Mathews et al, 1964):

To a 2-litre beaker containing 40ml of 0.5M-Na_2HPO_4, 200ml each of 0.5M-$CaCl_2.6H_2O$ and 0.5M-$Na_2HPO_4.12H_2O$ were added dropwise with continuous magnetic stirring. When all the calcium and phosphate had been added, the precipitate ($Ca_3(PO_4)_2$) as allowed to settle, the supernatant decanted off, and the gel washed 6 times with 2 litres of H_2O. Then it was applied to a column of 8 x 30 cm and washed with the equilibrating buffer.

(3.16) Sephadex columns

Sephadex gels consist of 3-dimensional networks of polymer chains which are prepared by cross-linking linear polymers. Too much cross linking gave rise to very small pores (gel particles), and little cross linking gives rise to large pores. Molecules, depending on their size and shape elute at different rates. Large molecules cannot penetrate the pores and elute rapidly and small molecules enter the pores so their elution is retarded. Molecules are thus eluted from the column in order of decreasing molecular weight.

To prepare the column, the necessary sephadex grade was used, depending on the molecular weight of the protein. The powder was washed with the necessary buffer, allowed to settle down, washed again and then applied to a long thin column (1.5 x 80 cm) and washed with

the equilibrating buffer, before the protein was applied to it and eluted.

(3.17) Carboxymethyl cellulose chromatography

(a) Equilibration:

The ion exchange was stirred into a volume of the buffer (15-30 ml/dry gm), allowed to settle for 60 minutes, and washed with 0.5N HCl (10 liters/100gm) over a buchner funnel, then washed with water until an intermediate pH of 4 was attained. The slurry was then washed with 10 liters of 0.5N NaOH and water until an intermediate pH of 8 was attained. This was carefully packed into the column allowing the effluent from the column to run to waste. When all the slurry was added, the column was washed overnight with water until a neutral pH of 7.0 was attained and then washed with the equilibrating buffer before the protein was applied.

(3.18) Salt precipitation

(a) Ammonium sulfate

Proteins vary in their solubilities in concentrated salt solutions like ammonium sulfate. Purification was achieved by adding sufficient salt to precipate contaminating proteins, centrifuged, discarded.

A further addition precipitates the protein of interest which might then be collected.

(b) Acetone fractionation

Organic solvents such as acetone and ethanol precipitate proteins selectively from the mixtures. These solvents often denature proteins, so the precipitation was carried out at very low temperatures such as that achieved by using an acetone bath which could reach temperatures of $-30^{\circ}C$.

(c) Isolectric precipitation

Suitable adjustment of pH might result in the precipitation of proteins of interest. Proteins have minimum solubility near their isolectric point which is defined experimentally as that pH which

the protein does not migrate in an electric field, that is the mean charge on the protein is zero.

V- Assay systems

(3.19) Acetoacetyl CoA assay

Acetoacetyl CoA was assayed by using β-hydroxyacyl CoA dehydrogenase thus following the oxidation of NADH at 340 nm which corresponded to the conversion of acetoacetyl CoA to β-hydroxy butyryl CoA according to the following reaction:

$$CH_3.CO.CH_2.CO.SCoA + NADH + H^+ \longrightarrow CH_3.CHOH.CH_2COSCoA + NAD^+$$

The concentration of acetoacetyl CoA can be calculated from the optical density change (ΔE) and the molar extinction coefficient of NADH at the assay pH which determined to be 6220 litre.$mol^{-1}cm^{-1}$

$$\left[\text{AcAcCoA}\right] \text{ mM} = \frac{\Delta E}{6.22} \times \frac{\text{reaction volume}}{\text{sample volume}}$$

Protocol

To a 1ml- 1cm light path curvette, add:

1ml 0.1M tris/HCl buffer, pH 8.2 containing 2.5 mM $MgCl_2$ and 10mM KCl.

50µl NADH, 5mM

10µl AcAcCoA, 28µM

zero the optical density at 340 nm on 0-1 O.D. against an air blank.

Start the reaction by adding 5µl of β-hydroxylacyl CoA dehydrogenase. The amount of NADH that was oxidized to NAD^+ corresponds to the concentration of acetoacetyl CoA that was converted to β-hydroxy butyryl CoA.

(3.20) Determination of the molar extinction coefficient of acetoacetyl CoA at different pH's

The absorption (E_{303}) of a sample of acetoacetyl CoA (10µl in 1ml

0.1M- Tris/MCl containing 2.5mM-$MgCl_2$ and 10mM-KCl) was measured at different pH values. The molar extinction coefficient was calculated as follows:

Example

at pH 8.8

10µl acetoacetyl CoA (2.8×10^{-3}M) in 1ml buffer gave an optical density change of 0.585 at pH 8.8,

$$\left[\text{Acetoacetyl CoA}\right] = \frac{\text{Absorption}}{E_{303}}$$

$$2.8 \times 10^{-3} = \frac{0.585 \times 100}{E_{303}}$$

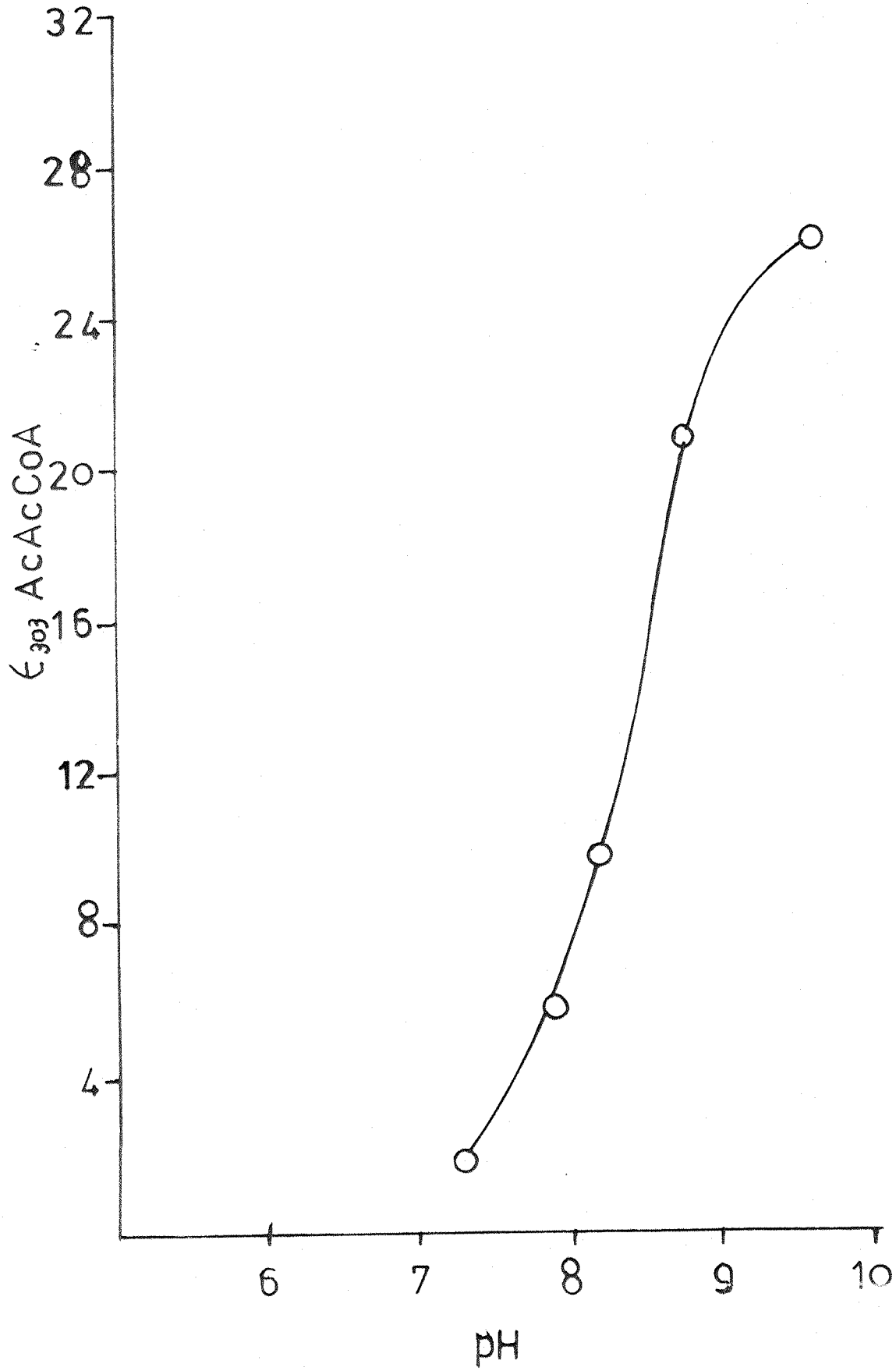
$$E_{303} = \frac{0.585 \times 100}{2.8 \times 10^{-3}} = \frac{2.08 \times 10^{4}}{}$$

The following values were obtained at different pH's:

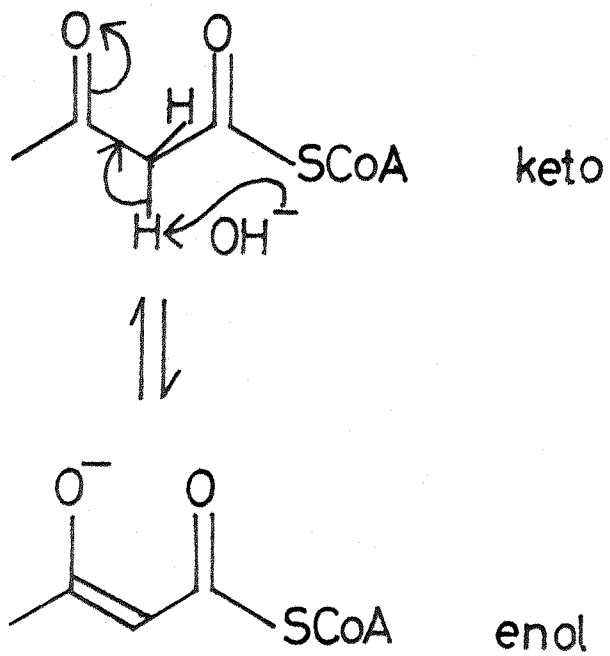
pH	E_{303} of 10µl Acetoacetyl CoA in 1ml buffer	Acetoacetyl CoA (litre.mol^{-1} cm^{-1})
7.3	0.064	2.28×10^3
7.9	0.164	5.86×10^3
8.2	0.275	9.82×10^3
8.8	0.585	20.8×10^3
9.15	0.62	22.1×10^3
9.6	0.73	26.0×10^3

Plotting the graph of E_{303} vs. pH (Fig.3), the extinction coefficient of acetoacetyl CoA could be determined at any pH. Note that as pH was increased, the absorption of acetoacetyl CoA increased due to the transformation of acetoacetyl CoA from the keto to the enol form as the medium became more basic, until at very alkaline pH (9.6) when the E_{303} became constant due to the formation of a complete enolate form which did not show any increase in absorption at 303 nm and hence any increase in E_{303}. Thus there was a different E_{303} at any pH between 7.3 - 9.5

Figure 3



due to the variation of the ratio of keto to the enol form of acetoacetyl CoA.



(3.21) Assay of Acetyl CoA and CoA

To a 1ml 1-cm light path cuvette, add:

- 1ml 0.1M-Tris/HCl, pH 8.2 containing 2.5 mM - $MgCl_2$, and 10 mM - KCl
- 0.1ml 5.5' - dithio bis-2-nitrobenzoic acid (DTNB), 2mM, pH 8.0
- 0.1ml oxaloacetate, disodium salt, 5 mM.

Zero the optical density at 412 nm on a 0-1 O.D units against an air blank.

Add a known volume of acetyl CoA (5-20µl), the total optical density change corresponds to the concentration of free CoA (the millimolar extinction coefficient of the thionitrobenzoate anion = 13.2.litre.m $mole^{-1}$ cm^{-1} at 412 nm).

Then add 5µl citrate synthase (contains 160 units/ml), and the new change in O.D. at 412 nm corresponds to the CoA which had been esterified to acetyl CoA.

Thus the ratio of CoA to acetyl CoA was measured as the ratio of

the O.D. change after its addition.

(3.22) Assay of β-ketoacyl CoA thiolase

Standard assay

This assay was described by Middleton (1973). The enzyme activity was determined at 30°C by following the stimulation of acetoacetyl CoA breakdown at 303 nm caused by the addition of CoA to the enzyme.

Protocol

To a 1ml, 1-cm light path cuvette, add:

1ml of 0.1 M-Tris/HCl, pH 8.2, buffer containing 10mM-KCl + 2.5mM-$MgCl_2$

10µl acetoacetyl CoA (2.8 mM by synthesis from 5mg CoA)

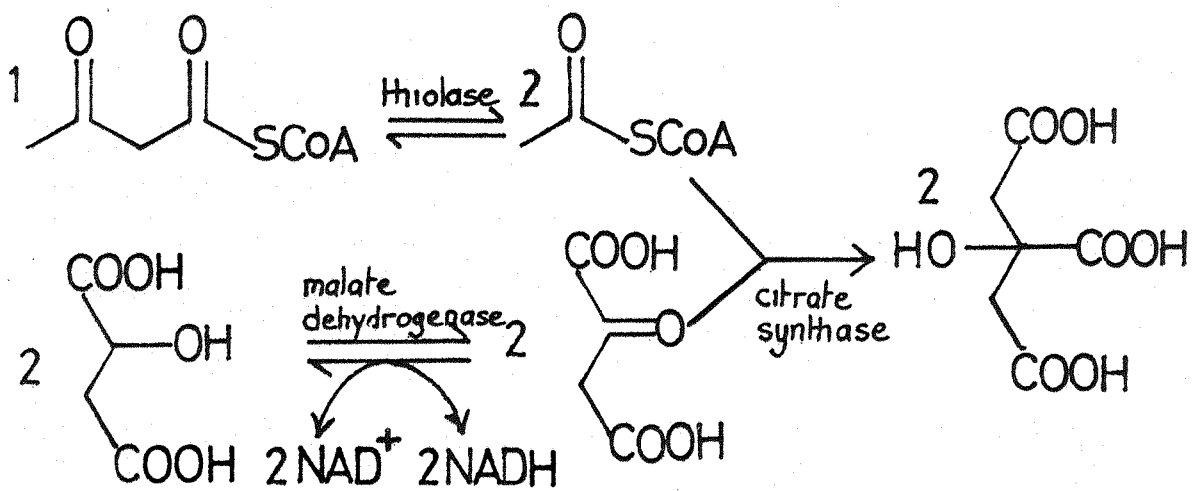
Adjust the optical density at 303 nm to give a deflection of 0.20.D against an air blank.

Add appropriate volume of enzyme, and start reaction by adding 25µl of CoA (5 mM) and follow the decrease in absorption of acetoacetyl CoA at 303 nm.

(3.23) Assay of liver cytoplasmic thiolase using coupled reactions sequence

Since the reaction being studied was towards the cleavage of acetoacetyl CoA forming two molecules of acetyl CoA, the following assay was done to show that the direct assay of thiolase results in acetyl CoA production.

This was done by using a coupled assay system of the type



To show that AcCoA was formed during the direct assay at 303 nm, two incubations were prepared:

Incubation 1	Incubation 2
1ml 0.1M-Tris HCl, pH8.2	1ml Tris HCl, 0.1M, pH8.2
acetoacetyl CoA (30µM)	10µl AcAcCoA (30µM)
5µl thiolase	no enzyme
CoA (20µM)	CoA (20µM)
E_{303} (1) = 0.280	E_{303}(2) = 0.050

Where the enzyme was present (1), there was an O.D. change of 0.280 corresponding to acetoacetyl CoA being cleaved to form acetyl CoA When enzyme was absent (2), there was a very small O.D. change (0.05) which meant that 97% of the acetoacetyl CoA remained uncleaved as it was in the absence of the enzyme, and the small optical density change could be due to:

(a) small contamination of acetoacetyl CoA with CoA supported by the observation that (b) $\frac{0.05}{0.16} \times 100 = 3\%$ O.D.change was due to the hydrolysis of acetoacetyl CoA at pH 8.2 liberating 3% CoASH. Both of these observations were dependent on K_m AcAcCoA $>$ K_m CoASH

Follow the above incubations with addition of:

10µl L-malate, 5mM, pH7

10µl DTT, 10mM

5µl citrate synthetase

E_{340} (incubation 1) = 0.480

E_{340} (incubation 2) = 0.04

which shows that:

(a) maximal acetyl CoA was produced only in the presence of thiolase, CoASH and acetoacetyl CoA

(b) increase in E_{340} of NADH was dependent on thiolase and acetoacetyl CoA which corresponded to the reduction of approximately 2 moles of NAD equivalent to the formation of 2 moles of citrate.

(c) in the absence of citrate synthetase, no reaction was observed.

(3.24) Synthesis of methyl methanethiosulphonate

Methyl disulphide (28g) was dissolved in 90ml of glacial acetic acid in a 250ml beaker cooled on ice. The mixture was fitted with a stirrer and thermometer. H_2O_2 (68.1g; 30% v/v) was added dropwise with stirring over 15 min and then the solution was stirred for 20 min. During this time the temperature should not exceed 10°C. The beaker was now transferred to a water bath and the temperature was raised to 60°C for 1h. The glacial acetic acid was removed in vacuo to yield an oil. This was treated with 50ml of sat $NaHCO_3$. The organic phase was collected by shaking three times with 50ml of chloroform. The chloroform was removed in vacuo and the product was purified by vacuum distillation (20g; bp 73 - 75° at 0.5mm Hg). The ^{1}Hn.m.r. showed two distinct and equal reasonances at δ 2.65 (singlet) and 3.22 (singlet).

CHAPTER 4

The main aim of this thesis is to show that β-ketoacyl CoA thiolase contains an amino group at the active site which participates in the catalytic activity. An essential thiol group is already known to be involved in the catalytic process of the enzyme (Chase and Tubbs, 1966; Gehring et al, 1968) and a comprehensive discussion of its role was included in the introductory sections. To prove the existence of the additional amino group, the approach chosen was to investigate the effect of anhydrides which modify amino groups on the activity of the enzyme. But since anhydrides may also react with the active site thiol it was necessary to develop a reagent which could reversibly protect the active site cysteine. The reaction chosen was thiomethylation using methyl methanethiosulphonate ($CH_3.S.SO_2.CH_3$; Smith et al, 1975; Nishimura et al, 1975; Bloxham and Wilton, 1977). This reagent blocks the thiol, so that when using the anhydrides they are restricted to modifying the amino at the active site. This could generate an enzyme which is doubly modified, i.e., alkylated at the amino and the thiol. The thiomethyl group could then be removed to yield an enzyme specifically modified in the amino group. Finally, the active enzyme could be regenerated at acid pH (Dixon and Perham, 1968; Gibbons and Perham, 1970; 1974).

(4.1) Purification of mitochondrial β-ketoacyl CoA thiolase from pig heart

The main source of enzyme used in this work was isolated from pig heart. The enzyme is unstable and difficult to work with however the following procedure yields an enzyme which is reasonably homogenous and in adequate amounts for kinetic experiments. The isolation procedure is based in part upon the method of Stern (1955) however the introduction of the chromotography steps is new. In addition to the pig heart enzyme some of the experiments were duplicated using rat liver cytosolic enzyme and the purification of this enzyme

is described in the next section.

Step I : Homogenisation

Eight pig hearts (1500gm) were ground in an electric mincer. 170gm portions were homogenized in a Waring blender with 250ml of 50mM-potassium phosphate buffer, pH 7.4, containing 0.02M-KCl and 0.5mM-mercaptoacetic acid, for 5 minutes at the low speed setting then for 3 minutes at the high speed setting. The deep red homogenate was centrifuged at 10,000 rpm for 20 minutes and the murky supernatant decanted through cheesecloth to remove any fat. About 3 litre of clear red supernatant was collected which contained 6500 units.

Step 2 : Ammonium Sulphate Fractionation (I)

With gentle mechanical overhead stirring, 245 gm of ammonium sulphate (enzyme grade) was added for every litre of enzyme from the previous step. This addition took 20 minutes and a further 20 minutes mixing time was allowed for precipitation of unwanted proteins. The solution was centrifuged at 10,000 rpm for 20 minutes and the precipitate was discarded. Another 210 gm of ammonium sulphate was added per litre of supernatant. After centrifugation at 10,000 rpm for 20 minutes, the supernatant was discarded and the pellet was suspended in the smallest possible volume of 0.017-M potassium phosphate, pH 6.8, containing 0.5mM-mercaptoacetic acid. The sample was dialysed against the same buffer overnight. The volume at this stage was 220ml and contained 3900 units.

Step 3 : Acetone fractionation

The potassium phosphate concentration was increased to 0.067M with 0.5M-potassium phosphate, pH 6.8. Acetone was cooled in an ethanol-dry ice bath in a metal beaker to $-6^{\circ}C$, and the enzyme was placed in an acetone bath at $-6^{\circ}C$. Acetone addition was started

immediately with overhead stirring to ensure even mixing. Over 20 - 30 min, the acetone was raised to 40.5%($^{v}/_{v}$). The mixture was stirred for a further 20 min and then the protein was collected at 10,000 rpm at -6°C for 20 min. The pellet was resuspended in 0.017M-potassium phosphate, pH 7.4, containing 0.5mM-mercaptoacetic acid, and dialysed against the same buffer overnight. At this stage, 90ml of enzyme was obtained which contained 1800 units.

Note: The acetone should be added dropwise in an even manner to ensure complete precipitation of enzyme, and the supernatant should be assayed for enzyme activity before it was discarded. In the unlikely situation of a fair amount of activity present, another acetone precipitation (60%) should be done.

Step 4 : Acid precipitation

The enzyme from (3) had 16mg protein/ml and this was decreased to 6mg/ml with the same buffer. The pH was adjusted to 5.3 in the cold with 1M-acetic acid with continuous magnetic stirring, then it was centrifuged at 12,000 rpm for 15 min, and the supernatant was neutralized to pH 7.4 with 2M-KOH. This process must be done quickly to avoid any loss of activity. The enzyme has 1450 units in 90ml.

Step 5 : Second ammonium sulphate addition

Enzyme grade ammonium sulphate was added in two stages with continuous magnetic stirring.

stage 1. 35.8gm ammonium sulphate per 100ml from acid stage was added, over a period of 20 min. After 20 min, the mixture was centrifuged at 10,000 rpm for 20 min and the precipitate was discarded.

stage 2. 13.6gm ammonium sulphate per 100ml was added in the same manner and the procedure was repeated. The precipitate was suspended in 0.01M-potassium phosphate, pH 6.9, containing 0.5 mM-mercaptoacetic acid, and dialysed against the same buffer overnight.

(7) Carboxymethyl sephadex chromatography

A column of 20 x 5 cm carboxymethyl sephadex was packed and equilibrated in 0.01M-potassium phosphate, pH 6.9, containing 0.5 mM-mercaptoacetic acid at 4°. The enzyme from the ammonium sulphate precipitation was applied to the column and washed with:

-100ml starting buffer

-500ml 0.1M-potassium phosphate, pH 6.9, containing 0.5mM-mercapoacetic acid.

-Gradient of 200ml each of 0.2M-potassium phosphate, pH 6.9, containing 0.5mM-mercaptoacetic acid, and 0.7M-potassium phosphate, pH 6.9.

6ml fractions were collected and scanned at 280nm. There were two main protein peaks before applying the gradient (tubes 9-19, and 41-59), and when the gradient was applied three protein peaks appeared (tubes 71-89, 91-122, 141-169). Assaying for thiolase activity, only tubes 91-122 were active with about 80% of the activity recovered (944 units/214ml). The other protein peaks showed no activity indicating different proteins. The enzyme was concentrated to 27ml and dialysed against 50mM-potassium phosphate buffer, pH 6.9, containing 25%($^{v}/_{v}$) glycerol and 0.5mM-mercaptoacetic acid. Upon concentration the enzyme lost about 10% of its activity retaining 833 units in 19ml.

(8) Sephadex G-200 column chromatography

A Sephadex G-200 column was equilibrated with 50mM-potassium phosphate buffer, pH 6.9, containing 1mM-mercaptoacetic acid, after packing the column it was washed with the same buffer and half the carboxy methyl enzyme was applied (420 units/9ml) and eluted with

the same buffer and 2ml fraction were collected and scanned at 280nm, one protein peak was observed which contained all the activity (tubes 15-33).

The enzyme was concentrated to 2.4ml and running 50µg on polyacrylamide gel electrophoresis in sodium dodecyl sulphate there was one main protein band(95%) corresponding to the enzyme.

The purification procedure is summarised in Table 1.

Table 1 : Purification of Pig Heart Mitochondrial Thiolase

Fraction	Volume(ml)	Units	Protein mg/ml	Specific Activity Units/mg
Phosphate extract	3080	6522	18	0.121
$(NH_4)_2SO_4$(I)	220	3900	6	3.24
Acetone ppt.	90	2200	6	4.07
Acid treated	90	1450	3	5.03
$(NH_4)_2SO_4$(II)	68	1200	2	10.9
Carboxymethyl-sephadex column	27	944	1.4	24.11
Sephadex G-200 column	2.4	670	4.8	140

4.2 Purification of Cytoplasmic β-ketoacyl CoA thiolase from rat liver

Step 1 pH 5.5 Supernatant

15 rat livers (250gm) were homogenized in a Waring blender in 2-litres of 0.25M-sucrose, centrifuged at 12000 g for 10 minutes and the supernatant adjusted to pH 5.5 with a 1M-acetic acid. The murky supernatant was centrifuged at 2000 g and clear supernatant rapidly adjusted to pH 8.2 withM-Tris base and made 50mM- with respect to Tris, pH 8.2.

Step 2 DEAE - cellulose chromatography

The supernatant fraction was applied to a column of DEAE cellulose (15 x 8cm) at a loading ratio of 200mg/10ml DEAE. The column was washed with 1 litre of equilibrating buffer (100mM-Tris HCl, pH 8.2 containing 0.5mM-dithiothreitol). Cytoplasmic thiolase was eluted with 200mM-Tris/HCl, pH 8.2, containing 0.5mM-dithiothreitol. Although absorption for protein at E_{280} was recorded in tubes 11-61 the enzyme activity was detected in tubes 19-47 (250ml). Middleton (1973) has shown that this method gives exclusively the cytoplasmic enzyme without any mitochondrial thiolase activity. This step was done at room temperature.

Step 3 Chromatography on calcium phosphate

The DEAE cellulose eluate was applied to a calcium phosphate column such that loading of enzyme did not exceed an activity of 1 μmole of acetoacetyl CoA/min per ml of column. The column was washed with 90ml 1mM-dithiothreitol after which no protein was eluted, then the cytoplasmic thiolase was eluted with 100mM-sodium phosphate, pH 6.6, containing 0.5mM dithiothreitol (elution range tube (5-29) = 300ml).

This was done at room temperature.

Step 4 Chromatography on cellulose phosphate

The calcium phosphate eluate was adjusted to 50mM with

1M-sodium phosphate buffer pH 6.6 and applied to a short column of cellulose phosphate (100ml) equilibrated with 50mM-sodium phosphate, pH 6.6, containing 0.5mM-dithiothreitol. The column was washed with 200ml of starting buffer before applying a gradient of (400ml total volume) 50 - 250mM-sodium phosphate, pH 6.6, containing 0.5mM-dithiothreitol. The purified cytoplasmic thiolase was eluted at about 150mM-phosphate concentration and had a specific activity of about 35 µmole of acetoacetyl CoA/minute/mg protein.

The enzyme was dialyzed overnight against the same buffer and concentrated in a concentration cell with very gentle mixing to about 8ml, in this stage the enzyme loses about 25% activity but the loss was reduced to 10% by adding 1mM-dithiothreitol, then it was stored at $0^{\circ}C$ with 25% glycerol. The activity was maintained for at least 6 months under these conditions. The data is summarized in table 2.

4.3 Effect of pH on the activity of liver cytoplasmic thiolase

The participation of ionisable groups in enzyme catalysis can often be demonstrated by investigating the effect of pH on the rate of the enzymic reaction. As an example, an amino group undergoes the following pH dependent equilibrium

$$R - NH_2 + H^+ \rightleftharpoons R - NH_3^+$$

For proteins, the pK_a value of ε-NH_2 groups in lysine is usually quoted between 9-10.5. This means that if one form of the amino group is catalytically active and the other inactive then the activity of the enzyme may be expected to change in the pH region 9 - 11.0. A full kinetic analysis of the influence of pH on enzyme activity is extremely complicated, however as a preliminary part of this project, the effect of varying the pH in the range 7 to 10 on the reaction velocity at a single concentration of acetoacetyl

Table 2 : Purification of Rat Liver Cytoplasmic Thiolase

Procedure	Volume(ml)	Protein (mg/ml)	Units	Specific Activity (units/mg)
pH5.5 supernatant	940	10	1165	0.124
DEAE-52	250	7.5	475	0.253
$CaPO_4$	300	2	242	0.40
Cellulose PO_4	8.4	0.45	130	34.21

CoA was studied (Fig. 4). The reaction velocity varied as a bell shaped curve and indicated that the reaction could be controlled by functional groups with pK_a values of 7.4 and 8.9. This could correspond to the participation of a cysteine - SH (pK_a 7.4 ; active as S^-) and an amino group (pK_a 8.9 ; active as NH_3^+). Although this proposal is attractive particularly when considered with the later experiments, it should be noted that no attempt has been made to eliminate changes in binding constants from effects on reaction velocity. Our own studies indicate the $K_{\text{Acetoacetyl CoA}}$ and K_{CoASH} vary in a complex manner on changing pH (Fig. 5).

An additional factor in controlling the activity of the enzyme on varying pH is the possibility of pH dependent inactivation of the enzyme. This is also important for the inactivation experiments on the enzyme reported later. To investigate the effect of pH on enzyme stability, cytoplasmic thiolase was incubated at 20^0 in 0.1M-Tris/HCl at a variety of pH values between 5.0 and 9.6 for 15 minutes. The pH was then adjusted to 8.2 by the addition of either alkali or acid. Following pH adjustment a sample of the enzyme was assayed by the standard procedure. The preliminary incubation between pH5 and 9.6 did not affect the activity of the enzyme showing that within this range the enzyme was reasonably stable. If the pH was decreased below pH 5 then the enzyme was extremely unstable. This factor was very important in determining the acid dependent reactivation of citraconylated-thiolase reported later.

(4.4) Inactivation of thiolase by methyl methanethiosulphonate

The reaction of the active site thiol in thiolase can be shown by the time dependent inactivation of the enzyme with methyl methanethiosulphonate. For this experiment mitochondrial thiolase (10µl ; 1mg/ml) was diluted with 200µl of 100mM-potassium phosphate buffer, pH 8.2 at 0^oC and 20µl was assayed to determine the standard enzyme activity. Then 20µl of methyl methanethiosulphonate solution

Fig. 4 Effect of pH on the activity of liver cytoplasmic thiolase

Cytoplasmic β-ketoacyl-CoA thiolase was incubated at 30°C in the standard assay buffer of the appropriate pH. 28μM-Acetoacetyl CoA and 50μM-CoASH were added to the assay mixture, and the enzyme activity (μmoles of AcAcCoA consumed/min) was determined. The reaction velocities were corrected for the pH dependent change in the molar extinction coefficient.

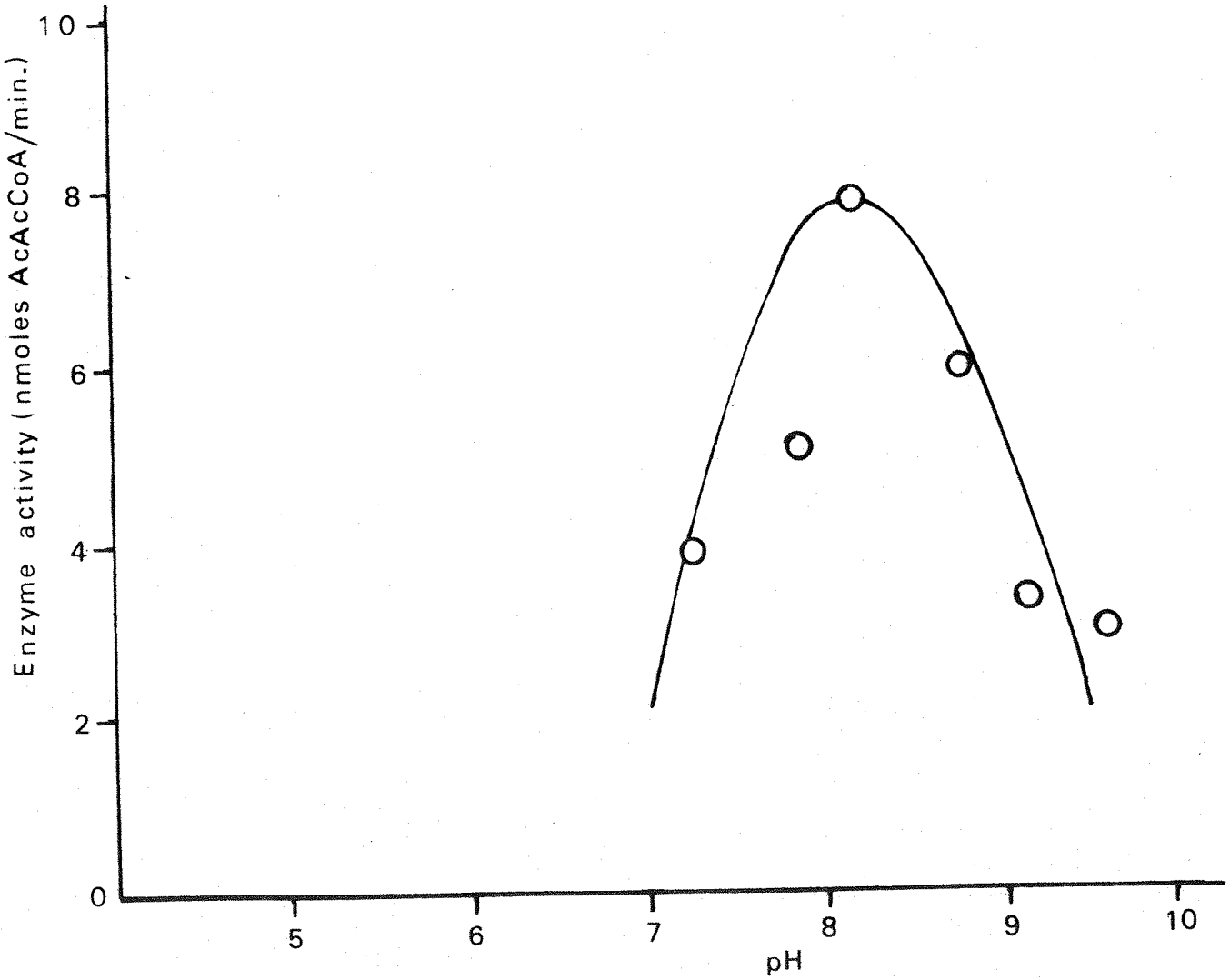
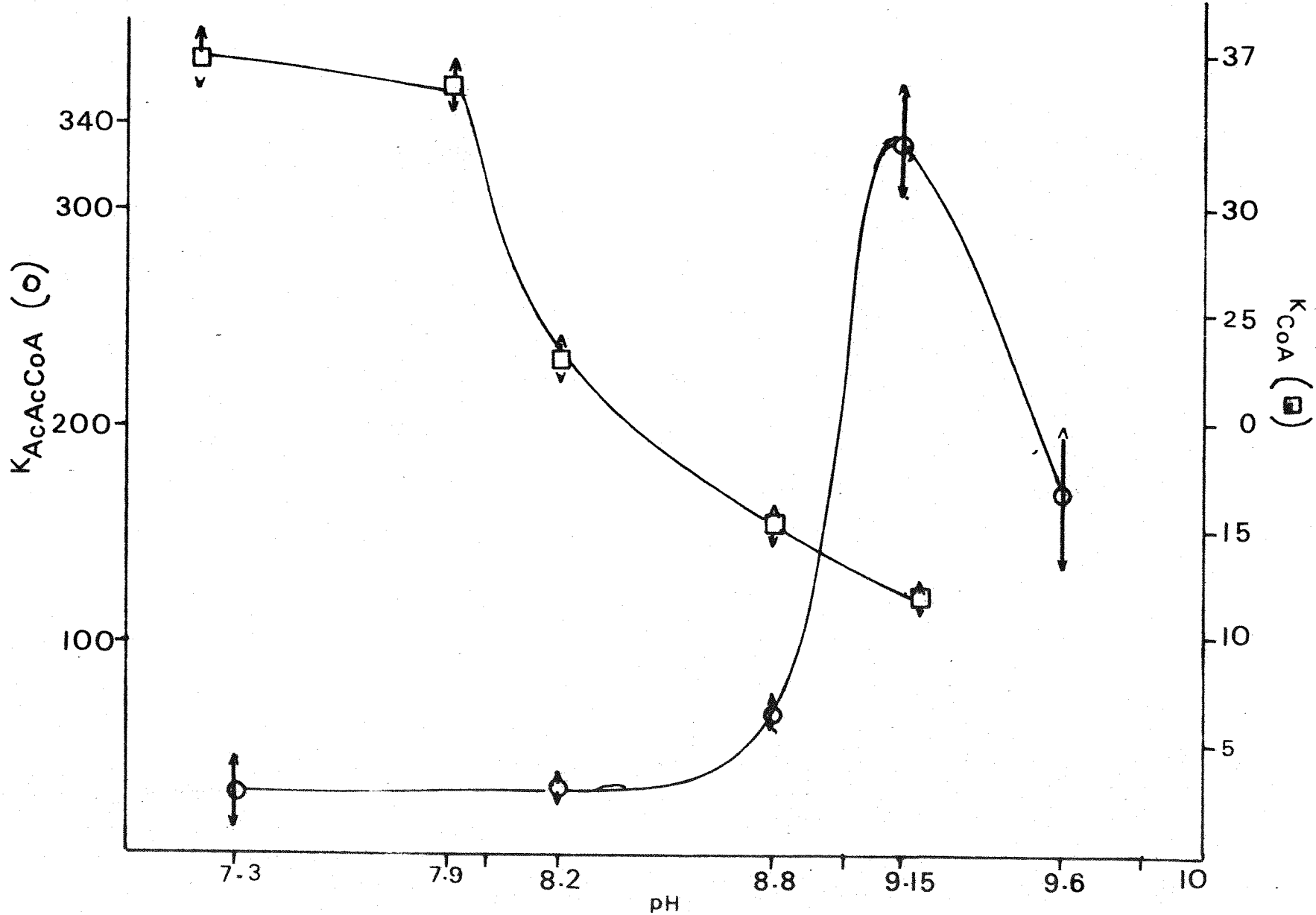

Enzyme activity (nmoles AcAcCoA/min.)
10
8
6
4
2
0
5
6
7
8
9
10
pH

Fig. 5 Determination of the pH dependent variation of Michaelis constant, K_m, for acetoacetyl CoA and CoASH

To determine $K_{\text{Acetoacetyl CoA}}$ a standard enzymic assay was performed as described in the method section using a constant concentration of CoASH (50μM) but using 4 different acetoacetyl CoA concentrations. For determing K_{CoASH}, the same procedure was followed but keeping the acetoacetyl CoA constant (28μM) and varying the CoASH concentration.


340
300
200
100
$K_{AcAcCoA}$ (○)
37
30
25
0
15
10
5
K_{CoA} (■)
7·3
7·9
8.2
8.8
9·15
9.6
10
pH

in water was added to the enzyme sample to a final concentration of 10, 50, 100, or 200μM. The decrease in enzyme activity was now followed over a time course (Fig. 6). In the absence of methyl methanethiosulphonate the enzyme was completely stable over the duration of the reaction, however the inclusion of the inhibitor resulted in a rapid loss of enzyme activity. The rate of loss of enzyme activity was increased by raising the concentration of the inhibitor. When the reaction was carried out with 200μM-methyl methanethiosulphonate the enzyme was completely inactivated and this demonstrates the essential role of the sulphydryl group in the catalytic process.

Plots of $\log_{10}\%$ activity versus time appeared to be linear in the time and concentration range studied here. For this type of result Kitz and Wilson (1962) have proposed the following model for covalent modification of the enzyme.

$$E + I \underset{k-1}{\overset{k+1}{\rightleftharpoons}} EI \xrightarrow{k+2} EI^*$$

For this system it is assumed that the inhibitor (I) binds to the enzyme through a rapidly equilibrating non-covalent intermediate (EI) and this is followed by the slower covalent reaction leading to inactivation of the enzyme (k+2). This process is apparently a first order process governed by the following relation:

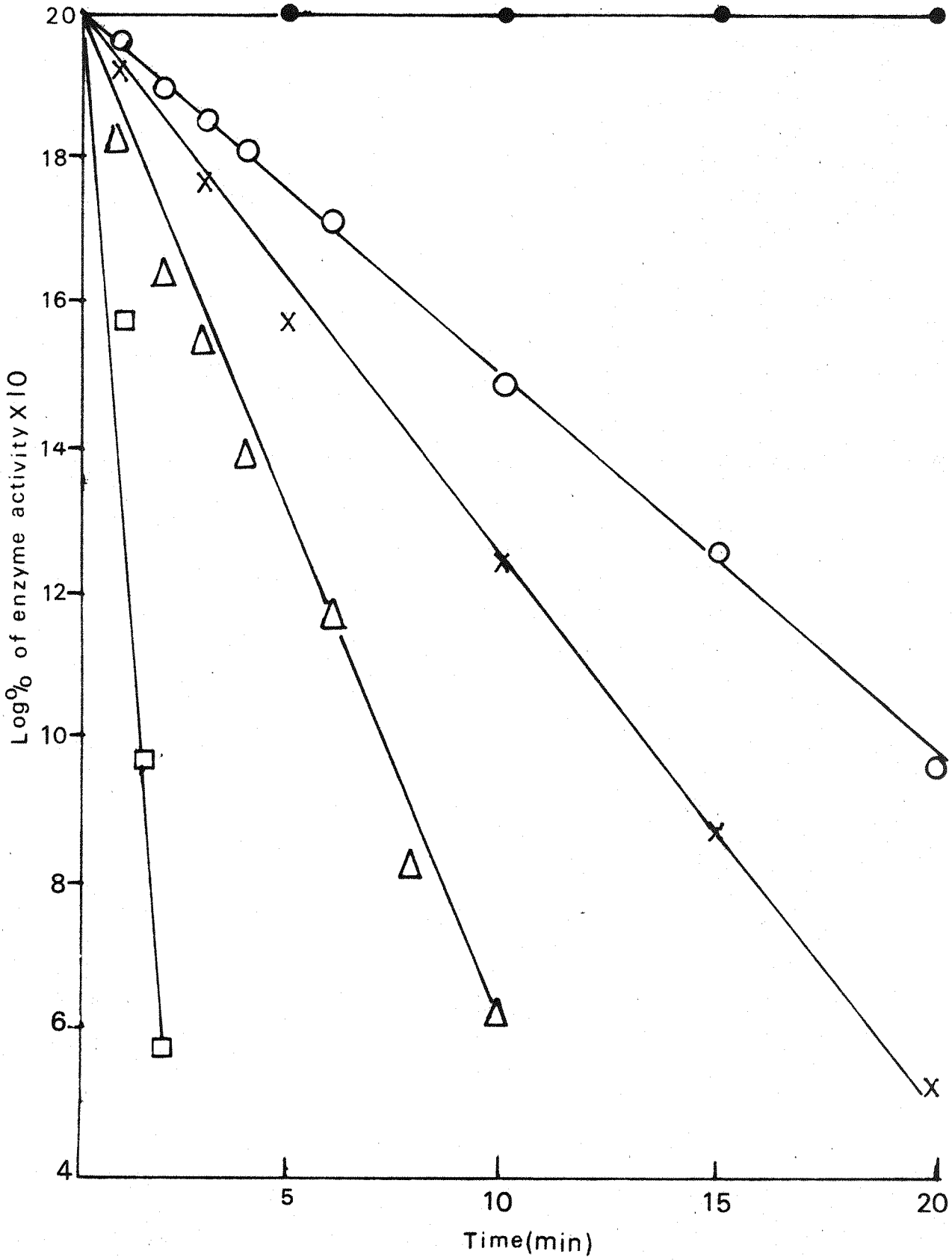
$$\log_{10}\% \text{ Activity} = \frac{-k_{+2} \cdot t}{2.303(1+K_I/I)}$$

where t is time, K_I is the dissociation constant for the inhibitor and I is the inhibitor concentration. This may be simplified to

$$\log_{10}\% \text{ Activity} = -k_{app} \cdot t$$

Fig. 6 Time dependent inactivation of mitochondrial thiolase at pH 8.2 by methyl methanethiosulphonate

Mitochondrial thiolase (10µg) was incubated at 0°C in 200µl of 0.1M potassium phosphate, pH 8.2, containing various concentrations of methyl methanethiosulphonate. At appropriate times 20ul was removed and assayed. The concentrations of methyl methanethiosulphonate were (□) 200µM; (△)100µM; (X) 50µM; (O)10µM; (●) 0.


20
18
16
14
12
10
8
6
4
Log% of enzyme activity X 10
5
10
15
20
Time(min)

A double reciprocal plot of kapp versus methyl methanethiosulphonate concentration may be used to estimate k_{+2} and K_I under certain conditions. However for the reaction of methyl methanethiosulphonate with thiolase the plot passed through the origin indicating that the process was not exactly following the simple model. As will be shown later the reaction of anhydrides with thiolase differs even more substantially from apparent pseudo first order kinetics.

(4.5) Substrate protection against inactivation by methyl methanethiosulphonate.

If methyl methanethiosulphonate modifies a group at the enzyme active site then clearly it should compete with substrates which also bind at the active site. Therefore the inclusion of substrates into the inactivation reaction mixture should prevent inactivation and protect the enzyme. Protection is usually taken as a critical experiment in establishing the active site character of a covalent enzyme inhibitor.

For the protection experiments, enzyme inactivation at 0° was measured in the presence of a fixed concentration (100μM) of methyl methanethiosulphonate and increasing concentrations of acetoacetyl CoA, or acetyl CoA or CoASH. Fig. 7 shows that acetoacetyl CoA provided excellent protection against inactivation and resulted in a decrease in both the initial rate of inactivation and the extent of inactivation. Maximum protection was obtained with 40μM-acetoacetyl CoA which is only three times the K_m concentration. In the next experiment (Fig. 8) acetyl CoA was used as a protecting ligand and it was clear that it was not so effective as acetoacetyl CoA. Inclusion of 50μM-acetyl CoA does reduce the rate constant for inactivation about four fold from 0.015 min^{-1} in the absence of substrate to 0.0039 min^{-1} in the presence of acetyl CoA. These experiments were limited by the fact that increasing the concentration of acetyl CoA above 50μM was

Fig. 7 Protection of mitochondrial thiolase by acetoacetyl CoA

Mitochondrial thiolase (10µg) was incubated at 0°C in 200µl potassium phosphate buffer, pH 8.2, in the presence of different concentrations of acetoacetyl CoA. The enzyme was assayed to determine the standard activity, and 100µM(20µl) of methyl methanethiosulphonate was added and 20µl was removed at appropriate times to determine the enzyme activity. Another sample containing only 100µM methyl methanethiosulphonate was assayed in parallel with the first to ensure the complete inactivation of the enzyme. The concentrations of acetoacetyl CoA were : (□) 40µM; (○) 25µM; (X) none.

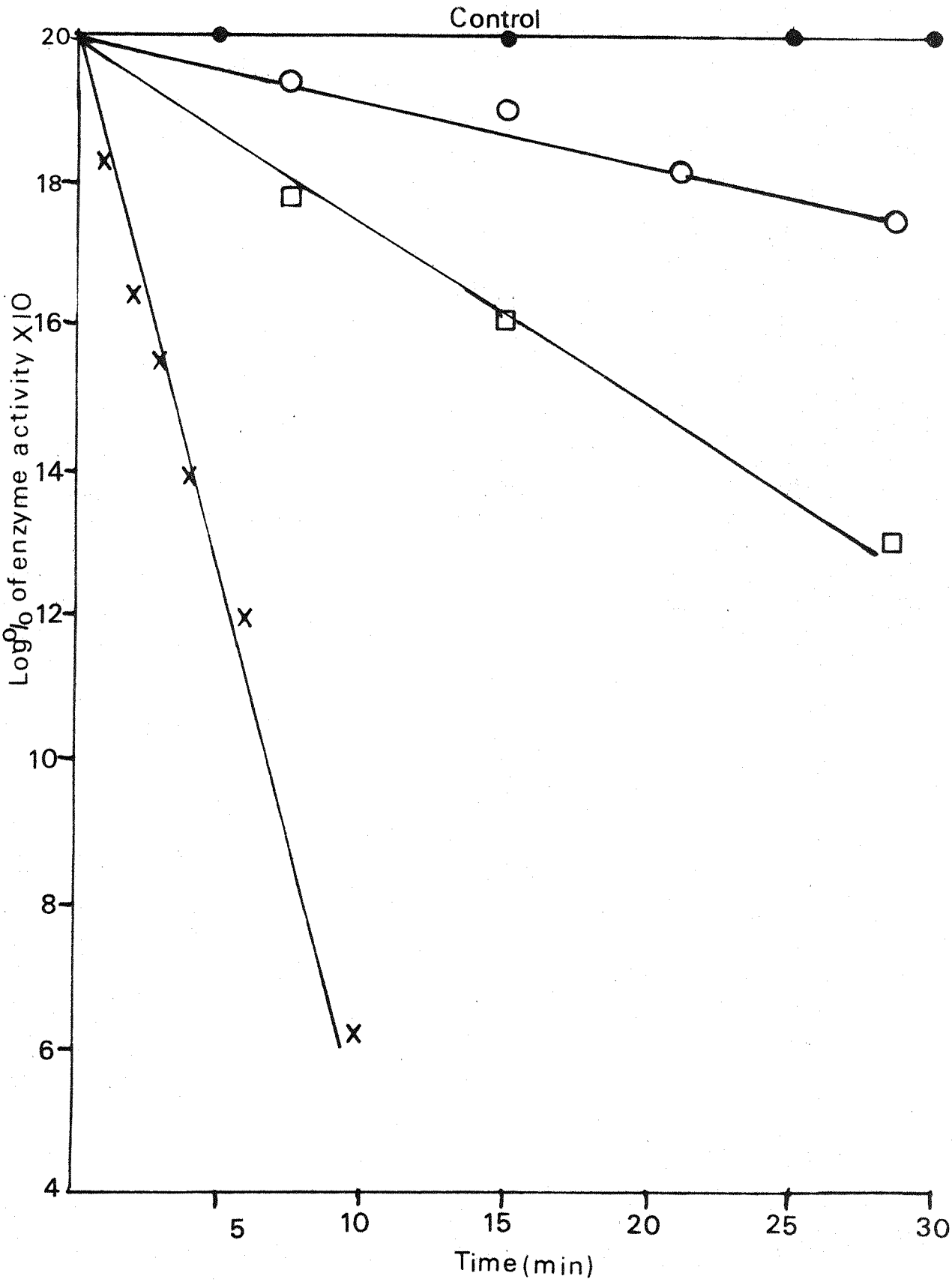
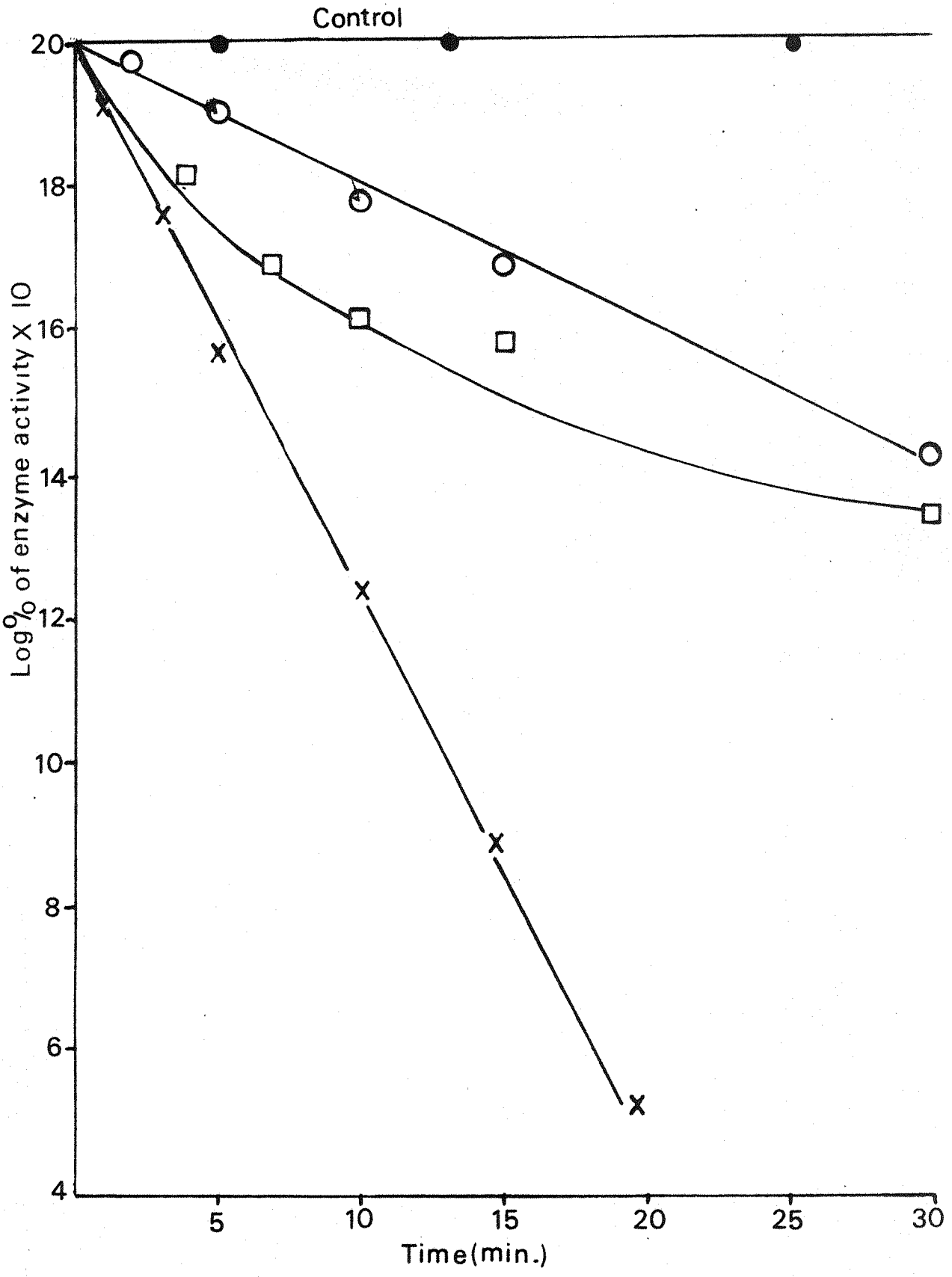

Control
20
18
16
14
12
10
8
6
4
Log10 of enzyme activity X10
5
10
15
20
25
30
Time (min)

Fig. 8 Protection of mitochondrial thiolase by acetyl CoA

This was performed as in Fig. 7 but the thiomethylation used 50μM-methyl methanethiosulphonate. The acetyl CoA concentrations were: (O) 50μM; (□)25μM; (X) none.


Control
20
18
16
14
12
10
8
6
4
Log% of enzyme activity X 10
5
10
15
20
25
30
Time(min.)

actually inhibitory.

For both the protection by acetoacetyl CoA or by acetyl CoA the result can be interpreted in terms of the reaction of the substrate with the enzyme to produce an acetyl-enzyme in which the enzyme in no longer able to react with methyl methanethiosulphonate. Since the catalytic mechanism of thiolase proceeds by a ping-pong mechanism in which CoASH binds to the acetyl enzyme it would not be required that CoASH binds to the free enzyme. Hence it might be anticipated that CoASH would provide poor protection in the modification reaction by methyl methanethiosulphonate. This was shown to be the case in Fig. 9 where the inclusion of CoASH (50μM; $2xK_{CoASH}$) resulted in a slight enhancement of the initial rate of inactivation and this rate was only protected once 45% of the enzyme was inactivated. We attribute this protection to formation of the mixed disulphide between CoASH and methyl methanethiosulphonate.

(4.6) Reactivation of thiomethylated thiolase

The reaction between methyl methanethiosulphonate and thiolase leads to the generation of a new disulphide bond within the protein. If advantage is to be taken of this protection, then it must be possible to cleave this bond to regenerate the enzyme fairly rapidly in good yield. Therefore, the effect of dithiothreitol and β-mercaptoethanol on the alkylated enzyme was studied. Dithiothreitol should regenerate active enzyme according to the following scheme:

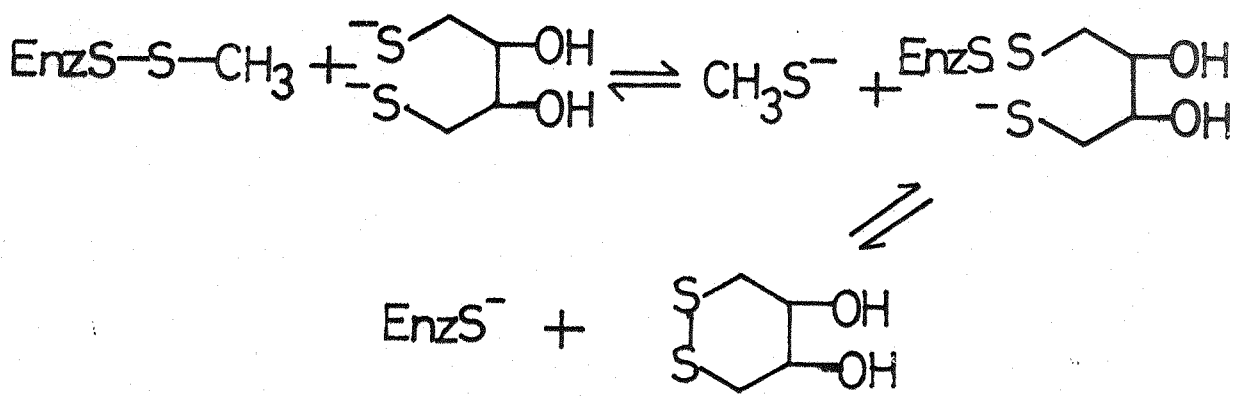
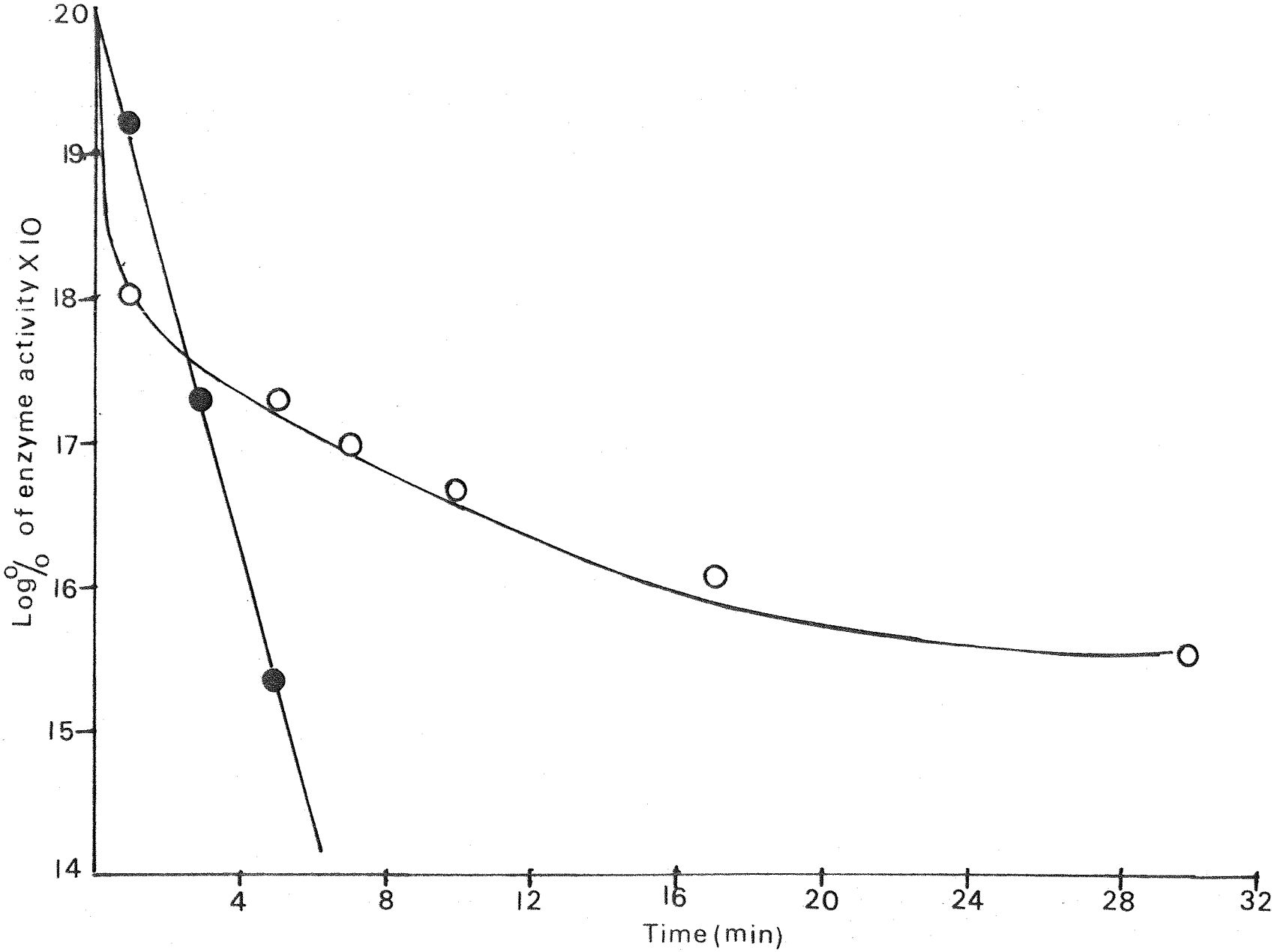


Fig. 9 Effect of CoASH on inactivation by methyl methanethiosulphonate

This was done as Fig. 8. The CoASH concentration added was: (o) 50μM.


20
19
18
17
16
15
14
Log% of enzyme activity X 10
4
8
12
16
20
24
28
32
Time (min)

For this experiment thiolase was initially inactivated completely by reaction with 50μM-methyl methanethiosulphonate for 25 min. The enzyme was then reacted with increasing concentrations of dithiothreitol and the recovery of enzyme activity was followed (Fig. 10) The time course for the reactivation was apparently biphasic and consisted of an initial rapid phase of reactivation in the first ten minutes followed by a much slower recovery of enzyme activity. The maximum recovery of enzyme activity was around 95% however 80% recovery was routinely obtained. The rate of reactivation was enhanced by increasing the concentration of dithiothreitol.

In addition to the experiments with dithiothreitol, β-mercaptoethanol was also found to reactivate the enzyme. The maximum reactivation with 150mM-, 20mM-, 10mM-, 5mM and 3.7mM was 95%, 66%, 46%, 34% and 10% respectively.

(4.7) Prevention of reactivation by acetyl CoA and acetoacetyl CoA

If thiomethylated-thiolase still contains an active site which resembles the configuration of the active site in the unmodified enzyme, then it might be expected to still bind substrates such as acetyl CoA or acetoacetyl CoA. Once the thiomethylated enzyme has bound the substrate then this could make the enzyme inacessible to reducing reagents and hence prevent reactivation of the enzyme. In order to examine this possibility thiolase was completely inactivated with methyl methanethiosulphonate and then the rate of reactivation was measured in the presence of a reducing agent (dithiothreitol or β-mercaptoethanol) either with or without the enzyme substrate. Fig. 11 shows that the inclusion of acetyl CoA in the reactivation mixture with dithiothreitol substantially decreased both the rate of reactivation and the extent of recovery of enzyme activity. A similar result with both acetyl CoA and acetoacetyl CoA was obtained when β-mercaptoethanol was used as the reducing agent (Fig. 12). This experiment appears to provide at least some evidence that although the thiomethylated enzyme is completely

Fig. 10 Time dependence of the reactivation of thiomethylated thiolase by dithiothreitol.

Thiolase (10μg/ml) was treated with 50μM methyl methanethiosulphonate as in Fig. 6 for 25 minutes, then assayed to ensure complete inactivation. The enzyme was then treated with dithiothreitol and the recovery of activity was followed at the indicated times. Additions of dithiothreitol were: (O) 50mM; (x) 10mM; (□) 5mM. The control line (●) shows the activity of enzyme which was not inactivated and treated with 50mM-dithiothreitol. Dithiothreitol had no effect on the activity of the control enzyme.

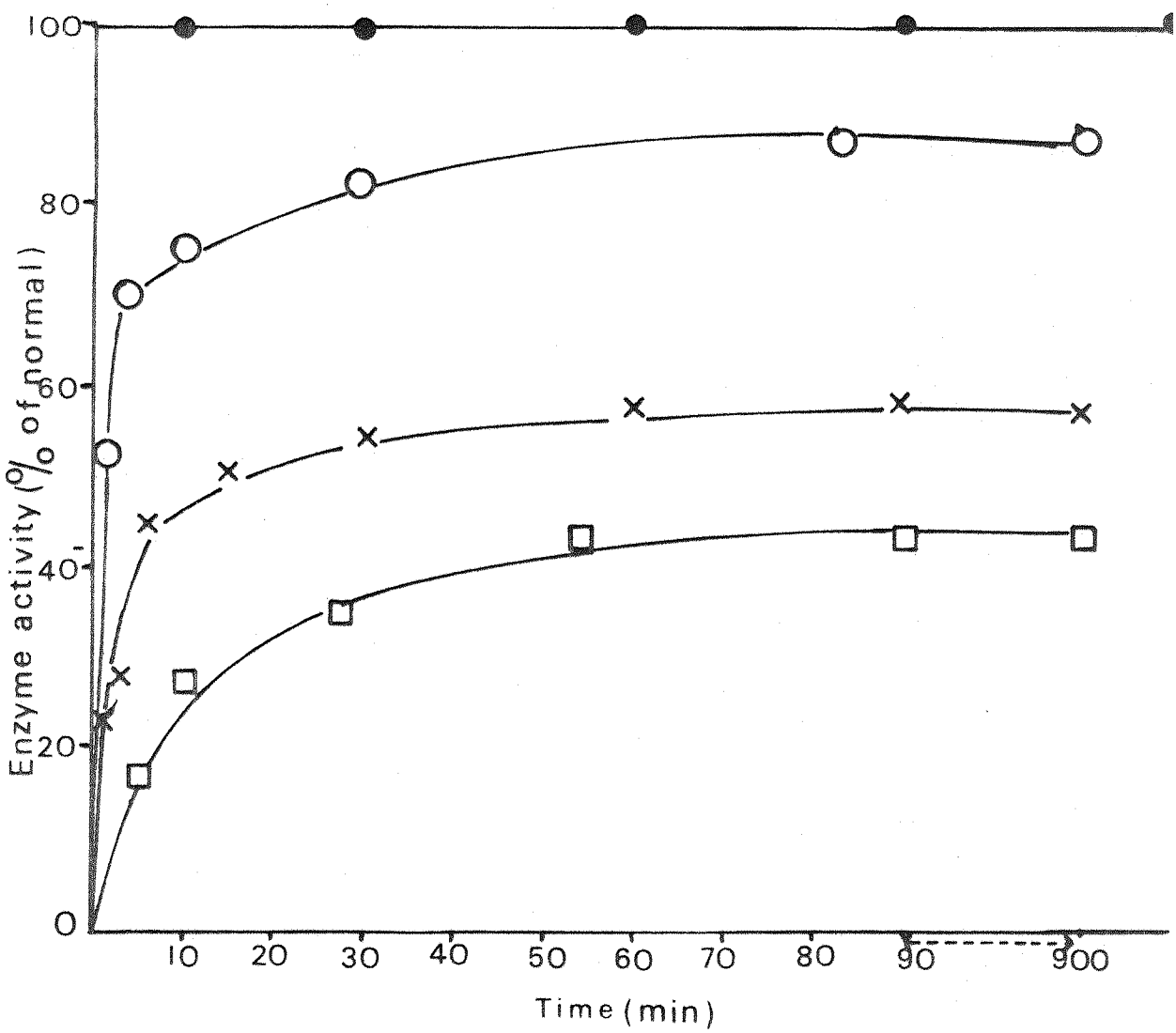

100
80
60
40
20
0
Enzyme activity (% of normal)
10
20
30
40
50
60
70
80
90
900
Time (min)

Fig. 11 Prevention of reactivation of thiomethylated-thiolase by acetyl CoA in the presence of dithiothreitol.

Thiolase (10µg/ml) was inactivated with 100µM-methyl methanethiosulphonate in potassium phosphate buffer, pH 8.2, for 25 minutes. Then it was treated with 50mM-dithiothreitol in the presence of 50µM-acetyl CoA, and the rate of reactivation was assayed at the indicated times. As a control, the rate of reactivation was assayed in absence of acetyl CoA. The additions were: (□) 50µM of acetyl CoA, 50mM-dithiothreitol; (O) 50mM-dithiothreitol.

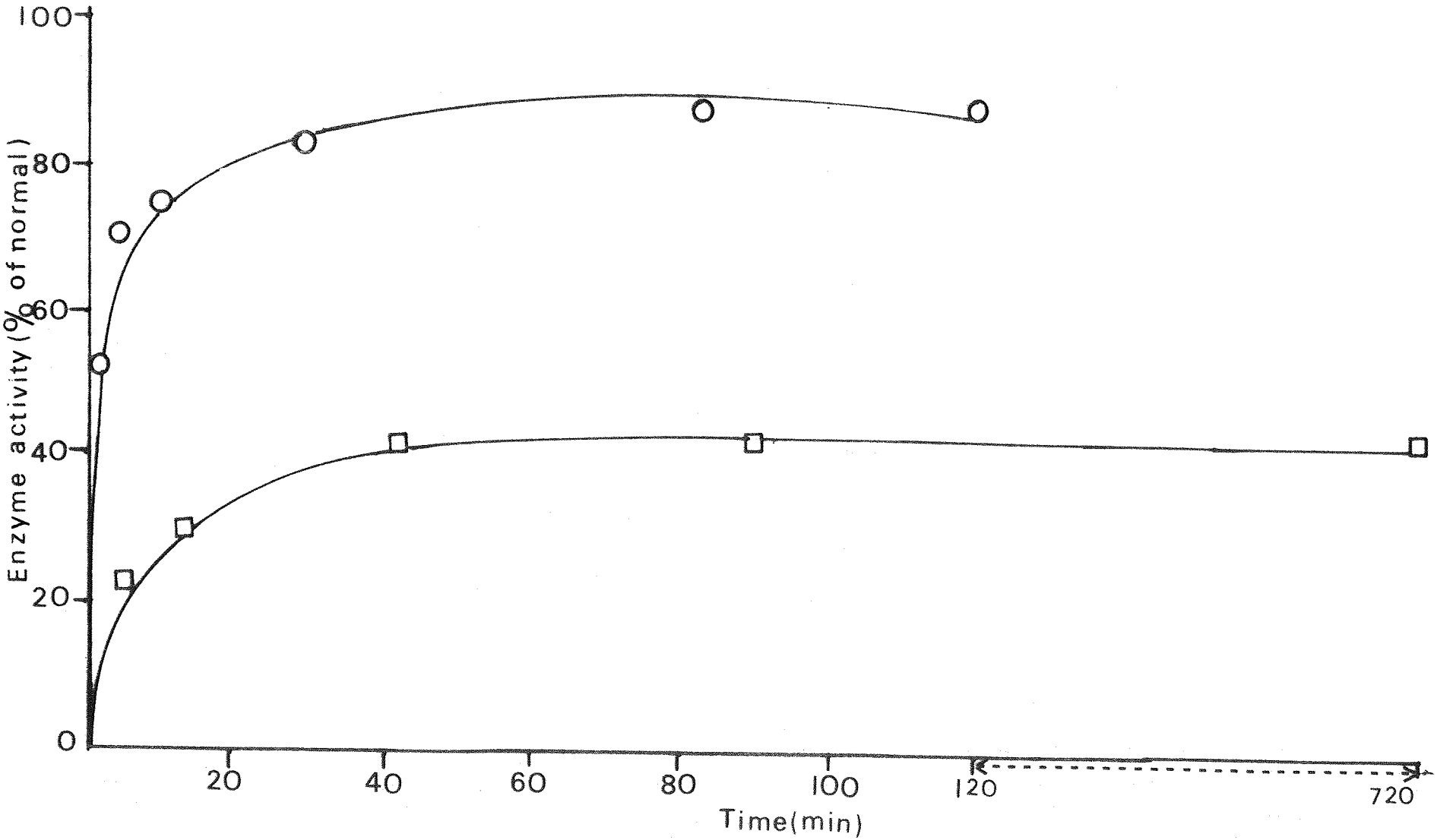
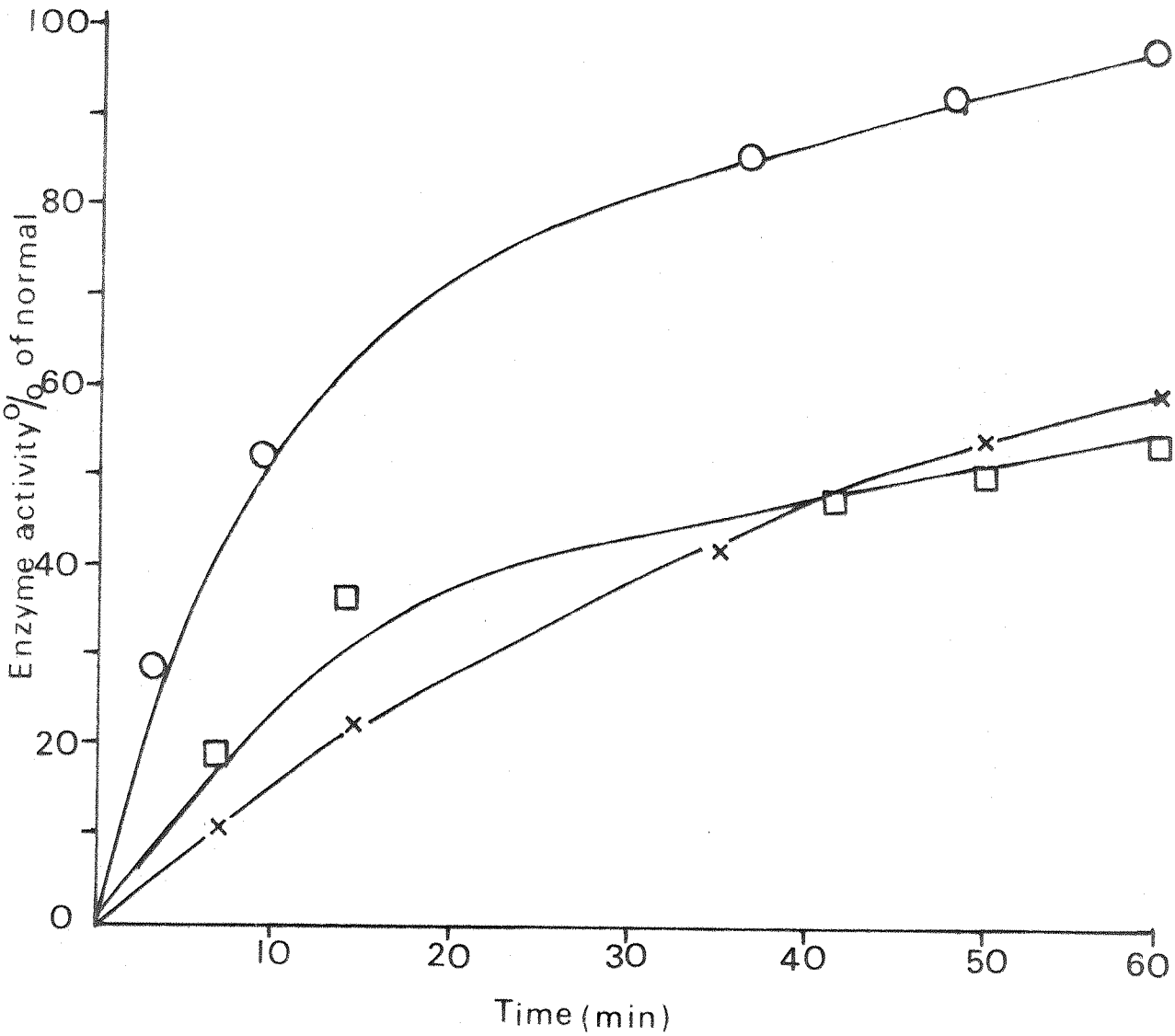

100
80
60
40
20
0
Enzyme activity (% of normal)
20
40
60
80
100
120
720
Time(min)

Fig. 12 Prevention of reactivation of thiomethylated thiolase by acetyl CoA and acetoacetyl CoA in presence of β-mercaptoethanol.

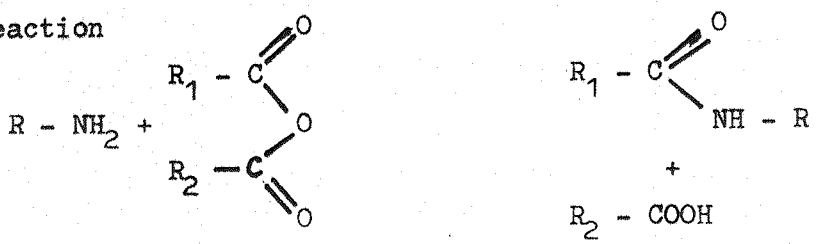
This was done as in Fig. 11, but two assay systems were prepared in which the rate of reactivation was prevented by acetyl CoA, or acetoacetyl CoA, along with a control reactivation with 150mM β-mercaptoethanol. The additions were: (O) 150mM β-mercaptoethanol; (□) 50μM-acetyl CoA, 150mM β-mercaptoethanol; (x) 50μM-acetoacetyl CoA, 150mM β-mercaptoethanol.


100
80
60
40
20
0
Enzyme activity % of normal
10
20
30
40
50
60
Time (min)

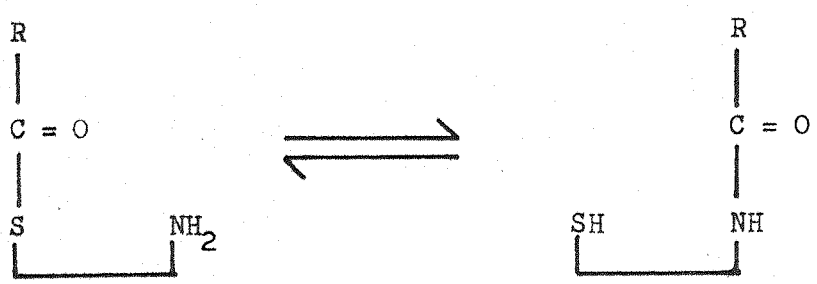
inactive yet it still possesses the ability to bind substrates. This is a very important consideration in view of the double modification experiments reported later since these are based on the assumption that insertion of the thiomethyl group into the enzyme active site does not grossly alter the arrangement of catalytically important groups.

(4.8) Inactivation of thiolase by anhydrides

In order to demonstrate the potential role of an amino group at the enzyme active site, the reaction of the enzyme with anhydrides was followed. Anhydrides could react with amino groups according to the general reaction

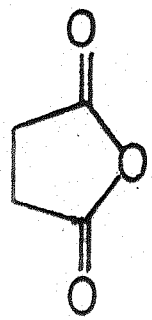


Although anhydrides may also react with thiols to generate an acyl-thioester, it should be noted that if an amino group is sufficiently close then an S- N acyl transfer will occur as follows

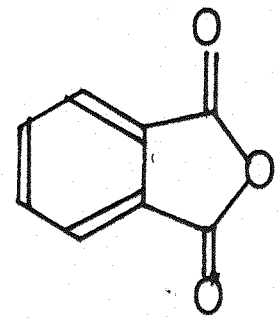


The equilibrium of this reaction markedly favours the amido-form (Martin et al, 1959; Barrett and Jencks, 1968). Therefore an amino group can still be modified even if the primary reaction is with a thiol group.

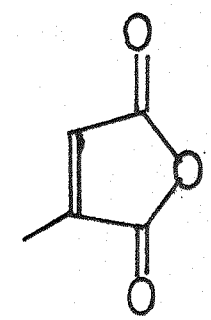
in these studies the actions of three anhydrides have been studied, namely succinic anhydride, phthalic anhydride and citraconic anhydride. Their structures are indicated below



succinic anhydride



phthalic anhydride



citraconic anhydride

The use of citraconic anhydride was introduced by Dixon and Perham (1968) since the amido group in unstable at acid pH and allows the modified enzyme to be unblocked fairly readily. In contrast the succinyl enzyme is quite stable at mildly acidic pH values.

For the experiments with anhydrides the reagent was dissolved in acetonitrile and added to the mixture so that the acetonitide concentration did not exceed 10% ($^{v}/_{v}$) in the reaction mixture. Fig. 13 shows that the presence of this concentration of acetonitrile had no effect on the enzyme activity over 50 min. incubation.

When succinic anhydride was included in the reaction mixture then the enzyme lost activity. Both the rate of loss of enzyme activity and the extent of inhibition were increased by raising the concentration of the inhibitor. The kinetics of inactivation did not follow the apparently simple pseudo first order inactivation process. Thus plots of $\log_{10}\%$ activity versus time were not linear (Fig. 14). Rather there was an initial rapid loss of activity followed by a much slower progress towards completion of the alkylation reaction. Childs and Bardsley (1975) have pointed out that the model originally proposed by Kitz and Wilson (1962)

Fig. 13 Effect of acetonitrile on the activity of mitochondrial thiolase at pH 8.2

Two incubations were performed in which thiolase (10µg/ml) was incubated in potassium phosphate buffer, pH 8.2, in the presence or absence ofacetonitrile(10% v/v) and the enzyme activity assayed as described in the method section.
(●) Free enzyme. (O) Enzyme in presence of acetonitrile

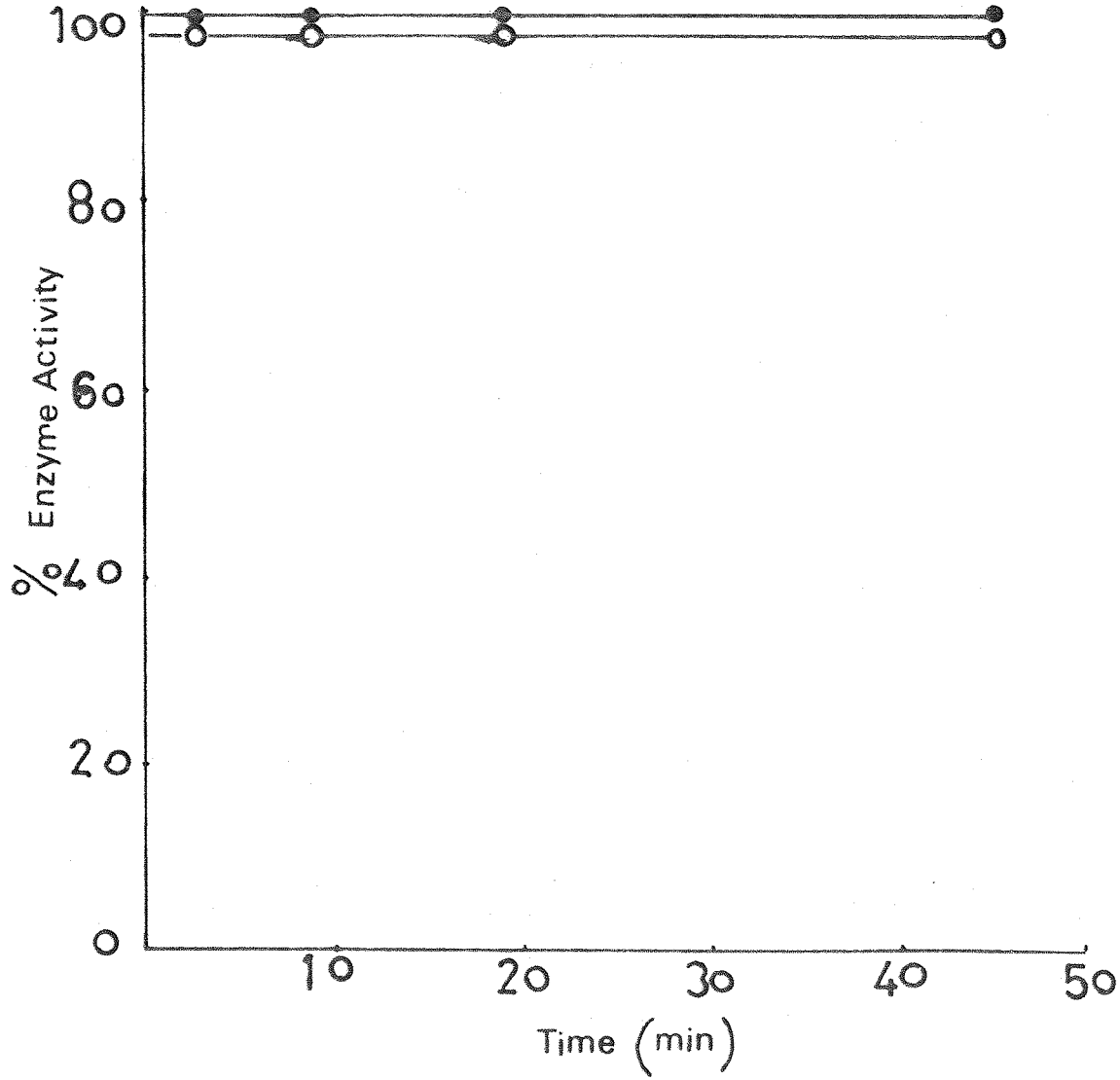
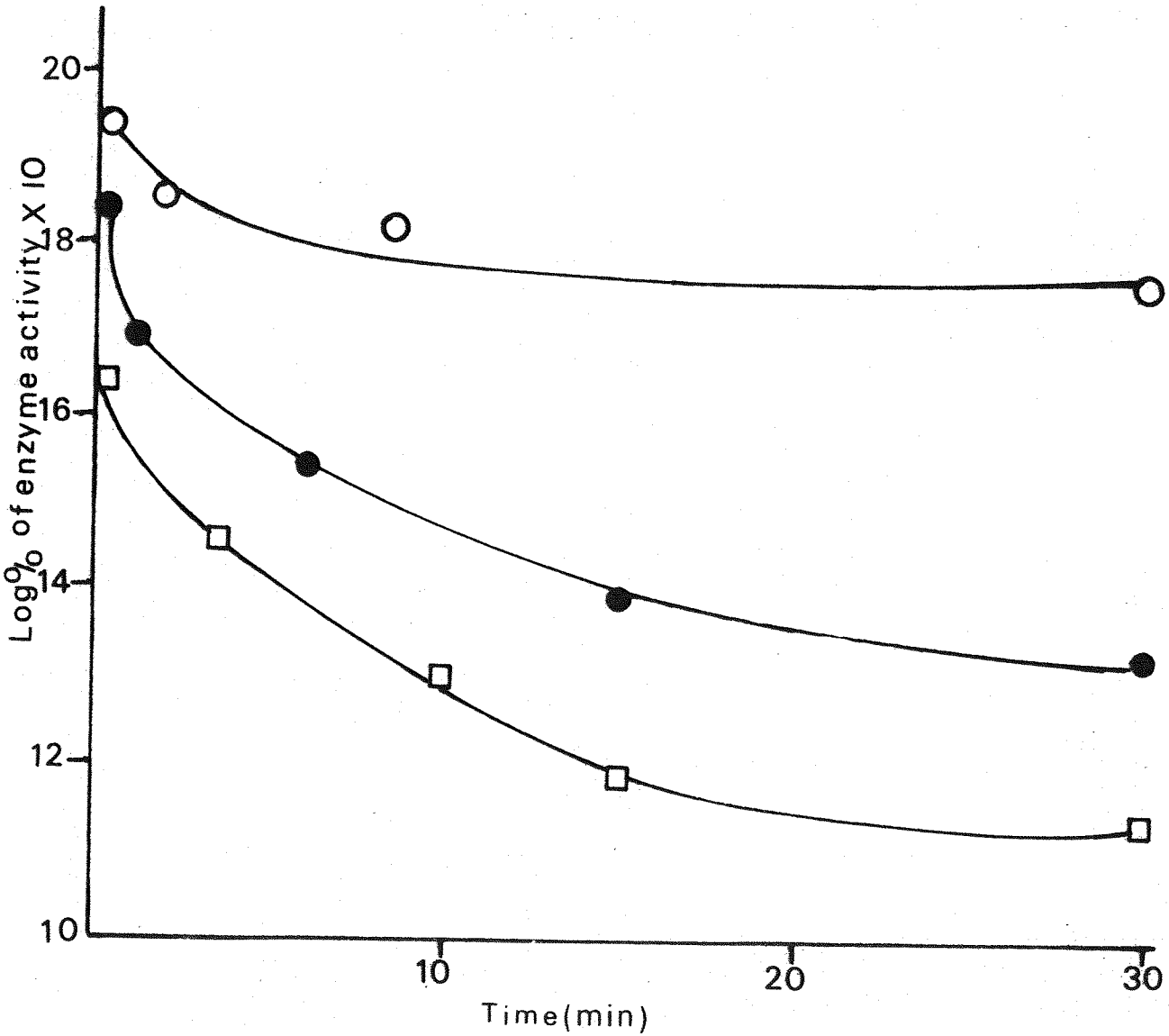

100
80
60
40
20
0
% Enzyme Activity
10
20
30
40
50
Time (min)

Fig. 14 Effect of succinic anhydride on the activity of thiolase

Thiolase (10µg/ml) was incubated at 0°C in potassium phosphate buffer, pH 8.2, and samples (20µl) were immediately assayed for enzyme activity at the indicated times after addition of the inhibitor. Succinic anhydride additions were; (□) 400µM; (●)200µM; (o) 50µM


20
18
16
14
12
10
Log% of enzyme activity X 10
10
20
30
Time(min)

may be deceptively simple. Rather they show that if the formation of the covalent complex was reversible (as the law of microscopic reversibility predicts it must be) then curves of the type shown in Fig. 14 will be obtained. Presumably this indicates that the amido bond between enzyme and succinyl groups must be moderately reversible.

The rate of reaction of thiolase with succinic anhydride was dependent upon the pH of the reaction mixture. As expected for a nucleophilic attack, the rate of inactivation increased as the pH of the mixture was raised (Fig. 15). The initial rapid reaction pattern was not changed by lowering the pH of the reaction medium. For the purposes of the latter experiments it was more convenient to use a pH near neutrality for the modification reaction. At lower pH values, it was possible to obtain increased inhibition of the enzyme simply by using higher concentrations of the inhibitor. This is shown in Fig. 16 using phthalic anhydride as an inhibitor at pH 6.5. This result also indicates that the pattern of the inhibition reaction was not influenced by the structure of the inhibitor (i.e. phthalic anhydride possesses an aromatic ring). A similar result was also obtained with citraconic anhydride in the concentration range 100μM to 1mM.

(4.9) Protection against inactivation of thiolase by succinic anhydride, phthalic anhydride and citraconic anhydride.

As discussed in an earlier section (4.5) a critical factor in demonstrating that an inhibitor modifies the active site region is to show that substrates prevent inactivation of the enzyme. Protection experiments were carried out for all of the anhydrides used in this work and these results are presented in this section. When acetoacetyl CoA was added to the reaction mixture the inactivation by succinic anhydride, citraconic anhydride and phthalic anhydride was markedly reduced. Fig. 17 shows a typical result using succinic anhydride as an inhibitor. At high concentrations of acetoacetyl CoA the enzyme was completely protected against inactivation. As the concentration of acetoacetyl

Fig. 15 pH dependence of the inactivation of thiolase by succinic anhydride.

Thiolase (10μg/ml) was incubated in 100mM-potassium phosphate solutions, pH as appropriate. 400μM succinic anydride was added and the enzyme activity was assayed at each pH used. The pH values used were:
(□) 8.2; (X) 7.55; (●) 7.0; (O) 6.45.

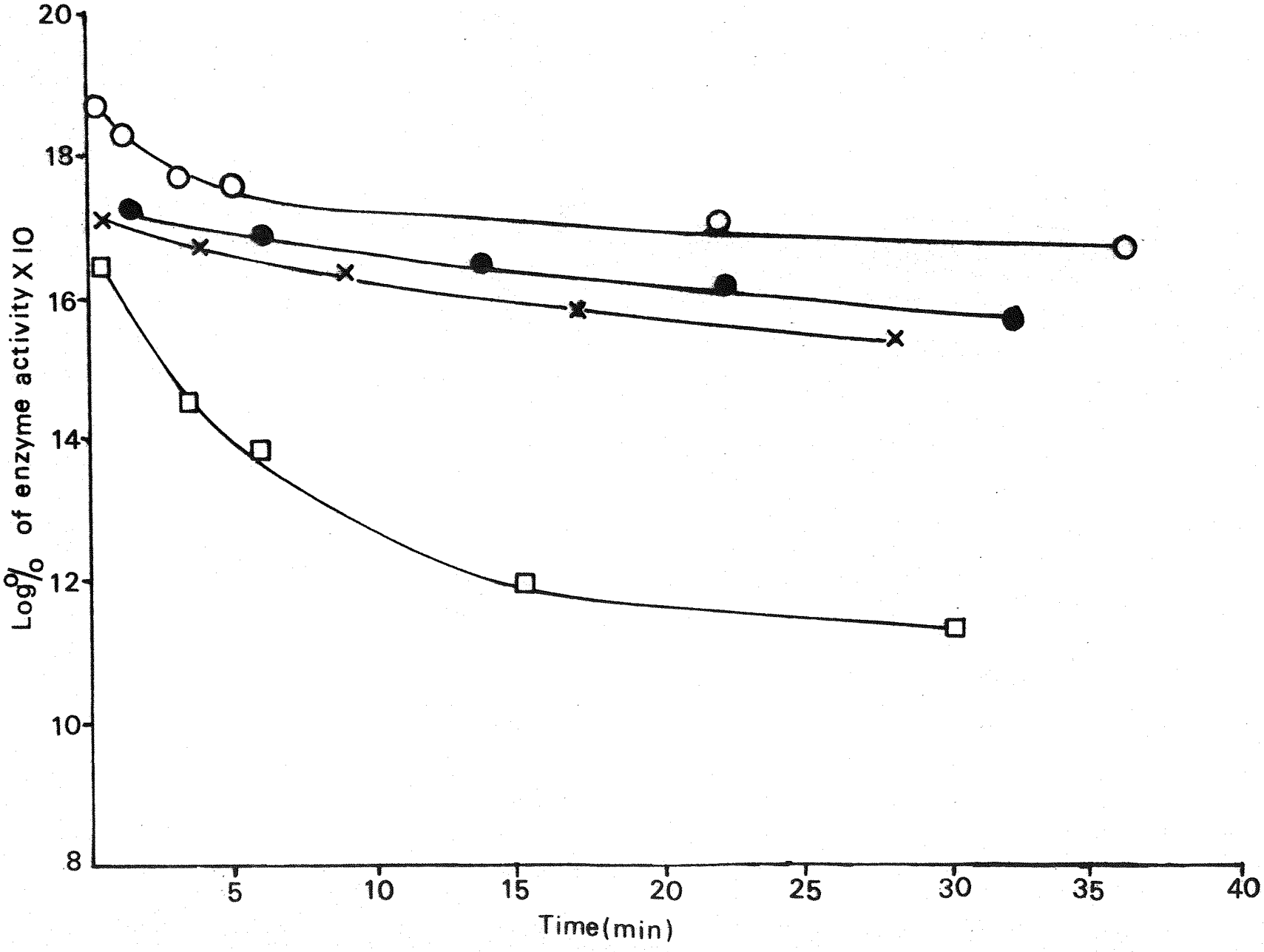

20
18
16
14
12
10
8
Log% of enzyme activity X 10
5
10
15
20
25
30
35
40
Time(min)

Fig. 16 Effect of phthalic anydride on the activity of thiolase.

Thiolase (10μg/ml) was treated at 0°C with phthalic anhydride in 0.1M potassium phosphate buffer, pH 6.5, and samples (20μl) were assayed for the loss of enzyme activity to determine the time-dependent inactivation of the enzyme. Phthalic anydride additions were:
(□) 2000μM; (●) 400μM; (○) 50μM.

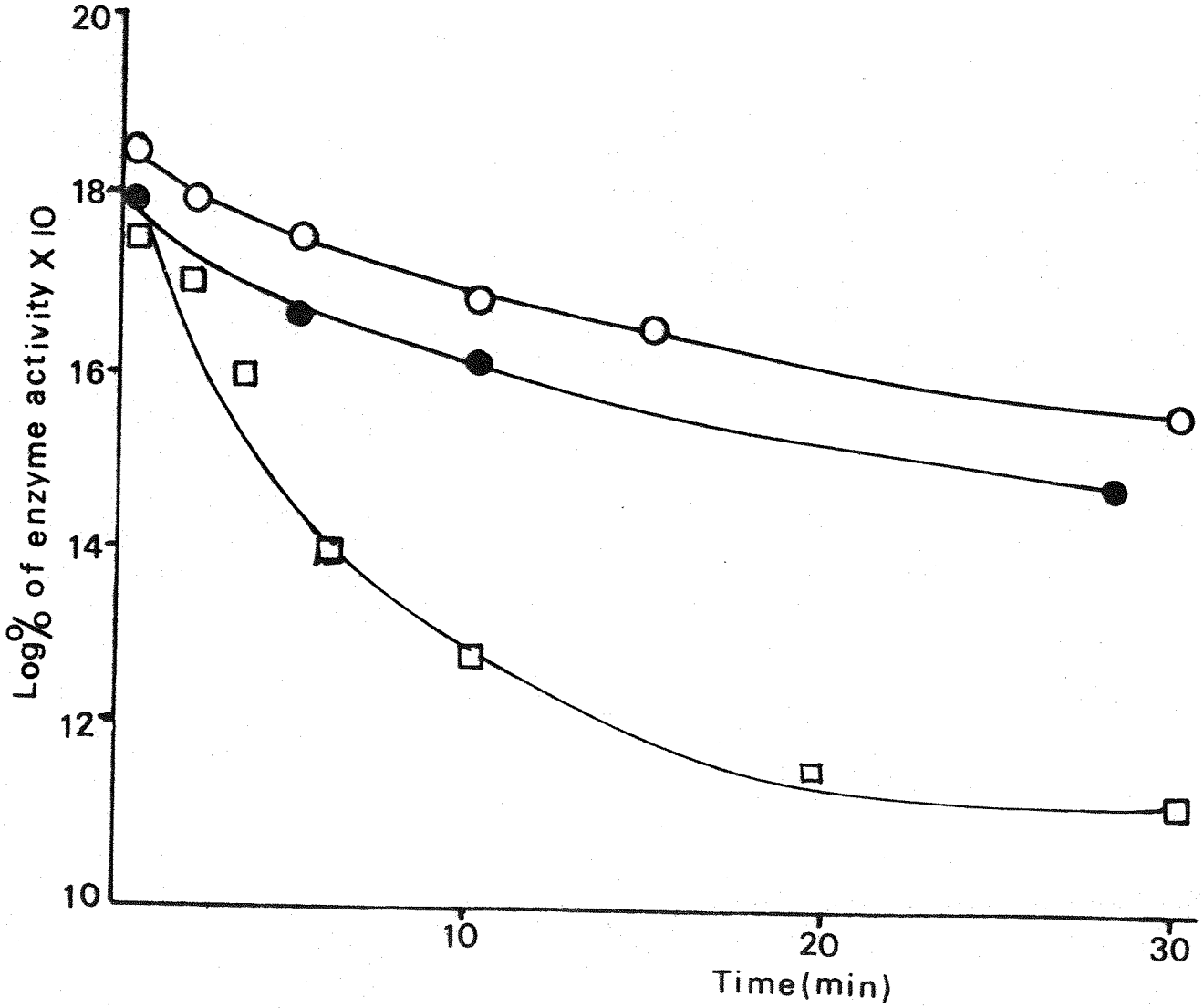
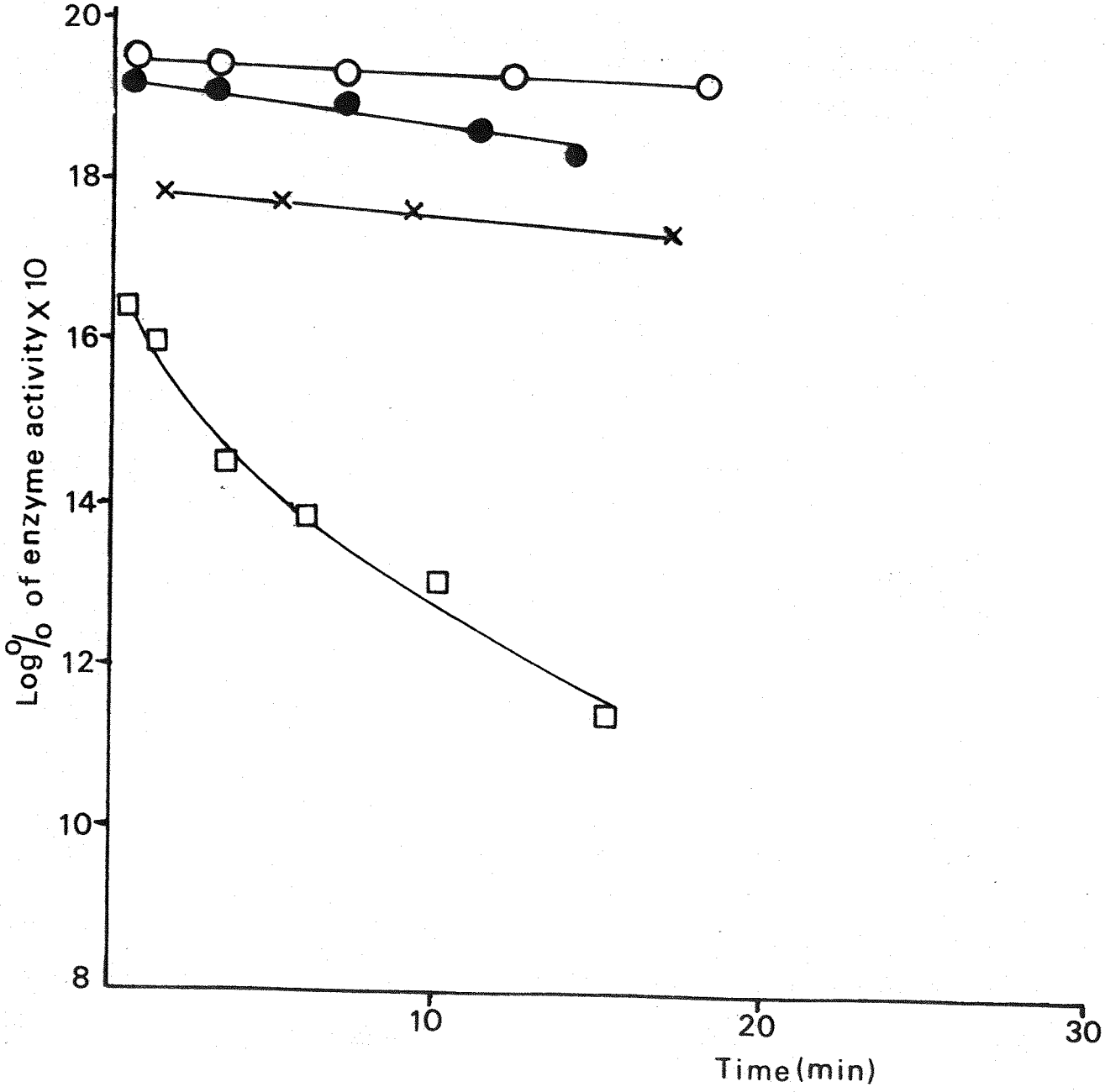

20
18
16
14
12
10
Log% of enzyme activity X 10
10
20
30
Time(min)

Fig. 17 Protection of thiolase by acetoacetyl CoA against the inactivation by 400μM succinic anhydride

Thiolase (10μg/ml) was inactivated at 0°C by 400μM succinic anhydride in 100mM potassium phosphate buffer, pH 8.2 The protection by acetoacetyl CoA was investigated by performing similar incubations but in the presence of the substrate before addition of they anhydride. Acetoacetyl CoA additions were:
(O) 56μM; (●)28μM; (X) 14μM; (□)0μM.


20
18
16
14
12
10
8
Log% of enzyme activity X 10
10
20
30
Time (min)

CoA was increased the pattern of inhibition remained roughly constant since there was still a rapid initial phase of inhibition followed by a much slower inactivation.

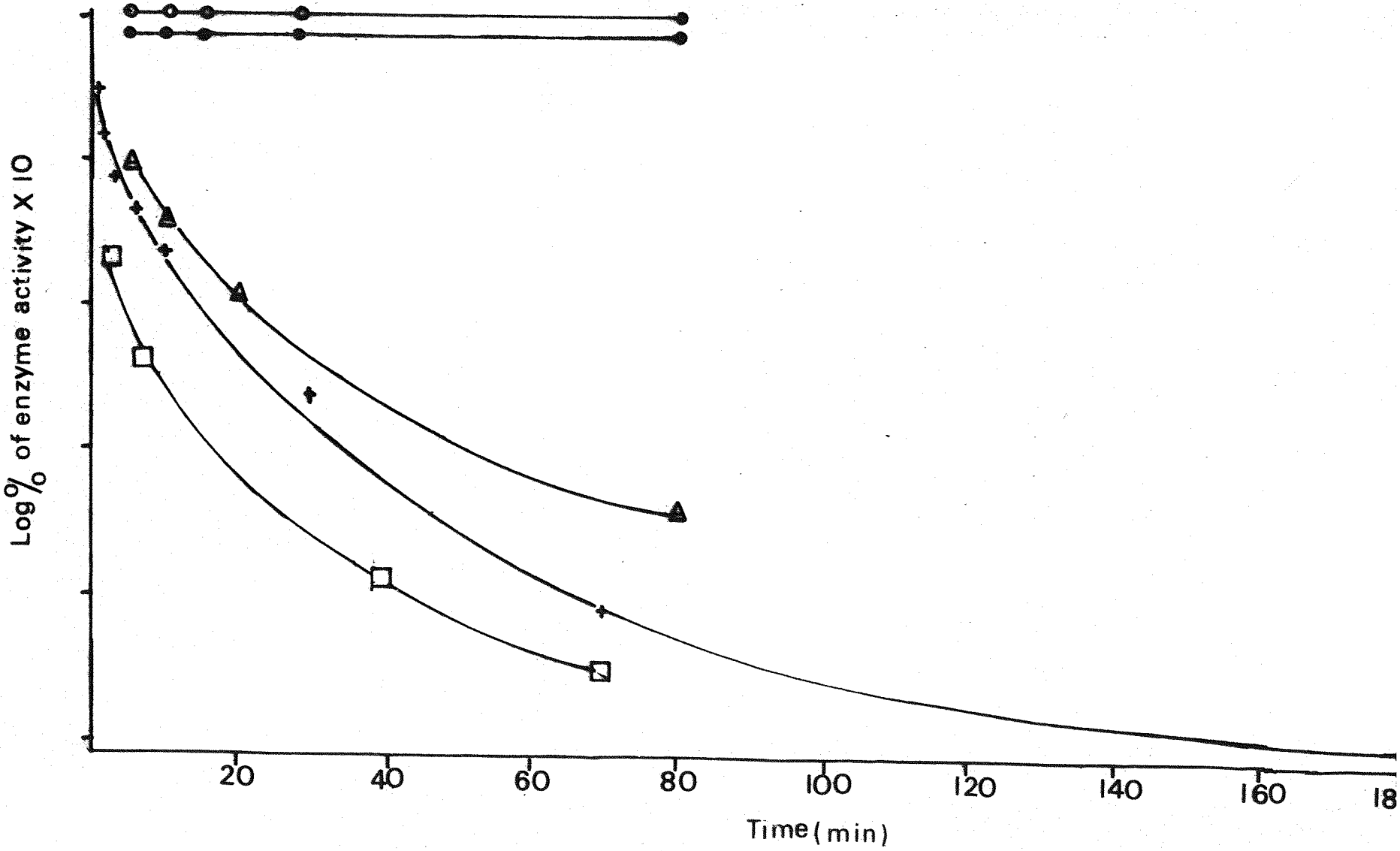
In contrast to the efficient protection against inactivation by acetoacetyl CoA, increasing the concentration of either acetyl CoA or CoASH appeared to have little effect on the inactivation of thiolase by anhydrides. This is shown in Fig. 18 for the inactivation of thiolase by phthalic anhydride. The result indicates that when 30μM-acetoacetyl CoA completely prevents inhibition by phthalic anhydride then neither acetyl CoA (50μM) nor CoASH provide significant protection against inactivation. In fact CoASH slightly enhances the rate of inactivation. This protection pattern clearly distinguishes the mechanism of inhibition of the anhydrides and methyl methanethiosulphonate. This is consistent with the suggestion that they may modify different functional groups on the protein. A similar difference has been noted previously by Holland et al (1973) who showed that whereas bromoacetyl CoA and bromocrotonyl CoA inactivation was protected by acetoacetyl CoA and acetyl CoA the alkylation by the suicide inhibitors, alk-3-ynoyl CoA esters, was only protected by acetoacetyl CoA and acetyl CoA was ineffective.

(4.10) Double modification of pig heart mitochondrial thiolase

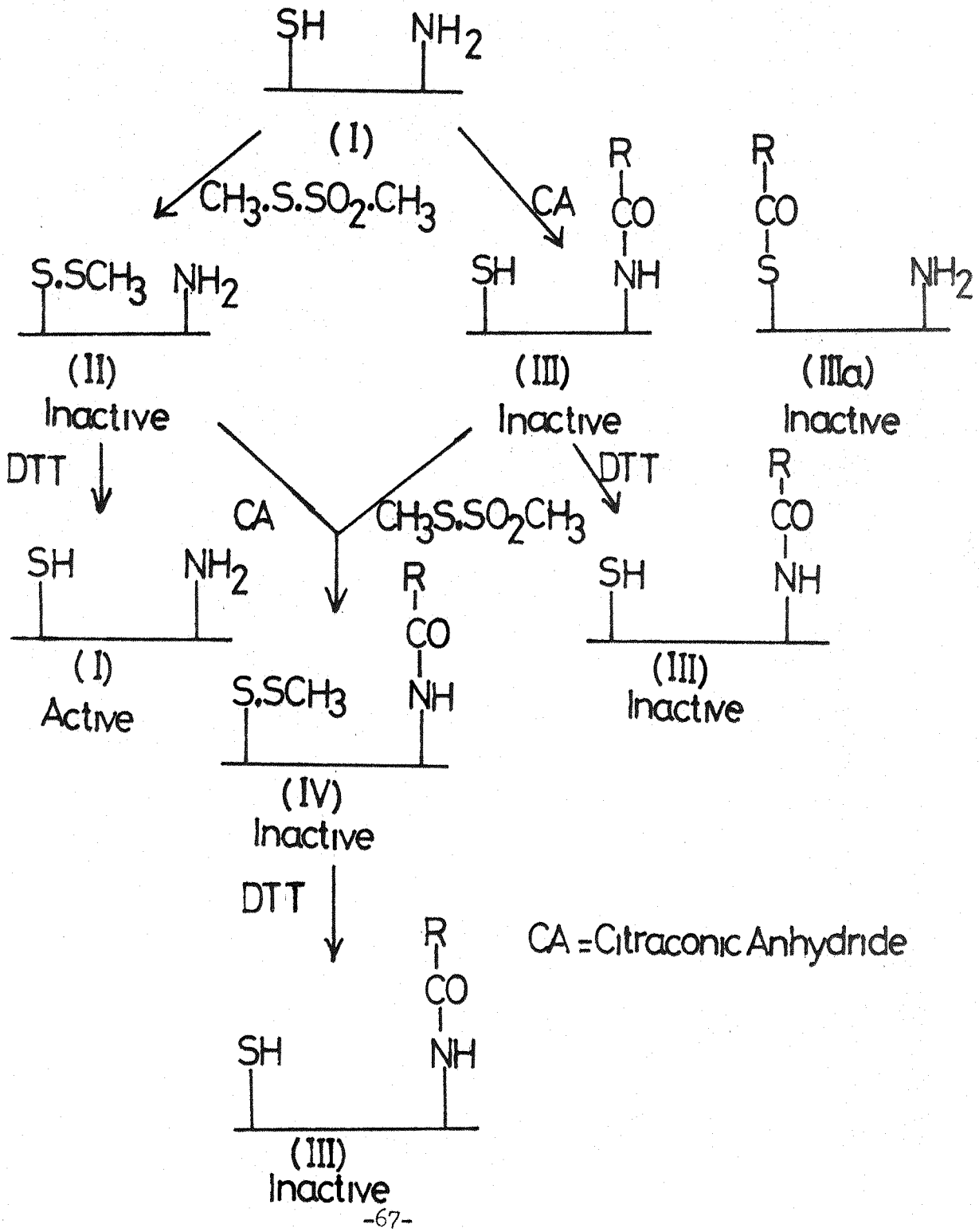
The preceding sections have described the reaction of thiolase with either methyl methanethiosulphonate or anhydrides individually. In this experiment we describe experiments involving double modification of the protein. The first experiment was designed to show that citraconic anhydride modifies a group which can be distinguished from the active site thiol group. In order to appreciate the design of this experiment the experiment is presented as a flow sheet below. In this

Fig. 18 Protection of thiolase by different substrates against inactivation by 500μM phthalic anhydride

Thiolase (10μg/ml) was inactivated at 0°C with 500μM phthalic anhydride in 100mM potassium phosphate buffer, pH 8.2 to ensure maximum inactivation. Similar incubations were done in the presence of different substrates before addition of inhibitor and the enzyme activity assayed at different times. Substrate additions were: (□) 75μM CoA; (▲)50 μM acetyl CoA; (●)30μM acetoacetyl CoA; (+) 0μM substrate.


Log% of enzyme activity X 10
20
40
60
80
100
120
140
160
18
Time (min)

scheme the enzyme is represented as containing a separate thiol and amino group at the active site



Double modification of the enzyme can be achieved by two routes. Either the enzyme can be reacted with methyl methanethiosulphonate to produce the thiomethyl enzyme (II) followed by reaction with citraconic anhydride to form thiomethyl-citraconyl-thiolase (IV). Alternatively, the reactions can be reversed. However, the citraconyl enzyme (III) is of limited value since clearly both the thiol and amino groups could have been modified to yield (IIIa).

For this experiment, thiolase (100µg) was reacted with 100µM-methylmethanethiosulphonate for 25 min in 1ml of 50mM-potassium phosphate, pH 7. The enzyme was then dialysed against 1 litre of 50mM-potassium phosphate, pH 7. The enzyme was now divided into two and one half was reacted with 500µM-citraconic anhydride for 50 min to produce the doubly modified enzyme. In a control experiment it was shown that a single modification with citraconic anhydride under these conditions resulted in 95% inhibition of the enzyme. The doubly modified (IV) and thiomethylated enzyme (II) were then reduced with 50mM-dithiothreitol (Fig.19). It is clear that whereas the singly modified enzyme was reactivated the doubly modified enzyme failed to regain activity. This result can only be obtained if there are two distinct groups involved in catalysis at the active site. Note that if only a single group was involved then the double modification of the thiomethylated enzyme would be inconsequential since the cysteine sulphydryl would be protected. The positive result shown in Fig. 19 seems conclusive evidence for the role of an amino group at the enzyme active site. A distinct problem with the use of anhydride modifications is that the ε-amino groups modified are polar groups and located at the surface of the protein. This means that citraconylation may result in multiple modification of the protein which could lead to substantial conformational changes in the protein i.e. dissociation into sub-units. This would mean that the apparent role of an amino group could be due solely to a non-specific action of the anhydrides rather than a specific

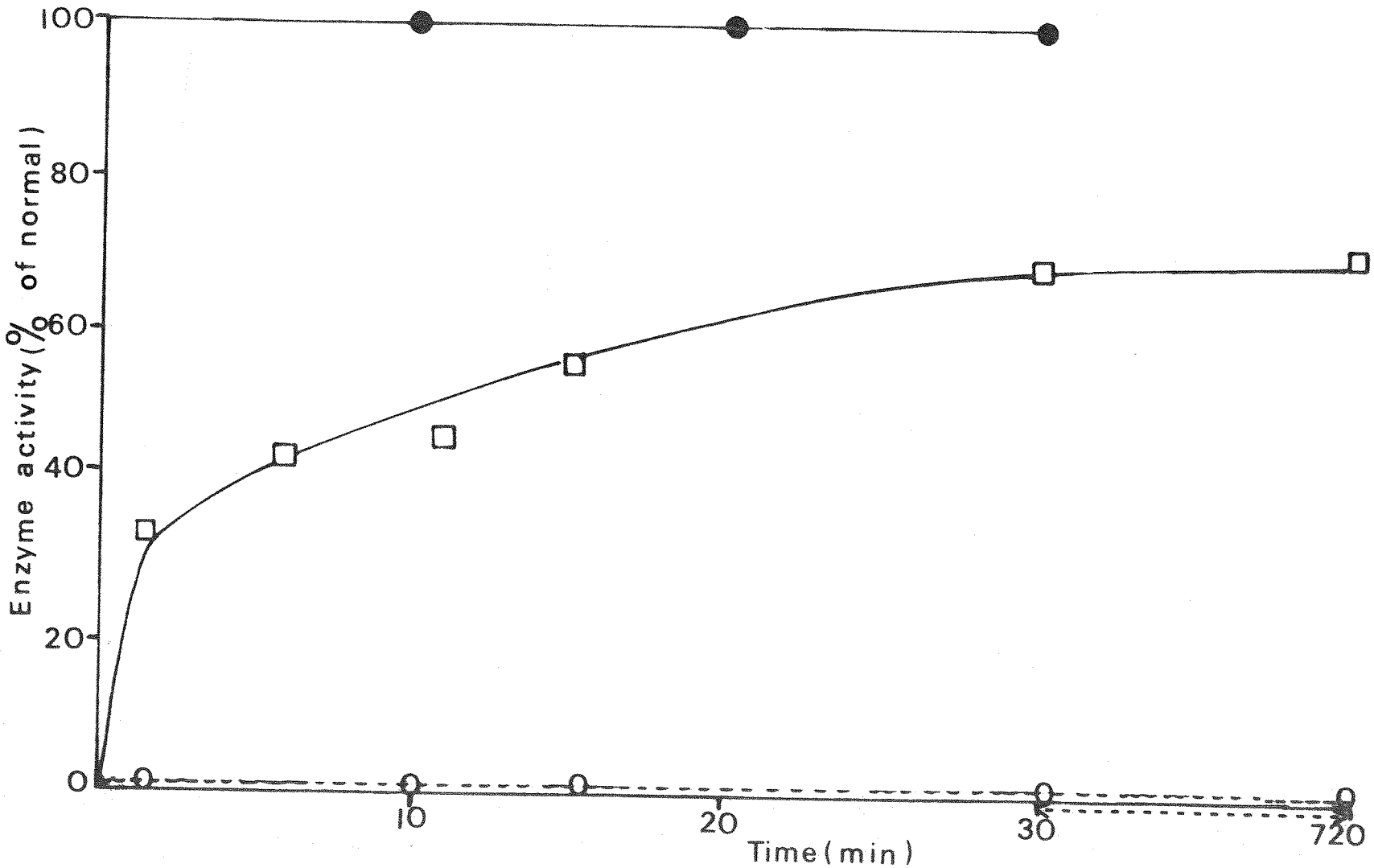
Fig. 19 Effect of dithiothreitol on different forms of covalently modified thiolase

(a) Thiomethylated enzyme.

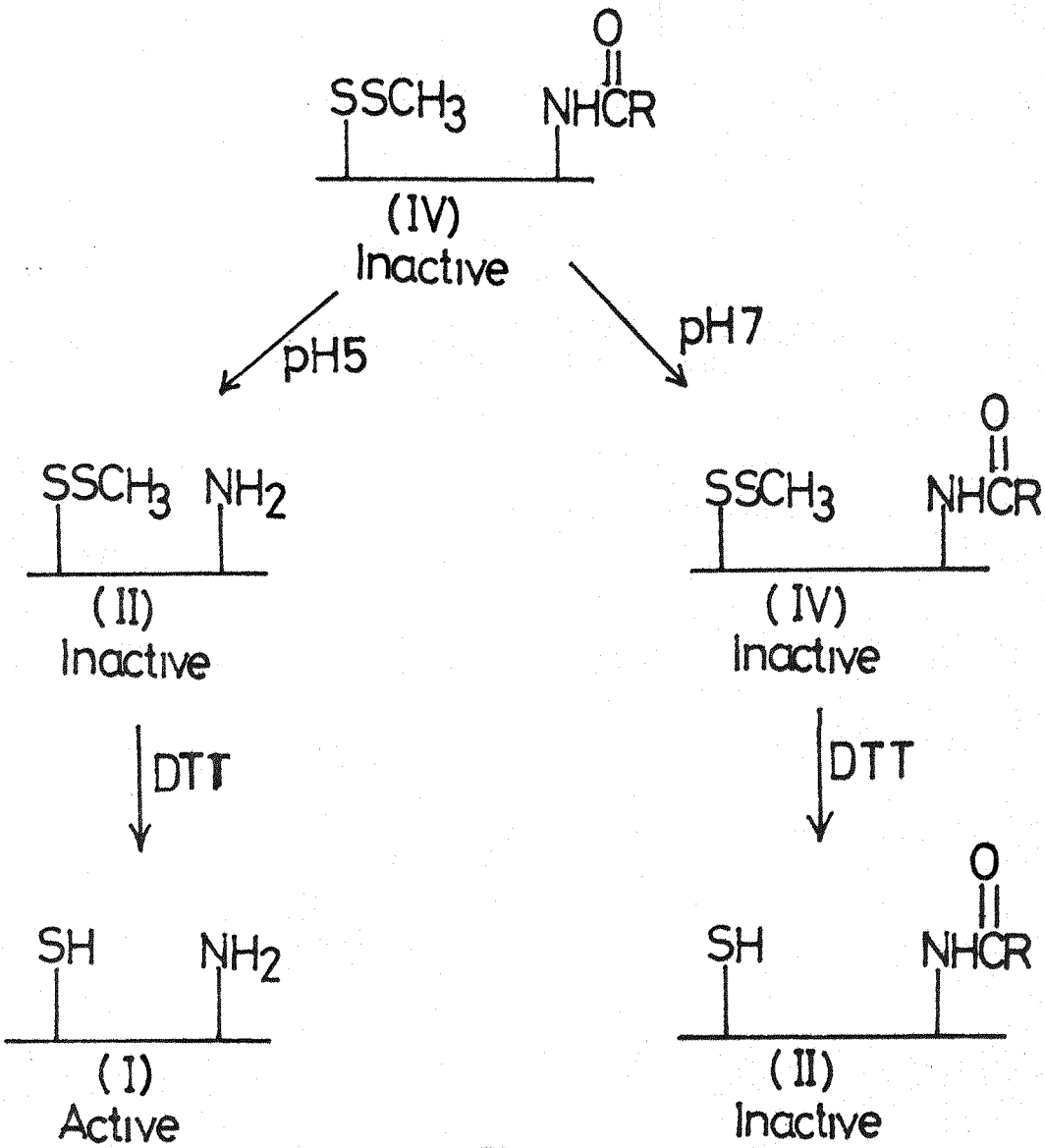
Mitochondrial thiolase (100µg/ml) in 50mM potassium phosphate, pH 8.2, was inactivated at 0°C with 100µM methyl methanethiosulphonate in water for 25 min until 95% inactivation was observed. The thiomethylated enzyme was dialysed for 5 hours against 50mM-potassium phosphate buffer, pH 7, to eliminate any unreacted methyl methanethiosulphonate, and the dialysed fraction was reassayed. The inactivation remained complete. 50mM dithiothreitol was added and the enzyme activity (20µl) was immediately assayed according to the standard assay in the method section.

(b) Thiomethylated and citraconylated enzyme

Another sample was modified in a similar fashion to the fraction (a) but the enzyme was modified by 500µM citraconic anhydride for 50 minutes (85% inactivation) after it had been inactivated with the 100µM methyl methanethiosulphonate. This was dialysed and assayed as before.
(●) control enzyme; (□) thiomethylated enzyme; (○) thiomethylated citraconyl enzyme.


100
80
60
40
20
0
Enzyme activity (% of normal)
10
20
30
720
Time (min)

modification of an essential amino group. In a final experiment on this point we decided to take advantage of the fact that the citraconyl group can be removed at acidic pH's, (Gibbons and Perham , 1974). The philosophy behind this experiment was that a specific modification would be reversible whereas non-specific dissociation would not be reversible. The protocol for the experiment is illustrated in the flow diagram below

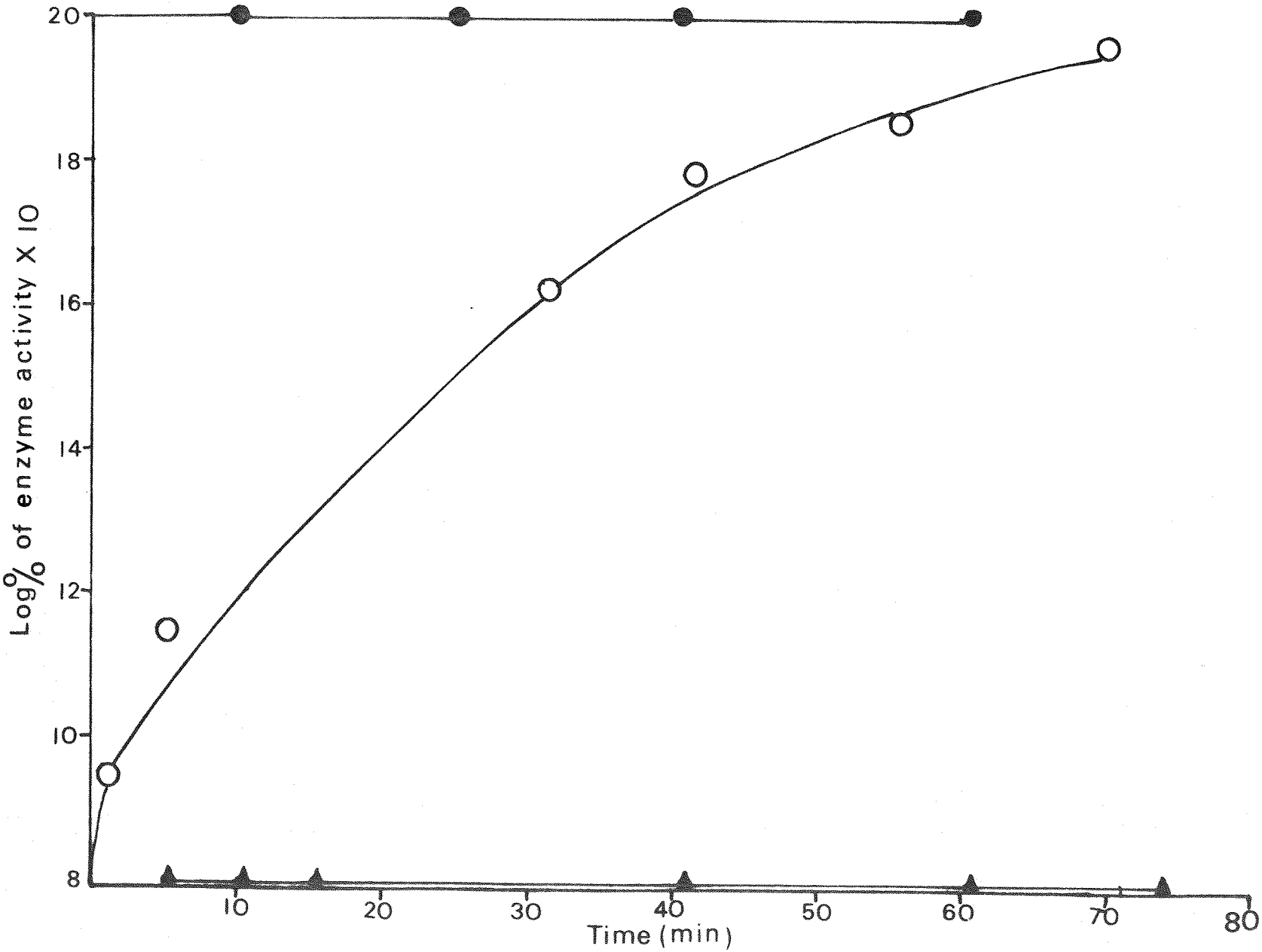


For this experiment the doubly modified enzyme was prepared as in Fig. 19. The enzyme was then divided into two portions, one of which was dialysed against 50mM potassium phosphate, pH 5.0, and the other was dialysed against 50mM potassium phosphate, pH 7.0. Normally pH 3.5 is used to remove the citraconyl group however the previous studies had already shown that pH 5.0 was the lowest pH the enzyme would tolerate. Following dialysis, both samples were adjusted to pH 8.2 and reduced with 50mM-dithiothreitol (Fig. 20). The result shows that the acid treated enzyme regenerated activity on reduction whereas the enzyme treated at neutrality showed no recovery of enzyme activity. This provides a final piece of evidence that citraconylation results in the specific and reversible modification of an amino group at the enzyme active site.

Fig. 20 Reactivation of thiomethyl-citraconyl-thiolase

Double modified enzyme was prepared as in Fig. 19. This was divided into two fractions, one was dialysed against 50mM-potassium phosphate buffer, pH 5.0 for 5 hours and the other against 50mM-potassium phosphate, pH 7.0 for the same period of time. The inactivation for both samples was insured by assaying after dialysis, and then 50mM dithiothreitol was added to each, and both were assayed for enzyme activity at the indicated times.

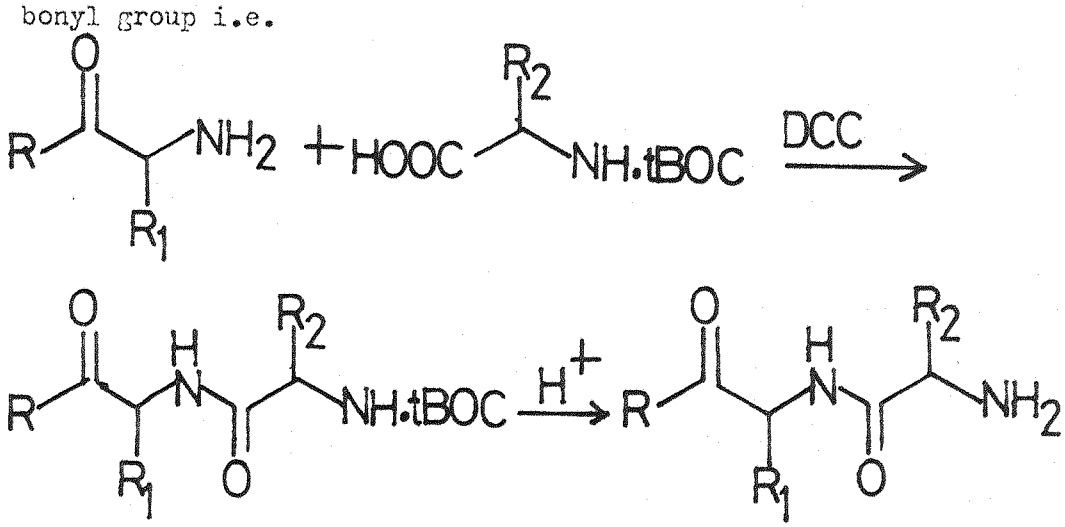
(●) Control enzyme dialysed at pH 5.0; (O) double modified enzyme, pH 5.0; (▲) double modified enzyme, pH 7.0.


20
18
16
14
12
10
8
Log% of enzyme activity X 10
10
20
30
40
50
60
70
80
Time (min)

CHAPTER 5

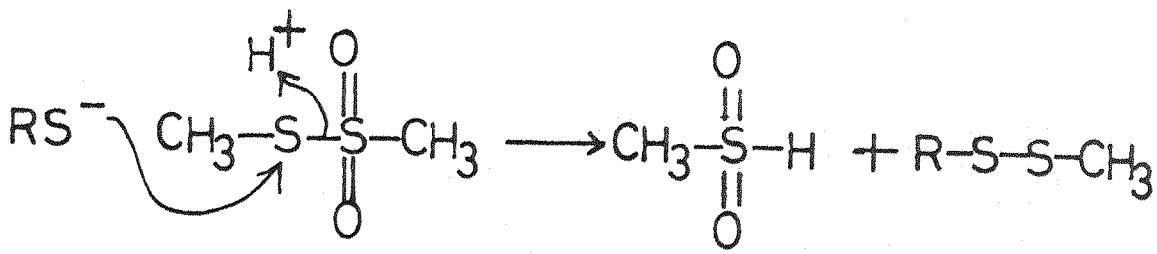
Discussion

The vital role of a cysteine in the catalytic mechanism of thiolase has been clearly demonstrated by the elegant studies of Lynen and his colleagues (Lynen, 1953; Gehring et al, 1968). The thiol group at the active site of thiolase appears to be highly reactive. This was shown by Holland et al (1973) who demonstrated that both 2-bromoacetyl-CoA and 4-bromocrotonyl-CoA reacted preferentially with thiolase rather than an externally added thiol such as dithiothreitol. A similar result may be deduced from the present work where the addition of a large molar excess of CoASH over the thiolase concentration did not protect the enzyme against the initial rate of inactivation by methyl methanethiosulphonate. Although the highly reactive thiol of thiolase makes this group susceptible to modification (Bloxham, 1975) it raises a problem in trying to identify subsidiary groups at the active site. However for a solution to these problems we can revert to one of the classic concepts of synthetic organic chemistry. Thus for synthetic reactions involving multifunctional compounds it has long been recognised that synthetic modifications at a single point required the ability to reversibly protect selective groups. As an example in the Merrifield solid phase synthetic system for complex peptides requires the use of the reversible modification of amino groups by the t-butoxycarbonyl group i.e.

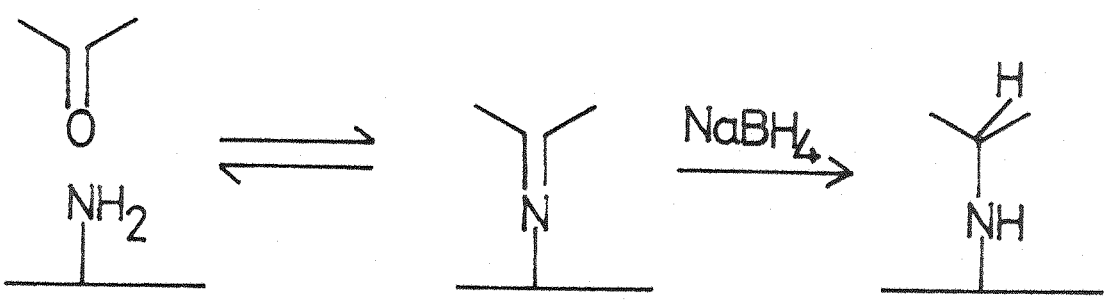


This type of approach is exactly the route we have attempted to use in demonstrating the role of an amino group at the active site of thiolase.

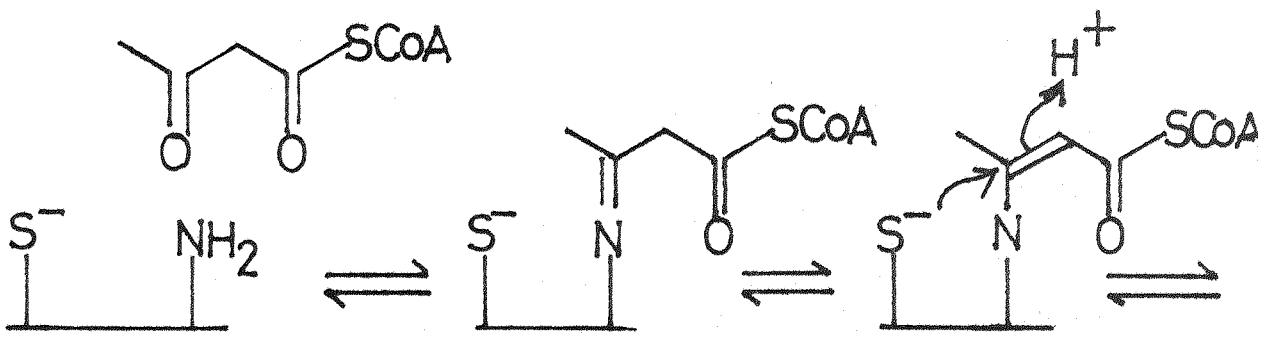
In this work methyl methanethiosulphonate has been used to reversibly modify a thiol. The presence of the thiosulphonate group should be advantageous in enhancing nucleophilic attack by a thiol group



A possible role for anamino group at the active site of thiolase was initially suggested by Kornblatt and Rudney (1971) who showed that the yeast enzyme was inativated in the presence of $NaBH_4$ and acetoacetyl CoA suggesting the formation of a secondary amine at the active site



These observations were subsequently confirmed by Holland et al (1973) who found that the mitochondrial enzyme was also inactivated in the presence of $NaBH_4$ and acetoacetyl CoA. This prompted them to suggest that the initial steps in the catalytic mechanism might proceed as follows



The mechanism involves a Schiffs base (step 1) to account for inactivation in the presence of $NaBH_4$ and was subsequently followed by formation of the enamine (step 2). This mechanism obligatorily requires the loss of a proton from C-2 in acetoacetyl CoA. However this route was recently eliminated by Willadsen and Eggerer (1975a,b) who showed that both protons from C-2 were incorporated intact into acetyl CoA. Therefore it seemed appropriate to try to identify a role for an amino group by using an alternative procedure.

The principle evidence that an amino-group could be located at the enzyme active site is as follows

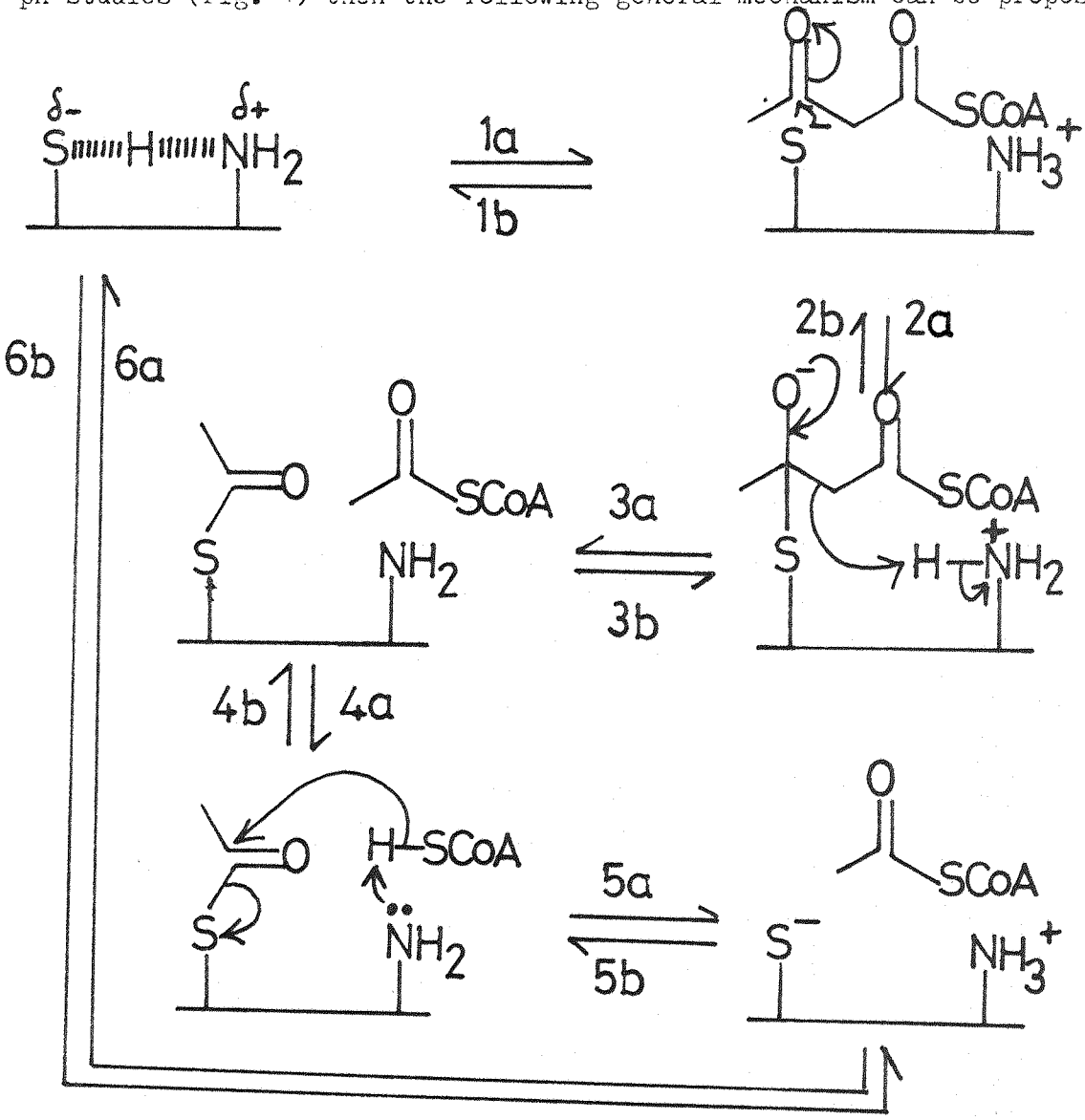
a) The enzyme is inactivated by reaction with anhydrides which are capable of modifying an amino group.

b) The amino group could be located at the active site since inactivation is prevented by acetoacetyl-CoA but not by acetyl CoA

c) The anhydrides still inactivate thiolase even when the active site cysteine is blocked as the thiomethyl group.

d) The thiomethyl-citraconyl-thiolase may be reactivated by sequential treatment at pH 5.0 followed by reduction at pH 7.0.

Assuming that an amino group could be involved in catalysis and that it's catalytically active form might be the NH_3^+ form based on the pH studies (Fig. 4) then the following general mechanism can be proposed.



In this mechanism the proposed roles for the amino functional group are as follows in the forward direction (a)

1. NH_3^+ functions as a proton donor to neutralise the carbanion on the leaving acetyl CoA group (3a).

2. NH_2 functions as a base to abstract a proton from CoASH to facilitate nucleophilic attack (5a).

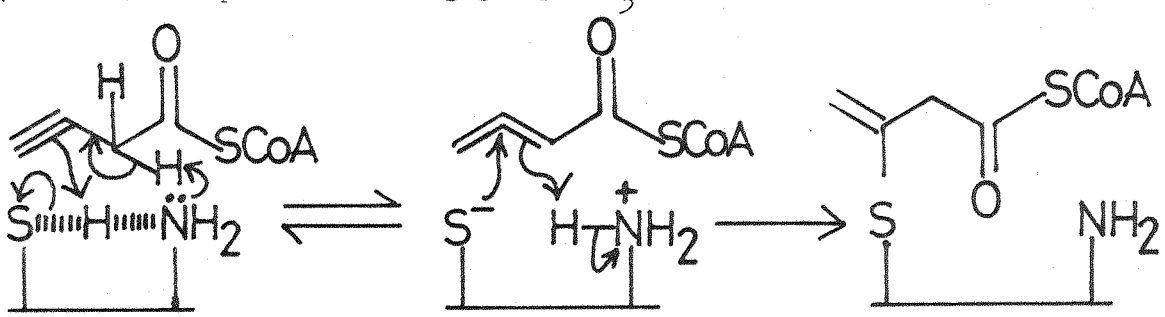
In the reverse direction (b)

3. NH_3^+ functions as a proton donor for the leaving $CoAS^-$ species (5b).

4. NH_2 functions as a base to abstract protons from the methyl group of acetyl CoA improving its nucleophilic character.

In the mechanism proposed here the amino group is essentially functioning either as a general base (NH_2 form) or general acid catalyst (NH_3^+ form). CoASH has been written in the protonated form for binding to the enzyme since we have shown that its affinity for the enzyme is increased at lower pH values. Furthermore, the inactivation of the enzyme in the presence of $NaBH_4$ and a carbonyl compound is probably a fortuitous observation which is not directly connected to the enzyme's reaction pathway.

The mechanism that I have proposed could help towards understanding two features of the thiolase catalytic process. Firstly, the 'hyperactivity' of the active site thiol could be rationalised by the close proximity of a proton withdrawing group (the NH_2; reaction 1a). Secondly, thiolase is highly susceptible to inactivation by alk-3-ynoyl CoA esters (Holland et al, 1973; Bloxham, 1975) which appear to inhibit the enzyme via a 'suicide inhibitor' type mechanism (Morisaki and Bloch 1972). This reaction requires the participation of both a nucleophile (i.e. S^-) and proton donating group (NH_3^+) and may proceed as follows



Although the proposal of a role for an amino group functioning as either a general acid or general base is quite attractive, it must be realised that there are alternative possibilities. Of these a plausible role is that the protonated amino may be required to neutralise one of the negative charges on the phosphate residues of the CoASH derivatives Examination of the binding of nucleotides to enzyme active sites by X-ray crystallography, usually reveals that this type of interaction takes place. Furthermore, the observation that acetoacetyl CoA prevents anhydride inactivation whereas acetyl CoA does not, suggests that there could be a role in binding selected substrates. However an identical result was found for alk-3-ynoyl CoA ester inactivation of the enzyme (Holland et al, 1973) which indicates that this protection result may have mechanistic implications.

In conclusion, this work has demonstrated the potential role of an amino group at the active site of thiolase which may participate in the catalytic mechanism. This has required the successful application of reversible protection of the active site thiol group. The story is far from complete. Clearly further work could proceed in a number of directions. These could include purifying the enzyme on a much larger scale and trying to identify the residues which are modified by the anhydrides. Furthermore the ability to demonstrate a base catalysed deprotonation of the mthyl group of acetyl-CoA would be consistent with the proposed mechanism.

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