14.2.79

PYRUVATE CARBOXYLASE:
THE MODE OF ACTION OF ACETYL CoA

A THESIS SUBMITTED BY

PETER ROY CLEMENTS, B.Sc. (HONS.) (ADELAIDE, 1973)

TO THE UNIVERSITY OF ADELAIDE

SOUTH AUSTRALIA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Department of Biochemistry
University of Adelaide
South Australia

December, 1977

Awarded July [illegible]

TABLE OF CONTENTS

PAGE NO.

TABLE OF CONTENTS i

SUMMARY vi

STATEMENT xi

ACKNOWLEDGEMENTS xii

ABBREVIATIONS xiii

CHAPTER 1 : GENERAL INTRODUCTION

1.1 PYRUVATE CARBOXYLASE AND ITS ACTIVATION BY ACETYL CoA 1

1.1.1 THE REACTION CATALYSED BY PYRUVATE CARBOXYLASE 1

1.1.2 THE EFFECT OF ACETYL CoA ON THE BIOTIN CARBOXYLATION STEP 2

1.1.3 THE EFFECT OF ACETYL CoA ON THE TRANS-CARBOXYLATION STEP 3

1.1.4 ENZYME CONCENTRATION DEPENDENCE AND THE ACETYL CoA-INDEPENDENT ACTIVITY 4

1.1.5 THE EFFECT OF ACETYL CoA ON THE INACTIVATION OF PYRUVATE CARBOXYLASE BY AVIDIN 5

1.1.6 THE EFFECT OF ACETYL CoA ON ACTIVATION BY MONOVALENT CATIONS 6

1.1.7 THE PYRUVATE CARBOXYLASE-DEPENDENT DEACYLATION OF ACETYL CoA 7

1.1.8 BINDING STUDIES OF ACETYL CoA TO PYRUVATE CARBOXYLASE 7

1.1.9 RECENT DEVELOPMENT OF AN ALTERNATIVE EXPLANATION FOR THE SIGMOID ACETYL CoA RESPONSE 8

1.1.10 THE EFFECT OF ACETYL CoA ON THE CATALYTIC VELOCITY 11

1.2 STRUCTURAL FEATURES OF ACETYL CoA AND SITE TOLERANCE 12

1.2.1 THE ACETYL CoA MOLECULE 12

1.2.2 STRUCTURE/ACTIVITY RELATIONSHIPS OF ACETYL CoA 13
1.2.3 THE CONFORMATION OF THE ACETYL CoA MOLECULE 14
1.3 APPROACHES TO THE STUDY OF THE MECHANISM OF ACTION OF ACETYL CoA 15
1.3.1 CHEMICAL MODIFICATION 15
1.3.2 AFFINITY LABELLING 16
1.3.3 PROTEIN SEQUENCING 18
1.3.4 ANALOGUE STUDIES 20
1.3.5 PHYSICAL TECHNIQUES 20
1.3.6 THE APPROACH ADOPTED 22

CHAPTER 2 : MATERIALS AND METHODS

2.1 MATERIALS 24
2.1.1 ENZYMES AND PROTEINS 24
2.1.2 RADIOACTIVE MATERIALS 24
2.1.3 GENERAL CHEMICALS 24
2.2 METHODS 25
2.2.1 PREPARATION AND PURIFICATION OF NUCLEOTIDES 25
2.2.2 PREPARATION AND PURIFICATION OF D-PANTETHINE DERIVATIVES 27
2.2.3 PURIFICATION OF PYRUVATE CARBOXYLASE 27
2.2.4 DETERMINATION OF RADIOACTIVITY 29
2.2.5 MEASUREMENT OF PYRUVATE CARBOXYLASE ACTIVITY 30
2.2.6 DETERMINATION OF BIOTIN CONCENTRATION 35

CHAPTER 3 : AFFINITY LABELLING

3.1 INTRODUCTION 37
3.1.1 OBJECTIVES IN THE DESIGN OF AN AFFINITY LABEL FOR THE ACETYL CoA BINDING SITE OF PYRUVATE CARBOXYLASE 37
3.1.2 THE APPROACH 38

3.1.3 SOME KINETIC CONSIDERATIONS 40

3.2 METHODS 41

3.2.1 THIN LAYER CHROMATOGRAPHY 41

3.2.2 DETECTION OF ALKYLATING ACTIVITY 41

3.3 THE ACETYL GROUP-DERIVED LABELS 42

3.3.1 2-BROMOACETYL-SCoA 42

3.1.1.1 INTRODUCTION 42

3.1.1.2 METHODS 44

3.3.1.3 RESULTS AND DISCUSSION 46

3.3.1.4 THE PROPERTIES OF 2-BROMOACETYL-SCoA 50

3.3.2 HALOETHYL CoA DERIVATIVES 51

3.3.2.1 INTRODUCTION 51

3.3.2.2 METHODS 52

3.3.2.3 THE EFFECT OF ETHYL-SCoA ON THE ACTIVITY OF PYRUVATE CARBOXYLASE 55

3.3.2.4 COVALENT MODIFICATION STUDIES WITH 2-IODO-ETHYL-SCoA 55

3.3.2.5 "AFFINITY INACTIVATION" STUDIES USING 2-IODO-ETHYL CYSTEINE 59

3.3.2.6 DISCUSSION 60

3.3.3 3-CHLOROACETONYL-SCoA 62

3.3.3.1 INTRODUCTION 62

3.3.3.2 METHODS 63

3.3.3.3 RESULTS 65

3.3.3.4 DISCUSSION 69

3.4 THE RIBOSE-3′-PHOSPHATE-DERIVED LABELS 70

3.4.1 ACETYL CoA DIAL 70

3.4.1.1 INTRODUCTION 70

3.4.1.2 METHODS 72

3.4.1.3 THE EFFECT OF ACETYLDEPHOSPHO CoA 73

3.4.1.4 COVALENT MODIFICATION STUDIES WITH ACETYL CoA DIAL 74

3.4.1.5 DISCUSSION 75

3.5 THE 6-AMINO GROUP-RELATED AFFINITY LABELS 77

3.6 FURTHER DISCUSSION 79

3.6.1 THE ACTIVE SITE OF PYRUVATE CARBOXYLASE 79

3.6.2 FURTHER DISCUSSION OF LABELS 80

CHAPTER 4 : ANALOGUES OF ACETYL CoA

4.1 STUDIES WITH ANALOGUES OF ACETYL CoA 82

4.1.1 INTRODUCTION 82

4.1.2 THE ROLE OF ISOTOPIC EXCHANGE REACTIONS IN THE STUDY OF PYRUVATE CARBOXYLASE 83

4.1.3 ANALOGUES OF ACETYL CoA 84

4.2 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE EXCHANGE, OVERALL AND ACETYL CoA-INDEPENDENT ACTIVITIES OF PYRUVATE CARBOXYLASE 85

4.2.1 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE ATP:ORTHOPHOSPHATE EXCHANGE REACTION

4.2.2 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE PYRUVATE:OXALOACETATE EXCHANGE 88

4.2.3 THE EFFECT OF ACETYL CoA ANALOGUES ON THE OVERALL REACTION CATALYSED BY PYRUVATE CARBOXYLASE 92

4.2.4 THE EFFECT OF THE ANALOGUES OF ACETYL CoA ON THE ENZYME ASSAYED UNDER ACETYL CoA - INDEPENDENT CONDITIONS 94

4.3 DISCUSSION 96

4.3.1 CONCLUSIONS AND HYPOTHESES 96

4.3.2 POINTS ARISING FROM THE RESULTS 97

CHAPTER 5 : DILUTION INACTIVATION

5.1 STUDIES ON THE PROTECTION AFFORDED BY ACETYL CoA AGAINST INACTIVATION OF PYRUVATE CARBOXYLASE ON DILUTION 101

5.2 METHODS 102

5.2.1 DILUTION INACTIVATION EXPERIMENTS 102

5.2.2 CIRCULAR DICHROISM SPECTRA 103

5.3 THE PHENOMENON OF INACTIVATION OF THE ENZYME ON DILUTION 103

5.3.1 THE EFFECT OF ACETYL CoA ON ENZYME ACTIVITY AFTER INACTIVATION BY DILUTION 104

5.3.2 THE EFFECT OF BUFFER IONS AND pH 104

5.3.3 THE EFFECT OF TEMPERATURE 105

5.3.4 THE EFFECT OF ACETYL CoA IN THE PRESENCE AND ABSENCE OF Mg^{++} 106

5.3.5 THE EFFECT OF APOLAR SOLUTES 107

5.3.6 THE EFFECT OF IONIC SOLUTES 108

5.3.7 THE EFFECT OF D_2O 108

5.3.8 THE EFFECT OF SOLUTES ON THE K_a, V and η_H VALUES FOR ACETYL CoA ACTIVATION OF THE ENZYME 109

5.3.9 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE RATE OF INACTIVATION AFTER DILUTION 110

5.3.10 THE POLYPEPTIDE BACKBONE STRUCTURE OF PYRUVATE CARBOXYLASE AND THE EFFECT OF SOLUTES WHICH INFLUENCE THE RATE OF INACTIVATION UPON DILUTION 111

5.3.11 THE EFFECT OF INACTIVATION UPON DILUTION ON THE ACTIVITY OF THE FIRST AND SECOND PARTIAL REACTIONS 115

5.3.12 THE EFFECT OF PYRUVATE CONCENTRATION ON THE OVERALL ENZYME ACTIVITY IN THE PRESENCE OF SOLUTES WHICH PREVENT INACTIVATION ON DILUTION OF THE ENZYME 116

5.4 DISCUSSION 118

CHAPTER 6 : GENERAL DISCUSSION

6.1 GENERAL DISCUSSION 123

6.2 THE MODE OF ACTION OF ACETYL CoA 124

6.3 THE ACTIVATION BY ACETYL PANTETHEINE 125

BIBLIOGRAPHY 128

PAPERS PUBLISHED OR IN PREPARATION 134

SUMMARY

In order to elucidate the mechanism of action of acetyl CoA activation of pyruvate carboxylase from sheep liver, two different approaches were adopted; (1) the technique of affinity labelling was used to study the structure of the enzyme at the acetyl CoA binding site; and (2) the molecular basis for activation was investigated by using molecules which could replace acetyl CoA in its effect on the first and second partial reactions and on the inactivation of the enzyme on dilution.

AFFINITY LABELLING

From previous kinetic studies, three moieties of the acetyl CoA molecule, the acetyl group, ribose-3'-phosphate and the 6-amino group of adenine, were considered to be important in the function of the molecule. Affinity labelling studies were carried out using reactive analogues of acetyl CoA keeping these three groups in mind. Three different analogues were tested for their ability to modify the enzyme in the acetyl group binding site:-

ACETYL GROUP-DERIVED AFFINITY LABELS

1. 2-bromoacetyl-SCoA

The major product of a published procedure for the synthesis of the compound, did not exhibit alkylating activity and was subsequently shown to be thiophenylcarboxymethyl-SCoA. Subsequently 2-bromoacetyl-SCoA was synthesised from 2-bromoacetylbromide and the tri-*n*-octylammonium salt of CoASH dissolved in acetonitrile. The product obtained

from this procedure was a very strong alkylating agent but was very unstable in neutral or alkaline solutions and hence was unsuitable as an affinity label.

2. 2-haloethyl-SCoA

It was found that the compound produced from the reaction between dibromoethane, CoASH and iodide ions in dimethyl formamide solution was 2-iodoethyl-SCoA. The results of covalent modification studies of pyruvate carboxylase, using 2-iodoethyl-SCoA were equivocal. A compound with no structural similarity to acetyl CoA, 2-iodoethylcysteine, gave a pattern of protection against inactivation similar to that obtained using 2-iodoethyl-SCoA. It was concluded that chemical modification of the enzyme in the presence of 2-iodoethyl-SCoA occurred with no apparent affinity for the acetyl CoA binding site.

3. 3-chloroacetonyl-SCoA

This compound possesses a stable C-S bond and moderate alkylating activity. The compound was synthesised from 1,3-dichloroacetone and CoASH in aqueous acetonitrile. In the presence of 3-chloroacetonyl-SCoA, the enzyme underwent specific covalent modification and the ligand which afforded the greatest degree of protection against inactivation was acetyl CoA. Using two different methods, the stoichiometry of enzyme inactivation in the presence of 3-chloroacetonyl-SCoA was determined as 4 molecules of label bound per enzyme tetramer.

RIBOSE-3'-PHOSPHATE GROUP-RELATED AFFINITY LABELLING

The ribose-3'-phosphate binding site was investigated using acetyl CoA dial, which was prepared from acetyldephospho CoA by periodate oxidation. The results of covalent modification studies of the enzyme using acetyl CoA dial showed that ATP was the only ligand which gave protection against inactivation. The unexpected specificity for the ATP binding site was attributed to covalent modification of the enzyme by adenosine dialdehyde which was a product of β-elimination of acetyl CoA dial in the presence of enzyme.

THE MOLECULAR BASIS FOR ACTIVATION OF PYRUVATE CARBOXYLASE BY ACETYL CoA

Three aspects of the mechanism of activation of pyruvate carboxylase by acetyl CoA were investigated in order to reconcile the diverse functions which had been previously observed, with the action of a single molecule of acetyl CoA.

1. Analogue Studies

The effect of analogues of acetyl CoA on the first and second partial reaction was investigated in order to locate the region of influence on the enzyme of moieties of the acetyl CoA molecule.

The results can be summarised as follows:-

Activator	ATP:Pi exchange reaction	Pyr:OAA exchange reaction	Overall Reaction
acetyl CoA	stimulates	stimulates	stimulates
CoASH	stimulates	no effect	stimulates
3'5' ADP	stimulates	no effect	no effect
acetylpantetheine	no effect	stimulates	not done
acetyl-2'4'-phosphopantetheine	no effect	stimulates	stimulates
acetyldephospho CoA	stimulates	stimulates	stimulates
acetyldesamino CoA	no effect	stimulates	no effect

It was concluded that the adenosine moiety of acetyl CoA activates the first partial reaction for which the 6-amino group is essential and the acetylpantetheine moiety of acetyl CoA activates the second partial reaction.

2. Studies on inactivation of the enzyme by dilution

The inactivation of the enzyme on dilution was used to further investigate the molecular basis of action of acetyl CoA. It was found that in preventing inactivation on dilution of the enzyme, acetyl CoA could be replaced by non-ionic or apolar solutes in the dilution buffer. The rate of inactivation was slower in buffers made with less polar compounds or in buffers made with D_2O in place of water. The inactivation process had its effect at the first partial reaction site since inactivation of the ATP: orthophosphate exchange reaction on dilution paralleled the loss in overall activity while the pyruvate:oxaloacetate exchange activity was unaffected. The inactivation on dilution was prevented by addition of CoASH and not by addition of acetyl 2'4'-phosphopantetheine. It was concluded that the adenosine moiety of acetyl CoA prevented inactivation of the first partial reaction by exclusion of water from this site. Hydration of the site in the absence of acetyl CoA resulted in the irreversible loss of enzyme activity.

3. Circular dichroism studies

Further work on the nature of inactivation of the enzyme on dilution was carried out using circular dichroism. It was found that dilution of the enzyme was accompanied by major changes in the structure of the polypeptide backbone. The spectra were interpreted as indicating α-helix formation after dilution which could be prevented by the presence of acetyl CoA or apolar solutes in the dilution buffer or enhanced by ionic solutes. It was concluded that hydration of the first partial reaction site in the absence of acetyl CoA resulted in the formation of an α-helix which destroyed the native conformation of the enzyme and was responsible for the loss of activity.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

PETER R. CLEMENTS

ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

I am grateful to my supervisors, Dr. D.B. Keech and Dr. J.C. Wallace for their valuable advice, criticism and encouragement throughout the course of this work and in the preparation of this thesis. I am grateful to Dr. A.J. Campbell for her valuable collaboration in the dilution inactivation experiments. In addition I wish to thank Dr. R.H. Symons for his guidance in the nucleotide chemistry. I would also like to thank Dr. M. Snow and Dr. T. Kurucsev of the Physical and Inorganic Chemistry Department for their help in the interpretation of the circular dichroism spectra and Dr. R.H. Prager of the Organic Chemistry Department for discussions concerning the organic syntheses. I am grateful to Mr. N. Goss and Ms. J. Duc for permission to discuss their unpublished results in this thesis.

I would like to thank Ms. J. Anderson for expert technical assistance and the preparation of the diagrams and Mr. D. Field for the preparation of the litho-masters.

I would like to thank my wife, Wendy, for typing this thesis and for her support and understanding throughout the course of this work.

I acknowledge the financial support of a University Research Grant from the University of Adelaide for the duration of the project.

LIST OF ABBREVIATIONS

In addition to those accepted for use in the Journal of Biological Chemistry, the following abbreviations are in use in this thesis:-

ANS	1-anilinonaphthalene-8-sulphonate
CoA,CoASH	Coenzyme A
DTE	dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
ESR	electron spin resonance
E.U.	1 international enzyme unit
DMF	dimethyl formamide
HEPES	N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
MOPS	morpholinopropane sulphonic acid
NBP	γ-(4-nitrobenzyl)-pyridine
NEM	N-ethylmorpholine
NMR	nuclear magnetic resonance
OAA	oxaloacetate
PEI	polyethylene imine
pyr	pyruvate
TNBS	2,4,6-trinitrobenzene sulphonic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 PYRUVATE CARBOXYLASE AND ITS ACTIVATION BY ACETYL CoA.

1.1.1 THE REACTION CATALYSED BY PYRUVATE CARBOXYLASE

Pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), EC 6.4.1.1) catalyses the ATP-dependent carboxylation of pyruvate by bicarbonate, (reaction 1.),

$$\text{ATP} + \text{HCO}_3^- + \text{pyruvate} \xrightarrow[\text{acetyl CoA}]{\overset{\text{M}^+}{\text{Mg}^{++}}} \text{oxaloacetate} + \text{ADP} + \text{orthophosphate} \qquad \text{...1.}$$

and releases the products, oxaloacetate, ADP and orthophosphate. The overall reaction can be divided into two partial reactions (reactions 2. and 3.).

$$\text{MgATP}^{2-} + \text{HCO}_3^- + \text{E.biotin} \rightleftharpoons \text{E.biotin.CO}_2 + \text{MgADP}^- + \text{orthophosphate} \qquad \text{...2.}$$

$$\text{pyruvate} + \text{E.biotin.CO}_2 \rightleftharpoons \text{E.biotin} + \text{oxaloacetate} \qquad \text{...3.}$$

There are species-specific differences in the requirements for these cations (Utter, Barden and Taylor, 1975). In addition the requirement for an acyl CoA activation varies widely with pyruvate carboxylases isolated from different species. The requirement varies from apparently absolute in the enzyme from chicken liver (Utter and Keech, 1963; Scrutton and Utter, 1967) to no response in the enzymes from *Pseudomonas citronellolis* (Seubert and Remberger, 1961) and *Aspergillus niger* (Bloom and Johnson, 1962).

The enzyme isolated from sheep kidney and liver is the subject of investigations in this laboratory and its requirement for the presence of an acyl CoA in the assay solution is dependent upon (a) enzyme concentration (b) pyruvate and (c) HCO_3^- concentrations. (See section

1.1.4 for details). Acetyl CoA is the most effective activator of sheep liver pyruvate carboxylase.

The purpose of this investigation was to elucidate the mechanism by which acetyl CoA activates the reaction catalysed by sheep liver pyruvate carboxylase. Several review articles (Moss and Lane, 1971; Scrutton and Young, 1972; Utter, Barden and Taylor, 1975) provide adequate background to the general properties of pyruvate carboxylase and its relation to other biotin dependent carboxylases. Advances in the field have been detailed in recent review articles (Wood, 1976; Wood and Barden, 1977).

It is believed that the initial step involves the formation of E.biotin.CO_2 from which carboxyl transfer to the acceptor occurs in the second partial reaction. Since it is now known that monomers of the enzyme from rat liver (Nakashima *et al.*, 1975) exhibit activity, it can be deduced from the above mechanism that the active site on each monomer of pyruvate carboxylase is composed of two functionally distinct subsites with the biotinyl prosthetic group acting as a mobile carboxyl carrier.

The enzyme is a tetrameric molecule and contains 4 biotin prosthetic groups per tetramer and 4 tightly bound manganese atoms. Besides the substrates listed in reaction 1, optimal enzymatic activity requires the presence of Mg^{++} and the presence of a monovalent cation.

1.1.2 THE EFFECT OF ACETYL CoA ON THE BIOTIN CARBOXYLATION STEP

The activity of the biotin carboxylation step (reaction 2.) can be observed by the use of the isotopic exchange reaction between orthophosphate and $MgATP^{2-}$ (Utter and Keech, 1963; McClure, *et al.*, 1970; Barden, Fung, Utter and Scrutton, 1971). The reaction was extensively characterised for sheep kidney enzyme in this laboratory (Ashman and Keech, 1975). The reaction was shown to be absolutely dependent upon the presence of acetyl CoA and since the pyruvate:oxaloacetate exchange reaction exhibits activity in the absence of acetyl CoA this led to speculation that acetyl CoA only acts at the biotin carboxylation step (Scrutton, Keech and Utter, 1965).

In addition to stimulation of the ATP:orthophosphate exchange reaction rate, acetyl CoA affects the biotin carboxylation step by lowering the apparent K_m value for HCO_3^- (Ashman, *et al.*, 1972) but does not affect the apparent K_m value for $MgATP^{2-}$.

1.1.3 THE EFFECT OF ACETYL CoA ON THE TRANSCARBOXYLATION STEP

The isotopic exchange reaction between pyruvate and oxaloacetate (reaction 3.) can be used to follow the activity of carboxyl group transfer from E.biotin.CO_2 to pyruvate, (Seubert and Remberger, 1961; Scrutton, Keech and Utter, 1965). The exchange reaction differs from that catalysed by the biotin carboxylation step in that it occurs in the absence of acetyl CoA although Ashman, *et al.*, 1973, showed that the reaction is stimulated three fold by its presence. Further work in this laboratory, (Ashman, *et al.*, 1972) has shown that acetyl CoA lowers the apparent K_m value for pyruvate. These two pieces of evidence demonstrated that acetyl CoA did influence events during the transcarboxylation process and that the biotin carboxylation step was not the only site of action of acetyl CoA.

1.1.4 ENZYME CONCENTRATION DEPENDENCE AND THE ACETYL CoA-INDEPENDENT ACTIVITY

Studies in this laboratory, (Ashman, Keech, Wallace and Nielsen, 1972) showed that at high enzyme concentrations, (above 4 E.U./ml.) the enzyme was stable and catalysed the reaction in the absence of acetyl CoA. The maximum velocity of the acetyl CoA-independent reaction was 25% of the overall rate measured in the acetyl CoA-dependent assay in the presence of acetyl CoA. At high enzyme concentrations (above 4 E.U./ml.) acetyl CoA still gave a marked stimulation of the enzyme catalysed reaction.

However, when the enzyme was diluted to concentrations below 4 E.U./ml, the enzyme underwent inactivation. Both the initial rate of inactivation and the extent of this process were dependent upon the final enzyme concentration. When enzyme was diluted in the presence of saturating levels of acetyl CoA, no inactivation was observed. One possible interpretation of the concentration dependence of inactivation was that a change occurred in an equilibrium situation such that active tetramers were dissociated to inactive monomers.

Three arguments against this hypothesis are that:- (a) the inactivation process was irreversible under all conditions including the addition of saturating levels of acetyl CoA. (b) Active enzyme monomers have been prepared from rat liver (Nakashima *et al.*, 1975) indicating that at least for rat liver pyruvate carboxylase dissociation does not result in inactivation of the enzyme and (c) assuming that pyruvate carboxylases isolated from rat liver and sheep liver are similar it might be expected that active monomers would form on dissociation of the sheep liver enzyme; however, active species sedimentation experiments carried out in this laboratory (Easterbrook-Smith *et al.*, 1978b) have shown that inactivation on dilution does not involve the formation of catalytically active dimers or monomers of the enzyme.

1.1.5 THE EFFECT OF ACETYL CoA ON THE INACTIVATION OF PYRUVATE CARBOXYLASE BY AVIDIN

Another effect of acetyl CoA on pyruvate carboxylase is the enhancement of the rate of inactivation of the enzyme by avidin (Scrutton and Utter, 1967). The inactivation rate was measured at varying concentrations of acetyl CoA and followed a biphasic pattern in which the rate increased up to 200µM acetyl CoA, ie. at saturating levels and decreased again up to 1.0mM. The conclusion drawn from these data was that acetyl CoA affected the environment of residues in the biotin binding site of the enzyme active site. A further conclusion made from this investigation was that non-productive binding of acetyl CoA to the enzyme occurred at levels above 200µM. The type of non-productive binding envisaged involved binding of an acyl thioester moiety from one molecule of acetyl CoA and an adenosine moiety from another molecule in the one acetyl CoA binding site on the enzyme. A significant point, overlooked in making this conclusion was that inhibition of the overall reaction did not occur at the high levels of acetyl CoA.

1.1.6 THE EFFECT OF ACETYL CoA ON ACTIVATION BY MONOVALENT CATIONS

Studies in this laboratory (Ashman *et al.*, 1972) and others (Barden and Scrutton, 1974) have shown that the presence of acetyl CoA alters the response of pyruvate carboxylase to the presence of monovalent cations. In the absence of acetyl CoA, the enzyme was weakly activated by Na^+ but not by $Tris^+$; in the presence of acetyl CoA, the enzyme was activated by $Tris^+$ but not by Na^+. In both cases however, NH_4^+ was the most effective monovalent cation and its binding was stimulated four fold in the presence of acetyl CoA as indicated by the change in its apparent K_a value.

1.1.7 THE PYRUVATE CARBOXYLASE-DEPENDENT DEACYLATION OF ACETYL CoA

A feature of the thioester bond in acetyl CoA is its susceptibility to hydrolysis particularly in the presence of ammonium ions or amines. Although during the time taken to carry out most kinetic studies the rate of deacylation, even at pH 8.4, is insignificant, pyruvate carboxylase itself catalyses the deacylation of acetyl CoA (Scrutton and Utter, 1967). Even though the rate (0.275n mol/min/E.U.) is not sufficient to interfere with most kinetic studies where 0.1 E.U. are used at saturating levels of acetyl CoA, the rate of deacylation becomes significant in longer term experiments such as in binding studies where high levels of enzyme (30-60 E.U.) are used. The deacylase activity was assumed to be at the acetyl CoA binding site on the basis of experiments (Scrutton and Utter, 1967; Ashman, 1973) which showed enhancement of the reaction rate in the presence of the substrates of the pyruvate carboxylase reaction. An acetylated enzyme species was ruled out by studies (Ashman, 1973) in this laboratory.

1.1.8 BINDING STUDIES OF ACETYL CoA TO PYRUVATE CARBOXYLASE

Since the first report of a sigmoidal reaction velocity response to increasing concentrations of acetyl CoA (Barritt *et al.*, 1966; Scrutton and Utter, 1967), it was assumed that the activator bound to the enzyme in a homotropic cooperative manner. The only direct evidence for cooperative binding of acetyl CoA was provided by Frey and Utter (1976) using a rapid flow dialysis technique. However, the rate of deacylation of acetyl CoA at the high enzyme concentration used by Frey and Utter was sufficient to make an unequivocal interpretation of their data, in terms of binding parameters, difficult to obtain. On the other hand, other evidence suggests that homotropic cooperativity of binding is not the explanation for the non-classical acetyl CoA kinetics. For example Nakashima *et al.*, (1975) reported that active monomers of rat liver pyruvate carboxylase exhibit a sigmoidal shaped velocity profile suggesting that inter-subunit cooperativity is not involved while the report of Utter *et al.*, (1975) suggests that only one molecule of acetyl CoA binds specifically to each monomer. In an experiment designed to measure the rate of deacylation of acetyl CoA catalysed by pyruvate carboxylase as a function of varying acetyl CoA concentration, Easterbrook-Smith *et al.*, (1978b) obtained a hyperbolic response suggesting that the binding of acetyl CoA to the enzyme under these conditions is a classical Michaelis-Menten type process.

The implication from this result was that explanations could be found for the sigmoidal response of the pyruvate carboxylase catalysed reaction to acetyl CoA which did not involve homotropic cooperativity of binding of acetyl CoA.

1.1.9 RECENT DEVELOPMENT OF AN ALTERNATIVE EXPLANATION FOR THE SIGMOID ACETYL CoA RESPONSE

Studies in this laboratory (Easterbrook-Smith, *et al.*, 1976) on the mechanism of the second partial reaction have led to further investigations (Easterbrook-Smith, *et al.*, 1978b) on the nature of the response of pyruvate carboxylase to increasing concentrations of acetyl CoA.

An examination was made of the factors that contribute to the sigmoidal velocity profiles obtained with varying concentrations of acetyl CoA. It was proposed that acetyl CoA exerts its action largely by affecting the degree of saturation of the enzyme with pyruvate and bicarbonate.

This was in contrast to the previously held view (Scrutton, *et al.*, 1965) that the sole locus of action of acetyl CoA was at the first partial reaction sub-site. However, as explained in section 1.1.3 acetyl CoA exerts a profound influence on the second partial reaction site through its influence on the apparent Km for pyruvate. This suggests that when acetyl CoA is the variable ligand the fixed concentration of pyruvate, although saturating at high levels of acetyl CoA, will become non-saturating as the level of acetyl CoA is decreased. The prediction from the above explanation that higher fixed levels of pyruvate would decrease the degree of sigmoidality while low fixed levels would increase sigmoidality, proved to be correct. It was further shown that in the presence of different levels of pyruvate the value of η_H for acetyl CoA varied. At low levels of pyruvate (0.95mM) the value was 3.4 but at high levels of pyruvate (19mM) the value decreased to 1.95. However, it was pointed out that the reason the η_H vlaue does not decrease to a limiting value of 1.0 is because, in addition to the influence of acetyl CoA on pyruvate binding, other factors are involved. Previously, Easterbrook-Smith, *et al.*, (1976) provided an explanation for the concave downward, double reciprocal velocity profile obtained when the initial velocity was determined by measuring oxaloacetate synthesis as a function of pyruvate concentration in the presence of a saturating concentration of acetyl CoA. They showed that the stoichiometry of the products, orthophosphate and oxaloacetate, varied as a function of pyruvate concentration. (See Fig. 1.1). It was proposed that this was due to a hydrolytic leak operating at the second partial reaction site, whereby in the presence of low levels of pyruvate, the immediate enzyme-carboxy-biotin complex underwent non-productive hydrolysis to form enzyme-biotin and HCO_3^- at a significant rate. However, as the pyruvate concentration was increased, the rate of hydrolysis decreased relative to the rate of oxaloacetate synthesis. To ascertain whether the hydrolytic leak of the enzyme-carboxy-biotin complex was a factor contributing to the production of the sigmoidal-shape of the velocity profile with varying concentrations of acetyl CoA, the reaction velocity was monitored by measuring both the orthophosphate released and oxaloacetate synthesised.

The η_H value obtained using the rate of oxaloacetate synthesis was 2.08± 0.04 while the value based on orthophosphate release was 1.68 ± 0.05. It was also shown that the ratio of orthophosphate released to oxaloacetate synthesised varied as a function of acetyl CoA concentration. The high ratio at low levels of acetyl CoA was attributed to the fact that at low levels of acetyl CoA the fixed concentration of pyruvate becomes non-saturating. Since the rate of non-productive breakdown of the enzyme-carboxy-biotin-pyruvate complex is at a maximum under these conditions, it was concluded that the hydrolysis of the enzyme-carboxy-biotin complex at low levels of acetyl CoA is a factor contributing to the sigmoid velocity profile.

Ashman *et al.*, (1972) showed that in addition to influencing the binding of pyruvate to the enzyme, acetyl CoA also decreased the apparent K_a value for bicarbonate by about an order of magnitude. Using reasoning analogous to that outlined above, this observation suggested that another factor contributing to the non-classical velocity profile was that the fixed bicarbonate concentration used in the assay solution became non-saturating at the low levels of acetyl CoA. It was again shown that the value of η_H for acetyl CoA varied depending upon the fixed concentration of bicarbonate. Similar experiments were carried out for $MgATP^{2-}$ and it was shown that the value for η_H did not vary depending upon the fixed concentration of $MgATP^{2-}$. This was in agreement with the findings of Ashman *et al.*, (1972) that acetyl CoA did not affect the K_a value for $MgATP^{2-}$.

Yet another factor contributing to the atypical velocity response curve is the phenomenon of irreversible inactivation of the enzyme on dilution (Ashman *et al.*, 1972). In an experiment where all reaction components were saturating regardless of the acetyl CoA concentration and the enzyme concentration was raised to a level where inactivation did not occur even in the absence of acetyl CoA, the reciprocal of velocity plotted as a function of the reciprocal of acetyl CoA was concave downward. This result demonstrated that provided the factors discussed above no longer operate, a sigmoidal velocity profile (ie. a concave upward double reciprocal plot) is not observed.

1.1.10 THE EFFECT OF ACETYL CoA ON THE CATALYTIC VELOCITY

A conclusion of the work of Ashman *et al.*, (1972) was that acetyl CoA affected the catalytic velocity *per se*, of the enzyme reaction ie. it had a V effect as well as a K_{py} and a $K_{HCO_3^-}$ effect. The V effect was demonstrated in the experiment outlined in section 1.1.9 (Easterbrook-Smith *et al.*, 1973b) where factors contributing to the sigmoidal response of enzyme activity to acetyl CoA were eliminated. Under the conditions used, viz. of high enzyme concentration and saturating levels of pyruvate and bicarbonate, the stimulation of the enzyme catalysed reaction was evidence of a V effect due to the presence of acetyl CoA.

One factor which could contribute to the V effect is stabilisation of the enzyme against hydrolysis of E.biotin.CO_2 in the presence of acetyl CoA (Easterbrook-Smith *et al.*, 1976). Stabilisation could involve facilitation by acetyl CoA of the movement by the biotin prosthetic group from the first partial reaction sub-site to the second.

1.2 STRUCTURAL FEATURES OF ACETYL CoA AND SITE TOLERANCE

1.2.1 THE ACETYL CoA MOLECULE

It is useful to discuss the biosynthetic route of acetyl CoA in order to (a) become familiar with its constituent moieties, (b) to divide them into three convenient chemical groups and (c) to consider the route as a possible means of synthesising analogues of acetyl CoA. (Abiko, 1970).

The first moiety, 4'-phosphopantetheine, is made up of the vitamin pantothenic acid, β-cysteamine and a phosphate group. The 4'OH group of pantothenic acid is phosphorylated by pantothenate kinase to form 4'-phosphopantothenic acid (reaction 5.) which is then coupled to cysteine by an amide linkage (reaction 6.). The cysteine carboxyl group is then removed in another enzyme catalysed step to give 4'phosphopantetheine (reaction ~~7~~6).

The second moiety, adenosine 3'5' diphosphate, is then attached by a multifunctional enzyme in two steps. Firstly dephospho CoA is formed by condensation of 4'-phosphopantetheine and ATP with the release of pyrophosphate (reaction 7.). The 3'phosphate group is then attached to the ribose moiety in a kinase catalysed step (reaction 8.) to complete the coenzyme A molecule.

The acetyl group is attached from one of three routes ie. via lipolysis in which CoA functions as a C_2 carrier or via glycolysis in which the pyruvate dehydrogenase complex catalyses the synthesis of acetyl CoA for utilisation in the citric acid cycle. Thirdly transport of acetate across the mitochondrial membrane involves acyl exchange to the carrier acetyl carnitine by the enzyme acetyl CoA:

carnitine transferase (EC 2.3.1.7) (Fig 1:2b. [redacted].) The process is reversed inside the mitochondrial membrane to reform acetyl CoA. The three groups or moieties can be used as points of reference in the review of structure/ function studies to follow.

1.2.2 STRUCTURE/ACTIVITY RELATIONSHIPS OF ACETYL CoA

The nature of acetyl CoA activation of pyruvate carboxylase has been investigated extensively by the use of structural analogues and homologues. It was found that acyl esters of CoA were the most effective activators and those with an acyl chain length greater than four carbon atoms were very much less effective than those with shorter chain length (Scrutton and Utter, 1967). The same pattern was obtained with the enzyme from rat liver (Scrutton, 1974), but longer carbon chain length acyl groups were also effective activators. Acetyl pantetheine did not activate the enzyme from either species and was found to inhibit the enzyme from both chicken and rat liver with respect to activation by acetyl CoA. Similarly analogues lacking the 3'phosphate group (acetyldephospho CoA), and lacking the 6-amino group (acetyldesamino CoA) were also found to be inhibitors and not activators of both the chicken and rat enzymes. (Scrutton, 1974; Fung and Utter (1970). Adenosine 3'phosphate 5'pyrophosphate was an activator of chicken liver pyruvate carboxylase (Fung, 1972) but had very low affinity for the acetyl CoA binding site.

Conclusions that can be made from the above data are that (a) an acyl moiety with carbon chain length no greater than four, is essential for significant activation

(b) the 3'phosphate group and 6-amino group are both essential for activation and (c) while the phosphorylated adenosine moiety produces some activation, the acetyl pantetheine moiety is required for more effective binding to the site.

1.2.3 THE CONFORMATION OF THE ACETYL CoA MOLECULE

The bound conformation of propionyl CoA in relation to a Co^{++} atom in the active site of transcarboxylase has been determined using relaxation measurements on the ^{13}C-NMR spectrum of propionyl CoA (Fung *et al.*, 1976). These authors were able to show that the coenzyme took up a "U" shape around the Co^{++} atom and that the substrate pyruvate completed a circular arrangement around the metal. Other investigators have shown that the solution conformation of CoA esters was a dynamic equilibrium between extended and folded configurations (Lee and Sarma, 1974) unless interaction between adenine and a hydrophobic acyl moiety occurred (Mieyal *et al.*, 1976).

Therefore, it can be concluded that the solution conformation of CoA esters bears no relation to the bound configuration and can only be a consideration in kinetic studies should the rate of interchange between solution and bound conformations be significantly slow. One factor which could influence the binding conformation of acetyl CoA is its interaction with Mg^{++}. Studies on monovalent and divalent cations using chicken liver pyruvate carboxylase (Barden and Scrutton, 1974) revealed an interaction between Mg^{++} and acetyl CoA at low levels of each indicating positive heterotropic cooperativity. Recent unpublished observations from this laboratory have shown that the K_a value of acetyl CoA is lowered by a factor of 5 in the presence of Mg^{++}. In a study of the enzyme isolated from rat liver (Seufert *et al.*, 1971) the activation of both the overall reaction and the pyruvate:oxaloacetate exchange reation by acetyl CoA was influenced by the presence of Mg^{++}. Each of these observations suggests that interactions between Mg^{++} and acetyl CoA cannot be ignored in a study of the mode of activation by acetyl CoA.

1.3 APPROACHES TO THE STUDY OF THE MECHANISM OF ACTION OF ACETYL CoA

1.3.1 CHEMICAL MODIFICATION

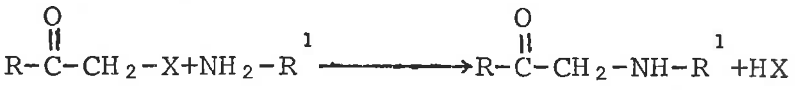
Many reagents have been used for the specific modification of amino acids in order to identify residues essential for catalytic activity (Means and Feeney, 1971; Glazer *et al.*, 1975). Chemical modification studies in this laboratory (Ashman, Wallace and Keech, 1973) identified an essential lysine residue in the binding site of acetyl CoA by the use of TNBS modification. These studies showed that TNBS inactivated the acetyl CoA-dependent activity but stimulated the acetyl CoA-independent activity. In other words the enzyme was found to be desensitised against acetyl CoA activation. Both the ATP:orthophosphate exchange reaction and the pyruvate:oxaloacetate exchange reactions were affected by TNBS modification of the enzyme. These data provided circumstantial evidence that the modified lysyl residue was in the acetyl CoA binding site. Further work (Scrutton *et al.*, 1977) has shown that the pattern of protection by analogues of acetyl CoA against TNBS modification did not implicate any part of the acetyl CoA molecule in binding to the modified lysine residue.

Chemical modification studies using other enzyme systems have led to the identification of residues essential for catalytic activity. In most of these studies, however, there has been no direct proof that the modified residue was in the ligand binding site under study. Conformational changes initiated from a site of modification distant from the ligand binding site could account for such phenomena as desensitisation against activation by the ligand. In such a case the only definitive evidence of common chemical modification and ligand binding sites would be from X-ray crystallographic studies of the enzyme both chemically modified and with bound ligand. In the absence of crystals of pyruvate carboxylase suitable for analysis by X-ray diffraction, other techniques for studying ligand binding sites are called for. The most successful of these has been affinity labelling.

1.3.2 AFFINITY LABELLING

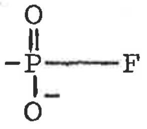
The technique of affinity labelling is ideal for the specific covalent modification of ligand binding sites. The principles and practice of the technique are described by Baker (1967) while a number of reviews have adequately covered the successes in the field (Shaw, 1970; Singer, 1970; Yount, 1975; Sigman and Mooser, 1975). The review of Yount, (1975) covers developments in the more specialised field of ATP binding sites while the review of Sigman and Mooser (1975) focuses attention on developments in the field of affinity labelling. This includes a recent development called "k_{cat} inhibition", or, alternatively, "suicide substrate labelling". This technique depends for its specificity on the catalytic process before modification of the enzyme occurs. Highly specific modification of enzymes is obtained since the compound only becomes reactive after catalysis by the target enzyme has occurred. In this way the problem of lack of specificity due to over reactivity of other reagents is overcome. These advantages are, unfortunately, not available for the study of receptor and effector site labelling since the catalytic processes of the enzyme do not affect these ligands.

The type of reactive group used in affinity labelling studies determines the reactivity and consequently the specificity of the compound. Several classes of reactive groups have been used but all depend upon the nucleophilicity of the target residue in the binding site and all are therefore electrophilic. The most common reactive group type, the α-haloketones acts by displacement of the α-halogen by a nucleophilic residue such as lysine or cysteine to result in covalent attachment of the label through a C-N or C-S single bond (Scheme 10.) eg. Jornvall *et al.*, (1975).



$$R-\overset{\overset{O}{\|}}{C}-CH_2-X+NH_2-R^1 \longrightarrow R-\overset{\overset{O}{\|}}{C}-CH_2-NH-R^1+HX \qquad ...10$$

Other reactive groups are more dependent upon the type of functional group available for attachment and are generally less versatile in placement and ease of synthesis. Examples of the types of groups which have been used are:-



$-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}-F$ fluoro phosphate (Pal *et al.*, 1975) and fluoro-sulphonyl groups (Anderson *et al.*, 1973).

Adehydes such as pyridoxal phosphate and the periodate oxidation product of ribose have been used with some success. (Hill *et al.*, 1975; Easterbrook-Smith *et al.*, 1976). Other reactive groups such as diazonium salts ($R\text{-}N_2^+$) (Cuatrecasas, 1970) and epoxides, or oxiranes, have also been used to good effect (Fee *et al.*, 1974).

Another type of reactive group which can be employed is found in photo-affinity labels. These reagents have the advantage that they are not reactive until subjected to irradiation and can be allowed to saturate the target site before being converted to the reactive compound (Knowles, 1972).

Photo-affinity labels based on CoA have been used successfully (Lau *et al.*, 1977 a&b). These investigators used two azide derivatives of acyl CoA, one of which had the reactive group attached to the acyl moiety and the other on the adenine moiety. The acyl labelled CoA derivative, p-azidobenzoyl-CoA, was found to be an alternative substrate for the target enzyme, acyl-CoA:glycine N-acyltransferase, prior to photolysis. The labelling studies were used to show that there was one active site per molecule of enzyme. The same enzyme was able to use the other photoaffinity label, S-benzoyl (3'dephospho-8-azido) CoA, as a substrate prior to photolysis and irreversible inhibition was observed after photolysis.

1.3.3 PROTEIN SEQUENCING

The ultimate goal of most affinity labelling studies is the isolation of a covalently modified peptide from the binding site of the reactive ligand. The information which can be gained from the peptide consists not only of the amino acid sequence of that peptide but in its relation to other peptides. The long term aim of the project on pyruvate carboxylase in this laboratory is to piece together as many peptides as possible in and around the active site. These can be obtained from the affinity labelling and chemical modification studies being carried out on the binding sites of each ligand. The biotin prosthetic group has also been the subject of peptide isolation studies and the sequence of the tryptic peptide is now known (Rylatt *et al.*, 1977). Other sites from which peptides will soon be isolated, are those modified by oxidised ATP (Easterbrook-Smith *et al.*, 1976) and bromopyruvic acid (Hudson *et al.*, 1975). In this way the relationship of each peptide to others in the overall sequence can give information about the active site structure and therefore about the possible mechanism of action of the enzyme. This approach has been used with sucrase-isomaltase (Quaroni and Semenza, 1976). An extension of this work is in model studies using synthetic peptides to make a reconstruction of the active centre and so gain information about the catalytic mechanism. This treatment has been attempted by several groups including a study of methyl malonyl CoA mutase (Flohr *et al.*, 1976). In one elegant study the synthetic 63-residue analogue of ribonuclease possessed full enzymic activity (Gutte, 1977) when compared with the native 124-residue enzyme.

1.3.4 ANALOGUE STUDIES

The results of studies using structural analogues of acetyl CoA were detailed in section 1.2.1. Other workers have used this technique to gain knowledge of the structure/function relationships of the ligand under study and its target protein.

A recent study of analogues of the coenzyme adenosyl cobalamin and their effect on the diol dehydrase system of *Klebsiella pneumoniae* (Toraya *et al.*, 1977) is very comprehensive in its approach. Transition state analogues have also been used to elucidate enzyme mechanisms (Schindler *et al.*, 1976; Wolfenden, 1972). These latter studies are however, of no use in the study of ligands which are not involved in the catalytic process.

1.3.5 PHYSICAL TECHNIQUES

Many physical techniques have been applied to the study of enzyme structure and function. Only those which have been used for the study of pyruvate carboxylase, or have been used in this thesis, will be introduced here.

(a) Nuclear Magnetic Resonance and Electron Spin Resonance

The application of resonance spectral techniques to biochemistry has been well covered in a recent book (Dwek, 1975). Except for problems associated with its large molecular weight, pyruvate carboxylase is well suited to these studies since the bound Mn^{++} ion can be detected in both NMR and ESR systems by its paramagnetic field.

One such study has revealed that changes in hydration of the enzyme-bound Mn^{++} occur in the presence of substrates and that chelation to the Mn^{++} by enzyme-bound ligands reflects a rhombic rather than axial symmetry (Reed and Scrutton, 1974). No information about the binding of acetyl CoA to the enzyme has yet been revealed by these studies. The information required could be obtained from studies of relaxation times in the same way that distances between pyruvate and the enzyme-bound Mn^{++} atom were calculated.

Further work has been done using a spin-labelled derivative of CoA in order to calculate distances between the coenzyme and bound pyruvate on transcarboxylase. (Fung *et al.*, 1976). This work could be readily repeated with pyruvate carboxylase provided the spin labelled CoA derivative is not sterically excluded from the acetyl CoA binding site of the enzyme.

(b) Fluorescence measurements

Information about conformational changes in pyruvate carboxylase upon binding of the effectors Mg^{++} and acetyl CoA has been obtained from perturbation of the ANS fluorescence spectrum (Bais and Keech, 1972; Ashman, 1973). These studies have revealed minor changes in enzyme conformation after acetyl CoA binding which suggested that allosteric control by conformation change was not a likely explanation for the mode of acetyl CoA activation of pyruvate carboxylase.

Another way in which fluorescence can be of use is the measurement of distances between two enzyme bound fluorescent centres. This technique has been used to calculate the distance between CoA and FAD on the surface of pyruvate dehydrogenase (Shepherd, Papadakis and Hammes, 1976) and a statistical treatment of the data from such studies has been published (Hillel and Wu, 1976). The centres on pyruvate carboxylase to which this treatment could apply are the binding sites of acetyl CoA and ATP.

(c) Circular dichroism

Many reviews have been written on the techniques of circular dichroism and optical rotatory dispersion and their application to biochemical systems (Tinoco and Cantor, 1970; Fasman, 1963; Timasheff, 1967; Adler *et al.*, 1973). The technique provides information about changes to both the polypeptide backbone structure and to changes in aromatic amino acid side chain position on a far more sensitive scale than ANS fluorescence measurements. No work had been done on pyruvate carboxylase using circular dichroism or optical rotatory dispersion prior to this thesis.

1.3.6 THE APPROACH ADOPTED

The initial thrust of this work was to affinity label the binding site of acetyl CoA in order to obtain information about the distance of the effector from other ligand binding sites. The labelling studies coupled with the isolation of active site peptides would be used to build up a picture of the three dimensional structure of the active site. The published procedure of (Chase and Tubbs, 1969) for the synthesis of 2-bromoacetyl CoA provided what was thought to be an ideal affinity label for use on this ligand binding site.

As other projects in this laboratory developed, it became clear that more fruitful methods could be employed to discover the mechanism of acetyl CoA activation. The results of Easterbrook-Smith *et al.*, (1976) which emphasised that the major effect of the acetyl CoA activation was to stimulate pyruvate binding renewed interest in the use of analogues of acetyl CoA to assign function to structure.

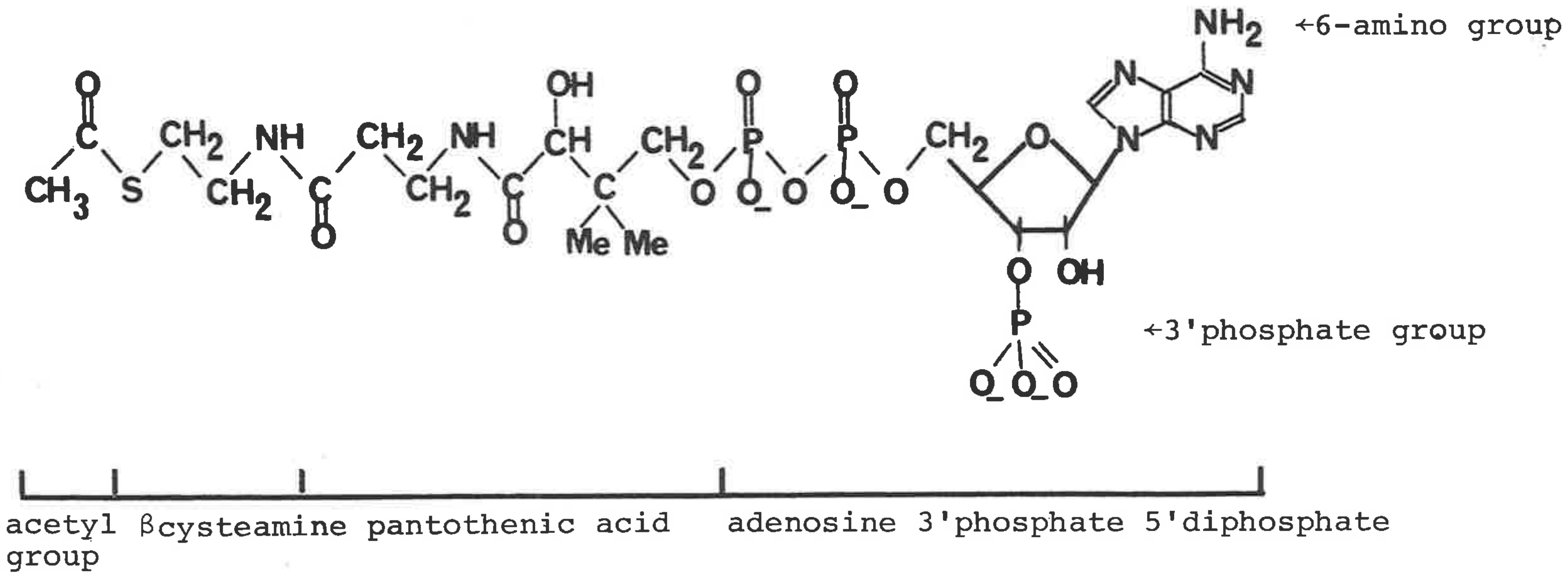
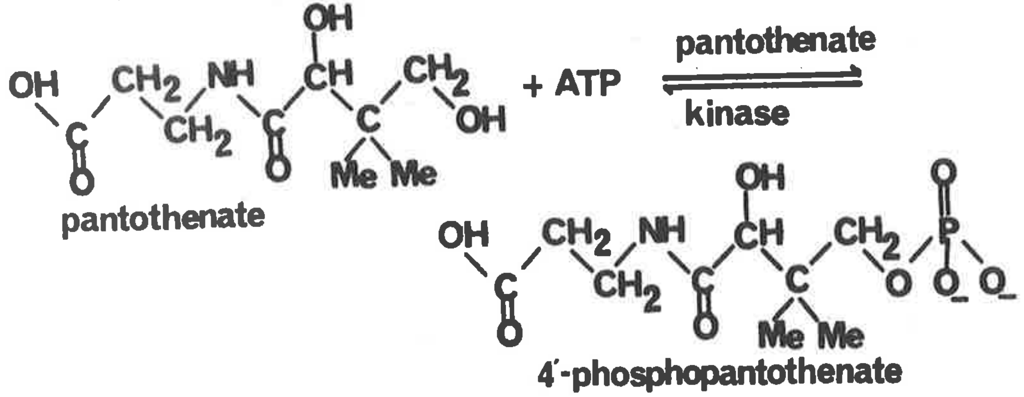

←6-amino group
←3'phosphate group
acetyl group
βcysteamine
pantothenic acid
adenosine 3'phosphate 5'diphosphate

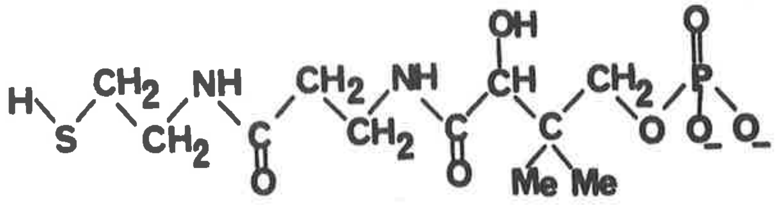
Fig. 1.1 acetyl CoA

Gp I 4′-phosphopantetheine


pantothenate
+ ATP
pantothenate
kinase
4′-phosphopantothenate

. . . 5

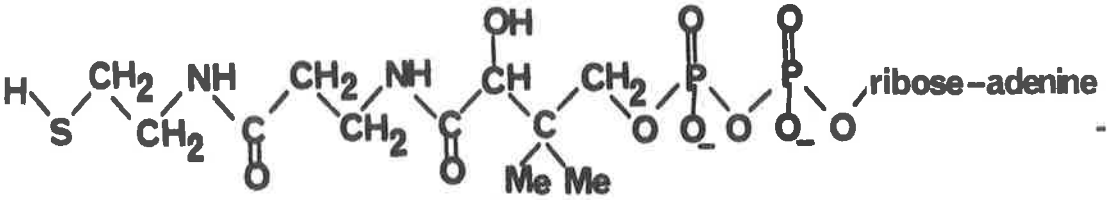
4′-phosphopantothenate + cysteine ⟶ ⟶



4′-phosphopantetheine . . . 6

Gp II 3′5′ ADP attachment

4-phosphopantetheine + ATP ⟶



dephosphoCoA . . . 7

dephosphoCoA + ATP $\xrightarrow[\text{kinase}]{\text{dephosphoCoA}}$ CoA + ADP . . . 8

Fig. 1.2a

Gp III The attachment of the acetyl group

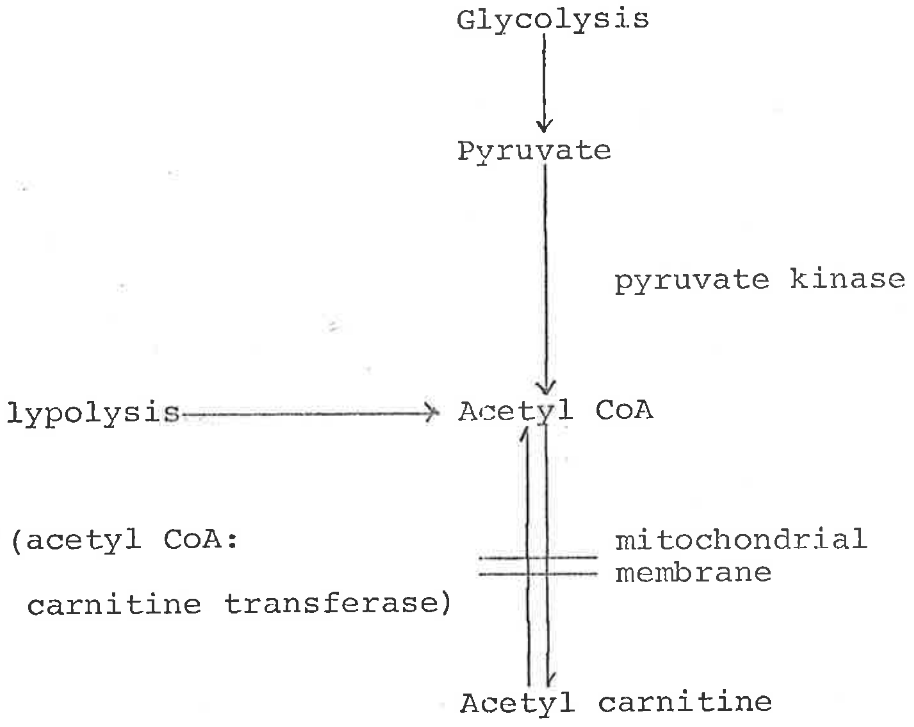

Glycolysis
Pyruvate
pyruvate kinase
lypolysis
Acetyl CoA
(acetyl CoA:
carnitine transferase)
mitochondrial
membrane
Acetyl carnitine

Fig 1.2b

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ENZYMES AND PROTEINS

Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37), from pig heart, lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) type I from rabbit heart, glutamate:pyruvate transaminase (L-alanine: 2-oxoglutarate amino transferase, EC 2.6.1.2) type I from pig heart, glutamate:oxaloacetate transaminase (L-aspartate: 2-oxoglutarate amino transferase, EC 2.6.1.1), type I from pig heart, citrate synthase (citrate oxaloacetate lyase [CoA-acetylating] EC 4.1.3.7) type III from pig heart and 3'-nucleotidase (3'-ribonucleotide phosphohydrolase; EC 3.1.3.6) type III from rye grass were supplied by the Sigma Chemical Co., St. Louis, Mo., U.S.A. Avidin was supplied by Worthington Biochemical Corporation, New Jersey, U.S.A.

2.1.2 RADIOACTIVE MATERIALS

Sodium [2-^{14}C] pyruvate, sodium [^{14}C] bicarbonate, [1-^{14}C] acetyl CoA, [U-^{14}C] ethylene dibromide, and [G-^{3}H] CoA were obtained from The Radiochemical Centre, Amersham, England. [^{32}P] orthophosphate was supplied by the Australian Atomic Energy Commission, Lucas Heights, Australia.

2.1.3 GENERAL CHEMICALS

ATP (disodium salt, Grade I), ADP (disodium salt, Grade I) NADH, CoA (Grade I), oxaloacetic acid, pyridoxal phosphate, sodium pyruvate (type II, dimer free), 2-mercaptoethanol, TNBS, tri-*n*-octylamine, D-pantethine, 1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene, 2,5-diphenyl-

oxazole, DTE and Trizma base were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. N-ethylmorpholine was obtained from Eastman Organic Chemicals and polyethylene glycol (molecular weight 20,000) from Union Carbide Corporation. Sodium glutamate, 1,3 dichloroacetone and L-lysine hydrochloride were obtained from British Drug Houses. $MgCl_2$ was prepared from Spec-pure magnesium (Hilger-Watts Ltd., London) and redistilled HCl, and was standardised by titration against EDTA, using Eriochrome Black as an indicator (Vogel, 1961), Triton X-100 was supplied by I.C.I. (Australia) Ltd., Melbourne. Polyethyleneimine thin layers were obtained from Machery-Nagel and Co., Duren, Germany. Cellulose and silica gel thin layers were obtained from Eastman-Kodak Co., NY., U.S.A.

2.2 METHODS

2.2.1 PREPARATION AND PURIFICATION OF NUCLEOTIDES

(a) Acetyl CoA

Acetyl CoA was prepared by a slight modification to the method of Simon and Shemin (1953) and purified by ascending chromatography on Whatman 3MM paper using isobutyric acid (57.7 ml) adjusted to pH 4.3 using tri-n-ethylamine and diluted to 100 ml with water. The band (Rf ca.0.5) was cut out and eluted with 10^{-5} M EDTA, concentrated, and the concentration adjusted to 5mM ($\varepsilon^{mM}_{260nm} = 16.4$; Dawson, *et al.*, 1969). The product obtained in 80-90% yield, had a ratio of extinction 232/260nm (thioester/adenine moiety) of 0.575, compared with the value of 0.53 from Dawson, *et al.*, (1969).

(b) Acetyldephospho CoA

To a solution of acetyl CoA (3 ml, 5 mM) was added an equal volume of 0.1M Tris Cl buffer (pH 7.4, containing 1.5 E.U. per ml of 3'-nucleotidase) at room temperature. The progress of the reaction was followed by determining the release of orthophosphate using the method of Lin and Morales (1977). After six hours the reaction was complete and the mixture was applied to a DEAE cellulose column (3 cm x 10 cm) and eluted using a linear LiCl gradient from 0.08M to 0.3M as described by Moffatt and Khorana (1961). A_{260nm} for each fraction was determined (Fig. 2.1) and fractions 24 to 26 were pooled and found to have a 232/260nm ratio of 0.55. The pooled material was lyophilised, desalted by BioGel P_2 (170 cm x 2 cm) column chromatography and the fractions containing the 260nm absorbing material were pooled and lyophilised. Using two different chromatographic systems, the material had an Rf value identical with an acetylated dephospho CoA sample obtained commercially. The compound was stable over long periods when stored in aqueous solution at -80^{0}C.

(c) Acetyldesamino CoA

Acetyldesamino CoA was prepared according to the method described by Kaplan (1956) for the preparation of inosine triphosphate from adenosine triphosphate. The $NaNO_2$ solution was added dropwise to the acetyl CoA solution. Immediately after addition of the nitrite, a suitable aliquot was removed and its A_{265nm} determined. When there was no further decrease in the reading the mixture was purified by Sephadex G_{10} chromatography.

2.2.2 PREPARATION AND PURIFICATION OF D-PANTETHINE DERIVATIVES

(a) Cyclic-2'4'-phosphopantethine

Cyclic-2'4'-phosphopantethine was prepared by a method similar to that of Baddiley and Thain (1953) using commercial D-pantethine as the starting compound. The product was identified using the chromatography system of Baddiley and Thain (1953).

(b) Acetyl-2'4'-phosphopantetheine

Acetyl-2'4'-phosphopantetheine was prepared from reduced 2'4'-phosphopantetheine by the method of Simon and Shemin (1953) and purified by Sephadex G_{10} chromatography. The product had thioester content from absorbance at 232nm and moved as a single spot by a thin layer chromatography in two different solvent systems.

(c) Acetyl pantetheine

Acetyl pantetheine was prepared from D-pantethine by reduction with sodium borohydride followed by acetylation using the method of Simon and Shemin (1953). The compound was purified by Sephadex G_{10} chromatography eluted with acidified water.

2.2.3 PURIFICATION OF PYRUVATE CARBOXYLASE

Pyruvate carboxylase was purified from freeze-dried sheep liver mitochondria by the following procedure. The mitochondria (120g) were suspended in 1750 ml of extraction buffer which contained 25mM Tris acetate, pH 6.7, 3.5mM$MgCl_2$, and 1.7mM ATP. The pH was maintained between 6.5 and 6.7 during addition of the mitochondria.

The suspension was stirred for twenty minutes, and undissolved material removed by centrifuging (23000g, for 20 minutes, at 4^{0}C).

Ammonium sulphate was added to the supernatant to give a final saturation of 33% (19.6g ammonium sulphate per 100 ml solution). . The pH was maintained between 6.9 and 7.1 during addition of the ammonium sulphate. The suspension was stirred for twenty minutes and precipitated protein collected by centrifuging (23000g for 20 minutes, at 4^{0}C). The precipitate was suspended in 26% saturated ammonium sulphate to a final volume of 5 ml per 10g of mitochondria. This procedure removed a substantial proportion of the contaminating glutamate dehydrogenase.

The precipitated protein was collected by centrifuging (23000g, for 10 minutes, at 4^{0}C) and suspended in 25mM potassium phosphate, pH 7.2, containing 1mM EDTA, 0.1mM DTE and 0.5% saturated with ammonium sulphate, to give a final protein concentration of 10 mg/ml. This suspension was stirred for thirty minutes and precipitated protein was then collected by centrifuging (23000g, for 20 minutes, at 4^{0}C). The protein was then suspended in 5-6 ml of the phosphate buffer described above, per 10g of mitochondria, and undissolved protein removed by centrifuging (23000g, for 10 minutes, at 4 C). The supernatant, containing essentially all the pyruvate carboxylase was loaded on to a DEAE-Sephadex A-50 column (14 x 5 cm) previously equilibrated with 25mM potassium phosphate, pH 7.2, containing 1mM EDTA, 0.1mM DTE, and 1% saturated with ammonium sulphate, in the above buffer. Pyruvate carboxylase of specific activity 15-20 units/mg protein was routinely obtained using this procedure. The major contaminant of this enzyme preparation was glutamate dehydrogenase. If necessary this contaminant was removed by affinity chromatography, using a NAD$^+$-Agarose column (1 x 5 cm) previously equilibrated with 50mM Tris Cl pH 7.4, containing 0.1mM DTE. Pyruvate carboxylase dissolved in 0.1M N-ethylmorpholine-Cl, pH 7.2, containing 1.6M sucrose, was applied to the column, which was then washed with 50 ml of 50mM Tris Cl, pH 8.4, containing 0.1mM DTE. The enzyme was eluted using a linear gradient of 0.05 - 0.5M Tris Cl, containing 0.1mM DTE. There was usually a 100% recovery of enzyme, with increase in specific activity to values approaching 30 E.U./mg. and was homogeneous as judged by SDS acrylamide gel electrophoresis (Fairbanks, *et al.*,1971).

(a) Storage

For long term storage the enzyme was kept at 200-800 E.U./ml. dissolved in 0.1M N-ethylmorpholine-Cl, pH 7.2 containing 1.6M sucrose and stored frozen at -80°C. However, for a series of experiments which required samples of enzyme with identical purity it was found that storage at -80°C in any of the usual buffers used with pyruvate carboxylase, was satisfactory and the enzyme maintained its original activity for long periods unless thawed and re-frozen at intervals.

2.2.4 DETERMINATION OF RADIOACTIVITY

Samples dried on to solid supports (2cm x 2 cm squares of Whatman 3 MM paper) were placed in vials containing 2 ml scintillation fluid (0.3% ($^w/v$) 2,5-diphenyloxazole, 0.03% ($^w/v$) 1,4-bis-2 (4-methyl-5-phenoxazolyl)-benzene, in sulphur-free toluene; Bousquet and Christian, 1960) and counted in a Packard Scintillation Spectrometer. When the samples contained coloured material, (as was the case in the radiochemical pyruvate carboxylase assay) correction was made for colour quenching using the channels ratio method, Baillie, 1960. Liquid samples were placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toluene scintillation fluid, as above, containing Triton X-100, 7:3 $^{v}/v$), and counted in a Packard Scintillation Spectrometer.

2.2.5 MEASUREMENT OF PYRUVATE CARBOXYLASE ACTIVITY

(a) The spectrophotometric assay system

This continuous assay procedure, based on that described by Utter and Keech (1963) involves reduction of the oxaloacetate produced by the pyruvate carboxylase reaction, using malate dehydrogenase, with concommitant oxidation of NADH to NAD^+. Assay solutions contained, (in μmoles) in a final volume of 1.0 ml; Tris Cl, pH 8.4, (100); ATP, (2.5); $MgCl_2$, (5); HCO_3^-, sodium salt (20); pyruvate, sodium salt, (10); acetyl CoA, (0.25),(unless otherwise stated); NADH, (0.125); malate dehydrogenase, (5 units); and pyruvate carboxylase (0.025-0.1 units).

The reaction was followed at 340nm, using either a Unicam SP800 spectrophotometer, or a Varian-Techtron 635-0 spectrophotometer. The cell block was thermostatted at 30°C. The rate of oxaloacetate synthesis was calculated assuming an extinction coefficient at 340nm for NADH of $6.22mM^{-1}$ cm^{-1} (Dawson, *et al.*, 1969).

A more accurate estimate of the extinction coefficient of $6.3mM^{-1}$ cm^{-1} has recently been published

(Bergmeyer, 1975) but the former value was retained for consistency.

(b) The radiochemical assay system

In this procedure [^{14}C] bicarbonate fixed in an acid stable form is measured, while unreacted bicarbonate is driven off by acidification and subsequent drying on paper squares. Assay solutions contained, (in μmoles), in a final volume of 0.5 ml; Tris Cl, pH 8.4, (50); ATP, (1.25); $MgCl_2$, (2.5); [^{14}C]HCO_3^-, sodium salt, 5 x 10^5 cpm/μmole, (5); pyruvate, sodium salt, (5); acetyl CoA, (0.125),(unless otherwise stated), and up to 0.06 units of pyruvate carboxylase. The reaction was initiated by addition of enzyme and allowed to proceed for a time up to five minutes at 30 ^{0}C before being quenched by addition of 50μℓ of a saturated solution of 2,4-dinitrophenylhydrazine in 6M HCl. As well as terminating the reaction, this reagent drives off unreacted [^{14}C] bicarbonate and stabilises the oxaloacetate formed as the dinitrophenylhydrazone. Triplicate samples (0.05 ml) were applied to 2 cm squares of Whatman 3MM paper, dried at 100 ^{0}C for five minutes, and the remaining radioactivity determined as described in Section 2.2.4. Recently, however, the 2,4-dinitrophenylhydrazine solution has been replaced by a solution of 2N semicarbazide in 2N HCl. The colourless semicarbazone solution was prepared for counting in the same way but quench correction by the channels ratio method was no longer necessary. This procedure was found to be more convenient than the spectrophotometric assay for experiments involving a large number of assays. Moreover, use of the radiochemical assay obviated the need to measure the reaction rate in the presence of a ligand (NADH) which was not essential for pyruvate carboxylase activity.

The acetyl CoA-independent assay system used was that developed by Ashman (1973). The assay solutions contained, (in µmoles), in a final volume of 0.5 ml; Tris Cl, pH 8.4, (50); $MgCl_2$, (4); $[^{14}C]HCO_3^-$, sodium salt, 5×10^5 cpm/µmole, (20); pyruvate, sodium salt, (20); NH_4Cl, (50), and 2 units of pyruvate carboxylase. Reactions were initiated by addition of ATP (1.25 µmole) and after 30 - 60 sec. $[^{14}C]$-oxaloacetate was determined by the procedure described above.

(c) ATP:Pi isotopic exchange reaction assay

Reaction mixtures contained, (in µmoles), in a final volume of 0.5 ml:Tris Cl, pH 8.4, (50); ATP, (1.25); ADP, (0.75); $MgCl_2$, (4); HCO_3^-, potassium salt, (1); acetyl CoA, (0.125), (unless otherwise stated); phosphate, potassium salt, (5); and pyruvate carboxylase, 0.5-1 units. In the controls, pyruvate carboxylase that had been specifically inactivated by previously treating with avidin was used. After incubating the reaction mixtures at 30 °C for ten min. the reaction was initiated by addition of carrier-free $[^{32}P]$ orthophosphate ($1-2 \times 10^6$ cpm) which had been purified by passage through a charcoal column. Samples (0.2 ml) were taken at various time intervals and quenched with 0.1 ml 5M formic acid, at 0 °C. The quenched exchange reaction mixtures were then treated in the following procedure which was developed after it was found that the polyethyleneimine paper procedure (Scrutton, Keech and Utter, 1963) was unsatisfactory for large numbers of exchange reactions.

Aliquots of the quenched exchange mixture were diluted into approximately 1 ml of H_2O in scintillation vials and Cerenkov radiation determined using a 50% gain setting. This gave the total amount of orthophosphate added to each exchange reaction. Larger aliquots (50 - 100µℓ) of the same exchange reaction mixtures were then diluted into approximately 1 ml, 0.1M KH_2PO_4 at 0^0C and stirred before the addition of a suspension of freshly activated charcoal (200µℓ:200mg/ml in 6N HCl. The mixture was stirred at intervals for 10 minutes and kept at 0^0C. Each mixture was then filtered through Whatman GF/A circles and washed three times with 0.1N HCl, 0^0C (20 - 50 ml) and given a final rinse with H_2O at 0^0C. The filters were then transferred to pyrex centrifuge tubes, covered with 3.0 ml 6N HCl and then tightly capped. The tubes were placed in a boiling water bath for 20 minutes, then cooled before centrifugation at 5000 x g for 5 minutes. An aliquot of the supernatant (2.0 ml) was removed for counting for Cerenkov radiation at the same time as the total orthophosphate counting. Using this procedure duplicate estimations agreed to within 5%. A similar procedure had been used previously (Priess and Wood, 1964).

(d) Pyruvate/oxaloacetate isotopic exchange reation assay

Assays contained, (in µmole), in a final volume of 0.5 ml:Tris Cl, pH 8.4, (50); oxaloacetate, (1); pyruvate containing sodium [2-^{14}C]pyruvate (0.005 µmole, 4 x 10^7 cpm/ µmole), (1), acetyl CoA, (0.125),(unless otherwise stated), and pyruvate carboxylase, 0.1 units. Usually the reaction was initiated, after five minutes incubation at 30 °C, by addition of oxaloacetate.

At various time intervals, samples (0.1 ml) were withdrawn and the reaction quenched with 0.01 ml avidin (10 mg/ml) in 0.02M sodium phosphate, pH 7.4). After two minutes, pyruvate and oxaloacetate were converted to alanine and aspartate by the addition of 0.05 ml of 0.05M sodium glutamate, 0.02 ml of 2mM pyridoxal phosphate, and 0.02 ml sodium phosphate, 0.1M, pH 7.4, containing one unit each of glutamate/pyruvate transaminase, and glutamate/oxaloacetate transaminase. The transamination reaction was allowed to proceed for fifteen minutes at 30 °C and then quenched with 0.025 ml of 5M formic acid. Alanine and aspartate were separated by electrophoresis on a water cooled flat-bed apparatus in 0.04M sodium acetate (pH 5.5). Samples (0.02 ml) were applied to Whatman 3 MM papers which included lanes for marker alanine and aspartate samples. It was found that the number of samples which could be applied to one electrophoresis paper could be doubled by the use of a simple procedure. Lines drawn on the electrophoresis paper before sample application were soaked along their length with a carefully applied saturated solution of paraffin wax in chloroform. This provided a non-wettable division between channels which prevented overlap of samples during electrophoresis. The paraffin did not interfere with mobility of samples during electrophoresis, nor with counting of the papers. Electrophoretograms were developed for 45 minutes at 2600 V, 60mA (40V/cm, 2mA/cm). After drying, the aspartate and alanine markers were located by spraying with 0.5% ninhydrin in acetone. Sample lanes were cut into 1 cm pieces and radioactivity determined as described in Section 2.2.4. Since contaminant parapyruvate moved very close to aspartate on the electrophoretograms, correction was made for it by subtracting controls from which enzyme was omitted. The rate of isotope exchange was calculated using the formula: (Wahl and Bonner, 1951)

$$\text{rate} = -\frac{[\text{pyr}]\,.\,[\text{OAA}]}{[\text{pyr}] + [\text{OAA}]}\;.\;\frac{\ln(1-F)}{t}$$

where t is time of exchange, and F, the fractional equilibrium, is given by the expression:

$$F = \frac{\%\ \text{label in oxaloacetate}}{\left(\frac{[\text{oxaloacetate}]}{[\text{pyruvate}] + [\text{oxaloacetate}]} \times 100\right)}$$

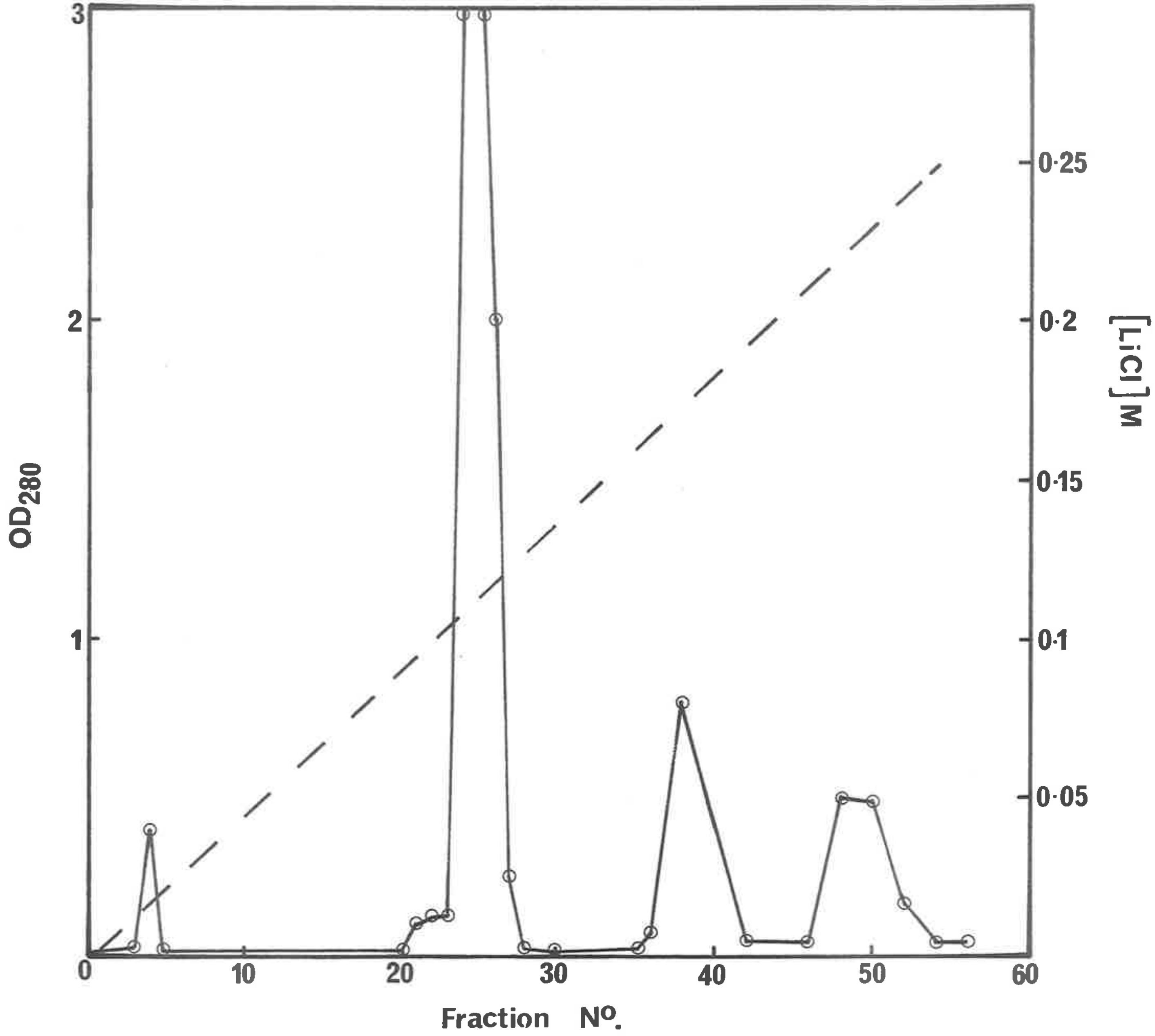
2.2.6 DETERMINATION OF BIOTIN CONCENTRATION

Biotin concentration was determined using the radiochemical assay developed by Rylatt, *et al.*, (1977). The assay solution contained, in a final volume of 0.5 ml; 0.1M potassium phosphate, pH 7.2 (50 µmole ~~biotin~~ avidin). After incubating for 10 minutes at room temperature, 0.05 ml [^{14}C] biotin (2.2 nmole, 10^5 cpm/nmole) was added, and 10 minutes later the avidin-biotin complex was precipitated by adding 0.2 ml 0.1M zinc sulphate, followed by 0.2 ml 0.1M sodium hydroxide. The precipitate was collected by centrifuging, and samples of the supernatant taken and their radioactivity determined using the Triton based scintillant (Section 2.2.4). The correlation between the radioactivity of the supernatant and the biotin content of the sample was established by constructing a standard curve using known amounts of unlabelled biotin as reference samples. There was a linear relationship between biotin content and radioactivity for the range 10 - 100pmoles of biotin.

Fig. 2.1 Elution profile of acetyldephospho CoA purification by DEAE cellulose column chromatography using a linear LiCl gradient

The concentrations of LiCl at which each compound began to be eluted were:-

1.	3' nucleotidase	0.013M
2.	acetyldephospho CoA	0.105M
3.	acetyl CoA	0.165M
4.	oxidised dephospho CoA	0.21M


3
2
1
0
OD280
0·25
0·2
0·15
0·1
0·05
[LiCl] M
10
20
30
40
50
60
Fraction Nº.

CHAPTER 3

AFFINITY LABELLING

3.1 INTRODUCTION

3.1.1 OBJECTIVES IN THE DESIGN OF AN AFFINITY LABEL FOR THE ACETYL CoA BINDING SITE OF PYRUVATE CARBOXYLASE

The objective of this study was to covalently modify the binding site of acetyl CoA on pyruvate carboxylase. This would facilitate a study of the relationship between other ligand binding sites and the modified site. Similar modification studies of all the ligand binding sites are currently being carried out in this laboratory and the sequences of the peptides isolated by these procedures could be used to construct at least the primary structure of the various binding sites. One such study has led to the modification of the acetyl CoA binding site using TNBS (Ashman *et al.*, 1973) and isolation of the TNP-peptide would be of further interest in its relationship to other affinity labelled peptides in the acetyl CoA binding site.

As outlined in the General Introduction, 1.2.2, the moieties of acetyl CoA which were found to be most important in activation, were (i) the acetyl group, (ii) the 3'phosphate group and (iii) the 6-amino group. The reactive compounds which were used in this chapter were chosen with the importance of these groups in mind. For a large molecule such as acetyl CoA the placement of a reactive group on or nearby an important moiety would yield the most useful information.

The acetyl group was considered to be the most important moiety for study and provided a convenient point of attachment for reactive groups. A published procedure for the synthesis of 2-bromoacetyl CoA, I (Fig. 3.1) provided access to a compound which seemed to be ideal for the specific modification of pyruvate carboxylase at the acetyl group region of the acetyl CoA binding site. Two other reactive analogues located at the acyl group position were also tested viz. 2-iodoethyl CoA II and 3-chloroacetonyl CoA III (Fig 3.1).

The 3'phosphate group was another important group in the activation by acetyl CoA and offered several possibilities for reactive group attachment. Those considered were 3'-fluoro sulphonyl and 3'-fluorophosphate groups but both would undergo cyclisation by nucleophilic attack from the 2'OH group of ribose ([illegible]). Another reactive group considered was 2'-bromoacetate similar to that used by Pincus *et al.*, (1975) but the 2'OH group of the pantetheine moiety would have been similarly modified. While this may ultimately prove to be an advantage in dual modification of the site in later studies, it was considered that as an initial study the identification of modified residues would have presented problems. Acetyl CoA dial IV was a reactive compound which could be conveniently synthesised and had precedent in the literature.

3.1.2 THE APPROACH

Three criteria discussed by Singer (1970) were used as the basis of the approach for the affinity labelling studies presented in this chapter. The criteria must be rigorously satisfied so that any reactive ligand analogue can be said to be specific and thus justify the title of "affinity label". The first of these criteria is that the target protein should undergo "stoichiometric inactivation". This requires that the number of molecules of inhibitor bound per active site should be equal to the fraction of binding sites inactivated. Singer states, however, that this criterion becomes less useful for complex receptor systems and would therefore place in doubt the relationship between inactivation and binding in an allosteric situation.

The second and more useful criterion is that the ligand should provide "specific protection against inactivation". This relies upon the affinity between the ligand and its binding site being able to prevent or at least greatly reduce the rate of the inactivation caused by the reactive analogue. Should any protection against modification occur in the presence of ligands which bind specifically to the enzyme other than the ligand under study, it could then be concluded that the affinity label lacked specificity for the target site.

The third criterion was that of "affinity inactivation" which involves inactivation of the target enzyme with a compound bearing the same reactive functional group but no specific affinity for the target site. Such a compound should not possess the properties expected of an affinity label as outlined above.

An interesting development in affinity labelling is the criterion of specificity called "catalytic competence" (Groman *et al.*, 1975). These authors were able to demonstrate that enzyme which had been covalently modified with a reactive substrate analogue could catalyse the conversion of the bound substrate analogue to product. This principle has been extended in the case of deazaflavin which can act as a coenzyme when bound covalently to the enzyme (Jorns and Hersh, 1976). This criterion of specificity could be applied equally well to any analogue of acetyl CoA which can either activate or prevent dilution inactivation after covalent attachment.

3.1.3 SOME KINETIC CONSIDERATIONS

Some useful information can be obtained from inactivation experiments before attempting to isolate the modified peptide and identify the modified residue. The first kinetic parameter to be determined is usually to show that the affinity label obeys saturation kinetics. This can be determined from modification of the enzyme in the presence of different concentrations of the label. The data plotted as the pseudo first-order inactivation constants ($k_{inact.}$) versus concentration of inhibitor reaches a plateau region at increased levels of inhibitor if the inactivation process obeys saturation kinetics. This indicates that only one site on the enzyme has undergone chemical modification. Determination of the second-order rate constant can also be made from the slope of such a plot. (Meloche, 1967).

Further information can be obtained if the same data is plotted in the form of log $k_{inact.}$ versus log concentration of inhibitor. The straight line obtained from this plot has a slope of 1.0 when one molecule of inhibitor is involved in the inactivation process. The rationale of this plot can be found in a report of work done in this laboratory (Keech and Farrant, 1968) in which FDNB was used to covalently modify pyruvate carboxylase.

Some interesting facts were obtained from the kinetic treatment of data wherein Pincus *et al.*, (1975) were able to demonstrate the contribution to the inactivation rate made by the binding of the label to the enzyme. The authors used an estimate of the first order rate constant for reactions carried out between inhibitor and enzyme and between inhibitor and free amino acids. These estimates also enabled comparisons to be made between the action of the inhibitor and of other alkylating reagents. The contribution to the reaction rate made by the binding factor was shown to be a factor of 25 for their particular affinity label.

3.2 METHODS

3.2.1 THIN LAYER CHROMATOGRAPHY

Cellulose plates containing fluorescent indicator were developed in isobutyric acid:sodium isobutyrate (57.7 ml isobutyric acid adjusted to pH 4.3 with NaOH and diluted to 100 ml with water). Nucleotides were detected by ultraviolet absorption and R_f values for acetyl-SCoA and CoASH were 0.80 and 0.72 respectively. An alternative solvent was acetonitrile:2% acetic acid (1:1, v/v) in which the R_f values for acetyl-SCoA and CoASH were 0.74 and 0.58 respectively. Chromatograms of these compounds using polyethleneimine plates were developed in either 0.8M NH_4HCO_3 or in 1.2M LiCl as indicated in the text.

3.2.2 DETECTION OF ALKYLATING ACTIVITY

To detect alkylating activity, a spray procedure was adapted from Epstein *et al.*, (1955) and consisted of successive spraying with potassium biphthlate (50mM); 2% γ(4-nitrobenzyl) pyridine in acetone; and 0.5M NaOH.

Drying at 100 ^{0}C for 5 mins. was performed between each step. Alkylating activity was displayed as a purple to blue colour which faded within a few minutes after the alkali spray. The colour could be regenerated briefly upon respraying the dry plate with alkali, This procedure, hereafter referred to as the NBP spray procedure, can detect 10^{-6} moles of an alkylating compound on a thin layer chromatography plate. However, this test for alkylating activity could not be used on polyethyleneimine plates because the spray also gives a positive reaction with polyethyleneimine.

3.3 THE ACETYL GROUP-DERIVED LABELS

3.3.1 2-BROMOACETYL-SCoA

3.3.1.1 INTRODUCTION

The use of 2-bromoacetyl-SCoA as an affinity label of acetyl-SCoA binding enzymes has been reported in the literature (Chase and Tubbs, 1969; Roskoski, 1974). A method for synthesising this CoASH derivative has been published by Chase and Tubbs (1969) and involves the synthesis of 2-bromoacetyl-thiophenol which was then expected to undergo reversible thiol exchange with CoASH to form 2-bromoacetyl-SCoA.

$$BrCH_2COS\phi \quad + \quad CoASH \rightleftharpoons BrCH_2CO\text{-}SCoA \quad + \quad \phi SH \qquad ...3.1.1$$

Since the product obtained using this procedure did not possess the properties expected of 2-bromoacetyl-SCoA, the work of Chase and Tubbs was extended.

Obviously the chemistry involved is not as straightforward as first suggested. For example, the proposed thiol group exchange is, by definition, a reversible process and thus creates an equilibrium situation. However, the presence of alkylating moieties and reactive thiol groups in a basic environment presents the strong possibility of irreversible alkylation reactions occurring. These would eventually compete successfully against the equilibrium reaction and the following reactions would occur to varying extents,

$BrCH_2COS\phi + HS\phi \rightarrow \phi SCH_2COS\phi + HBr$ ...3.1.2

$BrCH_2COS\phi + HSCoA \rightarrow CoAS\text{-}CH_2COS\phi + HBr$ ...3.1.3

$BrCH_2CO\text{-}SCoA + HSCoA \rightarrow CoAS\text{-}CH_2CO\text{-}SCoA + HBr$ ...3.1.4

$BrCH_2CO\text{-}SCoA + HS\phi \rightarrow \phi SCH_2CO\text{-}SCoA + HBr$ ...3.1.5

Evidence is presented here to indicate that the predominant sequence of events obtained by following the Chase and Tubbs procedure is a CoASH nucleophilic attack on 2-bromoacetyl-thiophenol resulting in alkylation rather than acylation. The major product of the reaction was identified as thiophenylcarboxymethyl-SCoA (reaction 3.1.3) Since the procedure for synthesising 2-bromoacetyl-SCoA by this method was not successful, the possibility of direct acylation of CoASH with 2-bromoacetyl bromide was examined. To achieve this aim, it was found that the attachment of the acyl group required anhydrous conditions and therefore, it was necessary to solubilize CoASH in an organic solvent. This required replacement of the lithium ions associated with the CoASH-phosphate groups with the hydrophobic tri-n-octylammonium cations. Under these conditions, 2-bromoacetyl-SCoA was produced but evidence presented here indicates that the extreme lability of the molecule in neutral and basic aqueous media restricts its use as an affinity label.

3.3.1.2 METHODS

(a) Synthesis of carboxymethyl-SCoA

To CoASH (0.5 mg) was added 5 µl of 1M 2-bromoacetic acid in acetonitrile followed by 15 µl of 0.1M NaOH. The solution was incubated for 30 minutes at 20 °C. Rf values in either solvent for CoASH and carboxymethyl-SCoA were 0.73 and 0.41 respectively.

(b) Electrophoresis

CoASH derivatives were electrophoresed on either thin layer cellulose plates or on Whatman 3 MM paper. The solvent system used was pyridine-acetic acid-water (25:1:224, v/v/v, pH 6.5) and separation was achieved using 17.5 V/cm and 2mA for 1.5 h.

(c) Preparation of the tri-n-ocylammonium salt of CoASH

Dowex AG-50W-X12 (1 g) was converted to the tri-n-octylamine form by regeneration with 50 ml washes of 1M HCl, H_2O, 1M NaOH, H_2O, 1M HCl and H_2O followed by equilibration with 1M tri-n-octylammonium acetate solution. An aqueous solution of CoASH (0.5 ml; 13 µmole) was passed slowly through a 2.0 x 1.0 cm column of the resin followed by 20 ml of H_2O. The eluate was collected but since this did not yield complete recovery of the nucleotide, the column was washed with 20 ml of acetonitrile. After removal of the solvent from each eluate under reduced pressure, oily liquid residues remained. The residue from the acetonitrile eluate consisted of two layers; the upper layer which was free of nucleotide, was assumed to be tri-n-octylamine and removed. The absorbance (260nm) of the pooled oily liquid residues indicated complete recovery of the nucleotide.

(d) Synthesis of 2-bromoacetyl-SCoA

The tri-n-octylammonium CoASH solution (13 µmole) in 250 µl of acetonitrile was dried by co-evaporation with three cycles of acetonitrile (dried over calcium hydride) under reduced pressure. The residue was dissolved in o.5 ml of dry acetonitrile, removed from the vacuum line and, if unused, was stored at 0^{0}C under anhydrous conditions. To the anhydrous preparation of tri-n-octylammonium CoASH was added a 1.4 molar excess (18 µmole) of 2-bromoacetyl bromide in 150 µl of dry dioxan and incubated at 20^{0}C for 30 minutes. Unreacted 2-bromoacetyl bromide, HBr and solvent were removed under reduced pressure, leaving the product as an amorphous white solid.

The synthesis of 2-bromoacetyl-SCoA was followed by thin layer cellulose chromatography in 58% sodium iso-butyrate (pH 4.3) and the product spot, detected by ultra-violet absorption, had an R_f value of 0.95 indicating the hydrophobic influence of the tri-n-octylammonium CoASH moiety. When sprayed with NBP solution, an intense purple colour, coincident with the product spot, was observed. A marker of 2-bromoacetyl bromide had an R_f value of 0.76 as detected by a blue colour reaction following the NBP spray procedure. Thin layer chromatography was repeated using acetonitrile-acetic acid as the developing solvent and similar results were obtained. Electrophoresis, however, was unsuccessful since the hydrolysis of the product was very rapid in the aqueous solvents used, and spots corresponding to CoASH and bromoacetic acid were observed by ultraviolet absorption and NBP spray respectively.

After removal of the organic solvents from the reaction product and subsequent addition of water, thin layer chromatography of the resultant solution revealed that the nucleotide spot no longer possessed any alkylating activity.

3.3.1.3 RESULTS AND DISCUSSION

For the following reasons, we believe that the product obtained using the Chase and Tubbs (1969) method for the synthesis of 2-bromoacetyl-SCoA, was thiophenyl-carboxymethyl-SCoA;

(a) The product lacked alkylating capacity

Although the R_f value (0.8) on thin layer cellulose was different from that of CoASH (0.72) indicating the formation of a CoASH derivative, it failed to give a positive alkylating test as determined by the NBP spray procedure. 2-bromoacetyl-SCoA would be expected to be a very strong alkylating reagent.

(b) Detection of a carboxymethyl-SCoA moiety in the product

Analysis of the charge properties of the product by electrophoresis at pH 6.5 and by thin layer chromatography on polyethyleneimine plates using lithium chloride as developing solvent revealed a mobility similar to acetyl-SCoA suggesting a net charge of -3.5 at pH 6.5. However, after base hydrolysis, electrophoresis at pH 6.5 showed the compound to have a mobility similar to carboxymethyl-SCoA (ie. a net charge of -4.5 at pH 6.5). Similarly, chromatography on polyethyleneimine plates using 0.8M NH_4HCO_3 (pH 8.0) as developing solvent showed that the hydrolytic product had a R_f value of 0.41 which was identical to the R_f value of carboxymethyl-SCoA.

(c) Detection of a thiophenol moiety in the product

After base hydrolysis of the reaction product, a substance, which exhibited an absorbance maximum at 240nm, was isolated by ether extraction. An ethereal solution of thiophenol exhibits a similar absorbance maximum. Furthermore, the detection of a stoichiometric amount of a thiol moiety in the product after base hydrolysis was observed by the use of DTNB and quantitated by its absorption at 412nm (Ellman, 1959). However, ether extraction of the base hydrolysed product completely removed the thiol compound from the aqueous phase indicating the association of the sulphydryl group with a hydrophobic moiety.

These data and the findings reported above suggest that the product was thiophenyl-carboxymethyl-SCoA and that base hydrolysis cleaved the thio-ester link to form thiophenol and carboxymethyl-SCoA.

(d) Reaction of the product with citrate synthase

To confirm the identity of their product, Chase and Tubbs followed the release of free thiol groups by observing the change in absorbance at 412nm when they reacted their product with citrate synthase, oxaloacetate and DTNB. The rationale for this procedure was that bromocitrate and CoASH should be formed and the reaction could be monitored by following the release of free thiol groups. However, it was observed that the compound gave a stoichiometric release of a thiol compound at pH 8.0 as determined by the DTNB assay in the absence of any enzyme. Therefore, the procedure was varied for following citrate synthase activity by replacing the oxaloacetate with malate, malate dehydrogenase and NAD^+ (Ochoa, 1957). Under these conditions, no NADH was formed as determined by measuring the change in absorbance at 340nm.

These results indicated that citrate synthase was not able to utilise the compound as a substrate and that the release of the thiol group was primarily a function of the pH. Finally, it was observed that freshly prepared product was odourless but after storage for seven days at $-15^{0}C$, the characteristic odour of thiophenol could be detected.

(e) Equivocal results obtained in the presence of various enzymes

i) Acetyl carnitine transferase

The linking of the reaction product to carnitine in the presence of acetyl carnitine transferase was observed by Chase and Tubbs (1969) and postulated to occur by the following sequence:-

$BrCH_2CO\text{-}SCoA + HO\text{-}carnitine \rightarrow CoASH + BrCH_2CO\text{-}O\text{-}carnitine$ ...3.1.6

$BrCH_2CO\text{-}O\text{-}carnitine + CoASH \rightarrow CoAS\text{-}CH_2CO\text{-}O\text{-}carnitine + HBr$ ...3.1.7

However, this result is just as easily explained if the compound was thiophenyl-carboxymethyl-SCoA, ie.,

$\phi\text{-}S\text{-}COCH_2\text{-}SCoA + HO\text{-}carnitine \rightarrow CoAS\text{-}CH_2CO\text{-}O\text{-}carnitine + \phi\text{-}SH$ ...3.1.8

It seems unlikely that the mechanism of bromoacetyl group exchange, as proposed by the above investigators, would occur without some alkylation of reactive groups on the surface of the enzyme. Furthermore, alkylation of carnitine may form an alternative inhibitor but this was not reported.

ii) Acetyl choline transferase

Similarly, the reversible inhibition of acetyl choline transferase was postulated by Roskoski (1974) to occur as follows:-

$BrCH_2CO\text{-}SCoA + HS\text{-}enz \rightleftharpoons BrCH_2CO\text{-}S\text{-}enz + CoASH$ ...3.1.9

However, the existence of a reactive 2-bromoacetyl group in close proximity to a reactive enzyme thiol group without some irreversible alkylation seems improbable. However, the reversible inhibition can be explained if the reaction product was thiophenyl-carboxymethyl-SCoA, ie.,

$CoASCH_2CO\text{-}S\text{-}\phi + HS\text{-}enz \rightleftharpoons CoASCH_2CO\text{-}S\text{-}enz + \phi\text{-}SH$ ...3.1.10

3.3.1.4 THE PROPERTIES OF 2-BROMOACETYL-SCoA

As indicated in section 3.3.1.2 (d), 2-bromoacetyl-SCoA was readily hydrolysed at pH values of 7.0 or above. The lability of the thioester bond in aqueous solution was predictable since the electron withdrawing effect due to bromine would produce a more stable bromoacetate leaving group upon nucleophilic attack by water. Although, the stoichiometry of 2-bromoacetyl-SCoA production was difficult to assess due to its inherent lability, on the basis of the following observations, it could be argued that no other species was produced. These were; (a) only one nucleotide spot was present on thin layer chromatograms developed in the isobutyric acid solvent and alkylating activity was associated with this spot, (b) at pH 8.0 there was a stoichiometric release of thiol groups as determined by DTNB analysis thus indicating that the sulphydryl group was neither alkylated nor oxidised, and (c) electrophoresis in aqueous medium eliminated alkylating activity associated with the nucleotide. As only thioesters would exhibit such lability, this indicates that no group (eg 2'-OH of ribose or 2'-OH of pantothenate) other than the thiol group was bromoacetylated. Therefore, these results suggest that 2-bromoacetyl-SCoA would have little value as an affinity label in an aqueous environment at or above neutral pH values although its stability in the sodium isobutyrate solvent indicates a possible use under acidic conditions.

Transcarboxylase, an enzyme which possesses an acyl CoA binding site and is stable at pH 5.5 presented the possibility of an application for 2-bromoacetyl-SCoA. The enzyme was known to possess two acyl CoA binding sites on each 12 S_H subunit and each 12 S_H subunit was known to be formed of a single polypeptide chain (Wood and Zwolinski, 1976). It was therefore of interest to identify and compare the two binding sites by covalent modification using 2-bromoacetyl-SCoA.

Using transcarboxylase kindly supplied by Professor H.G. Wood, all attempts at modification were unsuccessful, however, and it was assumed that either the acyl CoA binding sites do not possess reactive residues or that the pK_a values of any residues are such that they are not sufficiently reactive at pH 5.5 to allow covalent modification to occur. If the latter explanation is the correct one it would further suggest that 2-bromoacetyl-SCoA is completely unsuitable as an affinity label.

3.3.2 HALOETHYL CoA DERIVATIVES

3.3.2.1 INTRODUCTION

The lability to hydrolysis of the thioester bond in 2-bromoacetyl CoA precluded its use as an affinity label of pyruvate carboxylase. This conclusion prompted work towards the synthesis of a derivative of CoA with a stable C-S bond. One such derivative, identical in carbon chain length but lacking the carbonyl group was 2-bromoethyl-SCoA. Greater reactivity of this species relative to 2-bromoacetyl CoA was expected due to the electron withdrawing carbonyl group in the latter compound. This would result in greater effectiveness of the halogen as a leaving group. The greater alkylating activity was expected to result in lack of specificity in the compound but the specificity conferred by the CoA part of the molecule was expected to offset this problem to some extent. The advantages which would be afforded by a stable link between CoA and the enzyme favoured the investigation of the effects which 2-bromoethyl CoA might have on the enzyme. The non-reactive analogue ethyl CoA was first synthesised in order to establish competitive binding with respect to acetyl CoA, of a compound lacking the carbonyl group of the acetyl moiety.

3.3.2.2 METHODS

(a) Synthesis of ethyl-S-CoA

Ethyl iodide (0.08 ml, 1.0 mmole) was added to a solution of CoA (6 mg, 7.8 µmole) dissolved in 0.1M $KHCO_3$: acetone (2:5, $^v/_v$) in a total volume of 2.0 ml and the mixture was stirred at room temperature, for 6 h. and excluded from light. The reaction solution was extracted with ether and the aqueous phase was then desalted by Sephadex G_{10} chromatography. Nucleotide was detected by A_{260nm} determinations and iodide ion content was tested for using silver nitrate. Iodide-free nucleotide containing fractions were pooled and lyophilised. The product had an R_f value of 0.8 on cellulose thin layers developed with sodium isobutyrate.

(b) The synthesis of haloethyl-SCoA

To a solution of CoA (5 mg, 6.4 µmole) in 1 ml of anhydrous DMF was added dibromoethane (100 µl, 1.1 mmole) and tri-*n*-ethylamine (150 µl) and potassium iodide (20 µmole) and the solution was stirred at room temperature for 16 h. and excluded from light. The reaction was followed by chromatography on silica gel thin layers which were developed with tri-n-ethylammonium acetate (pH 5.5): acetonitrile (10:1 $^{v}/v$). The compound had an R_f value of 0.77 and was detected by its absorption of ultraviolet light. The compound gave a strong purple colour when sprayed with the NBP test which indicated that alkylating activity was associated with the nucleotide. The reaction solution was applied to Whatman 3 MM paper using 2% acetic acid:acetonitrile (1:5 $^{v}/v$) to develop the chromatogram. The material was again identified by its ultraviolet absorption and its reaction with the NBP spray test and was cut out, eluted with 0.1M acetic acid and lyophilised.

Although it was not determined directly, the halogen species responsible for the strong alkylating activity of the product was thought to be iodine by the following reasoning. In early experiments to synthesise bromoethyl-S-CoA a catalytic amount of iodide ion was added. This was found necessary since the symmetrical compound, dibromoethane, was completely unreactive toward nucleophilic attack by the sulphydryl group of CoA. The iodide ion was expected to replace one of the bromine atoms by nucleophilic substitution (reaction 3.2.1).

$$BrCH_2CH_2Br + I^- \longrightarrow BrCH_2CH_2I + Br^- \qquad ...3.2.1$$

The unsymmetrical dihalide was then susceptible to nucleophilic attack by the sulphydryl group (reaction 3.2.2) but the compound produced by this method was found to have no NBP positive activity, nor was it capable of inactivating the enzyme.

$$BrCH_2CH_2I + CoASH \longrightarrow BrCH_2CH_2S\text{-}CoA + I^- \qquad ...3.2.2$$

Later, when greater than catalytic concentrations of I^- were present, a compound with NBP positive activity which was capable of inactivating the enzyme, was formed and increased in amount with increase in I^- concentration, as determined empirically by the NBP spray procedure. These results provided circumstantial evidence that the reactive species produced by the above procedure was iodoethyl-SCoA and was formed by the scheme proposed in reaction 3.2.3.

$BrCH_2CH_2$-SCoA + I^- ⟶ ICH_2CH_2-SCoA + Br^- ...3.2.3

(c) The synthesis of 2-iodo [^{14}C]ethyl-SCoA

2-iodo [^{14}C]ethyl-SCoA was prepared by following the procedure for the synthesis of 2-iodoethyl-SCoA but using 1,2-dibromo[U - ^{14}C]ethane. The product was purified using the same procedure described for the unlabelled compound and its specific activity was determined to be 56,900 cpm/nmole.

(d) The synthesis of iodoethyl cysteine

Cysteine HCl (8.0 mg, 66 μmol) was dissolved in anhydrous DMF (1 ml) to which was then added dibromoethane (200 μl, 2.2m mol), triethylamine (200 μl) and potassium iodide (100 μmol). The solution was allowed to react at room temperature in the dark for 2 h. The reaction was followed by thin layer chromatography on silica gel in 2% acetic acid/acetonitrile (5:1 $^v/v$). After 2 h. a single spot with both NBP positive activity (sprayed first) and ninhydrin positive material was observed with R_f 0.82. The compound was purified by paper chromatography, the spots were located by their ninhydrin positive reaction, cut out and eluted with acidified H_2O. The eluate was lyophilised in 3 μmole aliquots.

3.3.2.3 THE EFFECT OF ETHYL-SCoA ON THE ACTIVITY OF PYRUVATE CARBOXYLASE

In order to determine whether ethyl-SCoA can replace acetyl CoA as an activator of pyruvate carboxylase its effect was tested on the overall reaction catalysed by the enzyme. The response obtained was sigmoidal as presented in Fig. 3.2. A double reciprocal plot of the data as presented in Fig. 3.2 (insert) was concave upward and the K_a value of 0.65mM was obtained from this plot. The V obtained in the presence of 2mM ethyl-SCoA was 100% of that obtained in the presence of 0.25mM acetyl CoA.

This result provided evidence that ethyl-SCoA could replace acetyl CoA as an activator of pyruvate carboxylase but that its binding was not as strong as indicated by the higher K_a value. It was concluded that an affinity label derived from ethyl-SCoA would bind to the acetyl CoA binding site of pyruvate carboxylase.

3.3.2.4 COVALENT MODIFICATION STUDIES WITH 2-IODOETHYL-SCoA

(a) The effect of different pH values on the rate of inactivation

The effect of pH on the rate of inactivation of pyruvate carboxylase was investigated in order to establish optimum conditions for subsequent experiments. The results presented in Fig. 3.3 show that inactivation was pH dependent and the process was non-linear at the lower pH values. This suggested that a second process was acting to either reduce the alkylating activity of the label or that a second site was being modified on the enzyme. Subsequent experiments led to the resolution of this problem.

(b) The stoichiometry of inactivation

The rate of inactivation of pyruvate carboxylase was determined in the presence of different concentrations of 2-iodoethyl-SCoA. The results presented in Fig. 3.4 show that although $t_{0.5}$ values were difficult to obtain, the tangent to the initial rates of inactivation could be used to provide approx. $t_{0.5}$ values. Reciprocals of these $t_{0.5}$ estimates were taken as the pseudo first-order rate constants of inactivation and were plotted against inhibitor concentration as presented in Fig. 3.5 to yield a second-order rate constant of 1.9 min^{-1} mM^{-1}. A replot of the data in the form of log k_{inact} versus log $[I]$, as presented in Fig. 3.6, when fitted to a straight line, gave a slope of 0.98 which as a first approximation, indicated that 1 mole of inhibitor was responsible for the loss of enzyme activity.

(c) The stability of 2-iodoethyl-SCoA

To determine whether the non-linear inactivation rates could be due to breakdown of the inhibitor, 2-iodoethyl-SCoA at different concentrations was either exposed to potassium MOPS buffer (pH 6.9) for one min. before addition to enzyme or added directly to the enzyme. Although there were slight differences in the initial rates of inactivation, the extent of inactivation was not significantly altered by exposure to buffer.

Of the possible reasons for the non-linear inactivation rates, breakdown of 2-iodoethyl-SCoA by hydrolysis seemed the most likely. Both the above experiment and the trend towards linearity at higher pH values argue against hydrolysis as an explanation. Subsequent experiments show that the second possibility of a second site of modification was the more likely explanation.

(d) Determination of stoichiometry by radioactive labelling studies

A direct determination of the number of moles of inhibitor bound was carried out with enzyme inactivated by 60% in the presence of 2-iodo [^{14}C] ethyl-SCoA. The number of molecules of inhibitor bound per mole of enzyme was calculated to be 59% of that expected for 1:1 stoichiometry. This encouraging result was followed by further studies into the specificity of the inactivation by 2-iodoethyl-SCoA using protection studies.

(e) Protection studies

In order to test for specific modification of the enzyme, inactivation experiments were carried out in the presence of selected ligands known to bind specifically to the enzyme.

i) Acetyl CoA

Enzyme was incubated in the presence of iodoethyl CoA (0.8 ml) at various concentrations of acetyl CoA. Aliquots of each incubation solution were taken at various time intervals and assayed using the radiochemical assay in the presence of saturating levels of acetyl CoA. The amount of inhibitor carried over into the assay mixture was insignificant in the presence of saturating acetyl CoA.

The results as presented in Table 3.1 show that acetyl CoA protection against iodoethyl CoA inactivation was concentration dependent. However, a plot of the pseudo first-order rates of inactivation against the reciprocal of acetyl CoA concentration as shown in Fig. 3.7 did not extrapolate to zero at infinite concentrations of the protector and was therefore an indication that more than one site of modification was responsible for the loss in enzyme activity in the presence of 2-iodoethyl-SCoA.

ii) Other ligands

The following ligands which bind to pyruvate carboxylase were tested for their effect on the rate of inactivation in the presence of 2-iodoethyl-SCoA:- pyruvate, ATP, ADP, oxaloacetate and a combination of ATP and HCO_3^- (to test for the effect of E.biotin.CO_2 on the rate of inactivation). The results as presented in Table 3.2 show that while acetyl CoA gave some degree of protection, pyruvate and E.biotin.CO_2, as generated by ATP and bicarbonate, were more effective in their protection of the enzyme against inactivation. ATP, ADP and oxaloacetate each stimulated the rate of inactivation.

It was clear that 2-iodoethyl-SCoA was modifying at least two sites on the enzyme which were the acetyl CoA binding site and probably the pyruvate binding site. The protection afforded by E.biotin.CO_2 could be explained on the basis that it may form part of the pyruvate binding site or alter the site in some way that prevents its modification. The non-linear inactivation plots can be readily explained on the basis that there are at least two sites of modification which are inactivated at different rates. Modification of the acetyl CoA site appears to be the slower rate and this suggests that the reactivity of a group in the pyruvate binding site is the primary site of modification. The most likely residue is the cysteine group which was modified previously (Hudson *et al.*, 1975) using 3-bromopyruvic acid. A second and less reactive site of modification was also observed at the higher levels of 3-bromopyruvic acid and this site was protected in the presence of acetyl CoA. The similarity of action of the two reagents suggests that 2-iodoethyl-SCoA is acting as a chemical modification reagent with no apparent specificity for the acetyl CoA binding site. In order to test this possibility the criterion of "affinity inactivation" was invoked.

3.3.2.5 "AFFINITY INACTIVATION" STUDIES USING 2-IODOETHYLCYSTEINE

As stated in the introduction to this chapter, a means of distinguishing between specific and unspecific enzyme modification was in application of the affinity inactivation criterion. This involves the use of a reactive compound with no structural basis for affinity to the target site. Such a compound was 2-iodoethylcysteine which can be seen to have little in common with the structure of acetyl CoA but which contained the same reactive moiety present in 2-iodoethyl-SCoA. The compound would therefore be expected to behave as a chemical modifier.

(a) The effect of different concentrations of 2-iodoethylcysteine on the rates of inactivation of pyruvate carboxylase.

The results presented in Table 3.3 show that enzyme inactivation rates were dependent upon the concentration of 2-iodoethylcysteine. The second-order rate constant was calculated to be 0.049 $\text{min}^{-1}\ \text{mM}^{-1}$ which was 39 fold lower than that determined for 2-iodoethyl-SCoA indicating that binding to the enzyme contributed to the rate of inactivation of the enzyme.

(b) The effect of different substrates on the rates of inactivation of the enzyme in the presence of a fixed concentration of 2-iodoethylcysteine

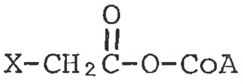
The results, as presented in Table 3.4 show that both acetyl CoA and pyruvate gave protection against inactivation of pyruvate carboxylase by 2-iodoethylcysteine. However, ATP and bicarbonate which had given protection against 2-iodoethyl-SCoA showed no protection in this instance. MgATP^{2-} and MgADP^{-} did not give any protection at the levels used. This result was sufficiently similar to the pattern of protection against 2-iodoethyl-SCoA inactivation to suggest that no basis for specificity of the proposed affinity label could be found and that the compound had failed to satisfy the criterion of "affinity inactivation".

3.3.2.6 DISCUSSION

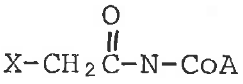
It was expected that problems encountered with the excessive reactivity of the iodoethyl group would be offset to some extent by the affinity of the CoA molecule for its enzyme binding site. Studies with the ethyl derivative had shown that ethyl CoA was capable of stimulating the enzymic activity and that therefore ethyl-SCoA had affinity for the activator binding site. Encouraging preliminary estimates of a 1:1 stoichiometry of covalent attachment of label were followed by unfavourable results in the protection studies in which pyruvate and ATP/HCO_3^- gave better protection against inactivation by 2-iodoethyl-SCoA than did acetyl CoA. An attempt to show that the non-linearity of inactivation was due to breakdown of the reactive iodoethyl moiety was equivocal and led to a possible conclusion that more than one site of modification was responsible for the curved lines. This was in contrast to the 1:1 stoichiometry of labelling obtained earlier. The result which confirmed that 2-iodoethyl-SCoA was not acting in a specific manner was the pattern of protection against modification in the presence of 2-iodoethylcysteine. This compound had no structural basis for specificity of binding to the acetyl CoA binding site and the protection pattern with ligands suggested that both compounds were acting as non-specific chemical modification agents of pyruvate carboxylase. The unfavourable results encountered with the protection studies suggested that it would be more important in future experiments to test the specificity before doing the stoichiometry determinations.

Testing the specificity of 2-iodoethyl-SCoA by application of Singer's "affinity inactivation" criterion proved invaluable in resolving the conflicting results obtained.

Other compounds which would have more stable linkages between the carbonyl group of the haloacetate moiety and the CoA moiety are those in which the unstable thioester linkage had been replaced by either the CoA-O ester (1) or the CoA-N amide (2).



$\mathrm{X{-}CH_2\overset{O}{\overset{\|}{C}}{-}O{-}CoA}$ ...(1)



$\mathrm{X{-}CH_2\overset{O}{\overset{\|}{C}}{-}N{-}CoA}$ ...(2)

Both of these compounds would require lengthy syntheses starting by replacement of the cysteamine component of CoA in order to replace the sulphur atom and were not considered for this project particularly in view of several other possibilities for labels.

The results obtained using one of these, 3-chloroacetonyl-SCoA, are presented in the following section.

3.3.3 3-CHLOROACETONYL-SCoA

3,3.3.1 INTRODUCTION

The failure of 2-bromoacetyl-SCoA as an affinity label emphasised the need for a stable C-S bond in the design of further reagents. The extreme reactivity of 2-iodoethyl-SCoA emphasised the need for mild alkylating activities in affinity labels. The compound which combined these features was a haloacetone derivative of CoA, viz. 3-chloroacetonyl-SCoA III, the direct synthesis of which was possible starting from 1,3-dichloroacetone and CoASH.

The alkylating activity of 3-chloroacetonyl-SCoA was expected to be mild due to the reduced effectiveness of the chlorine atom as a leaving group relative to the higher atomic weight halogens and also as a result of the electron withdrawing influence of the carbonyl group which tends to strengthen the C-Cl bond. The reduced alkylating activity was expected to be a feature in favour of the specific modification of the acetyl CoA binding site by 3-chloroacetonyl-SCoA since the binding component of the modification reaction would play a much larger part in the inactivation rate.

A point of interest which could be inferred from the binding constant of 3-chloroacetonyl-SCoA is the importance which the carbonyl group has in the acetyl group of acetyl CoA. The extra carbon atom in the chain could easily be tolerated by the acetyl CoA binding site on the basis that propionyl CoA affords a V of 100% when compared with that afforded by acetyl CoA. However, the K_a value obtained for propionyl CoA was five times larger than the K_a value for acetyl CoA indicating a reduced affinity for the activator site.

On the other hand, if the carbonyl group interaction with a site on the enzyme is a reference point for the binding of the acyl group, then the acetonyl group would be expected to provide a similar binding constant to that of acetyl CoA.

3.3.3.2 METHODS

(a) Synthesis of 3-chloroacetonyl-SCoA

CoA (10mg, 13 μmole) and 1,3 dichloroacetone (15 mg, 119 μmole) were dissolved in 1 ml aqueous acetonitrile (50%, $^{v}/v$, containing 13 μmole of N-ethylmorpholine base) and the mixture was stirred at room temperature in the dark for 40 min. The progress of the reaction was followed by applying 1 μl aliquots of the reaction solution on thin layer silica gel plates and eluting these with acetonitrile to remove unreacted 1,3-dichloroacetone. The insoluble nucleotide remained at the origin and the extent of its conversion to product was judged empirically by the intensity of reaction after spraying with NBP spray reagent. The product was also checked on thin layer cellulose plates in the 58% sodium isobutyrate system (as described in section 3.2.1). No free CoASH was found in the reaction mixture after 40 min. This latter thin layer separation procedure was too slow to be used as a check of the progress of the reaction. DTNB could not be used as a measure of remaining SH content since the anion of DTNB was found to react with alkylating reagents. The reaction was terminated by freezing in a dry ice/ethanol bath and volatile components were removed by lyophilisation. This had the added advantage that the majority of unreacted 1,3-dichloroacetone, a volatile solid, was removed. The pale pink powder was purified by three washes with dry ether acidified with glacial acetic acid. The compound could be stored under anhydrous conditions for several weeks at room temperature away from light.

The stability of 3-chloroacetonyl-SCoA in aqueous solution was determined by removal of a sample of the solution at various time intervals for treatment by the acetonitrile wash as described above followed by the NBP spray. Approximately one half of the alkylating activity remained after 2 h. as judged by this method. The reagent was more stable in solutions with low pH values and a $t_{0.5}$ value of 11 h. was obtained for the rate of loss of alkylating activity of a solution made with 20mM HCl. Further purification by Sephadex G_{10} chromatography in 20mM HCl resulted in the loss of most of the alkylating activity and was not used in subsequent preparations. The loss of activity was attributed to the interaction of the nucleotide with the Sephadex G_{10} beads.

(b) Synthesis of acetonyl CoA

CoA (10mg, 13 nmole) was dissolved in 0.5 ml aqueous acetonitrile (50% $^{v}/v$) to which was added chloroacetone (12 mg, 124 nmole) and 10 μmole N-ethylmorpholine base. The reaction was followed by thin layer cellulose chromatography in sodium isobutyrate and after 60 min. a single compound was detected by its absorption of ultraviolet light and had an R_f value of 0.86. After lyophilisation the dry compound was washed three times with acetonitrile in order to remove unreacted chloroacetone and the compound was purified by Sephadex G_{10} chromatography.

3.3.3.3 RESULTS

(a) The effect of acetonyl-SCoA on the activity of pyruvate carboxylase

The effect of acetonyl-SCoA on the activity of the enzyme was investigated in order to establish that the change in structure of the acyl group did not affect its capacity to bind to the activator site. The results presented in Fig. 3.8 show that acetonyl CoA had a high affinity for the enzyme and at a concentration of 30nM afforded a reaction velocity which was 48% of that obtained in the presence of saturating levels of acetyl CoA. This result suggests that acetonyl CoA binds very tightly to the activator site and that the addition of an extra carbon atom between the carbonyl group and the sulphur atom of

CoASH did not adversely affect the binding of that compound. The inhibition obtained at higher levels of acetonyl CoA suggests that there was unproductive binding of the activator as was suggested to occur (Scrutton and Utter, 1967) in the case of acetyl CoA in its effect on the rate of inactivation of the enzyme by avidin.

(b) The effect of different concentrations of 3-chloroacetonyl-SCoA on the pseudo first-order rates of inactivation of pyruvate carboxylase

The results, as presented in Fig 3.9, show that the rate of inactivation of the enzyme increased linearly with increasing concentration of 3-chloroacetonyl-SCoA. The second-order rate constant obtained from this data was calculated to be 0.0001 min^{-1} mM^{-1}. Replotting the data in the form log k_{inact} versus log $[I]$ (Fig 3.10) gave a line with a slope of 0.90±0.12. This was consistent with a stoichiometry of 1:1 for the number of moles of inhibitor responsible for the loss of activity per enzyme active site.

Two points at the low concentrations of 3-chloroacetonyl-SCoA were not on the line which passed through its origin in Fig 3.9 and were disregarded for the calculation of the slope of the line from Fig. 3.10.

(c) Protection studies

The effect of 3-chloroacetonyl-SCoA on the activity of pyruvate carboxylase was investigated in the presence of substrates and effectors of the enzyme. The results presented in Table 3.5 show that while acetyl CoA affords a substantial degree of protection against modification, there was a slight reduction in the rate of inactivation when

20mM pyruvate was present.

Oxaloacetate (5mM) afforded an equivalent degree of protection when compared with 0.25mM acetyl CoA which suggests that there are probably two sites of modification, one in the acetyl CoA binding site and one in the pyruvate/ oxaloacetate binding site. The result is not unexpected in view of the results obtained with 3-bromopyruvate (Hudson *et al.*, 1975) in which the modification of pyruvate carboxylase was protected against in the presence of acetyl CoA as well as by oxaloacetate. Modification of a single sulphydryl group by 3-bromopyruvate in the pyruvate binding site was, however, possible in the presence of saturating levels of acetyl CoA. The above results suggest that the reverse situation of modification of the acetyl CoA binding site as well as the pyruvate/oxaloacetate binding site has occurred. From the two studies it can be concluded that two reactive residues, one of which has been identified as cysteine, exist in close juxtaposition in the binding sites of acetyl CoA and pyruvate. This result is in accord with the findings presented in chapter 4 that the acetyl end of acetyl CoA and pyruvate bind in close association which results in the lowering of the apparent K_m value for pyruvate and the stimulation of the pyruvate:oxaloacetate exchange reaction.

The other ligands which were used in the protection studies viz. 20mM HCO_3^-, 5mM $MgCl_2$ and 10mM ATP all enhanced the rate of inactivation of the enzyme in the presence of 0.26mM 3-chloroacetonyl-SCoA. These results suggest that ligands of the first partial reaction site can influence events occurring in the acetyl group binding site. A possible mechanism for this influence is via the biotin prosthetic group since each of the ligands may influence the amount of time spent by biotin in each of the two partial reaction sub-sites. Promotion of a biotin shift towards the ATP binding site would prevent the prosthetic group from being protective in the pyruvate binding site. The influence of ligands on the movements of biotin will be discussed further in the following chapter.

In order to further characterise the protection by acetyl CoA, 3-chloroacetonyl-SCoA inactivation of pyruvate carboxylase was carried out in the presence of increasing concentrations of acetyl CoA. The results presented in Fig. 3.11 were plotted in the form used by Scrutton and Utter, (1967) for protection against inactivation by avidin in the presence of ATP. The Y-intercept of the line can be used to calculate the residual rate of inactivation in the presence of infinite concentrations of acetyl CoA. For complete protection the rate would be zero but a rate of 0.05 min^{-1} was obtained indicating that modification of the second residue occurred at a slow but significant rate.

A value of the K_d for the protection by acetyl CoA of 25 μM was obtained from the slope of the plot. This was comparable with the K_a value of 9.6 μM for acetyl CoA obtained in the absence of Mg^{++} by protection against dilution inactivation.

(d) Determination of the number of inhibitor molecules bound to the enzyme after inactivation by 3-chloroacetonyl-SCoA

In order to confirm the kinetic result of a 1:1

stoichiometry for the number of bound inhibitor molecules per active site, a more direct method was employed. The amounts of nucleotide bound to the modified enzyme after removal of the unbound inhibitor were calculated from the difference spectra of the enzyme after various degrees of inactivation. One example of the difference spectra obtained is shown in Fig. 3.12. The sensitive biotin assay developed in this laboratory (Rylatt *et al.*, 1977) was used to obtain estimates of the number of moles of enzyme in each sample. The results, as presented in Fig. 3.13, show that the number of moles of inhibitor bound per mole of biotin increases with increase in the extent of inactivation of the enzyme. Extrapolation to 100% inactivation, when fitted using linear regression analysis of the points below 80% gave a value of 0.78±0.06 for the number of moles of inhibitor bound per enzyme monomer. This was close to the 1:1 stoichiometry obtained by kinetic methods and taken together with the protection studies indicates that 3-chloroacetonyl-SCoA is an affinity label for the acetyl CoA binding site. These results also indicate that the second residue is modified at a slower rate than the first and a resultant curve from the addition of the two rates can be discerned from the points plotted in Fig. 3.13.

3.3.3.4 DISCUSSION

The results presented in this section show that 3-chloroacetonyl-SCoA is an affinity label of the acetyl CoA binding site. The modification of a residue in the pyruvate binding site was indicated by the protection studies and was in accord with the two sites of modification obtained using 3-bromopyruvate. The specific modification of a residue in the acetyl CoA binding site would therefore be possible in the presence of oxaloacetate. In the same way, using 3-bromopyruvate, modification of a single sulphydryl residue in the pyruvate binding site was possible in the presence of saturating levels of acetyl CoA. Once modified, the residue could be identified by the use of high voltage paper electrophoresis in 10% acetic acid (pH 1.9). This procedure would allow the charge of zero conferred by the secondary amino group of the 3-chloroacetonyl-SCoA-lysyl adduct to be used to separate it from the -cysteinyl adduct which would have a net charge of -1 at pH 1.9.

The implications of two sulphydryl residues in close juxtaposition in the active site will be considered in the General Discussion. Time did not permit the identification of the modified residue.

3.4 THE RIBOSE-3′-PHOSPHATE-DERIVED LABELS

3.4.1 ACETYL CoA DIAL

3.4.1.1 INTRODUCTION

The possibility that the essential lysine, previously modified by TNBS (Ashman *et al.*, 1973), could be associated in an ionic bond with the 3'phosphate group of acetyl CoA led to the proposal that a reactive aldehyde group could be used to modify the lysine residue as shown in Fig. 3.14. Periodate oxidation of the ribose ring of acetyl dephospho CoA would provide a dialdehyde moiety capable of forming an imine bond with the proposed lysyl residue. This could be converted through a series of steps to a stable morpholine adduct using sodium borohydride as shown in Fig. 3.14.

The formation of an imine bond between the aldehyde group of pyridoxal phosphate and lysyl residues in active sites of enzymes which bind the cofactor has been used to advantage by many workers including (Greenwell *et al.*, 1973; and Colombo and Marcus, 1974). In these studies the unstable imino linkage was converted to a stable C-N bond by sodium borohydride reduction thus altering the enzyme activity by irreversible covalent modification.

Other workers have used the periodate oxidisation products of ribose moieties in nucleotides as reactive analogues of those nucleotides. One example was the oxidation of UTP to UTP dial (Powell and Brew, 1976) and the structure of the periodate oxidation products of nucleotides have also been extensively investigated (Hansske *et al.*, 1974). Studies in this laboratory (Easterbrook-Smith *et al.*, 1975) in which the periodate oxidation product of ATP was used show that a reactive lysyl residue in the ATP binding site of pyruvate carboxylase was covalently modified.

Two labile bonds in the dialdehyde form of acetyl CoA were regarded as possible sources of ineffectual binding, namely, the thioester bond and the 5'ribose-phosphate ester bond. Deacylation of the compound would render radioactive acetyl group labelling useless but could be circumvented by using NaB^{3}H$_4$ during the reduction of the imino linkage. The possibility of β-elimination resulting in cleavage of the 5'ribose-phosphate bond would, however, be less readily overcome. The base catalysed elimination reaction, (Schwartz and Gilham, 1972) could be minimised by using acidic conditions during its preparation, but would not be as well protected in the pH range of pH 6.8 - 9.0 necessary for enzyme stability.

3.4.1.2 METHODS

(a) Synthesis of acetyl CoA dial

To a solution of acetyl dephospho CoA (8 μmole) dissolved in 2 ml, 0.1M potassium phosphate, pH 6.5, was added sodium metaperiodate (8 μmole) and the reaction was allowed to proceed at room temperature, in the dark, for one h. The reaction was quenched by adding ethane-1,2-diol (10 μl) after which the reaction mixture was desalted by gel filtration using Sephadex G_{10} in acidified water at $4^{0}C$. The concentration of acetyl CoA dial was determined by measuring the absorbance at 258nm, using a value of 14.9 mM for the extinction coefficient (Hannske *et al.*, 1973). The purity of acetyl CoA dial was checked by thin layer chromatography using two different solvent systems. Thin layer chromatography was performed on polyethylene imine sheets using 0.5M LiCl as the developing solvent and ultraviolet absorption to locate the nucleotide spots. The chromatogram revealed only one comound with an R_f value of 0.01 and no acetyldephospho CoA (R_f 0.93) was detectable. The low R_f value was indicative of a reaction between the aldehyde groups of acetyl CoA dial and the amine groups of the thin later chromatography sheet. Ascending chromatography was performed on thin layer cellulose plates using 58% sodium isobutyrate, pH 4.3, and only one compound was detected (R_f 0.89). The colourless adduct formed by addition of 2,4-dinitrophenylhydrazine to acetyl CoA dial was checked by thin layer chromatography on polyethyleneimine sheets using 1.2M LiCl. The product was detected by ultraviolet absorption and had an R_f value of 0.82, compared with acetyl CoA dial (Rf 0.01) and 2,4-dinitrophenylhydrazine (R_f 0.99). This was taken as evidence of the presence of reactive aldehyde groups on acetyl CoA dial.

(b) The synthesis of $[^{14}C]$ acetyl CoA dial

The preparation of $[^{14}C]$ acetyl CoA dial was carried out using the procedure described above for the synthesis of the unlabelled compound. The starting material was 1-$[^{14}C]$acetyl CoA at a specific activity of 57 mCi/mmole. An aliquot of the product of the procedure for the synthesis of 1-$[^{14}C]$ acetyl CoA dial was analysed by thin layer cellulose chromatography in the sodium isobutyrate solvent system. The developed chromatogram was tested for nucleotide-containing material by absorbance under ultraviolet light and for radioactivity by liquid scintillation counting of strips cut from the chromatogram. Radioactive counts were found to be associated with the nucleotide material. No other radioactive spots were detected.

3.4.1.3 THE EFFECT OF ACETYLDEPHOSPHO CoA

In order to establish that the inhibitor, acetyl CoA dial was derived from a compound with affinity for the acetyl CoA binding site, acetyldephospho CoA was tested as an inhibitor of pyruvate carboxylase. However, it was found that acetyldephospho CoA was an activator of the enzyme and the results are presented in conjunction with the effect of other analogues of acetyl CoA in section 4.
It was concluded that acetyl dephospho CoA had affinity for

the acetyl CoA binding site since it was capable of activating the enzyme.

3.4.1.4 COVALENT MODIFICATION STUDIES WITH ACETYL CoA DIAL

(a) Concentration dependence

The enzyme was incubated with varying acetyl CoA dial concentrations and samples with and without borohydride reduction were assayed for enzyme activity. The results presented in Fig. 3.15 show that the extent of inhibition increased with increasing concentrations of inhibitor concentration. The presence of borohydride made no difference to the extent of inactivation. These results were indication of irreversible inhibition of the enzyme by the inhibitor and indicated the formation of a stronger bond with the enzyme than that afforded by a single imine bond.

(b) Protection studies

In order to determine the specificity of the covalent modification of the enzyme afforded by acetyl CoA dial, inactivation studies in the presence of substrates were carried out. The effect of increased acetyl CoA levels in the presence of varied levels of acetyl CoA dial on the activity of pyruvate carboxylase was investigated. The results presented in Fig. 3.16 show that acetyl CoA dial modification acted to reduce the extent of activation by acetyl CoA but the K_a for acetyl CoA remained unaltered at 53μM. This result was consistent with irreversible inhibition by acetyl CoA dial since the extent to which acetyl CoA could activate the enzyme velocity was substantially reduced. The result gave no indication, however, that the inhibition was specific for the acetyl CoA binding site. In order to determine the specificity of inhibition with respect to acetyl CoA, enzyme was incubated in the presence of a fixed concentration of acetyl CoA dial together with increasing concentrations of acetyl CoA. The experiment was repeated with increasing concentrations of pyruvate and $MgATP^{2-}$. The results presented in Fig. 3.17 show that while acetyl CoA and pyruvate did not afford any protection against inactivation, $MgATP^{2-}$, did protect against the inhibitor. This provided strong evidence that the site of covalent modification of the enzyme was the ATP binding site and not the acetyl CoA binding site.

(c) Radioactive labelling studies

In an experiment to determine whether covalent attachment of the labelled inhibitor to the enzyme could be demonstrated, enzyme was incubated with 1-[^{14}C]-acetyl CoA dial for sufficient time for modification to occur, after which the enzyme was precipitated with trichloroacetic acid and its radioactivity determined. No significant counts were found to be associated with the precipitated protein. The result suggested that radioactive label which was in the acetyl group of acetyl CoA dial had been lost from the inhibitor during its incubation with the enzyme.

3.4.1.5 DISCUSSION

Taken in conjunction with the modification of the ATP binding site as indicated by the protection studies, the results presented above indicate that the inactivating species was probably a breakdown product of acetyl CoA dial which lacked the acetyl group. The most likely mechanism by which breakdown could have occurred to give a compound capable of specific modification of the ATP binding site was β-elimination at the ribose-5'-phosphate-ester bond. The resultant adenine dialdehyde compound as shown in Fig. 3.14 has features more in common with the structure of ATP than of acetyl CoA. One possible explanation for the irreversible inhibition afforded by this compound in the absence of borohydride reduction is that the conjugated system present in the compound would be capable of reaction with a nucleophilic group in a Michael addition reaction as presented in Fig. 3.14 which could result in stable bond formation. This was in contrast to the reversible inactivation which occurred when pyruvate carboxylase was incubated in the presence of oATP (Easterbrook-Smith *et al.*, 1975). The reversibility of oATP inactivation could have been due to the formation of a single imine bond with a lysine residue of the enzyme. The formation of a more stable morpholino adduct as obtained by Schwartz and Gilham, (1972) could possibly have been precluded by steric considerations in the case of oATP. Another factor which could have contributed to the unexpected specificity of inactivation by acetyl CoA dial was the lack of the 3'phosphate group. Absence of this moiety could have resulted in the inability of the enzyme to discriminate between the two types of adenosine-phosphate esters.

Further affinity labels based on the adenosine moiety of acetyl CoA could be designed with the above discussion in mind. One such compound was the cyanogen bromide-activated derivative of acetyldephospho CoA V. The cis diol group of the ribose ring provides an ideal site for attachment of the imine group in a similar mechanism to the preparation of activated sepharose for affinity chromatography as described by Porath (1974). The cyanogen bromide-activated derivatives of both ATP and [2-^{3}H]-acetyldephospho CoA were synthesised and the latter compound was found to covalently modify pyruvate carboxylase. The rate of inactivation of the enzyme was extremely slow and the incorporation of label onto the enzyme indicated a stoichiometry of 3.1 for the number of moles of inhibitor bound per enzyme monomer. This suggested that investigation of the compounds as affinity labels would not be worthwhile.

The lack of a 3'-phosphate group in the acetyldephospho CoA derived compound could possibly result in a lack of specificity in covalent modification studies.

3.5 THE 6-AMINO GROUP-RELATED AFFINITY LABELS

The third group of affinity labels considered were those derived from the 6-position of the adenine ring in acetyl CoA. This group was considered as being of most importance as a site for orientation of the adenine ring since the lone pair of electrons on the amino nitrogen atom would be less delocalised than those in the rings. This would make the amino group the most likely point for hydrogen bonding interactions with the enzyme which would be a most effective way of arresting the rotation of the adenine ring about the ribose-adenine C-N bond.

The group is easily replaced by treatment with nitrous acid to form a diazonium salt which breaks down by hydrolysis to give the hydroxyl derivative of adenine, hypoxanthine. The possibility of trapping the reaction at the diazonium salt form by doing the diazotisation at 0^{0}C was tested but the product was far too unstable for use as an affinity label. Replacement of the diazonium salt by azide ion was a possible extension of this method and the 6-azido acetyl CoA VI product could be activated by irradiation with ultraviolet light to produce a highly reactive nitrene *in situ*. One possible problem with the synthesis of this compound would be the amount of diazonium salt which was hydrolysed in competition with nucleophilic attack by the azide ion. The acetyldesamino CoA product would be difficult to remove from the 6-azido compound without causing more breakdown of the desired product. The presence of acetyl desamino CoA in the product mixture would lead to competitive inhibition for the activator site and reduce the effectiveness of the photo-affinity label.

Another consideration in any photolysis experiment with CoA derivatives would be that the adenine ring itself becomes reactive upon photolysis. The photo-reactivity of adenine was demonstrated for cyclic AMP by carrying out photolysis in the presence of cyclic AMP-binding proteins (Antonoff and Ferguson, 1974) which resulted in significant levels of incorporation of the label.

The possibility that acetyl CoA may act in the same way as cyclic AMP was tested and inactivation of pyruvate carboxylase was found to occur in the presence of acetyl CoA after irradiation with ultraviolet light. When enzyme was irradiated in the presence of $[^{14}C]$-acetyl CoA some radioactivity co-chromatographed with protein after gel filtration. Further attempts to characterise the binding of acetyl CoA, however, produced erratic and non-reproducible results and no conclusions could be drawn from these studies. One difficulty which arose was that the inactivation could not be unequivocally proven to be specific for the acetyl CoA binding site.

3.6 FURTHER DISCUSSION

3.6.1 THE ACTIVE SITE OF PYRUVATE CARBOXYLASE

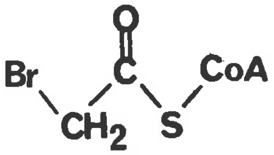
The multi-faceted approach to the study of the active site of pyruvate carboxylase, used in this laboratory has resulted in the covalent modification of residues in each substrate binding site on the enzyme (Hudson *et al.*, 1975; Easterbrook-Smith *et al.*, 1975; Easterbrook-Smith, 1977). The activator binding site has also been covalently modified (Ashman *et al.*, 1973) using the chemical reagent TNBS, and results presented in this chapter show that the acetyl CoA binding site has been covalently modified using the specific reagent 3-chloroacetonyl-SCoA. Furthermore, studies in this laboratory (Rylatt *et al.*, 1977) have revealed the sequence of the tryptic peptide to which the biotin prosthetic group is attached. The isolation of tryptic peptides or peptides from CNBr cleavage, which have the above covalently attached labels will enable the acquisition of an extensive array of sequences around the active site. Information about the structure of the active site which can be obtained from these sequences will allow predictions to be made about the enzyme mechanism and will allow estimates of the distances between the ligands to be made.

A further development could be the synthesis of each of the peptides to enable model studies and structure determinations to be done. Once the entire amino acid sequence of pyruvate carboxylase has been obtained the modified peptide sequences will allow comparisons of structure and function to be made within the enzyme and with peptides isolated from the other biotin carboxylases by modification with similar reagents. One such comparison (Wood and Barden, 1977) has already been made of the biotin-containing peptides from biotin-dependent carboxylases. A comparison of the TNB-peptide and the 3-chloroacetonyl-SCoA modified peptide will be useful for the purposes of understanding the action of acetyl CoA in more depth.

3.6.2 FURTHER DISCUSSION OF LABELS

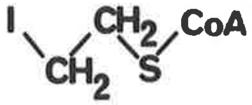
One further possibility of an affinity label for the acetyl CoA binding site would make use of a possible ionic interaction between the β-phosphate of CoA and an amino group in the enzyme binding site. Acetyl pantetheine could be treated by the mild oxidation mixture, CrO_3/pyridine, to form the aldehyde derivative of acetyl pantetheine. This compound would be capable of forming an imine bond with the postulated amino residue and could be stabilised by borohydride reduction.

In the affinity labelling studies presented in this chapter the assumption was made that residues in the acetyl CoA binding site would be reactive towards electrophilic reagents. This may not be the case for an allosteric activator binding site especially near the hydrophobic sections of the molecule. For this reason it may be more productive in further studies of this type, to concentrate on reagents which do not rely on the reactivity of enzyme residues. The photoaffinity labels are of this type and since two CoA-derived compounds have been used successfully to affinity label enzymes (Lau *et al.*, 1977 a & b) this may be a profitable direction in which to move. However, the acyl derivative of CoA which these workers used, p-azido benzoyl-SCoA, would not be expected to bind effectively to the acetyl CoA binding site since similar acyl derivatives of CoA have high binding constant values. The other affinity label used by Lau *et al.*, (1977 b) S-benzoyl (3' dephospho-8-azido) CoA would not possess the specificity conferred by the 3'phosphate group but could be enzymatically converted to the 3'-phospho-derivative using dephospho CoA kinase. This enzyme cannot be obtained in a pure form, however, and the CoA pyrophosphorylase activity which is co-purified with this enzyme is likely to cleave the CoA molecule until an equilibrium mixture is reached. Chemical phosphorylation of the derivative would be too vigorous to guarantee the stability of the azido group.



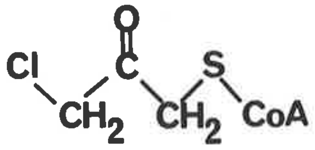
O
Br
C
CoA
CH2
S

I 2-bromoacetyl-SCoA



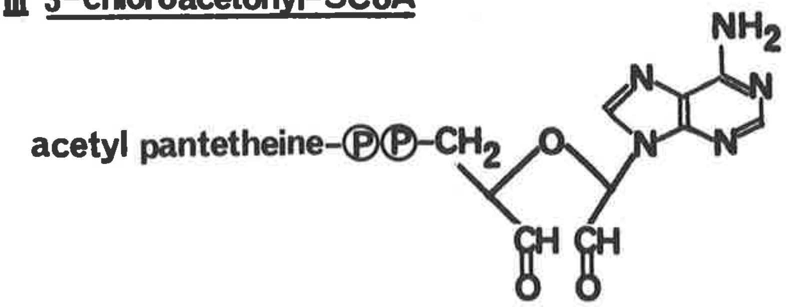
I
CH2
CoA
CH2
S

II 2-iodoethyl-SCoA



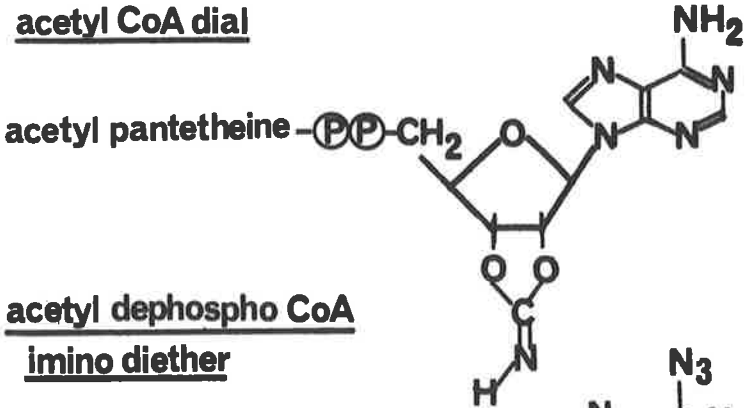
O
Cl
C
S
CH2
CH2
CoA

III 3-chloroacetonyl-SCoA



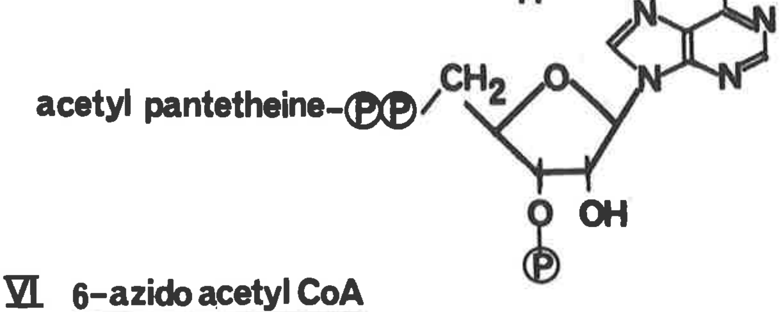
NH2
N
N
acetyl pantetheine-ⓅⓅ-CH2
O
N
N
CH
CH
O
O

IV acetyl CoA dial



NH2
N
N
acetyl pantetheine-ⓅⓅ-CH2
O
N
N
O
O
C
N

V acetyl dephospho CoA imino diether



N
N
acetyl pantetheine-ⓅⓅ
CH2
O
N
N
O
OH
Ⓟ

VI 6-azido acetyl CoA

Fig. 3.1

Fig. 3.2 The effect of ethyl CoA on pyruvate carboxylase activity

Enzyme activity was determined in the radiochemical assay as described in section 2.2.5b, in the presence of various concentrations of ethyl CoA (● - ●), and acetyl CoA was omitted from these assay solutions. Activity was also determined in the presence of 0.25mM acetyl CoA as shown (■).

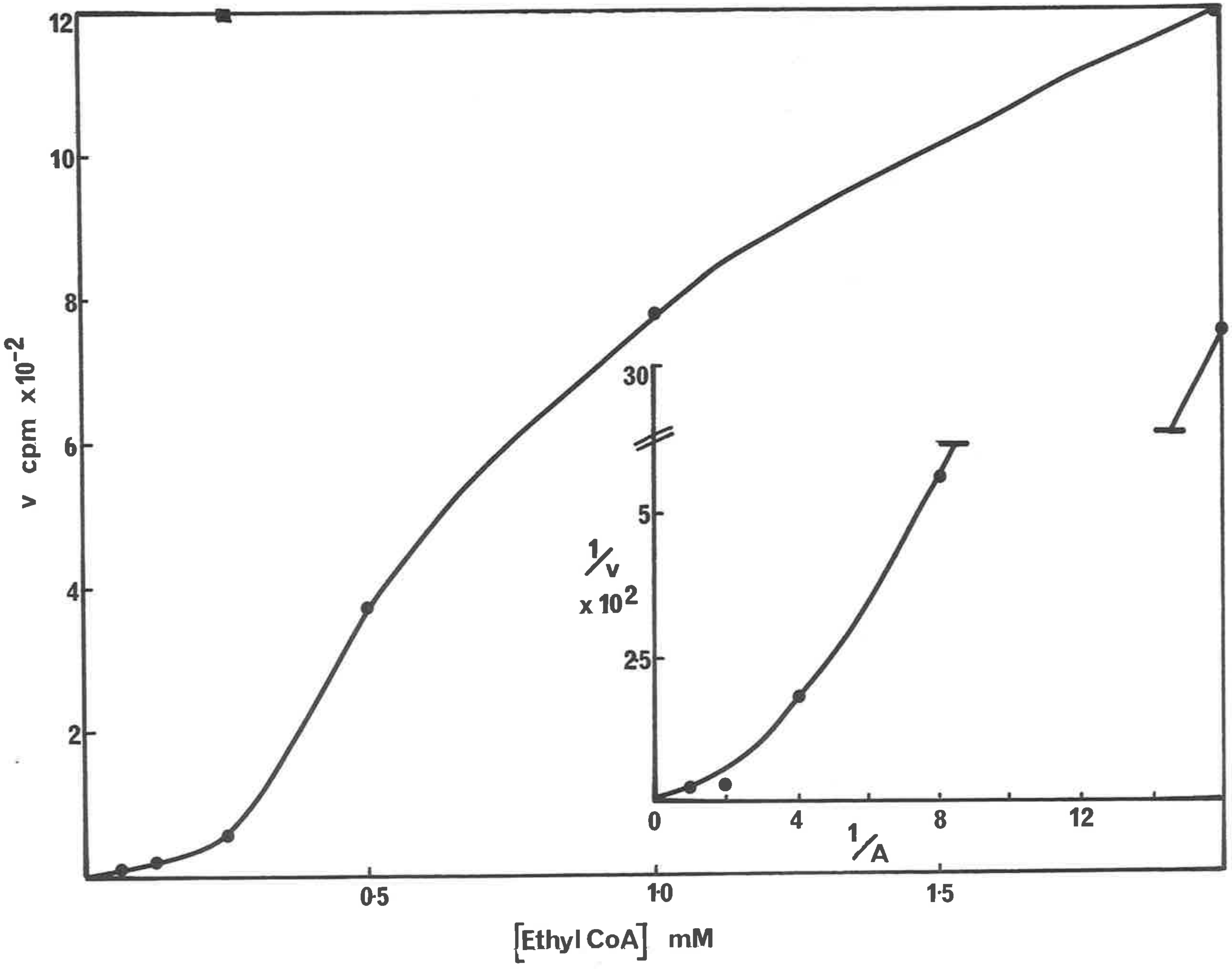

12
10
8
6
4
2
v cpm x10^-2
0·5
1·0
1·5
[Ethyl CoA] mM
30
5
2·5
1/v
x 10^2
0
4
8
12
1/A

Fig. 3.3 The effect of different pH values on the rate of inactivation of pyruvate carboxylase in the presence of 2-iodoethyl-SCoA.

Enzyme at a final concentration of 8 E.U./ml was incubated in the presence of 0.3mM 2-iodoethyl-SCoA in 0.1M potassium phosphate buffer at the following pH values:- 8.4 (● - ●), 7.2 (□ - □) and 6.5 (■ - ■). Aliquots (10μl) were withdrawn at various times for the determination of residual enzyme activity in the radiochemical assay, as described in section 2.2.5b, in the presence of 0.25mM acetyl CoA.

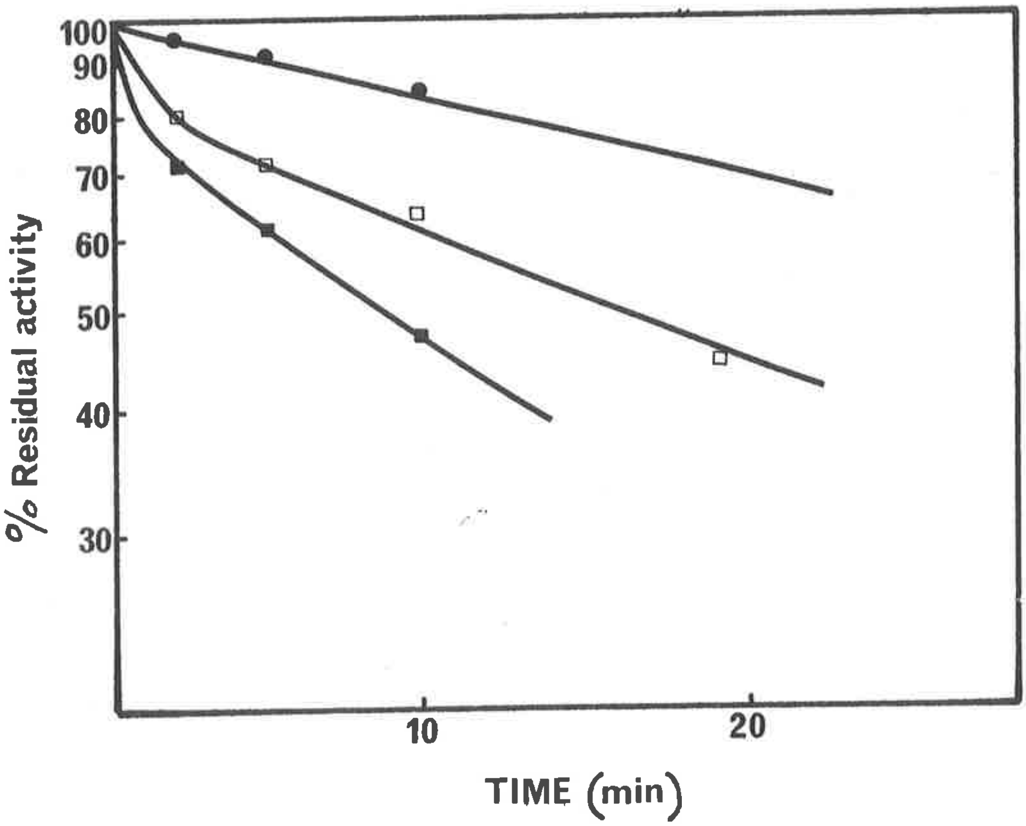

100
90
80
70
60
50
40
30
% Residual activity
10
20
TIME (min)

Fig. 3.4 The effect of 2-iodoethyl-SCoA concentration on the rate of inactivation of the enzyme.

Enzyme, 9 E.U./ml. in 0.1M potassium phosphate buffer, pH 7.2, was incubated in the presence of the following concentrations of 2-iodoethyl-SCoA:- 0.12mM, (● - ●); 0.3mM, (■ - ■) and 0.57mM, (▼ - ▼). Aliquots were withdrawn at the times indicated and activity was determined immediately in the radiochemical assay as described in section 2.2.5b, in the presence of 0.25mM acetyl CoA.

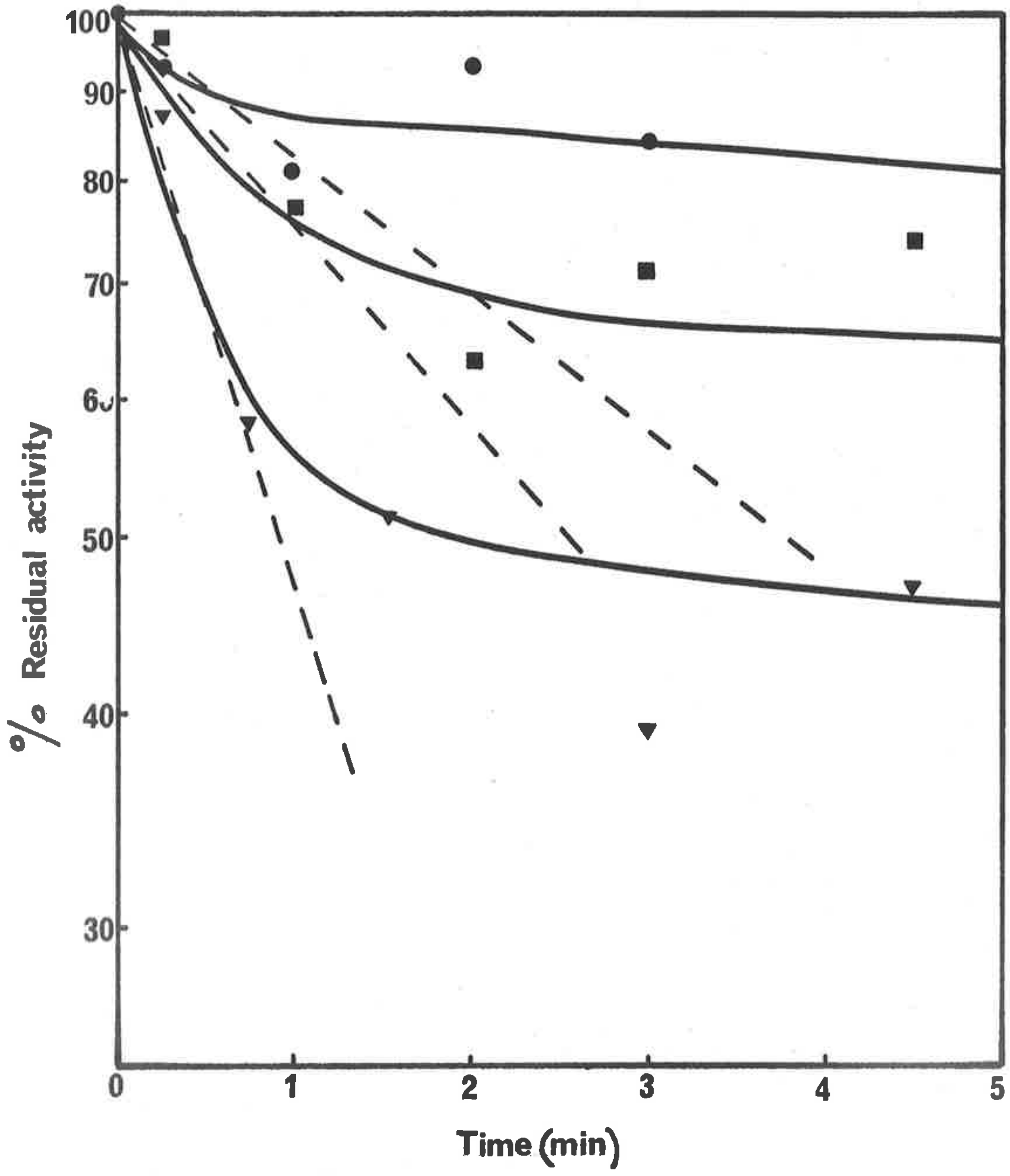
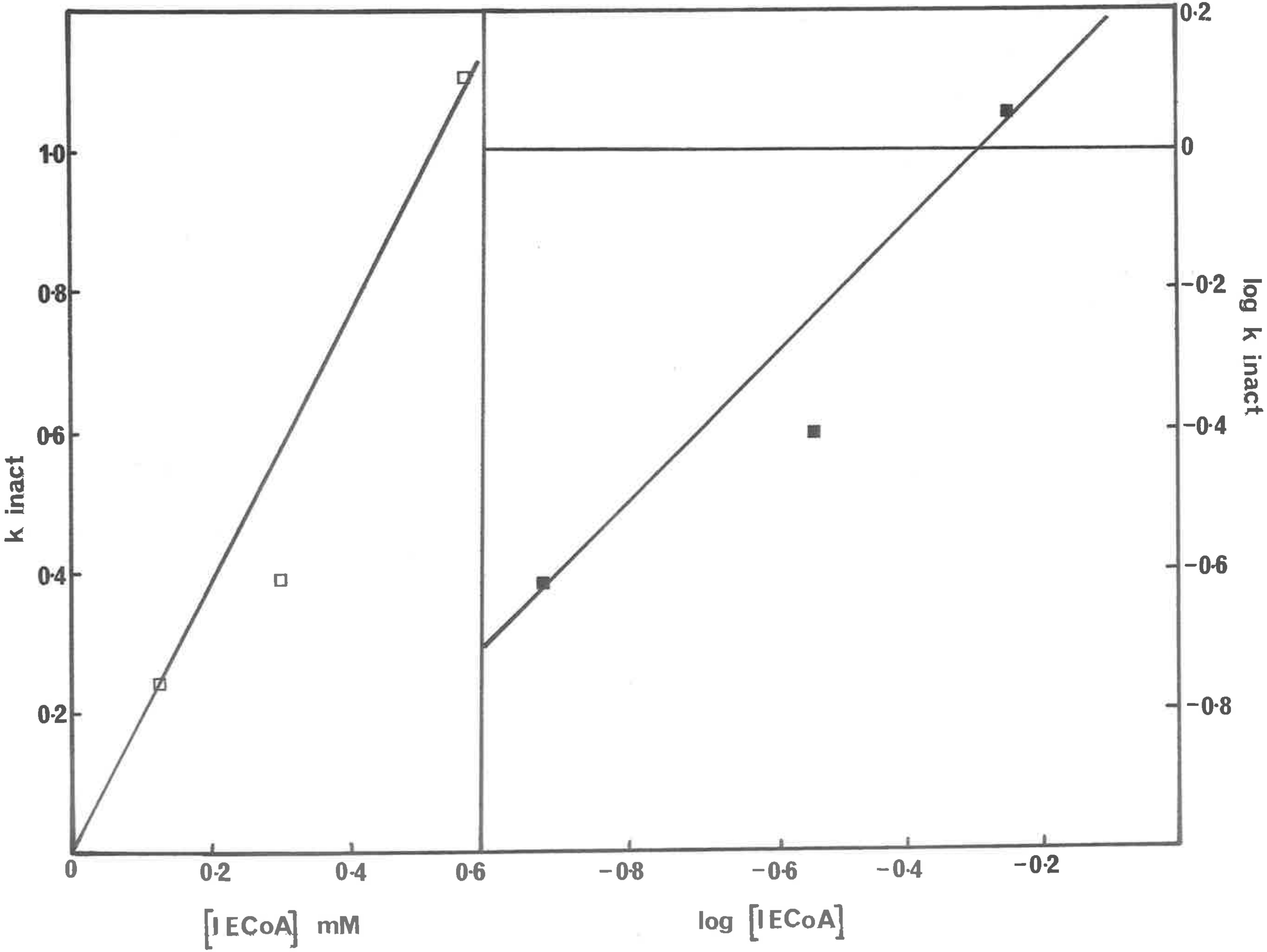

100
90
80
70
60
50
40
30
% Residual activity
0
1
2
3
4
5
Time (min)

Fig. 3.5 Pseudo first-order rates of inactivation as a function of 2-iodoethyl-S-CoA concentration. The K_{inact} data was obtained from the initial rates of inactivation as shown in Fig. 3.4.

Fig 3.6 Log pseudo first-order rates of inactivation as a function of log 2-iodoethyl-SCoA concentration.


k inact
1·0
0·8
0·6
0·4
0·2
0
0·2
0·4
0·6
[IECoA] mM
0·2
0
−0·2
−0·4
−0·6
−0·8
log k inact
−0·8
−0·6
−0·4
−0·2
log [IECoA]

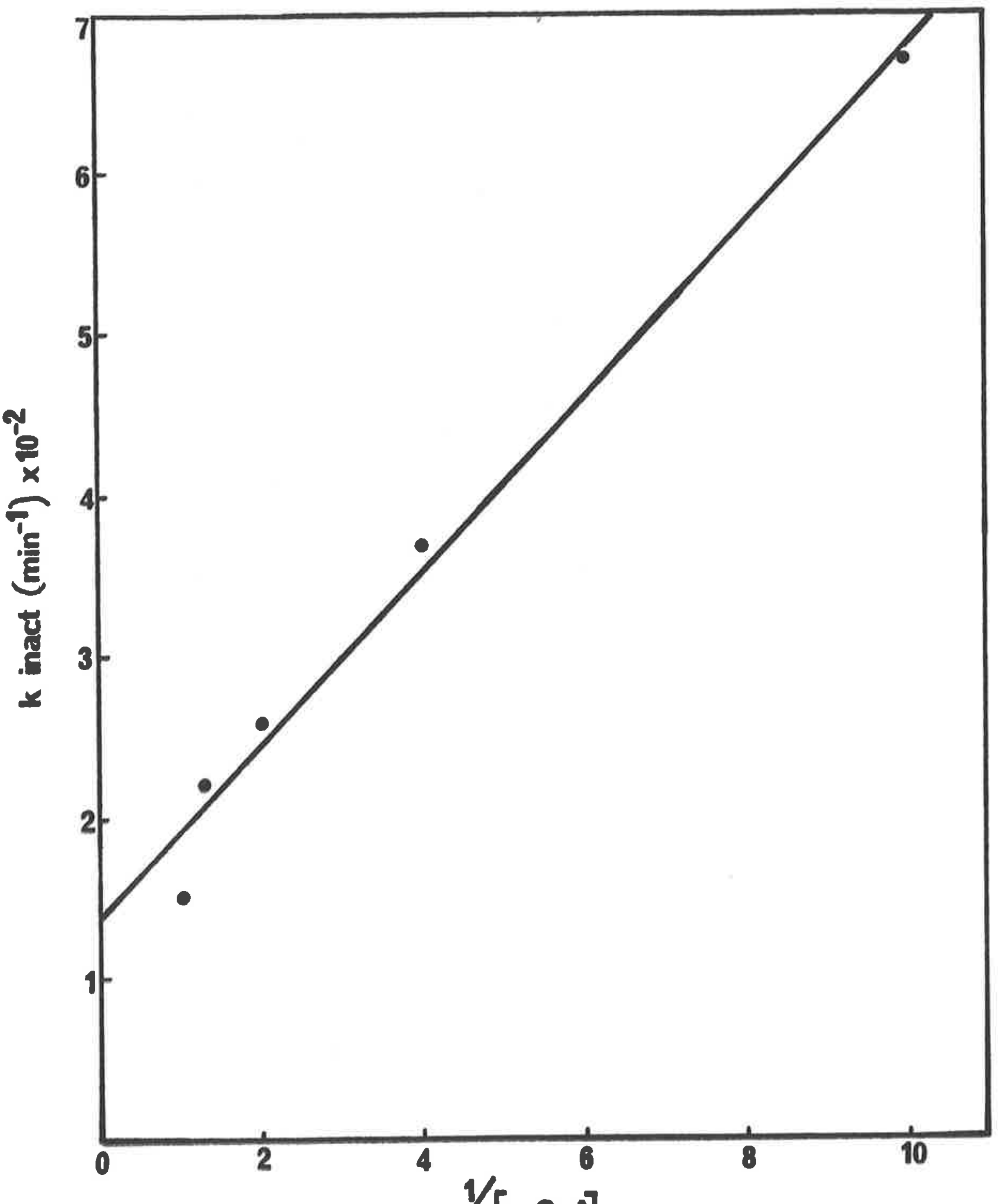
acetyl CoA concentration	K_{inact} (min^{-1})
0	0.11
0.1mM	0.067
0.25	0.037
0.75	0.022
1.0	0.015

Table 3.1 The effect of acetyl CoA on the activation of pyruvate carboxylase in the presence of 2-iodoethyl-SCoA

Enzyme at 10 E.U./ml. was incubated in potassium MOPS buffer (0.05M, pH 6.9) in the presence of 0.19mM 2-iodoethyl-SCoA and the final concentrations of acetyl CoA as shown in the table. Aliquots were removed at various times and enzyme activity was immediately determined in the radiochemical assay in the presence of 0.25mM acetyl CoA.

Fig. 3.7 Protection by acetyl CoA against inactivation of pyruvate carboxylase in the presence of 2-iodoethyl-SCoA

Enzyme 5 E.U./ml. was incubated in the presence of 0.88mM 2-iodoethyl-SCoA and the concentrations of acetyl CoA shown, in 0.1M potassium phosphate buffer pH 7.2. Aliquots were removed at various times for determination of activity in the radiochemical assay. K_{inact} values were determined from the slopes of the plots of log % residual activity against time.


k inact (min^{-1}) x 10^{-2}
1
2
3
4
5
6
7
0
2
4
6
8
10
1/[acCoA]

	ligand	concentration	k_{inact} (min^{-1})
(a)	0	0	0.067
	ATP/HCO_3	10mM	0.001
	pyruvate	20mM	0.015
	acCoA	1mM	0.045
(b)	0	0	0
	OAA	10mM	0.045
	$MgATP^{2-}$	8mM	0.059
	$MgADP^{-}$	8mM	0.083

Table 3.2a

The effect of substrates and products on the rate of inactivation of enzyme in the presence of 2-iodoethyl-SCoA

Enzyme at 41 E.U./ml in 0.1M potassium phosphate buffer pH 7.2 was incubated with 0.8mM 2-iodoethyl-SCoA in the presence of the concentrations of substrates and products indicated. Aliquots were taken at various times and quenched in a mixture containing 0.1M Tris Cl. (pH 8.4) and 15mM β-mercaptcethanol after which enzyme activity was determined radiochemically in the presence of o.25mM acetyl CoA.

(b) The values in part (b) were obtained from a different experiment performed under identical conditions except that the incubation mixtures each contained $MgCl_2$ at a final concentration of 0.08M.

[2-iodoethylcysteine] mM	k_{inact} (min^{-1})
0.3	0.0086
0.6	0.024
1.5	0.067

Table 3.3 The rates of inactivation of pyruvate carboxylase in the presence of different concentrations of 2-iodoethylcysteine

Enzyme at 24.7 E.U./ml. in 0.05M potassium MOPS buffer (pH 6.9) was incubated in the presence of the concentrations of 2-iodoethylcysteine indicated. At various times aliquots were quenched by addition of 0.1M Tris Cl buffer (pH 8.4) containing 20mM β-mercaptoethanol and 0.25mM acetyl CoA. Enzyme activity was determined radiochemically in the presence of 0.25mM acetyl CoA.

ligand	mM	k_{inact} (min^{-1})
0		0.077
acCoA	0.5	0.048
$MgATP^{2-}$	5	0.077
$MgADP^{-}$	5	0.077
$MgATP^{2-}/HCO_3$	5	0.42
pyruvate	50	0.036

Table 3.4 The effect of different substrates on the rates of inactivation of enzyme in the presence of 2-iodoethylcysteine

Inactivations were carried out under conditions identical to those described in the legend to table 3.5 except that a final concentration of 1.5mM 2-iodoethylcysteine was used in the presence of 0.08M $MgCl_2$ and the final concentrations of substrates were as shown in the table.

Fig. 3.8 The effect of acetonyl-SCoA on the activity of pyruvate carboxylase

The activity of pyruvate carboxylase was determined in the spectrophotometric assay in the presence of various concentrations of acetonyl CoA in lieu of acetyl CoA (● - ●). The activity was also determined in the presence of 0.25mM acetyl CoA (■).

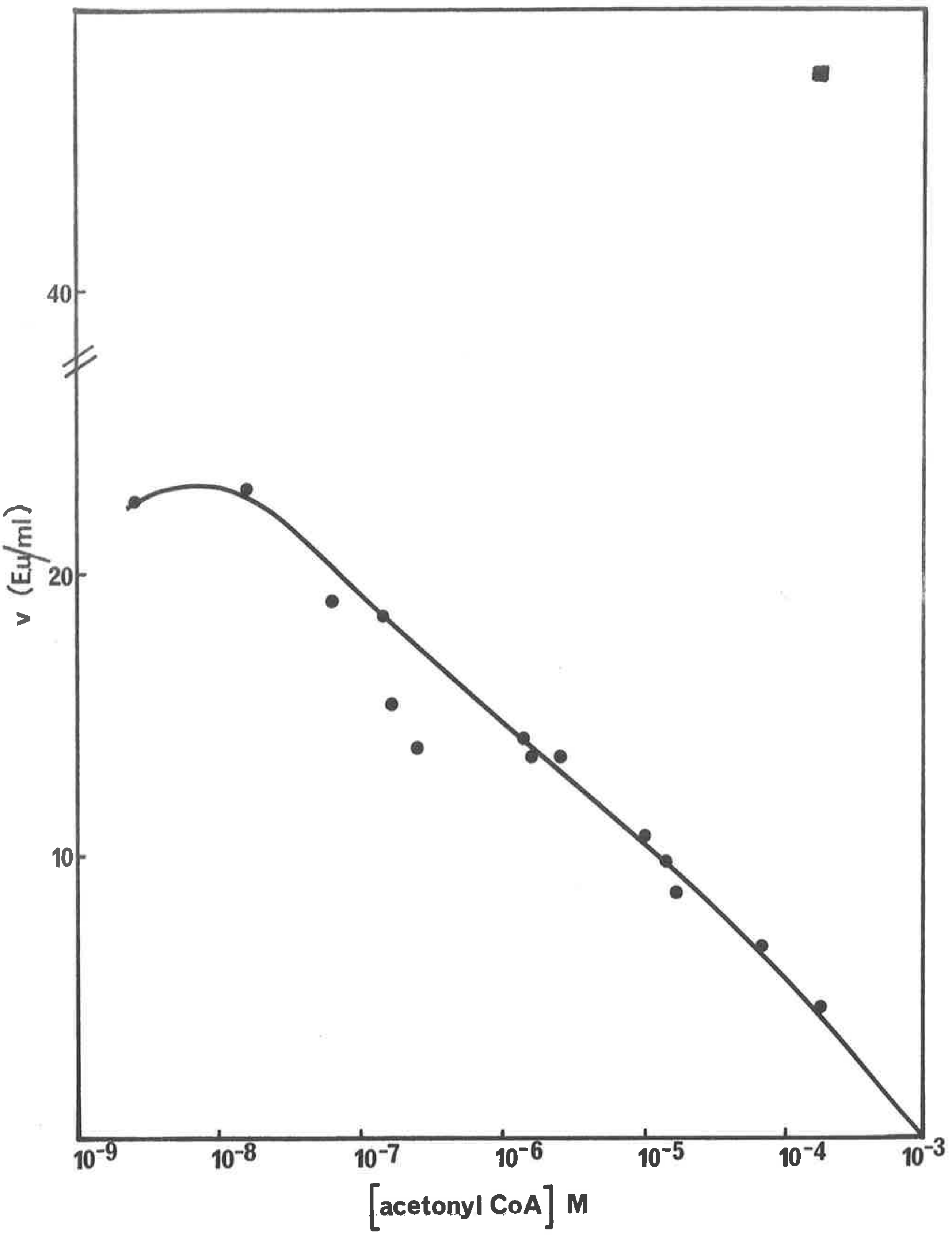
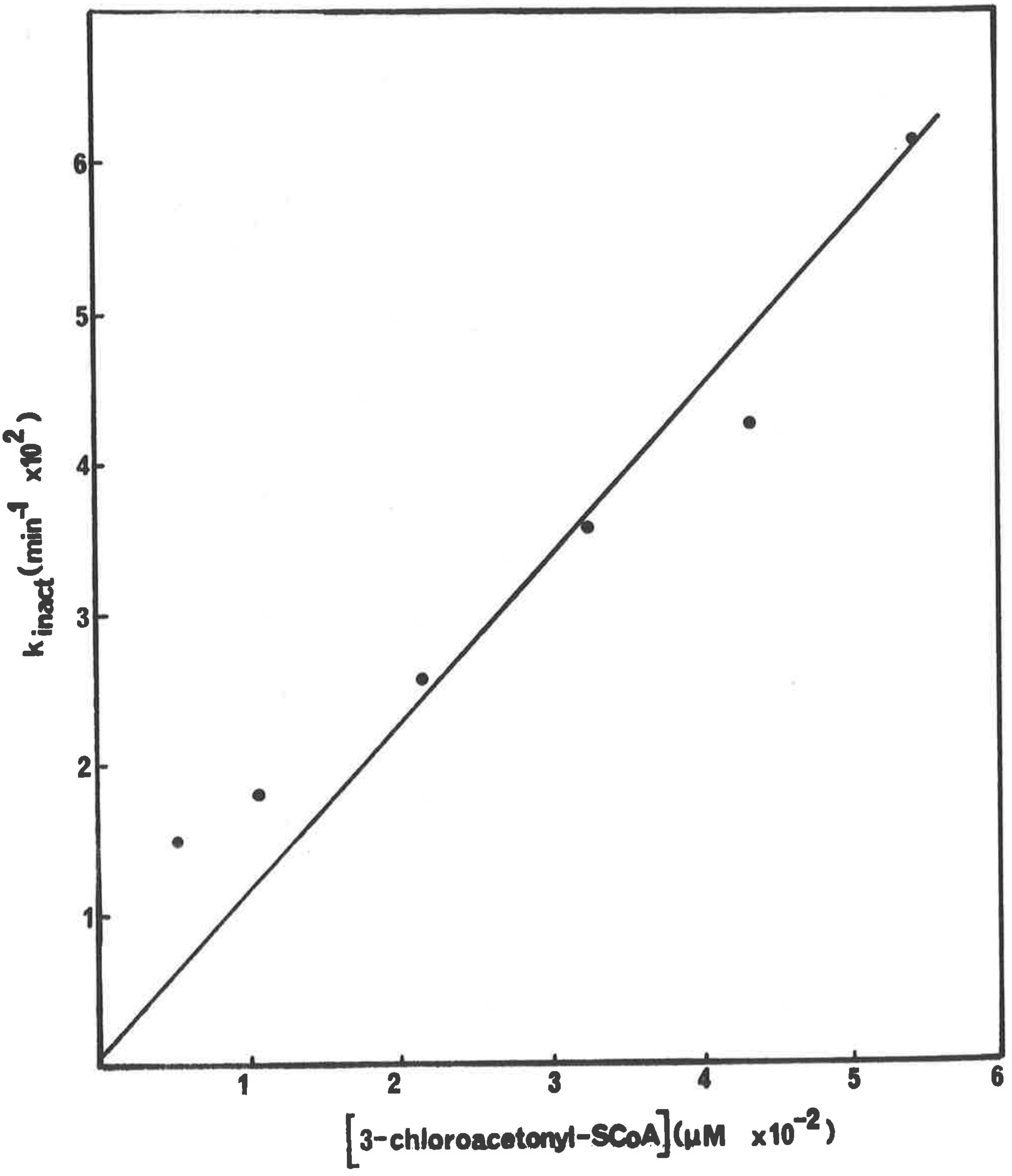

40
20
10
v (E.u/ml)
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3
[acetonyl CoA] M

Fig 3.9 The rate of inactivation of pyruvate carboxylase in the presence of varying concentrations of 3-chloroacetonyl-SCoA

Enzyme, 9.6 E.U./ml., was incubated in 0.15M NEMCl buffer pH 8.4 in the presence of varying concentrations of 3-chloroacetonyl-SCoA. Aliquots (10 μl) were removed at various times and residual activity was determined radiochemically. The K_{inact} values were obtained from plots of log % initial activity against time.


k inact (min^{-1} x10^{2})
1
2
3
4
5
6
1
2
3
4
5
6
[3-chloroacetonyl-SCoA] (µM x10^{-2})

ligand	(mM)	k_{inact}(sec^{-1})
	0	0.172
acCoA	0.25	0.083
pyruvate	20	0.154
OAA	5	0.085
HCO_3^-	20	0.238
ATP	10	0.222
$MgCl_2$	5	0.238

Table 3.5 The effect of substrates and procucts on the rates of inactivation of pyruvate carboxylase in the presence of 3-chloroacetonyl-SCoA

Enzyme 7.5 E.U./ml. in 0.25M NEMCl buffer (pH 8.4) was incubated in the presence of 0.25mM 3-chloroacetonyl-SCoA and the concentrations of each ligand shown in the table. Aliquots were withdrawn at various times and enzyme activity was determined immediately in the spectrophotometric assay in the presence of 0.25mM acetyl CoA.

Fig. 3.10 Logarithmic plot of the data from Fig. 3.9.

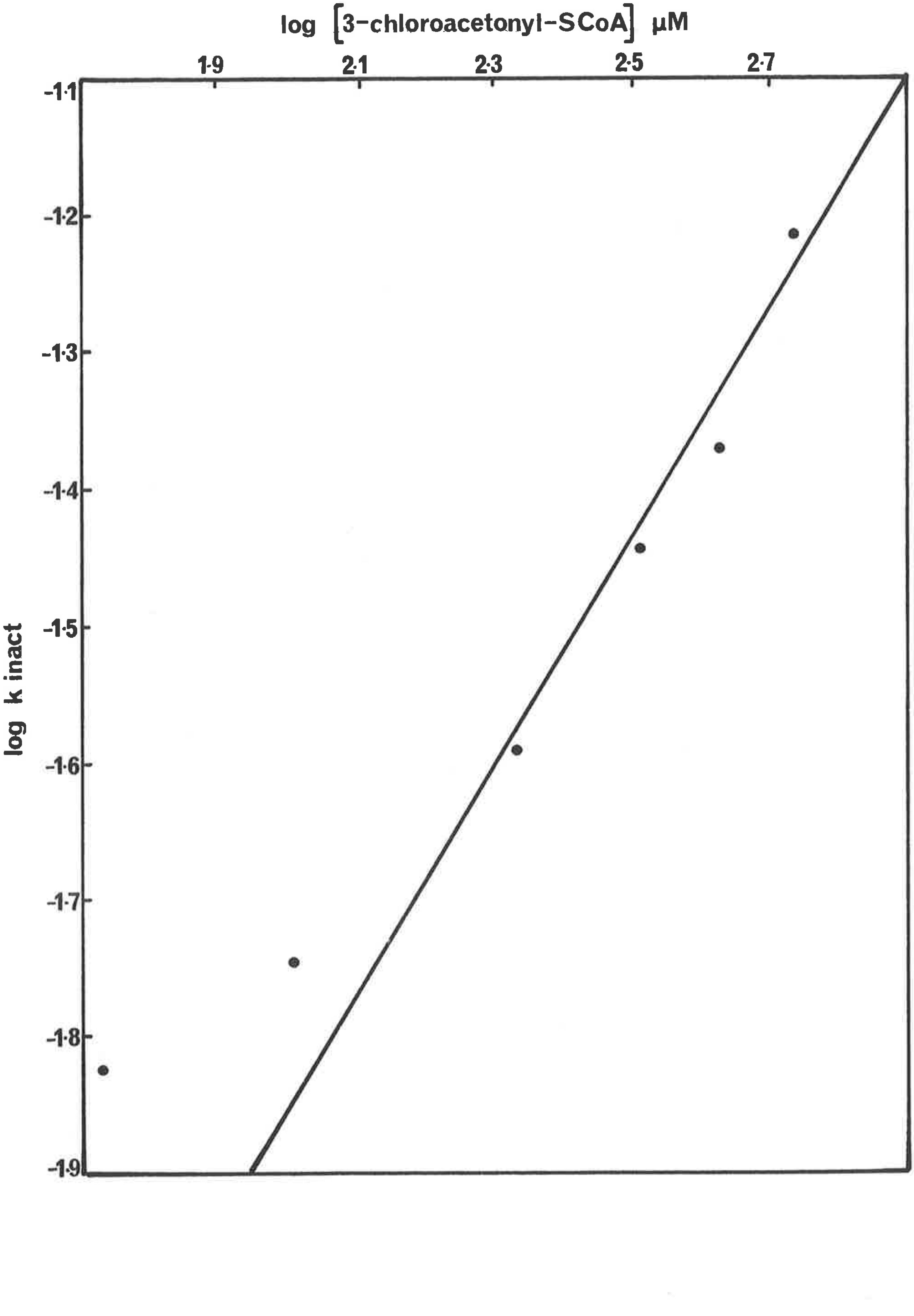

log [3-chloroacetonyl-SCoA] μM
1·9
2·1
2·3
2·5
2·7
-1·1
-1·2
-1·3
-1·4
-1·5
-1·6
-1·7
-1·8
-1·9
log k inact

Fig. 3.11 Determination of the residual rates of inactivation and K_d for the enzyme-acetyl CoA complex in the presence of 3-chloroacetonyl-SCoA.

Enzyme, 10.3 E.U./ml, was incubated in 0.25M NEMCl buffer pH 8.4 in the presence of 0.26mM 3-chloroacetonyl-SCoA and varying concentrations of acetyl CoA. Aliquots (10µl) were withdrawn at various times and residual activity was determined spectrophotometrically. K_{inact} values were determined from plots of log % initial activity against time.

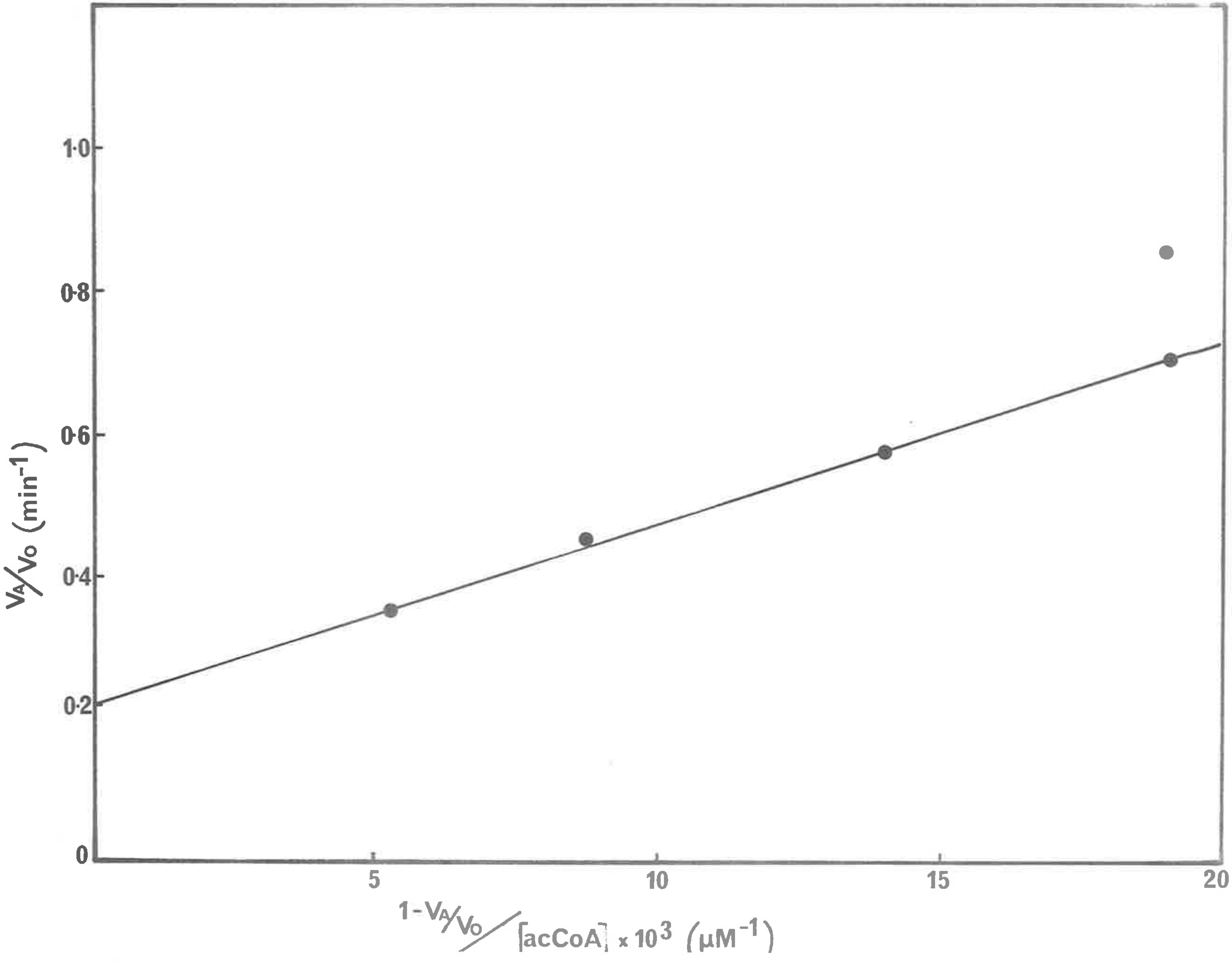

1·0
0·8
0·6
0·4
0·2
0
5
10
15
20
VA/Vo (min⁻¹)
1-VA/Vo / [acCoA] x 10³ (μM⁻¹)

Fig. 3.12 Difference spectrum of acetonyl CoA bound to pyruvate carboxylase after modification in the presence of 3-chloroacetonyl-SCoA

Unmodified pyruvate carboxylase used in the reference cell was adjusted to a concentration which afforded the same A_{280nm} value as the modified enzyme sample.

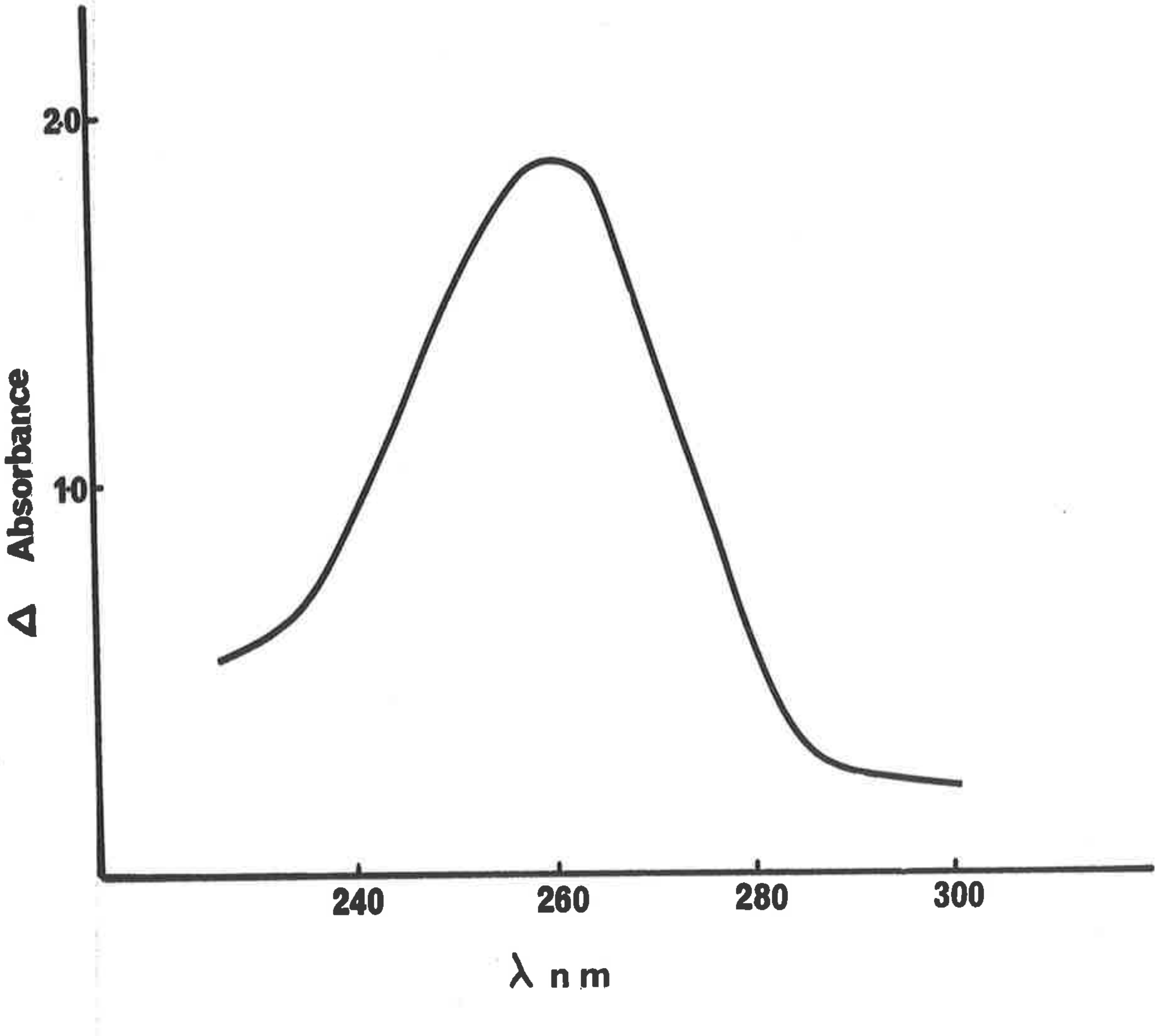
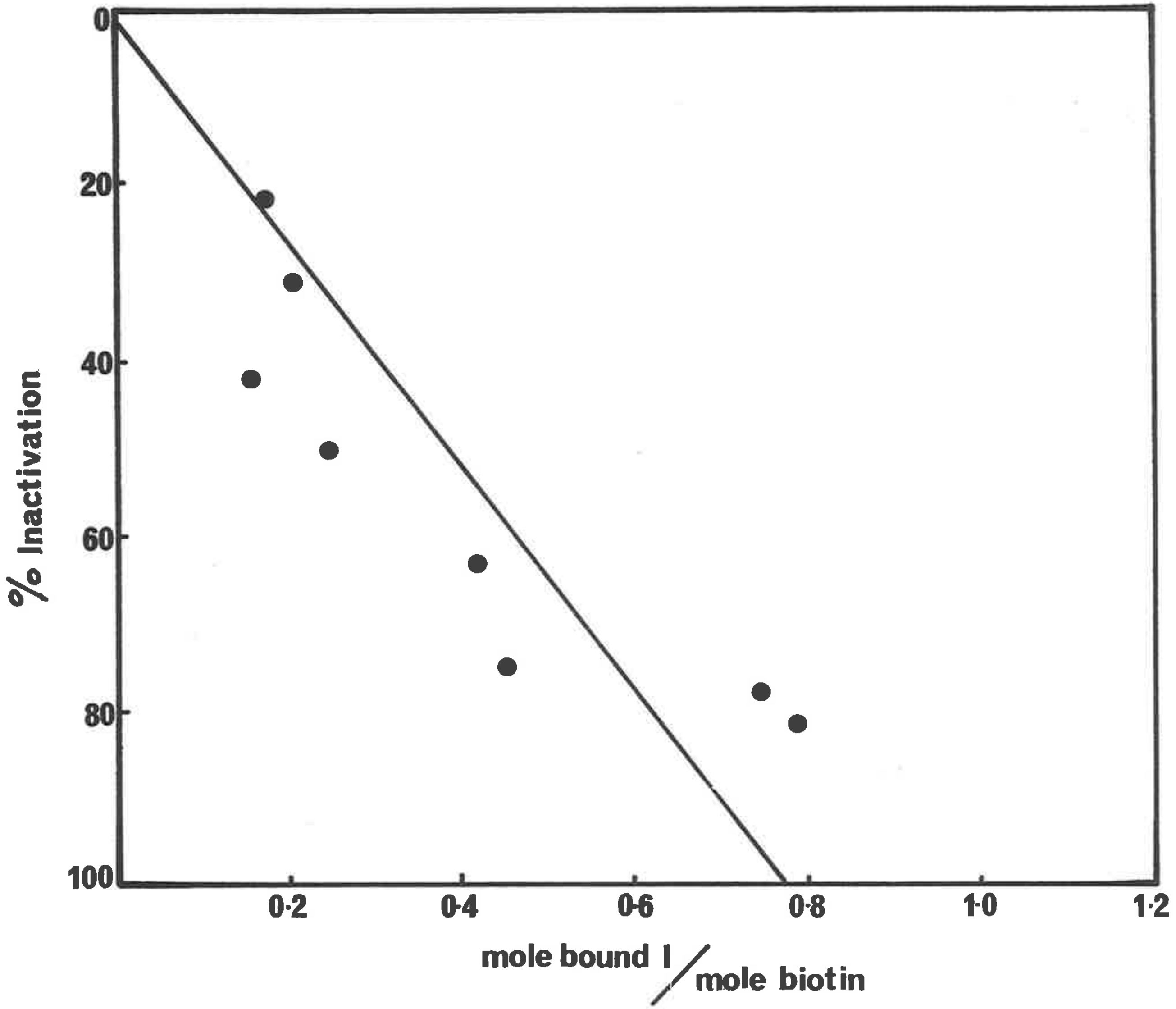

2·0
1·0
Δ Absorbance
240
260
280
300
λ nm

Fig. 3.13 Residual activity plotted as a function of the number of moles of 3-chloroacetonyl-SCoA bound per mole of enzyme biotin

Enzyme, 13 E.U./ml, was incubated with 88μM 3-chloroacetonyl-SCoA in 0.1M NEMCl pH 8.4. Aliquots (1.5 ml) were removed at various times and the inactivation quenched by addition of acetyl CoA and lysine to final concentrations of 0.25mM and 8mM respectively. The residual activity of the quenched enzyme samples were determined spectrophotometrically. The enzyme was precipitated by the addition of ammonium sulphate to a final saturation of 50%. The suspensions were centrifuged for 5 min. at 5000xg, resuspended twice in 50% ammonium sulphate solution and redissolved in 0.5M NEMCl buffer, pH 8.4. The difference spectra of the protein solutions were recorded using unmodified pyruvate carboxylase in the reference cell. The samples were then treated by pronase for 24 h. prior to the determination of biotin content by the procedure described in section 2.2.6.


% Inactivation
0
20
40
60
80
100
0·2
0·4
0·6
0·8
1·0
1·2
mole bound I / mole biotin

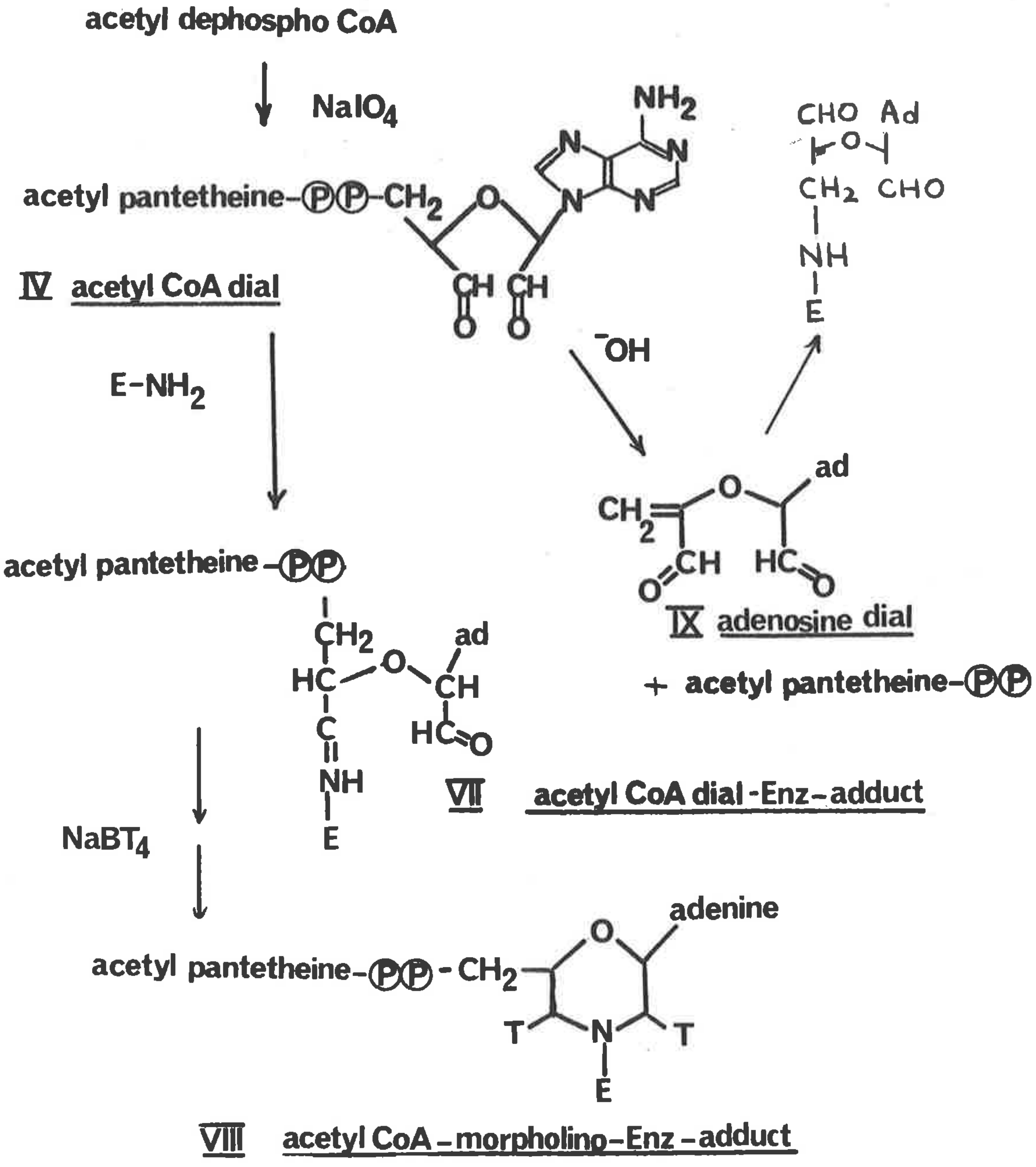

acetyl dephospho CoA
NaIO4
acetyl pantetheine-ⓅⓅ-CH2
NH2
CHO Ad
CH2 CHO
NH
E
IV acetyl CoA dial
E-NH2
⁻OH
ad
CH2
CH
HC
IX adenosine dial
+ acetyl pantetheine-ⓅⓅ
acetyl pantetheine-ⓅⓅ
CH2
HC
C
NH
E
ad
CH
HC
VII acetyl CoA dial-Enz-adduct
NaBT4
adenine
acetyl pantetheine-ⓅⓅ-CH2
T
N
T
E
VIII acetyl CoA-morpholino-Enz-adduct

Fig.3.1 4

Fig 3.15 The rate of inactivation of pyruvate carboxylase in the presence of acetyl CoA dial with and without borohydride reduction.

Enzyme, 26E.U./ml, was incubated with various concentrations of acetyl CoA dial for 15 min. The inactivation was terminated by the addition of lysine at a final concentration of 10mM. Each incubation mixture was divided into two and to one half was added sodium borohydride (▽-▽) at a final concentration of 6mM and an equivalent volume of water was added to the remaining half of the enzyme samples (●-●). Residual activity was determined radiochemically.

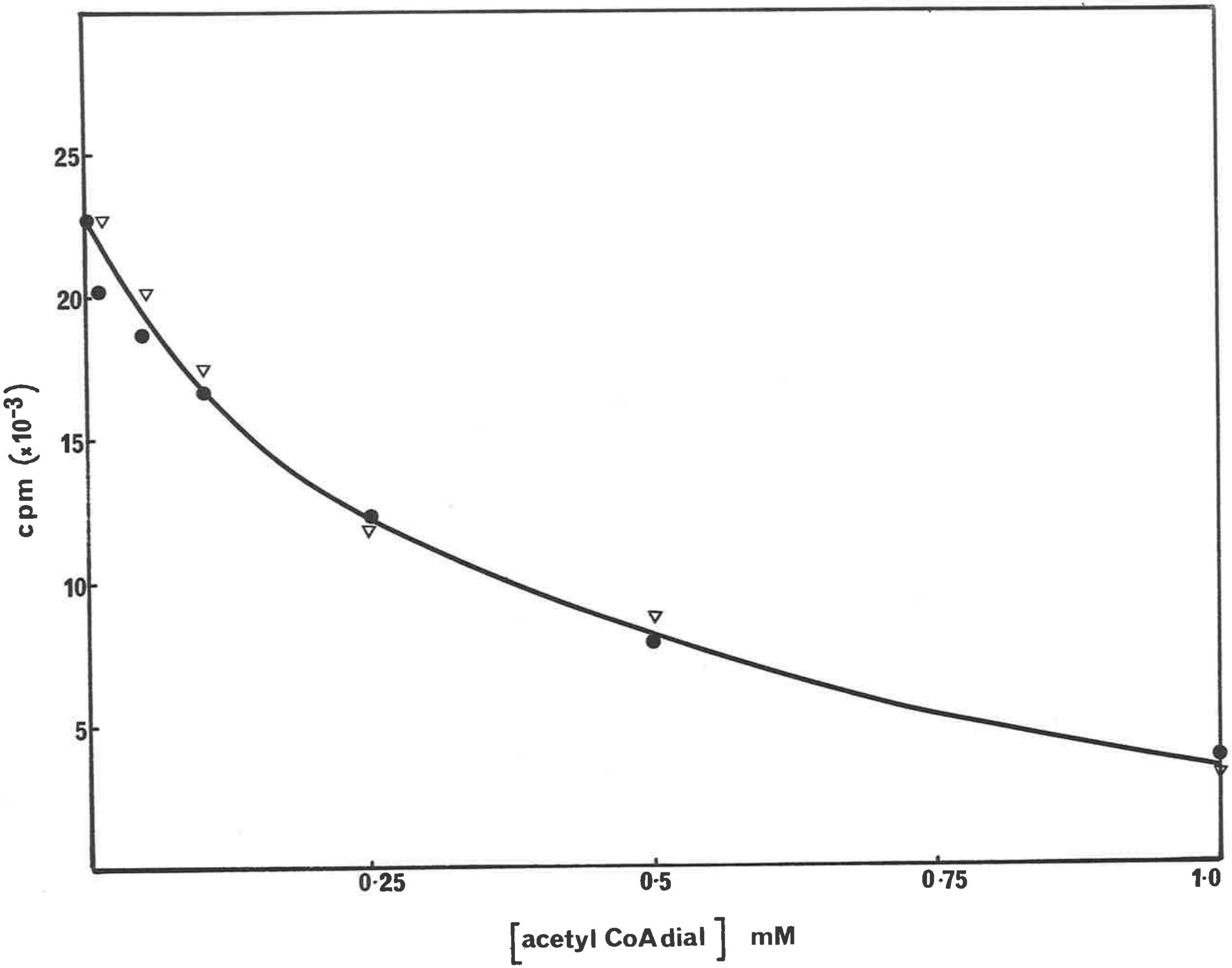

25
20
15
10
5
cpm (×10⁻³)
0·25
0·5
0·75
1·0
[acetyl CoA dial] mM

Fig 3.16 The effect of various concentrations of acetyl CoA dial on the activation of pyruvate carboxylase by acetyl CoA

Enzyme, 0.1 E.U./ml. was incubated for 5 min. in the radiochemical assay in a volume of 0.5 ml. and in the presence of varying acetyl CoA concentrations and the following fixed concentrations of acetyl CoA dial:- O (● - ●); 0.25mM (○ - ○); 0.5mM (x - x) and 1.0mM (□ - □).

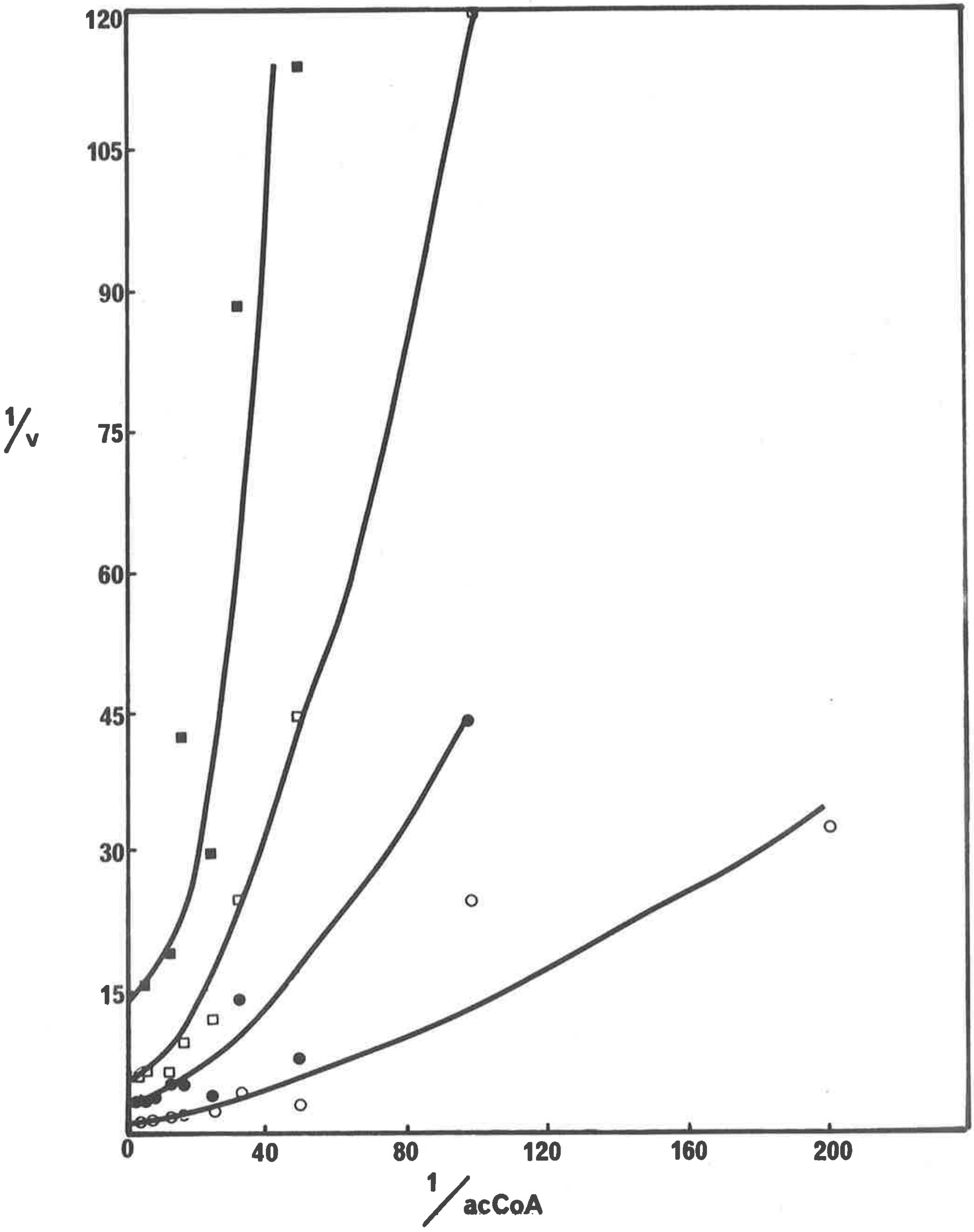
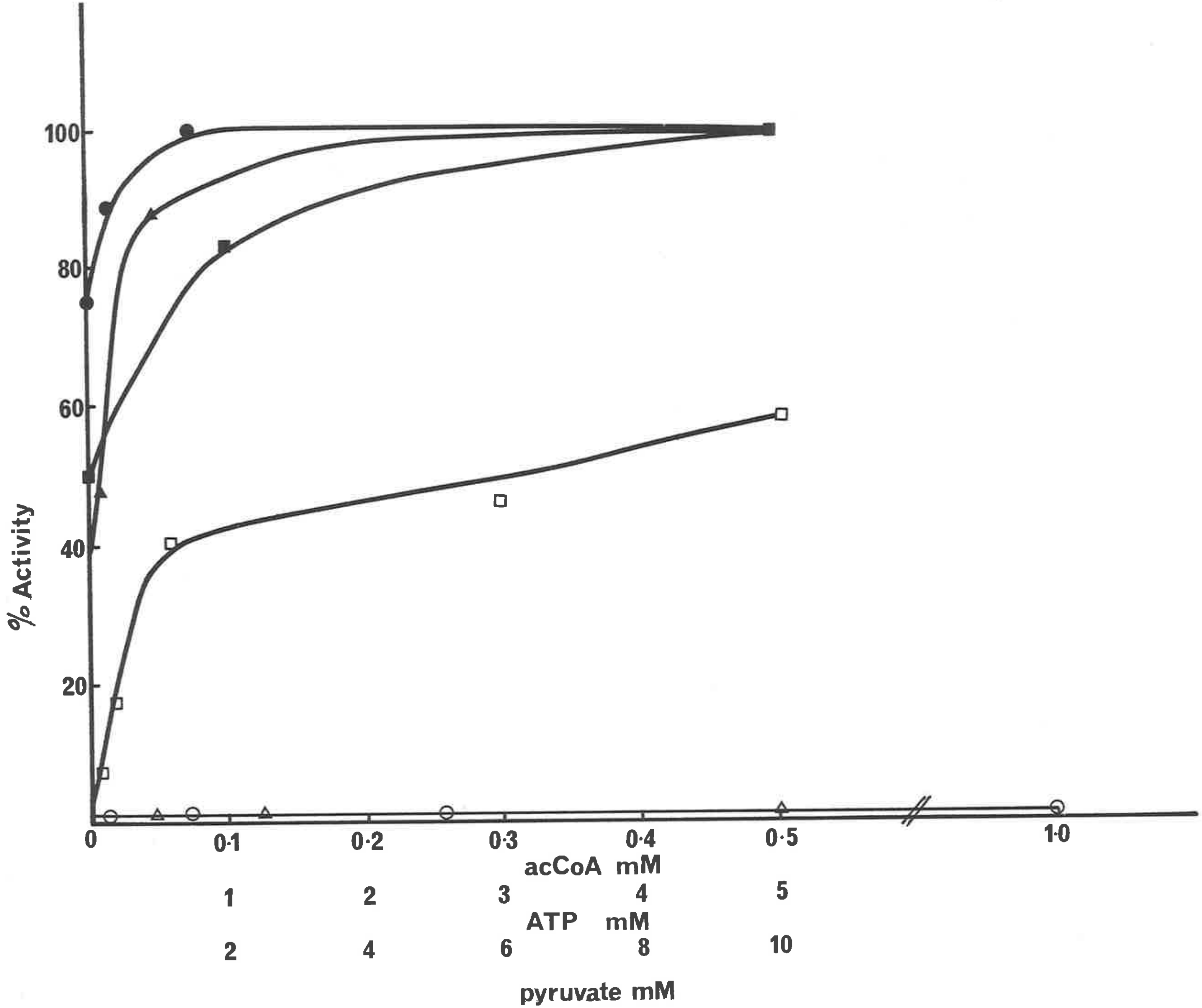

120
105
90
75
60
45
30
15
0
1/v
0
40
80
120
160
200
1/acCoA

Fig. 3.17 The effect of different substrates and acetyl CoA on the rate of inactivation of pyruvate carboxylase in the presence of acetyl CoA dial

Enzyme, was incubated in the presence and absence of acetyl CoA dial (1.5mM) and the following concentrations of:- pyruvate, 1mM (△-△), control (▲-▲); acetyl CoA, 1mM (o - o), control (● - ●); and ATP, 1mM (□ - □), control (■ - ■). After 10 min. the residual activity of the enzyme was determined radiochemically. Controls were in the absence of acetyl CoA dial.


100
80
60
40
20
% Activity
0
0·1
0·2
0·3
0·4
0·5
1·0
acCoA mM
1
2
3
4
5
ATP mM
2
4
6
8
10
pyruvate mM

CHAPTER 4

ANALOGUES OF ACETYL CoA

4.1 STUDIES WITH ANALOGUES OF ACETYL CoA

4.1.1 INTRODUCTION

Since the discovery of pyruvate carboxylase (Utter and Keech, 1960; Utter and Keech, 1963; Keech and Utter, 1963), the locus of action and the manner by which acetyl CoA exerts its activating effect on pyruvate carboxylases isolated from higher animals, has been the subject of much speculation (Utter *et al.*, 1964; Scrutton *et al.*, 1965). These latter studies showed that acetyl CoA was necessary for the exchange reaction between $MgATP^{2-}$ and orthophosphate while the isotopic exchange reaction between pyruvate and oxaloacetate proceeded in the absence of acetyl CoA. This led to the conclusion that the locus of action of acetyl CoA was at the first partial reaction site.

However, more recently, acetyl CoA has been shown to influence events occurring at the second partial reaction site. For example, Ashman *et al.*, (1973) showed that acetyl CoA decreased the apparent K_m value for pyruvate while Seufert *et al.*, (1971); McClure *et al.*, (1971c) and Ashman *et al.*, (1973) all showed that the pyruvate:oxaloacetate exchange reaction rate was stimulated by acetyl CoA. These observations provided compelling evidence that the influence of the allosteric activator was not restricted to the first partial reaction sub-site. Taken together with the observations discussed in section 1.1.5 that four molecules of acetyl CoA bind to each enzyme tetramer, the observations discussed above suggested that a single molecule of acetyl CoA influences events at both partial reaction sub-sites. The studies undertaken in this chapter were designed to provide a molecular basis for the above observations.

4.1.2 THE ROLE OF ISOTOPIC EXCHANGE REACTIONS IN THE STUDY OF PYRUVATE CARBOXYLASE

The application of isotopic exchange reactions to the study of many different enzymes has been detailed in the review of Clark *et al.*, (1974) which provides a good background to the general field. The isotopic exchange reactions catalysed by pyruvate carboxylase have been used to distinguish between possible mechanisms (Barden *et al.*, 1972; Ashman and Keech, 1975). As discussed in the General Introduction (section 1.1.1) the independent functioning of the exchange reactions have been taken as an indication of spatially distinct sub-sites and the different requirement for acetyl CoA has been taken as indication of the site of action of acetyl CoA.

Since they were first described, one difficulty in the interpretation of data provided by exchange reaction rates (Utter and Keech, 1963; Scrutton *et al.*, 1965) has been the observation that the rates of the ATP:ADP and the ATP:orthophosphate exchange reactions are very much slower than the rate of the pyruvate:oxaloacetate exchange reaction. For sheep liver enzyme the rate of the ATP:ADP exchange reaction in the presence of acetyl CoA was shown to be 1.95% of the forward rate (Ashman, 1973) and the ATP:orthophosphate exchange rate was 1.32% while the pyruvate:oxaloacetate exchange rate was 46.9% of the forward rate. The slow rates of the exchange reactions catalysed at the first partial reaction site were taken to indicate that the reactions were not valid as measures of events occurring at the first partial reaction site (Warren and Tipton, 1974a). However, an explanation for the slow rates had been put forward by Barden *et al.*, (1972) in which the formation of the dead-end complexes $E.HCO_3^-.MgADP^-$ and $E.HCO_3^-.Pi$ had the effect of titrating out available enzyme for catalysis of the exchange reaction. This, however, was not the complete explanation as recent work in this laboratory (Easterbrook-Smith *et al.*, 1978a) suggested that in the sequential mechanism proposed for pyruvate carboxylase, products of the first partial reaction are not released at a significant rate until after the binding of pyruvate.

This conclusion, taken together with the possibility of dead-end complex formation provided an explanation for the slow rates observed for the exchange reactions catalysed by the first partial reaction and argues strongly for the validity of the exchange reactions in their use as measures of events at the first partial reaction subsite. Exchange reactions were used in a similar way for the study of acetyl CoA carboxylase (Polakis *et al.*, 1974) as measures of the partial reaction activities catalysed by the different subunits of the enzyme. These workers were able to demonstrate that the two partial reactions were catalysed by different subunits and that a third subunit carried the biotin prosthetic group.

4.1.3 ANALOGUES OF ACETYL CoA

From the observations that one molecule of acetyl CoA binds per enzyme active site and that it exerts its influence on both the first partial reaction and the second partial reaction, a working model was proposed which requires that acetyl CoA spans across the active site into both subsites. To test this model and to orientate the positioning of the activator molecule, various components of the acetyl CoA molecule were examined for their influence on each of the exchange reactions. The main compounds used in this study were acetylpantetheine and acetyl-2'4'-phosphopantetheine representing the acetyl end of acetyl CoA and CoASH and 3'5' ADP to test for the adenyl moiety function.

4.2 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE EXCHANGE, OVERALL AND ACETYL CoA -INDEPENDENT ACTIVITIES OF PYRUVATE CARBOXYLASE

4.2.1 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE ATP:ORTHOPHOSPHATE EXCHANGE REACTION

(a) The effect of acetyl CoA

In order to establish the nature of acetyl CoA activation of the ATP:orthophosphate exchange and to provide a basis for comparison of the responses obtained, the response of the exchange rate to changes in the acetyl CoA concentration was first investigated. The results presented in (Fig. 4.1) show that the response of the exchange rate to acetyl CoA concentration appeared to be hyperbolic. However, a plot of the data in the double reciprocal form (Fig. 4.1, inset) shows that the line was concave downwards, from which a K_a value of 67µM was obtained. The type of response observed here does not support positive homotropic cooperativity of binding of acetyl CoA and is consistent with the evidence for lack of cooperativity observed when deacylation was used as a measure of acetyl CoA binding (Easterbrook-Smith *et al.*, 1977).

(b) The effect of CoASH

The effect of CoA on the ATP:orthophosphate exchange reaction was investigated and, like acetyl CoA, gave an apparently hyperbolic response (Fig. 4.2a) with a maximum velocity which was 42% relative to the maximum velocity in response to acetyl CoA. The double reciprocal plot was a straight line (Fig. 4.2b) which confirmed that the response was hyperbolic. The response reached a peak at 3mM above which some inhibition occurred. There are at least two possible explanations for the observed inhibition of the ATP:orthophosphate exchange reaction at high levels of acetyl CoA,

i) at high levels of CoA at which inhibition occurred, binding of the coenzyme to the ATP binding site could occur, and
ii) formation of a disulphide link with an enzymic sulphydryl residue to CoASH. Attempts to demonstrate this by the presence of enzyme bound nucleotide after exposure to high levels of CoASH were unsuccessful. The lower V elicited by CoASH could be explained on the basis of the inhibition that occurs at high levels of the activator although other explanations are possible.

(c) The effect of 3'5' ADP

In order to further characterise the portion of the acetyl CoA molecule responsible for stimulating the ATP:orthophosphate exchange reaction, the effect of 3'5' ADP was examined. The response, as presented in Fig. 4.3a, was hyperbolic but the V of the exchange reaction was only

7% relative to the maximum velocity obtained in response to saturating concentrations of acetyl CoA. The double reciprocal plot, Fig 4.5b, was a straight line. Again the exchange reaction was inhibited at high levels of activator and this was interpreted as indicating that 3'5' ADP was binding non-specifically to the ATP binding site as suggested for CoASH inhibition.

(d) The effect of acetylpantetheine and acetyl-2'4'-phosphopantetheine

The analogues of the acetyl end of acetyl CoA were tested for their effect on the ATP:orthophosphate exchange reaction and neither analogue was found to stimulate at the concentrations used (0-10mM) for each compound. This result indicated that the acetyl end of acetyl CoA was not involved in stimulating the first partial reaction.

Taken together with the previous results the results presented above were strong evidence that stimulation of the first partial reaction is a function of the adenosine moiety of acetyl CoA.

(e) The effect of acetyldephospho CoA

In order to further characterise the nature of the activation of the ATP:orthophosphate exchange by the adenosine moiety of acetyl CoA, the effect of acetyldephospho CoA and acetyldesamino CoA was investigated. The results presented in Fig 4.4 show that acetyldephospho CoA stimulated the exchange with a similar K_a value (0.034mM) to that of acetyl CoA as determined by its effect on this exchange reaction. The velocity of the reaction was 24%

of that obtained in the presence of acetyl CoA which suggests that although the analogue binds to the enzyme tightly the absence of the 3'-phosphate group results in a reduced stimulation of the first partial reaction.

The double reciprocal plot of the data, Fig 4.4 insert, shows that the response of the enzyme catalysed exchange rate to acetyldephospho CoA obeys Michaelis-Menten kinetics. This observation together with the similar K_a values for both acetyl CoA and acetyldephospho CoA suggests that the 3'-phosphate group of acetyl CoA may play a role in the overall catalytic process of the enzyme. This will be discussed more fully at the end of this chapter.

(f) The effect of acetyldesamino CoA

The effect of acetyldesamino CoA on the ATP: orthophosphate was investigated and no stimulation was observed at the concentrations used (0-5mM). This result indicates that the 6-amino group is essential for the activation of the first partial reaction by acetyl CoA.

4.2.2 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE PYRUVATE:OXALOACETATE EXCHANGE

The results in the preceding section provide strong evidence that the adenosine moiety of acetyl CoA stimulates the first partial reaction of pyruvate carboxylase and that the presence of the 6-amino group of adenosine is essential for this activation. In order to complete the assignment of function to acetyl CoA moieties the analogues were investigated for their effect on the second partial reaction as measured by the pyruvate: oxaloacetate exchange reaction. Studies on the effect of

Mg^{++} being carried out in this laboratory indicated that there was interaction between acetyl CoA and Mg^{++}. It was also found that the presence of Mg^{++} lowered the K_a value for acetyl CoA by a factor of 5-10. The inclusion of Mg^{++} in the pyruvate:oxaloacetate exchange reaction solution was approached with caution, however, since it had been found that at levels of Mg^{++} above 1mM, the reaction was strongly inhibited. For this reason the effect of each analogue in the pyruvate:oxaloacetate exchange was investigated first in the absence of Mg^{++} and then in the presence of 0.5mM Mg^{++}.

(a) The effect of acetyl CoA in the presence and absence of Mg^{++}

The results presented in Fig. 4.5 show that in the absence of Mg^{++} the response to acetyl CoA exhibits inhibition at low concentrations, an apparent plateau of activation from 0.1 to 0.23mM and is further stimulated at 0.5mM. A double reciprocal plot of the data was concave downwards. The results presented in Fig 4.6 show that in the presence of Mg^{++} the inhibition at low acetyl CoA levels was almost eliminated but the stimulation by acetyl CoA is reduced from a factor of 3.6 in the absence of Mg^{++} to a factor of 1.5 in its presence. When plotted in the double reciprocal form as presented in Fig. 4.6 (insert), the curve is concave downwards.

The effect of Mg^{++} in eliminating the inhibition which occurred at low levels of acetyl CoA could be explained by the effect of these activators on the rate of decarboxylation of oxaloacetate. It has recently been shown in this laboratory (Duc, 1977) that the presence of Mg^{++} reduces the rate of decarboxylation of oxaloacetate. Therefore, if it is assumed that the apparent inhibition at low levels of acetyl CoA is due to the rate of decarboxylation of oxaloacetate being faster than the exchange rate, then the presence of Mg^{++} would eliminate the observed inhibition by preventing the decarboxylation.

(b) The effect of acetylpantetheine in the presence and absence of Mg^{++}

The results in the absence of Mg^{++} as presented in Fig 4.7 show that acetyl pantetheine stimulates the pyruvate:oxaloacetate exchange with a biphasic response similar to that observed for acetyl CoA in the absence of Mg^{++}. The response did not show inhibition at low levels of the activator and the double reciprocal plot as presented in Fig. 4.7 (insert) was concave downwards. As shown in Fig 4.8, the exchange reaction was inhibited in the presence of Mg^{++} at all levels of acetyl pantetheine which was taken as indication of an unfavourable interaction either between Mg^{++} and acetylpantetheine or between Mg^{++} and the enzyme in the presence of acetyl pantetheine.

This result was consistent with the observation discussed at the beginning of this section that Mg^{++} inhibits the rate of the pyruvate:oxaloacetate exchange reaction. Since no phosphate group was present in the analogue to interact with Mg^{++} it could be concluded that Mg^{++} was acting to effect the exchange reaction as if no activator were present but that the presence of acetylpantetheine stimulated the effect of Mg^{++} in its inhibition of the exchange rate.

(c) The effect of acetyl-2'4'-phosphopantetheine in the presence and absence of Mg^{++}

The results presented in Fig. 4.9 show that in the absence of Mg^{++} the response of the exchange is complex and that inhibition occurred at low levels of the analogue as observed previously with acetyl CoA. The effect of Mg^{++} on the response of the exchange as presented in Fig. 4.10 shows that the inhibition which occured at low concentrations had been eliminated. The curve exhibits two maxima and in the double reciprocal form is concave downwards.

It can be seen that the rate of the exchange reaction is much greater in the presence of high levels of acetyl 2'4'-phosphopantetheine than in the presence of saturating levels of acetyl CoA. The results suggest that the presence of the adenosine 3'5'phosphate moiety in acetyl CoA may inhibit stimulation of the exchange reaction rate by the acetyl pantetheine moiety of acetyl CoA.

(d) The effect of CoASH

CoASH inhibited the exchange reaction in the absence of Mg^{++} as shown in Fig 4.11. In the presence of Mg^{++}, as shown in Fig 4.12, CoASH had no significant effect on the exchange rate. This allows the unequivocal assignment of a function for each moiety at the ends of acetyl CoA. The adenosine moiety allows the exchange between orthophosphate and ATP to occur while the acetyl pantetheine moiety stimulates only the exchange between pyruvate and oxaloacetate.

(e) The effect of acetyldephospho CoA

The results presented in Fig. 4.13 show that in the absence of Mg^{++}, acetyldephospho CoA did not stimulate the exchange reaction to the same extent as did acetyl CoA. In the presence of Mg^{++}, however, the results presented in Fig. 4.14 show that the rate of the exchange reaction in response to acetyldephospho CoA increased by a factor of 3 over the rate in the presence of acetyl CoA. This result implies that the 3'phosphate group in some way limits the extent of stimulation of the exchange by the acetyl pantetheine moiety.

(f) The effect of acetyldesamino CoA

The results presented in Fig. 4.15 show that in the absence of Mg^{++} acetyldesamino CoA weakly stimulates the exchange reaction rate and exhibits the characteristic 'dip' at low levels of the activator. The results presented in Fig 4.16 show that in the presence of Mg^{++} the exchange reaction is stimulated at a 5.5 fold greater rate than that afforded by saturating levels of acetyl CoA. This result can be compared with the acetyldephospho CoA and acetyl-2'4'-phosphopantetheine responses, in magnitude. This result also suggests that for a particular activator the lack of capacity to activate the first partial reaction is approximately matched in degree by the capacity to activate the second partial reaction.

4.2.3 THE EFFECT OF ACETYL CoA ANALOGUES ON THE OVERALL REACTION CATALYSED BY PYRUVATE CARBOXYLASE

It was useful to compare the activation of each partial reaction by the acetyl CoA analogues with their effect on the overall reaction catalysed by pyruvate carboxylase.

(a) The effect of CoASH

The results presented in Fig 4.17 show that the velocity response curve was sigmoidal and had a maximum velocity which was 63% ± 5% of the response in the presence of acetyl CoA. The K_a value for CoA was calculated to be 2.0mM (±0.2) from the double reciprocal plot as presented in Fig 4.17 (insert) using the value of the CoA concentration which afforded half of the maximum stimulation of the enzyme.

(b) The effect of acetyl-2'4'-phosphopantetheine

The results presented in Fig 4.18 show that the response curve was also sigmoidal and that the velocity was 6.2% of the response in the presence of acetyl CoA. A K_a value of 11.6mM could be calculated for acetyl 2'4'-phosphopantetheine from the double reciprocal plot as presented in Fig 4.18 (insert) using the value of the activator concentration which afforded half of the maximum rate over the rate in the absence of acetyl-2'4'-phosphopantetheine.

(c) The effect of acetyldephospho CoA

The results as presented in Fig. 4.19 show that the response was sigmoidal and the velocity was 12% of the response in the presence of acetyl CoA and the K_a value for acetyldephospho CoA was calculated to be 1.11mM from the double reciprocal plot of the data as shown in Fig 4.19 (insert).

(d) The effect of acetyldesamino CoA

No stimulation of the overall reaction was observed in the presence of 0-10mM acetyldesamino CoA. This result provided further evidence that the 6-amino group is essential for activation of the first partial reaction and that as a result stimulation of the overall activity was not observed.

(e) The effect of 3'5'ADP

At a concentration of 5mM, 3'5'ADP stimulated the reaction by 6.7% of the rate observed in the presence of acetyl CoA. The response was of the same magnitude as the stimulation of the ATP:orthophosphate exchange by 3'5'ADP.

4.2.4 THE EFFECT OF THE ANALOGUES OF ACETYL CoA ON THE ENZYME ASSAYED UNDER ACETYL CoA-INDEPENDENT CONDITIONS

The effect of each analogue has been examined on both the first and second partial reactions and on the overall reaction. If these effects are due to the lowering of the apparent K_m for HCO_3^- by the adenosine moiety and lowering of the apparent K_m for pyruvate by acetyl pantetheine then under the conditions which were found to be saturating for each of these substrates when assayed under acetyl CoA-independent conditions, the analogues should have no stimulatory effect on the enzyme catalysed reation rate.

(a) The effect of acetyl-2'4'-phosphopantetheine and of CoASH

The effect of acetyl-2'4'-phosphopantetheine and the effect of CoASH was compared with the effect of acetyl CoA on the enzyme-catalysed overall reaction assayed in the acetyl CoA -independent assay system. The results show that CoASH has no effect on the activity while acetyl CoA markedly stimulates the reaction. Acetyl-2'4'-phosphopantetheine had a slightly inhibitory effect over the concentration range used.

(b) The combined effect of CoASH and acetyl-2'4'-phosphopantetheine

The results presented in Fig. 4.21 show that the separate moieties did not have any effect on the enzyme catalysed reaction rate compared with that in the presence of acetyl CoA. These results allow the effect of the complete acetyl CoA molecule to be compared with moieties which separately enhance the two partial reactions. To avoid the criticism that overlapping regions of CoASH and acetyl-2'4'-phosphopantetheine may have resulted in the reduced binding of either activator, the experiment was repeated with acetyl-2'4'-phosphopantetheine and 3'5'ADP. The results presented in Fig. 4.21 show that the combination of activators did not stimulate the enzyme. It was concluded that the intact acetyl CoA molecule was necessary for enhancement of the enzyme catalysed reaction under conditions of saturating substrate levels and high enzyme concentration.

(c) The effect of acetyldephosphoCoA and acetyl-desamino CoA

The results presented in Fig. 4.22 show that both acetyldephospho CoA and acetyldesamino CoA inhibited the rate of the acetyl CoA -independent reaction. This was further evidence that the complete molecule of acetyl CoA was necessary for the V enhancement effect.

A possible explanation for the observed inhibition is that at the elevated concentrations of each activator which were used, non-productive binding to the ATP binding site could easily occur, particularly in view of their reduced affinity for the acetyl CoA binding site.

4.3 DISCUSSION

4.3.1 CONCLUSIONS AND HYPOTHESES

The overall conclusion to be drawn from the results presented in this chapter is that an explanation can now be provided for the stimulation by acetyl CoA of both the first and second partial reactions catalysed by pyruvate carboxylase. The role of stimulation of the first partial reaction, in which the lowering of the apparent K_m for HCO_3^- is a feature, can be assigned to the adenosine moiety of acetyl CoA. Similarly, the role of stimulation of the second partial reaction, including the lowering of the apparent K_m for pyruvate, can be assigned to the acetyl pantetheine moiety.

These findings provide a molecular explanation for the findings of Ashman *et al.*, (1972) that the acetyl CoA molecule influenced not only the first partial reaction but also the second partial reaction. The findings also provide further evidence to support the contention of Easterbrook-Smith *et al.*, (1978b), that the effects are due to a single molecule of acetyl CoA which does not bind cooperatively to the enzyme.

A further conclusion which can be drawn is that since the two partial reaction sub-sites are believed to be spatially distinct (Barden *et al.*, 1972) then the acetyl

CoA molecule must therefore span the two sub-sites. This assumes that the molecule acts on these sub-sites from a proximal binding site and evidence presented in the following chapters will provide some indication that this is the case.

The distance which the acetyl CoA molecule must span is not difficult to accommodate with the above hypothesis since the 14Å biocytin prosthetic group can commute between sites which are up to 28Å apart. This is coincidentally the same as the length (28Å) of the acetyl CoA molecule. The evidence of Fung *et al.*, (1976) for the "U" shape of propionyl CoA bound to transcarboxylase tends to indicate that given a probable evolutionary similarity between the coenzyme A binding sites of biotin carboxylases, the distance between sub-sites need not be as large as 28Å.

The possibility that the biotin prosthetic group resides in a cleft of the enzyme which contains the partial reaction sub-sites on opposite faces, can be easily visualised. The findings of Rylatt (1976) in which a cleft was proposed in which the peptide to which biotin was attached, formed the junction, support the above conception of the active site. This allows the proposal of a model illustrated in Fig 4.23, for acetyl CoA action where the molecule binds to the enzyme forming a "cap" spanning the active site cleft and protecting the substrates and the enzyme within from hydration by the medium. Further evidence for this model will be presented in the following chapter.

4.3.2 POINTS ARISING FROM THE RESULTS

Another conclusion which can be drawn from the results is that in its activation of the first partial reaction, the adenosine moiety requires both the 3'phosphate group and the 6-amino group for maximum effect. The 6-amino group appears to be essential for the activation process which could be interpreted to mean that specific ionic interaction between the enzyme and the group is directly involved in the activation process. This may be necessary for the orientation of the adenine group since free rotation around the ribose-adenine C-N bond would allow an infinite number of possible conformations. The 3'phosphate group did not appear to be essential since its absence afforded a reduced activation of the first partial reaction. The reduced V and the high K_a value obtained from the overall reaction in the presence of acetyldephospho CoA suggested that the 3'phosphate group was involved in binding rather than the activation process.

The effect of the 6-amino group was obviously more fundamental since no stimulation of the overall reaction was observed in its absence. Other analogues which could yield information in a similar type of investigation are adenosine-3'-phosphate-5'-pyrophosphate-o-pantothenate (CoAOH) which would not have the free sulphydryl moiety in its structure. Alternatively desulpho CoA prepared by the method of Stewart *et al.*, (1968) could be used. The effect of the analogues on the acetyl CoA -independent pyruvate carboxylase activity was as expected since at the levels of pyruvate and HCO_3^- used in the assay, no further saturation could be afforded by either acetyl CoA or by analogues which stimulate either partial reaction. The stimulation of the acetyl CoA -independent activity by acetyl CoA remains the unexplained factor in the mode of action of acetyl CoA which was alluded to in the General Introduction (section 1.1.10) on the V effect of acetyl CoA. One possible explanation of this effect and of the greater enhancement of the pyruvate:oxaloacetate exchange reaction by the analogues than by acetyl CoA, is that the adenosine moiety causes a change in the time spent by the biotin prosthetic group in either of the two sub-sites. Since there is only one biotin residue serving both partial reaction sites, it could be argued that in the absence of acetyl CoA or at least its adenosine moiety, the biotin residue spends more of its time in the second partial reaction site, thus making the biotinyl moiety more readily available for the exchange reaction. In the presence of the adenosine moiety, however, the biotin residue is induced to spend more of its time in the first partial reaction site thus having the effect of reducing the second partial exchange reaction rate.

Experiments designed to test this hypothesis could be carried out using the effect of analogues of acetyl CoA on the rate of inactivation of the enzyme by avidin based on the findings of Scrutton and Utter (1967) that acetyl CoA promotes the inactivation of the enzyme by avidin. If acetyl CoA causes the biotinyl residue to spend more time in the first partial reaction sub-site, which in turn causes the prosthetic group to be more exposed to interaction with avidin, then analogues which stimulate the first partial reaction ought to enhance the rate of inactivation by avidin.

Preliminary experiments of this type indicated that acetyl 2'4'-phospho pantetheine protected against the inactivation by avidin which was in agreement with the above hypothesis. Time did not permit the further investigation of this hypothesis.

It is evident from the results that the interaction between acetyl CoA and Mg^{++} is important in the binding of the coenzyme but has some catalytic role since in the absence of acetyl CoA, Mg^{++} has been shown to inhibit the pyruvate:oxaloacetate exchange reaction. The effect of Mg^{++} on the pyruvate:oxaloacetate exchange can be partially understood if it is accepted, as unpublished observations from this laboratory suggest, that Mg^{++} binds first to the enzyme and then to acetyl CoA. In other words there is a previously undescribed enzyme - Mg^{++} interaction.

Further work in this laboratory (Duc, 1977) using ^{31}P-NMR, has shown an interaction between Mg^{++} and the 3'-phosphate group of acetyl CoA and a second, weaker interaction between Mg^{++} and the β-phosphate group of acetyl CoA.

Fig. 4.1 The effect of acetyl CoA on the ATP: orthophosphate exchange reaction

ATP:orthophosphate exchange activity was determined, as described in section 2.2.5c, in the presence of the concentrations of acetyl CoA indicated.

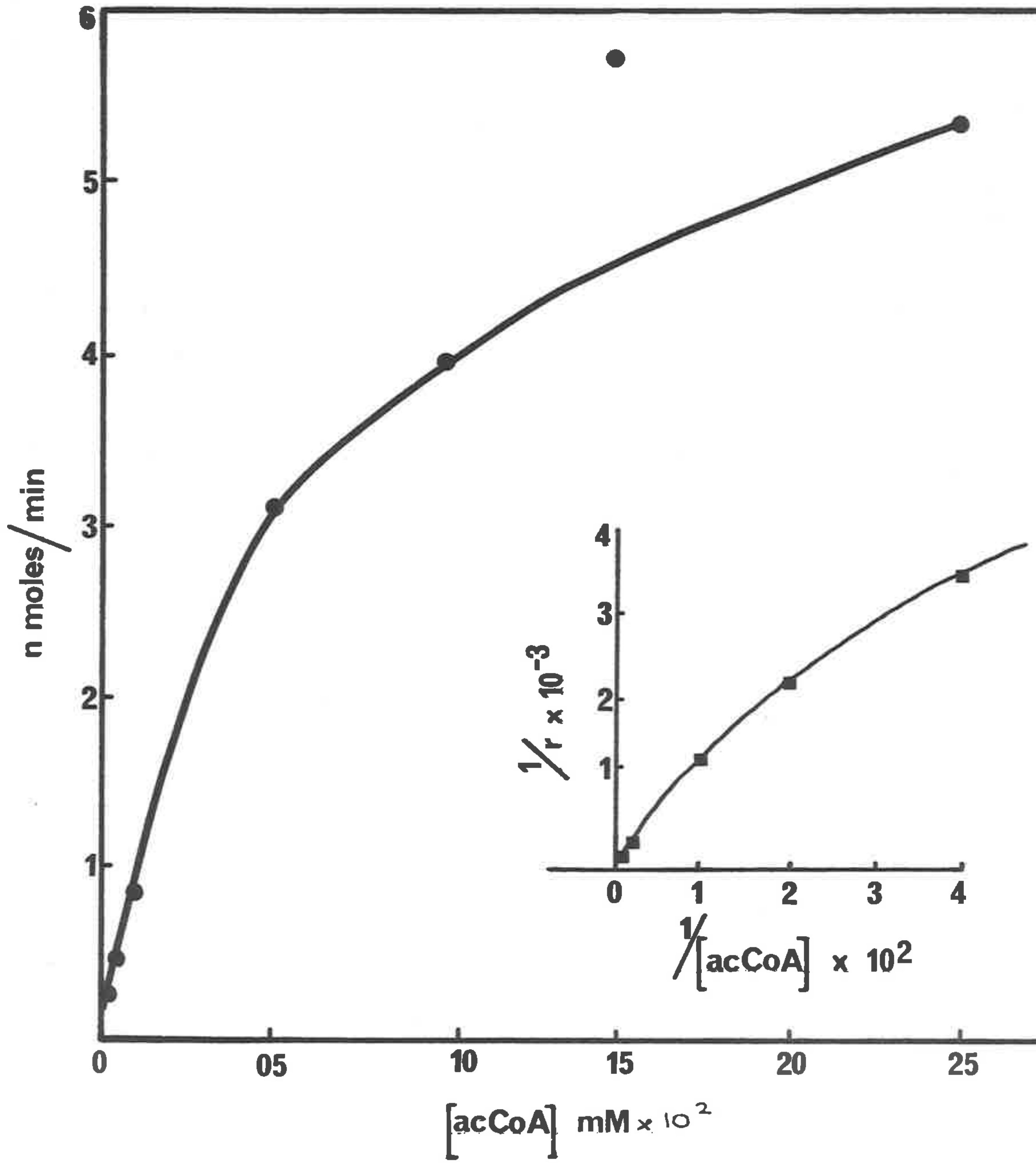

n moles/min
6
5
4
3
2
1
0
05
10
15
20
25
[acCoA] mM x 10²
1/r x 10⁻³
4
3
2
1
0
1
2
3
4
1/[acCoA] x 10²

Fig. 4.2 The effect of CoASH on the ATP:orthophosphate exchange reaction

ATP:orthophosphate exchange activity was determined, as described in section 2.2.5c, in the presence of the concentrations of CoASH indicated (●-●). Exchange reaction activity was also determined in the presence of 0.25mM acetyl CoA (■).

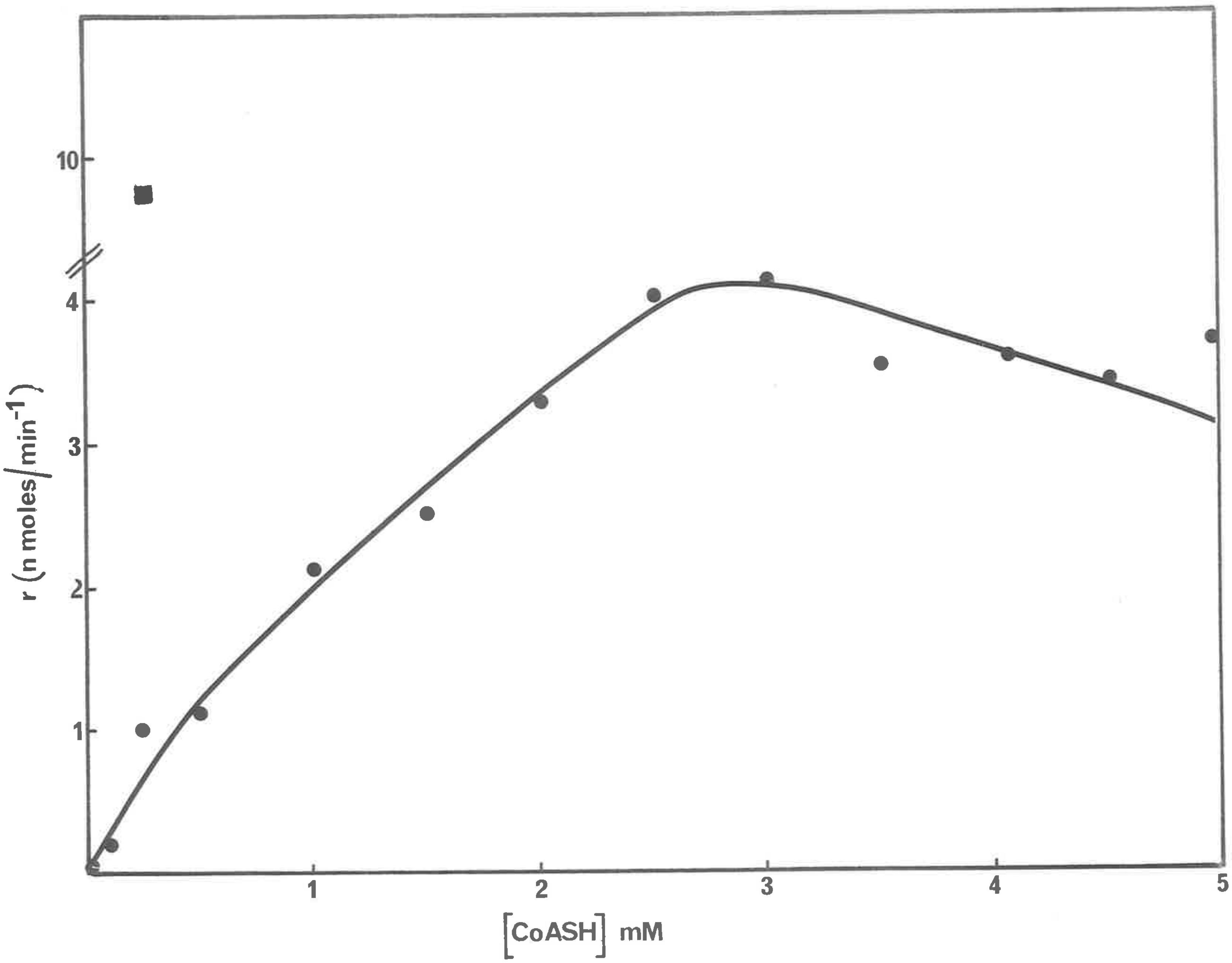
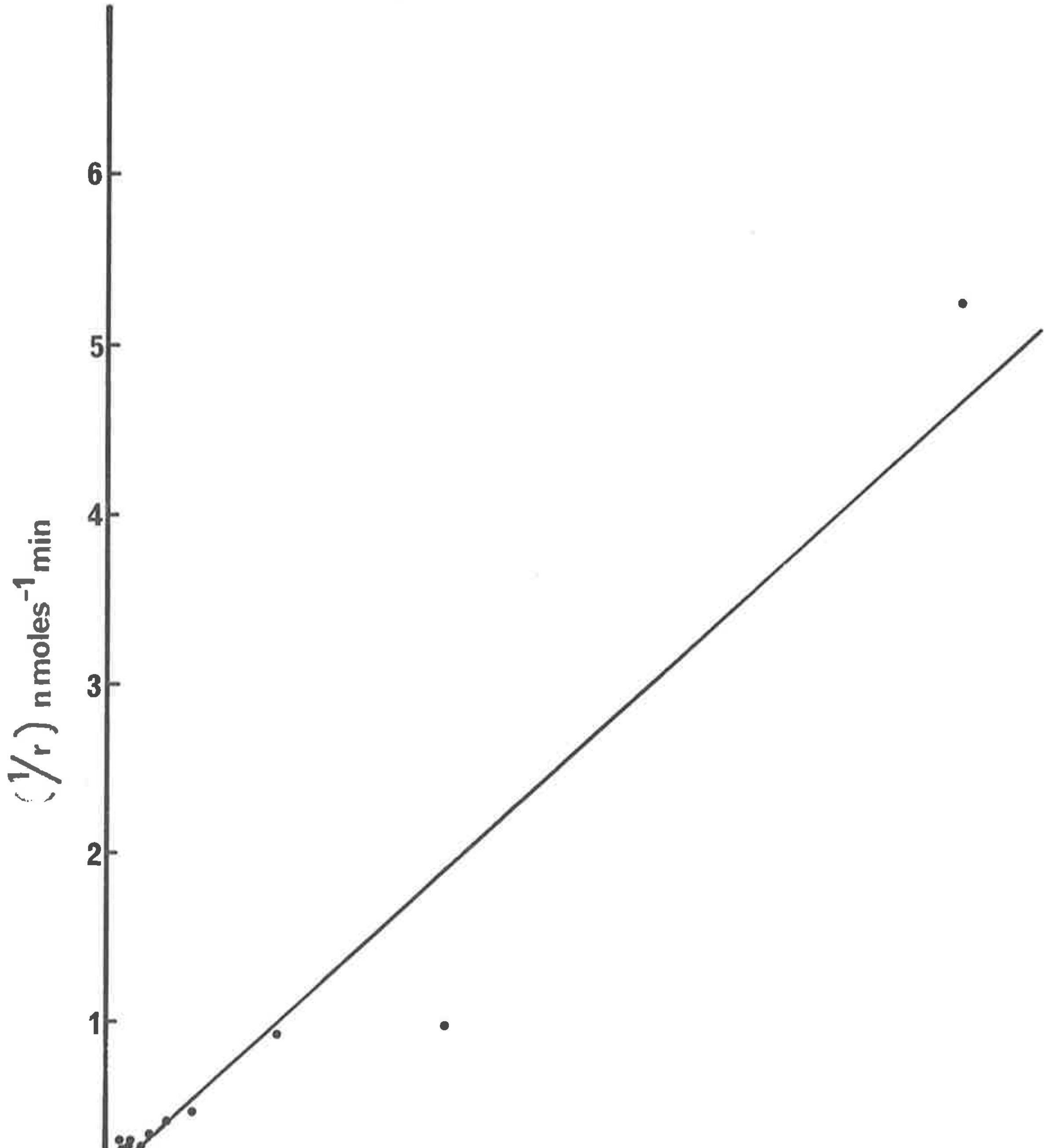

10
4
3
2
1
r (n moles/min^{-1})
1
2
3
4
5
[CoASH] mM

Fig. 4.2a Double reciprocal plot of data from Fig. 4.2.



(1/r) n moles^{-1} min
6
5
4
3
2
1
0
2
4
6
8
10
1/[CoASH] mM

Fig. 4.3 The effect of 3'5' ADP on the ATP:orthophosphate exchange reaction

ATP:orthophosphate exchange activity was determined, as described in section 2.2.5c, in the presence of the concentrations of 3'5'ADP indicated (●-●). Exchange reaction activity was also determined in the presence of 0.25mM acetyl CoA (■).

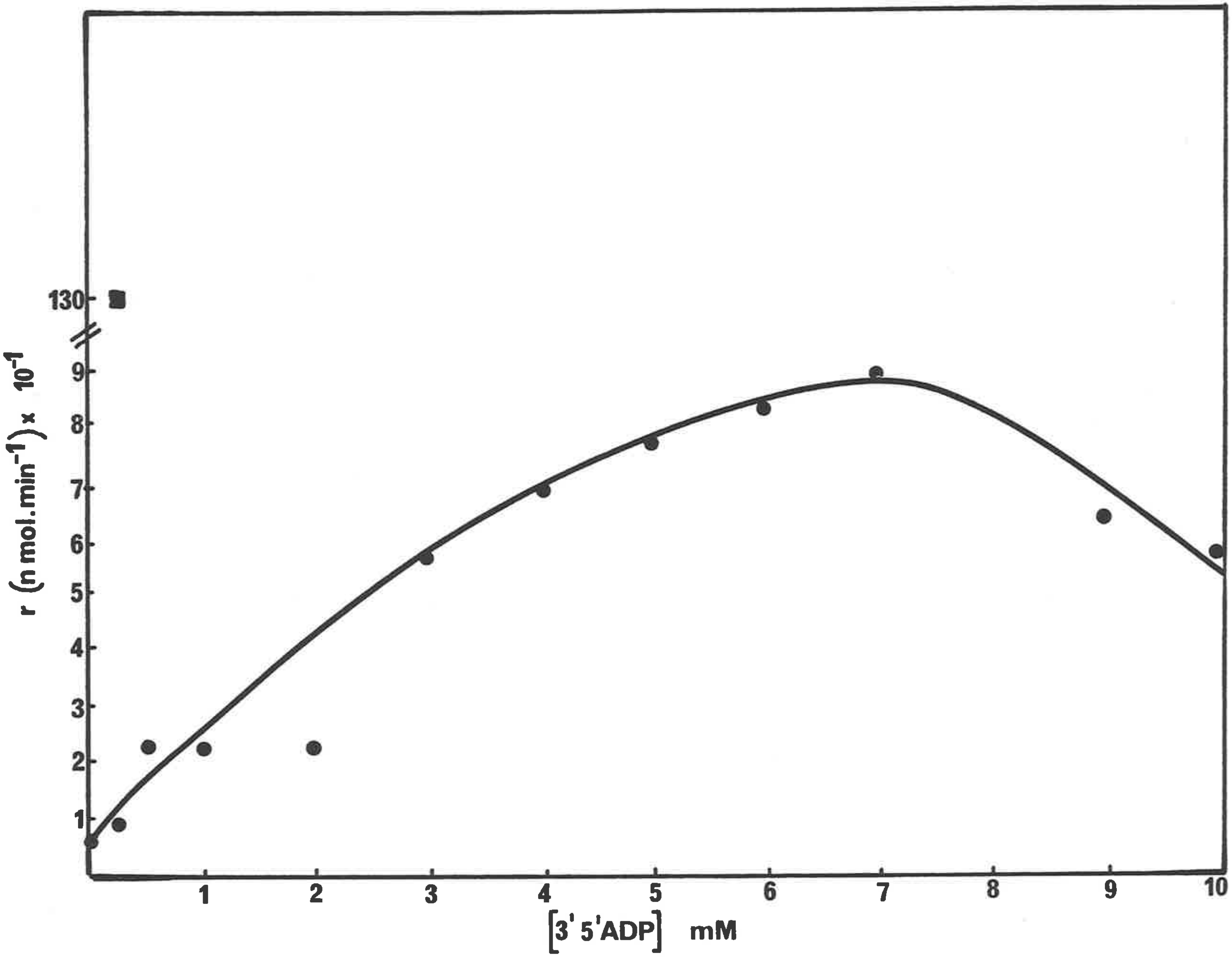

r (n mol.min^{-1}) x 10^{-1}
130
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
[3' 5'ADP] mM

Fig. 4.3a Double reciprocal plot of data from Fig. 4.3.

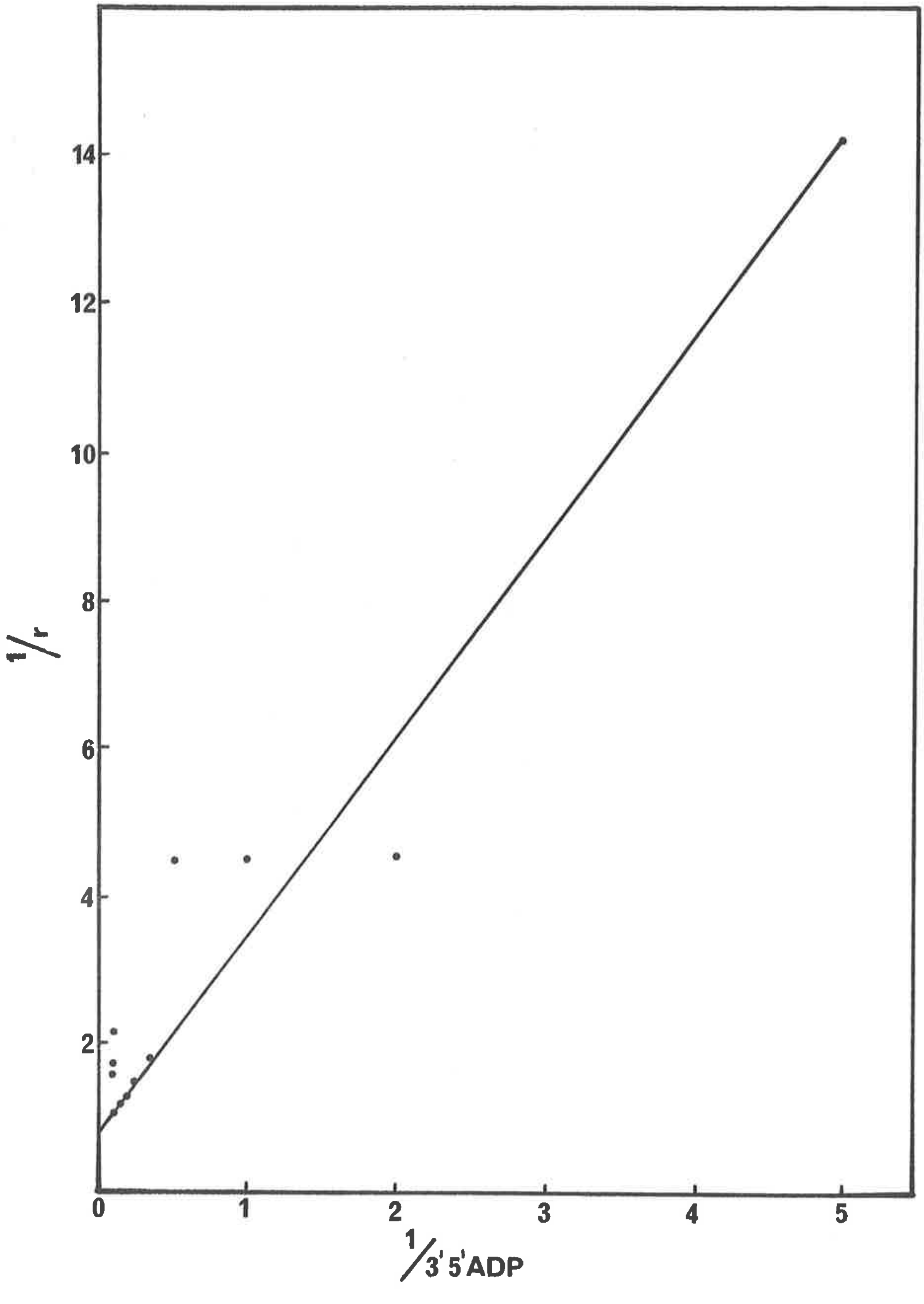

14
12
10
8
6
4
2
0
1
2
3
4
5
1/r
1/3'5'ADP

Fig. 4.4 The effect of acetyldephospho CoA on the ATP:orthophosphate exchange reaction

ATP: orthophosphate exchange activity was determined, as described in section 2.2.5c, in the presence of the concentrations of acetyldephospho CoA indicated (● - ●). Exchange reaction activity was also determined in the presence of 0.25mM acetyl CoA (■).

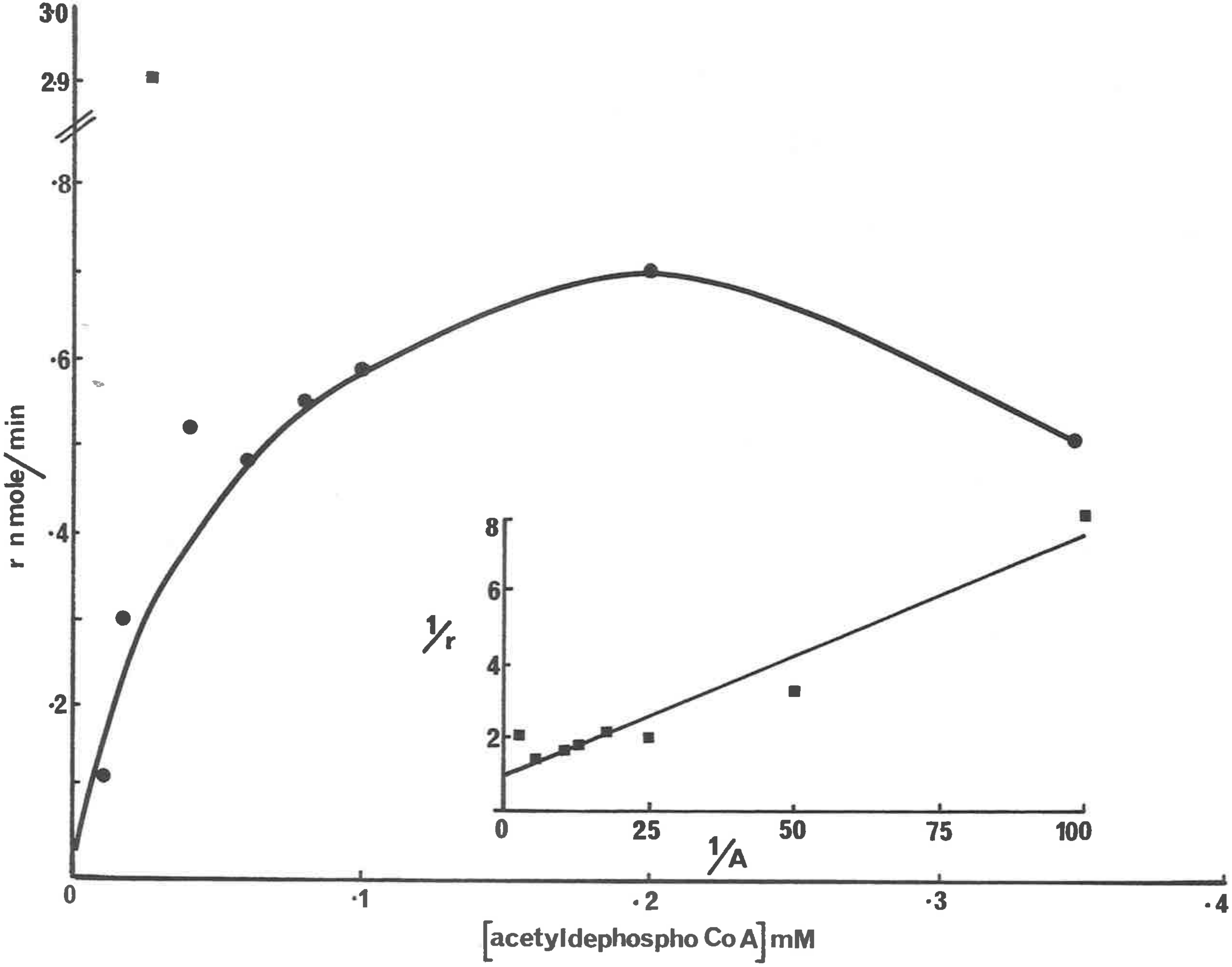

3·0
2·9
·8
·6
·4
·2
r n mole/min
0
·1
·2
·3
·4
[acetyldephospho CoA] mM
8
6
4
2
1/r
0
25
50
75
100
1/A

Fig. 4.5 The effect of acetyl CoA on the pyruvate: oxaloacetate exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of the concentrations of acetyl CoA indicated (●-●).

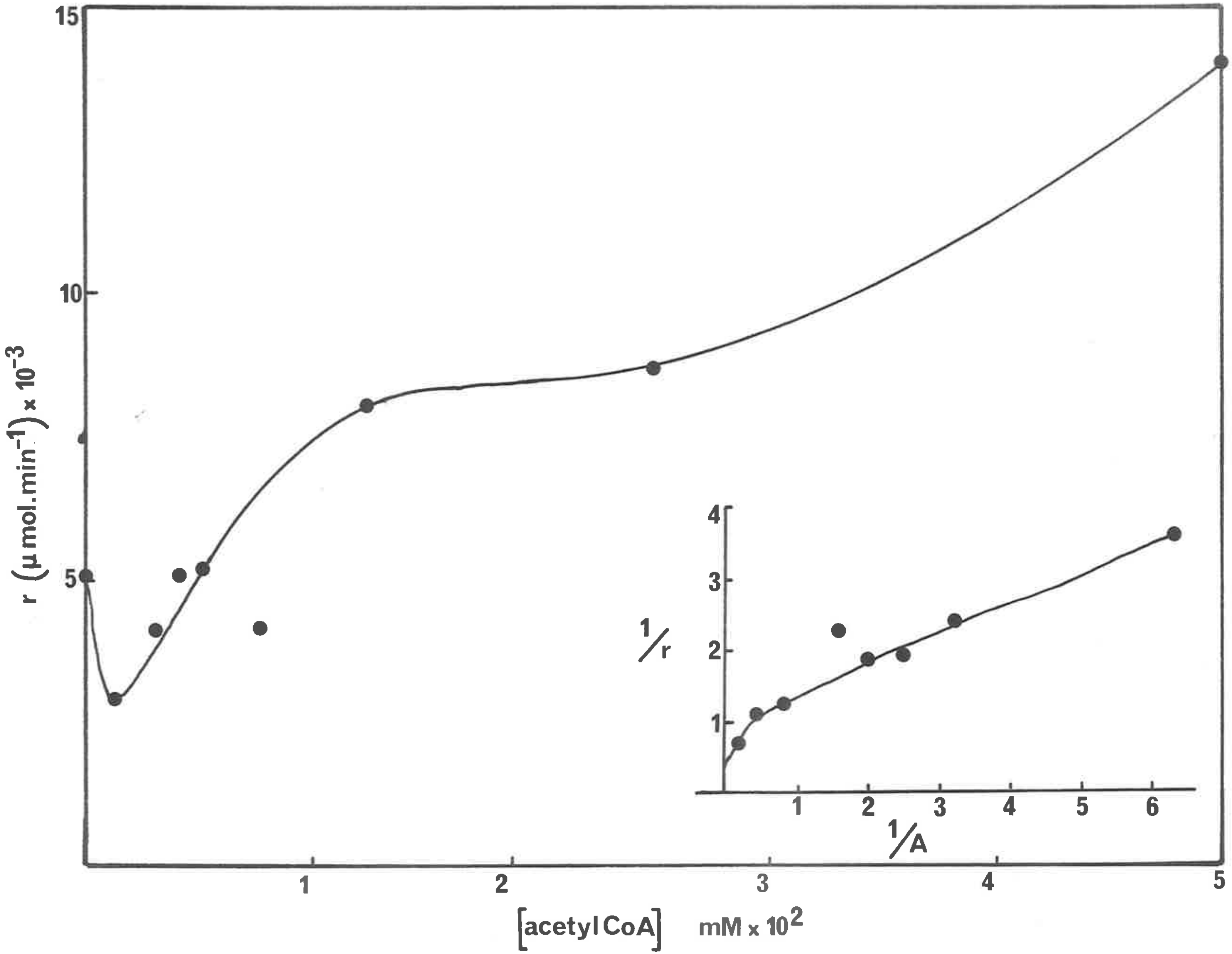

15
10
5
r (μ mol.min^{-1}) x 10^{-3}
1
2
3
4
5
[acetyl CoA] mM x 10^2
4
3
2
1
1/r
1
2
3
4
5
6
1/A

Fig. 4.6 The effect of acetyl CoA on the pyruvate: oxaloacetate exchange reaction in the presence of Mg^{++}

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of 0.5mM $MgCl_2$ and the concentrations of acetyl CoA indicated (●-●).

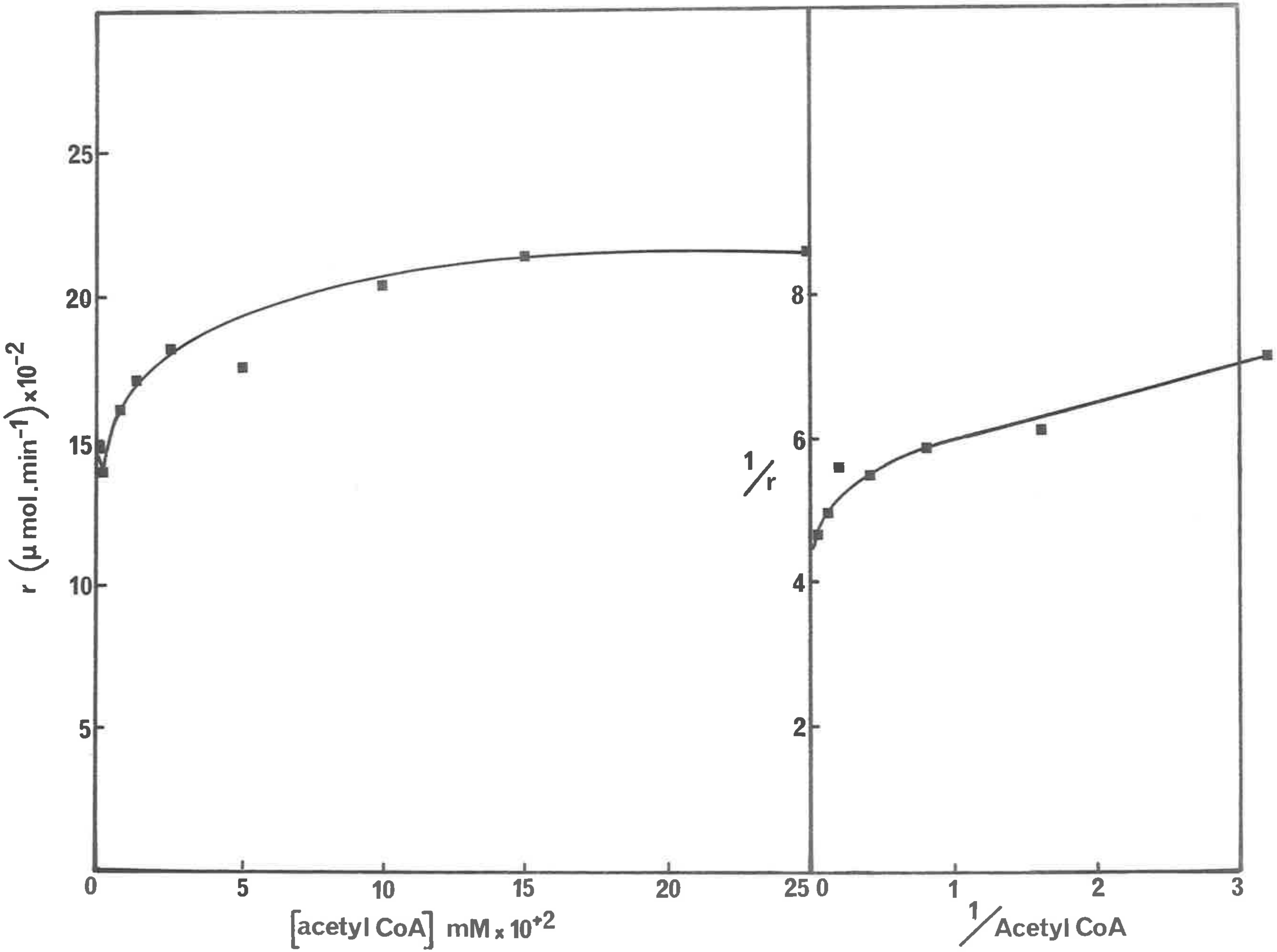

r (μ mol.min^{-1}) x 10^{-2}
25
20
15
10
5
0
5
10
15
20
25
[acetyl CoA] mM x 10^{+2}
1/r
8
6
4
2
0
1
2
3
1/Acetyl CoA

Fig. 4.7 The effect of acetylpantetheine on the pyruvate:oxaloacetate exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of the concentrations of acetylpantetheine indicated (● - ●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA (■).

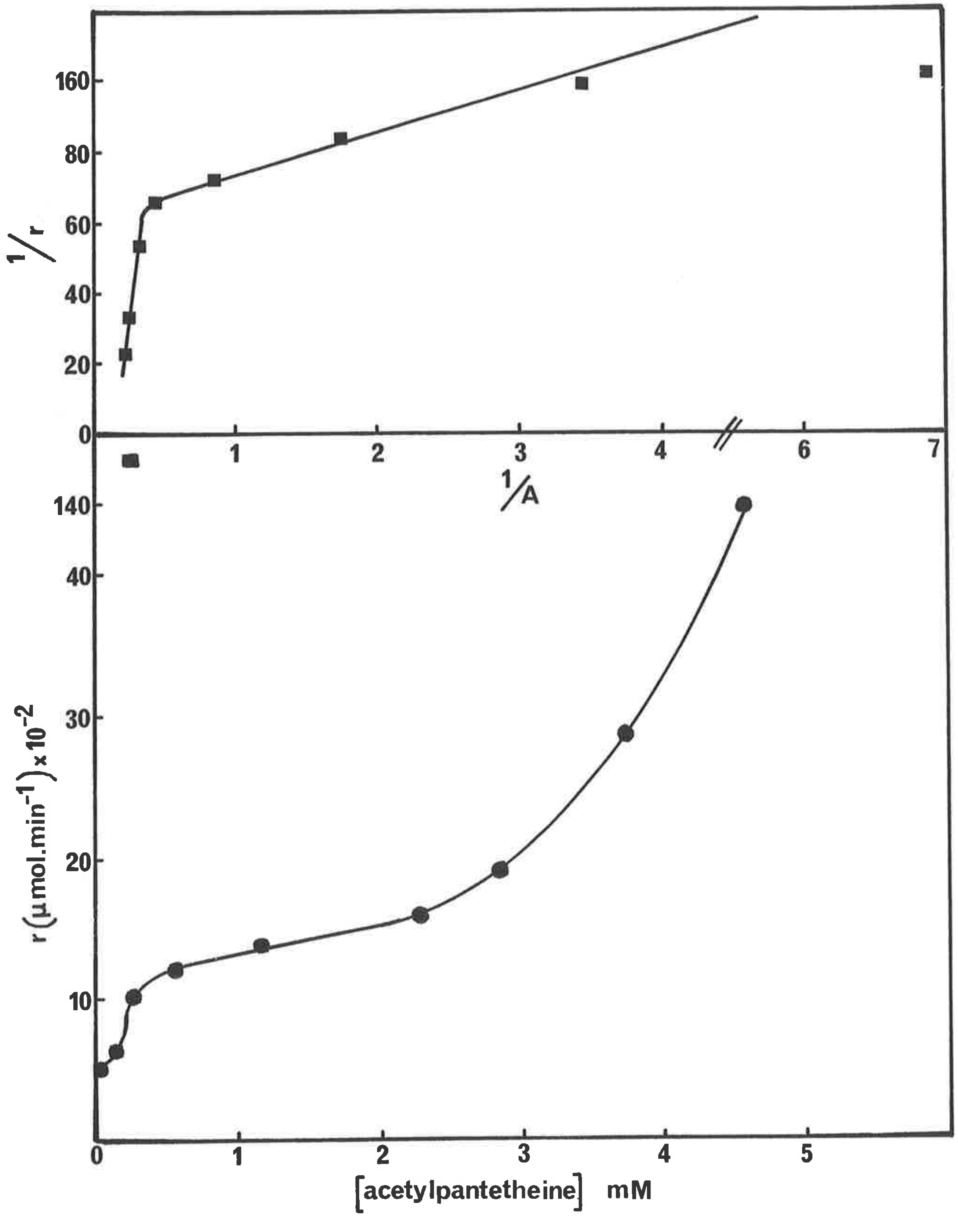

160
80
60
40
20
0
1/r
1
2
3
4
6
7
1/A
140
40
30
20
10
r (μmol.min⁻¹) x 10⁻²
0
1
2
3
4
5
[acetylpantetheine] mM

Fig. 4.8 The effect of acetyl pantetheine on the pyruvate:oxaloacetate exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of 0.5mM $MgCl_2$ and the concentrations of acetylpantetheine indicated (● - ●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA and 0.5mM $MgCl_2$ (■).

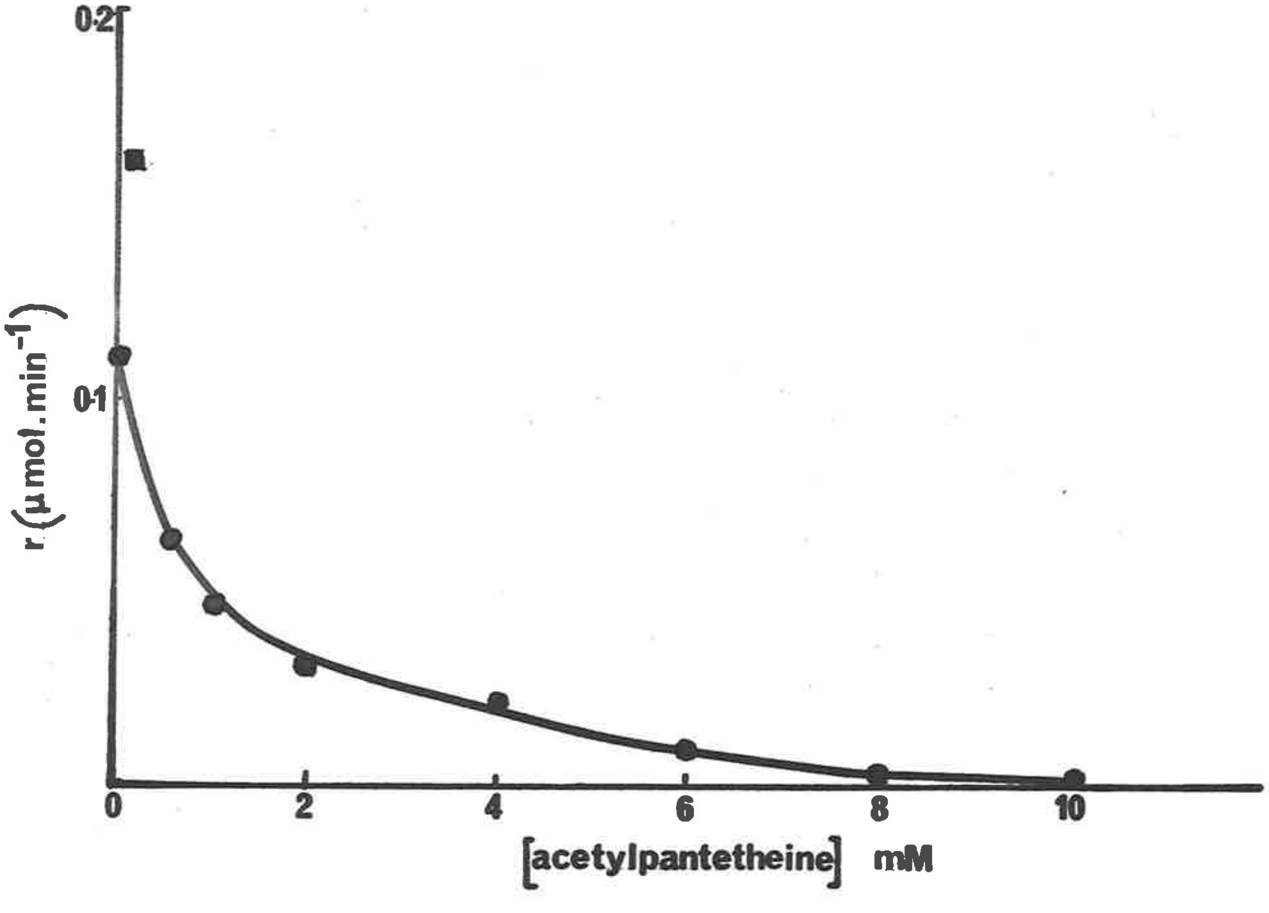

0·2
0·1
r (μ mol. min⁻¹)
0
2
4
6
8
10
[acetylpantetheine] mM

Fig. 4.9 The effect of acetyl-2'4'-phosphopantetheine on the pyruvate:oxaloacete exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of the concentrations of acetyl-2'4'-phosphopantetheine indicated (● - ●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA (■).

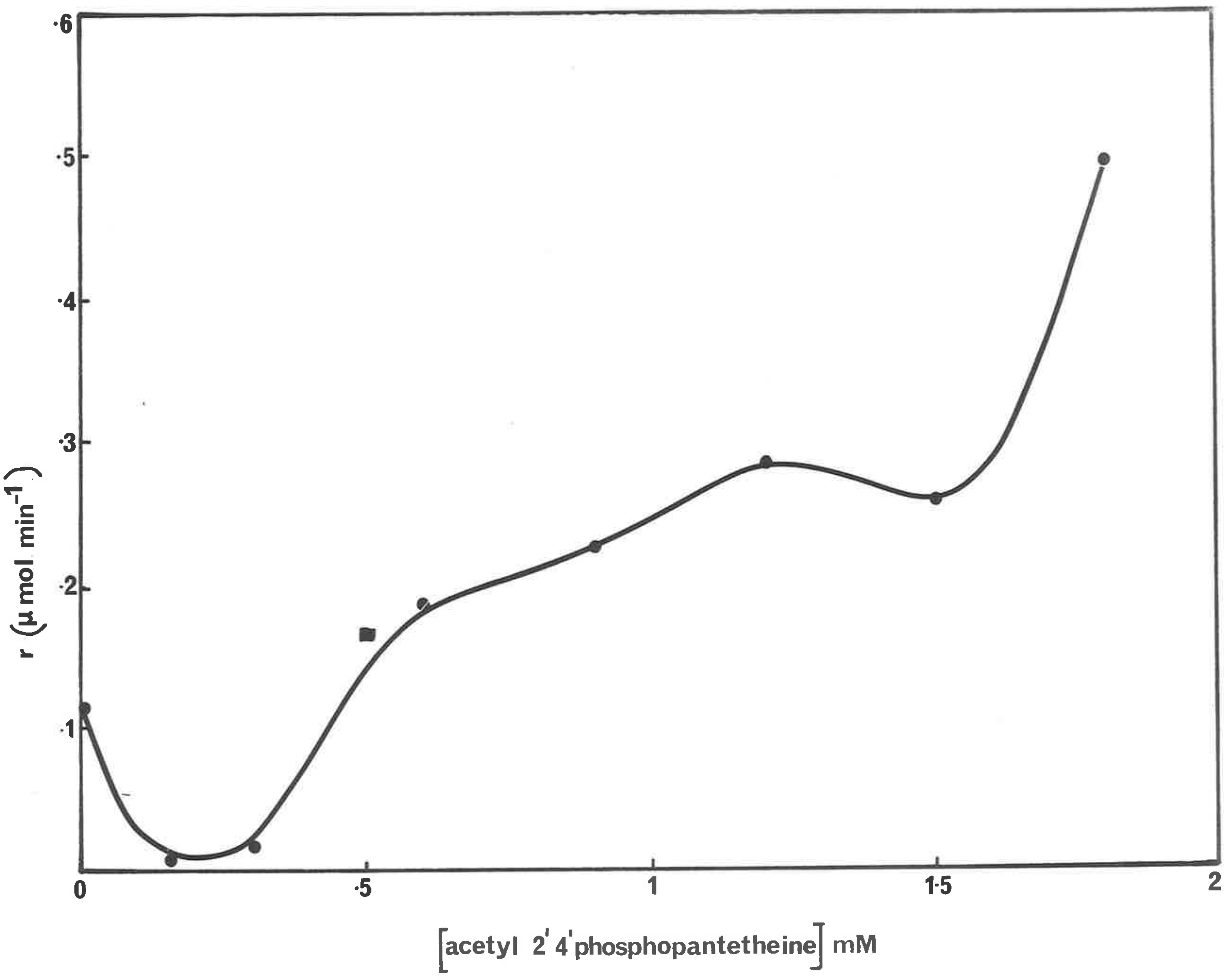
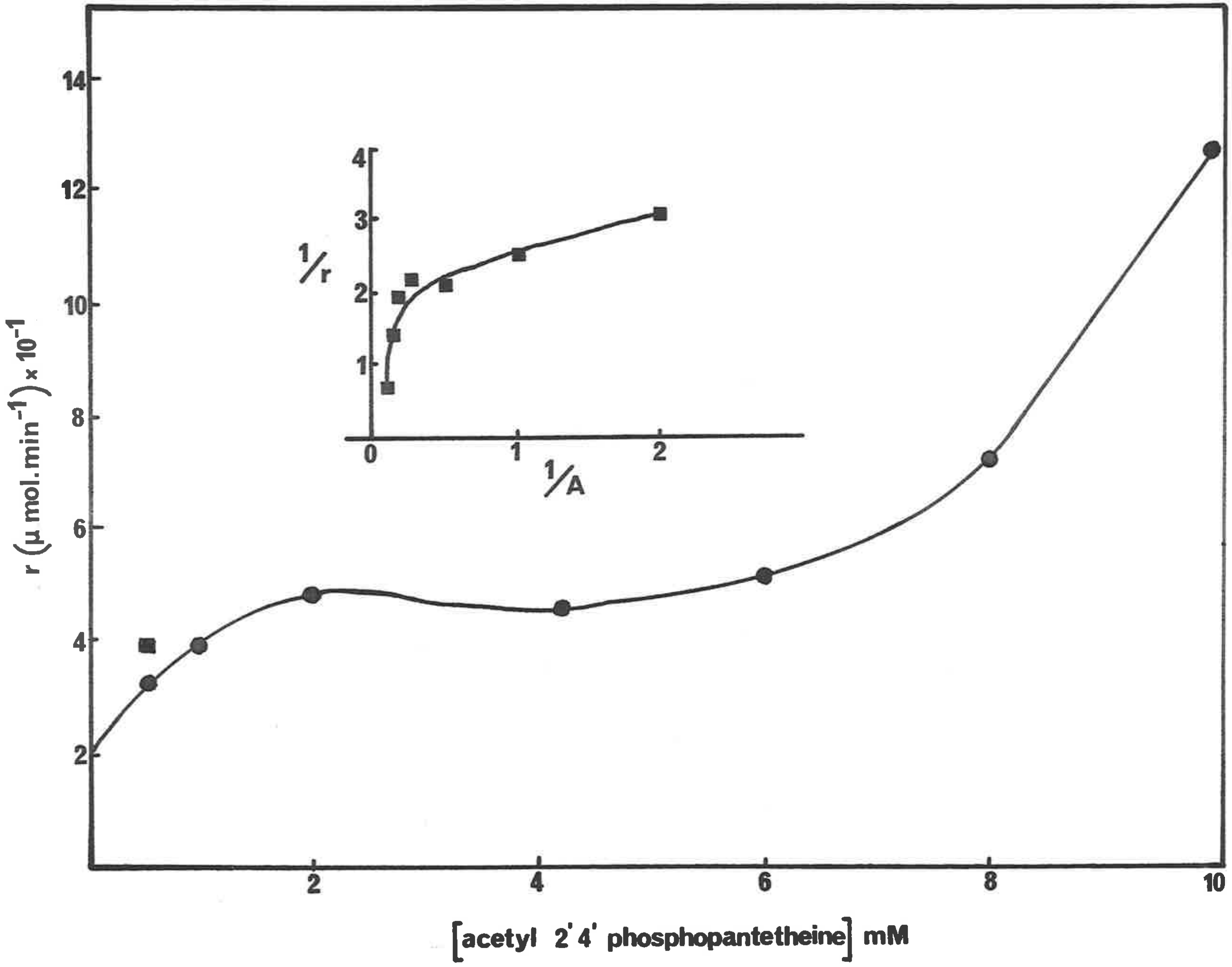

·6
·5
·4
·3
·2
·1
r (μ mol min^{-1})
0
·5
1
1·5
2
[acetyl 2' 4'phosphopantetheine] mM

Fig. 4.10 The effect of acetyl-2'4'-phosphopantetheine on the pyruvate:oxaloacetate exchange reaction in the presence of Mg^{++}.

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of 0.5mM and the concentrations of acetyl-2'4'-phosphopantetheine indicated (● - ●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA and 0.5mM $MgCl_2$ (■).



r (μ mol.min^{-1}) x 10^{-1}
14
12
10
8
6
4
2
2
4
6
8
10
[acetyl 2' 4' phosphopantetheine] mM
1/r
4
3
2
1
0
1
2
1/A

Fig. 4.11 The effect of CoASH on the pyruvate: oxaloacetate exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of the concentrations of CoASH indicated (● - ●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA (■).

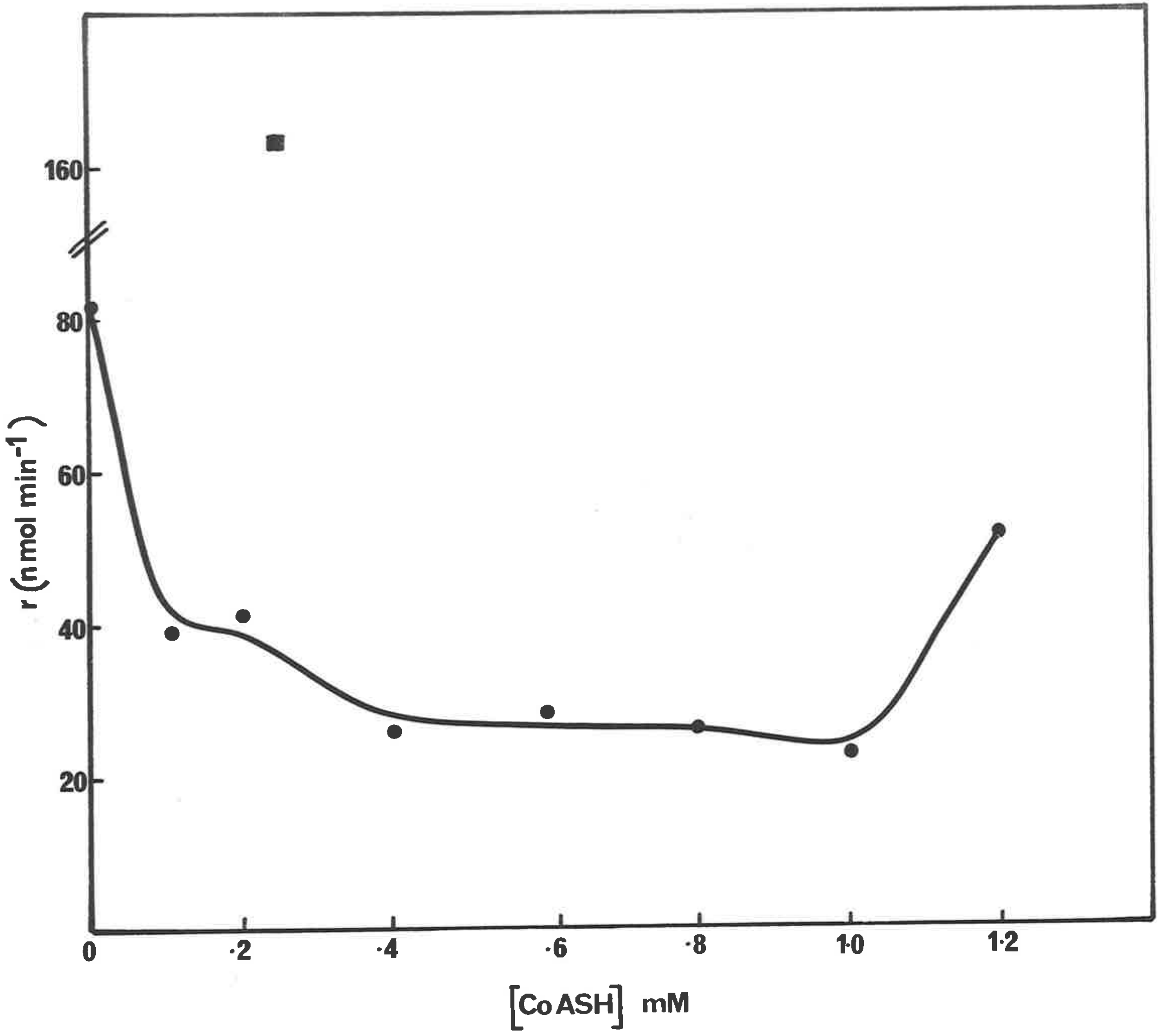

160
80
60
40
20
r (n mol min^{-1})
0
·2
·4
·6
·8
1·0
1·2
[Co ASH] mM

Fig. 4.12 The effect of CoASH on the pyruvate: oxaloacetate exchange reaction in the presence of Mg^{++}

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of 0.5 mM $MgCl_2$ and the concentrations of CoASH indicated (● - ●). Exchange activity was also determined in the presence of o.25mM acetyl CoA and 0.5mM $MgCl_2$ (■).

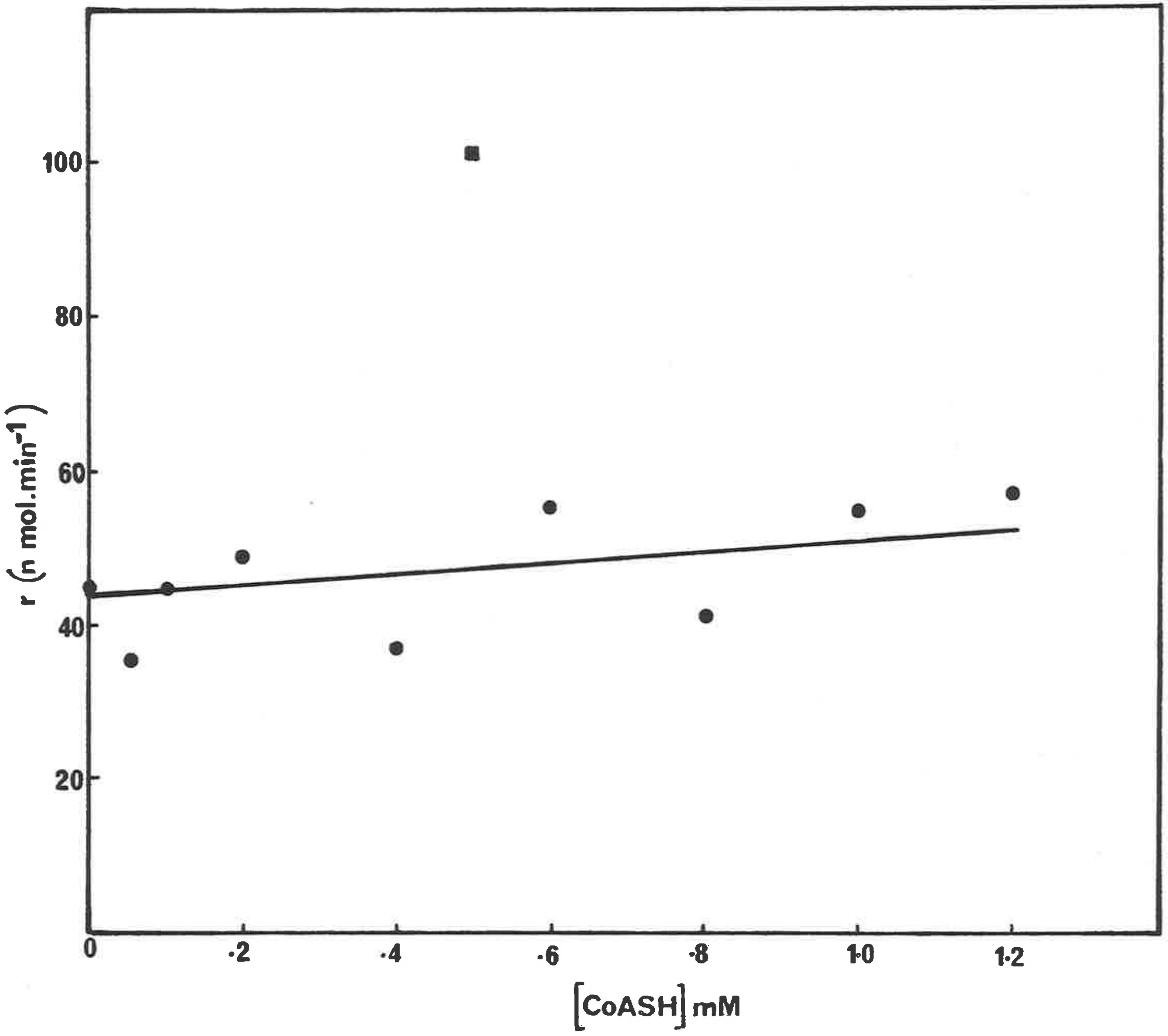

100
80
60
40
20
r (n mol.min^{-1})
0
·2
·4
·6
·8
1·0
1·2
[CoASH] mM

Fig. 4.13 The effect of acetyldephospho CoA on the pyruvate:oxaloacetate exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of the concentrations of acetyldephospho CoA indicated (●-●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA (■).

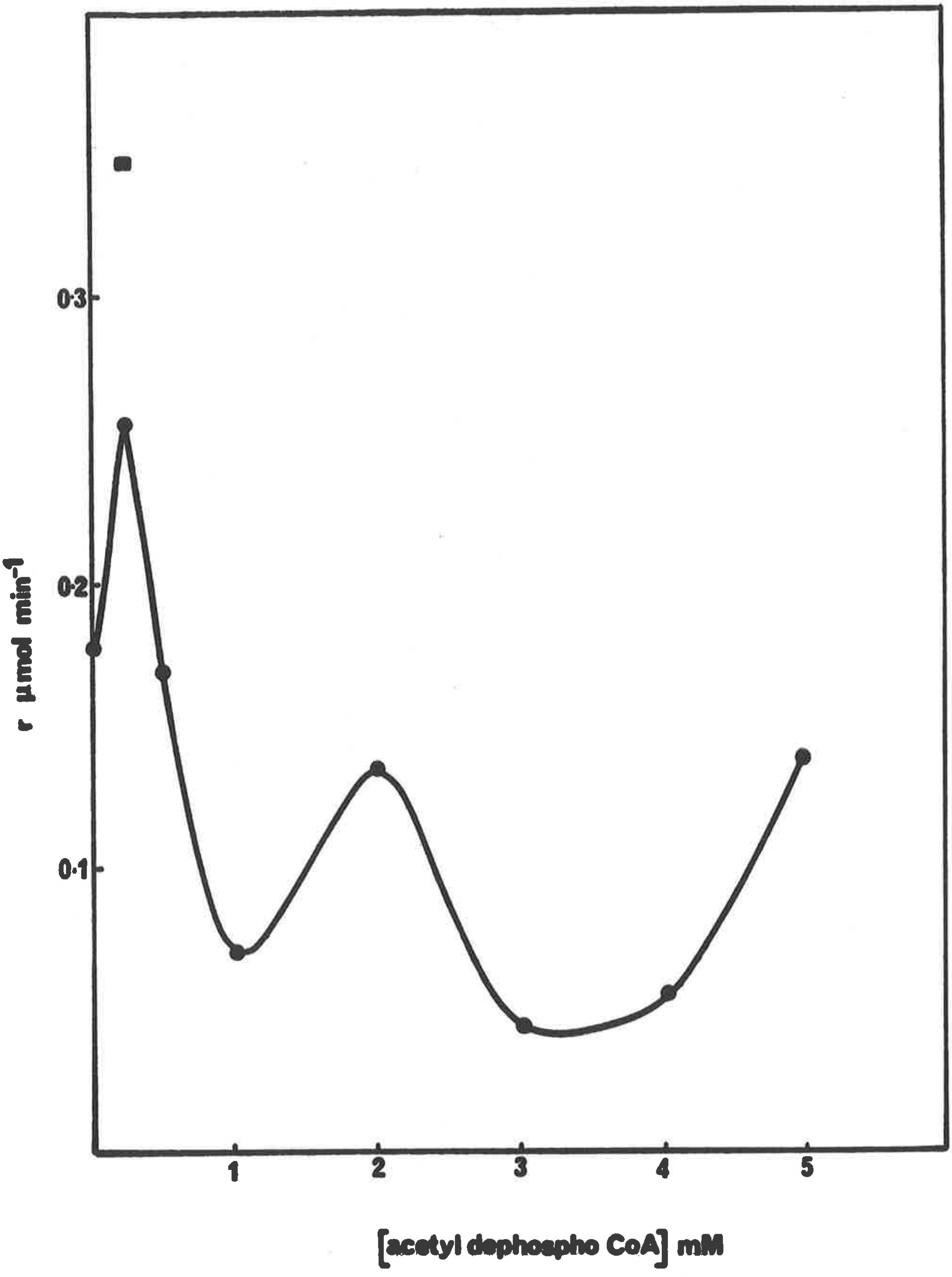

0·3
0·2
0·1
r µmol min^{-1}
1
2
3
4
5
[acetyl dephospho CoA] mM

Fig 4.14 The effect of acetyldephospho CoA on the pyruvate:oxaloacetate exchange reaction in the presence of Mg^{++}

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of 0.5mM $MgCl_2$ and the concentrations of acetyldephospho CoA indicated (● - ●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA and 0.5mM $MgCl_2$ (■).

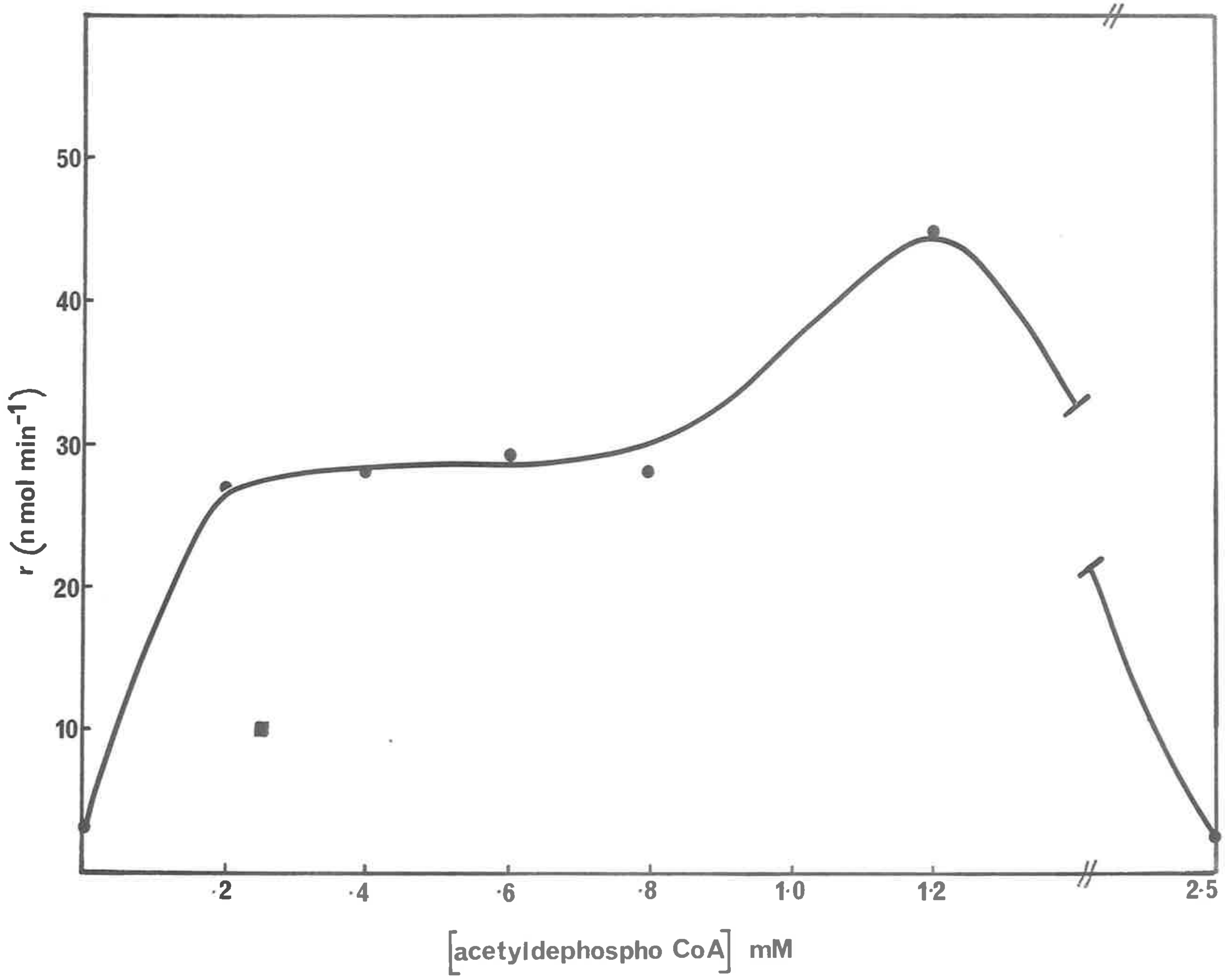

50
40
30
20
10
r (n mol min^{-1})
·2
·4
·6
·8
1·0
1·2
2·5
[acetyldephospho CoA] mM

Fig 4.15 The effect of acetyldesamino CoA on the pyruvate:oxaloacetate exchange reaction

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of the concentrations of acetyldesamino CoA indicated (●-●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA (■).

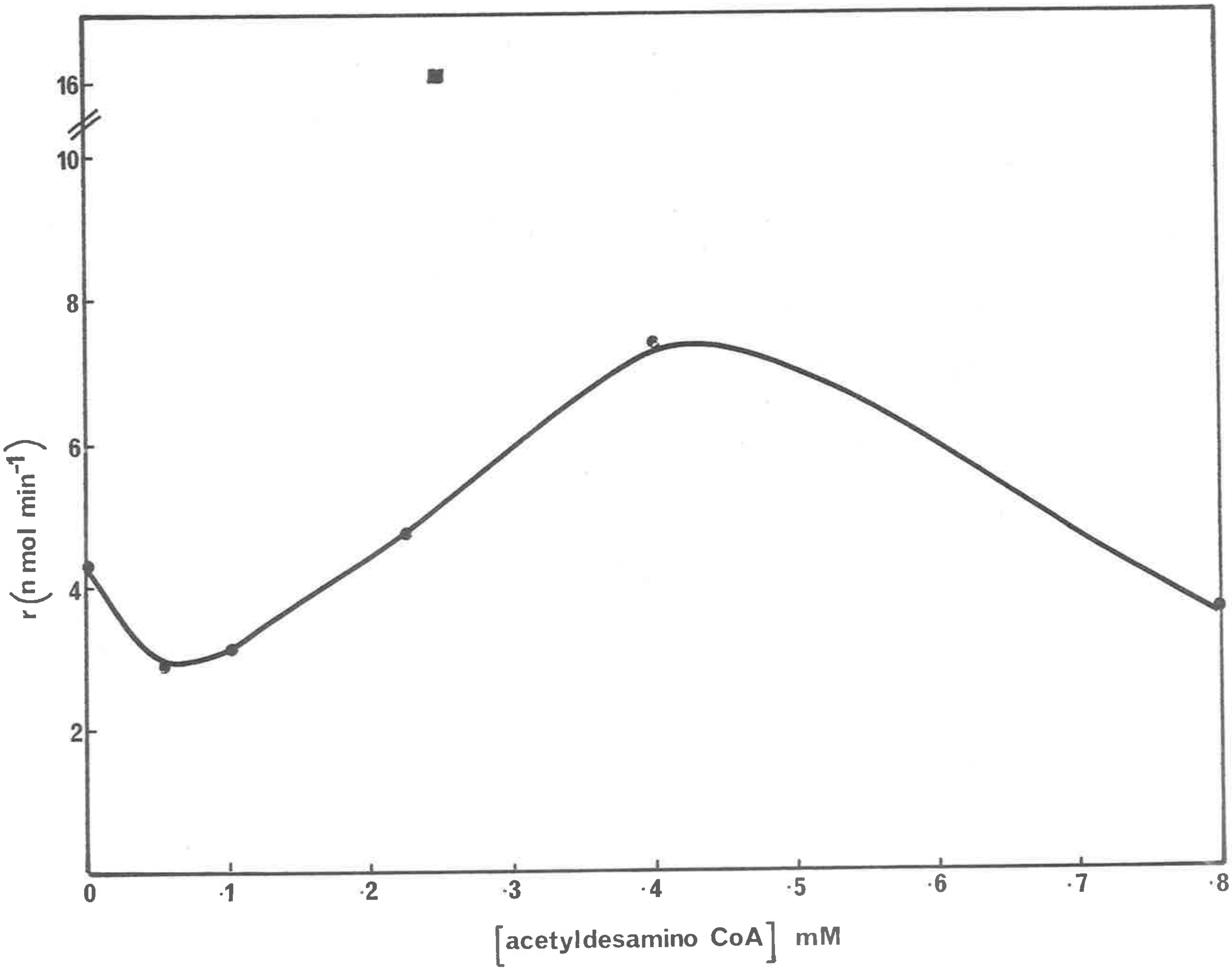

16
10
8
6
4
2
r (n mol min^{-1})
0
·1
·2
·3
·4
·5
·6
·7
·8
[acetyldesamino CoA] mM

Fig. 4.16 The effect of acetyldesamino CoA on the pyruvate oxaloacetate exchange reaction in the presence of Mg^{++}

Pyruvate:oxaloacetate exchange activity was determined, as described in section 2.2.5d, in the presence of 0.5mM $MgCl_2$ and the concentrations of acetyldesamino CoA indicated (●-●). Exchange activity was also determined in the presence of 0.25mM acetyl CoA and 0.5mM $MgCl_2$ (■).

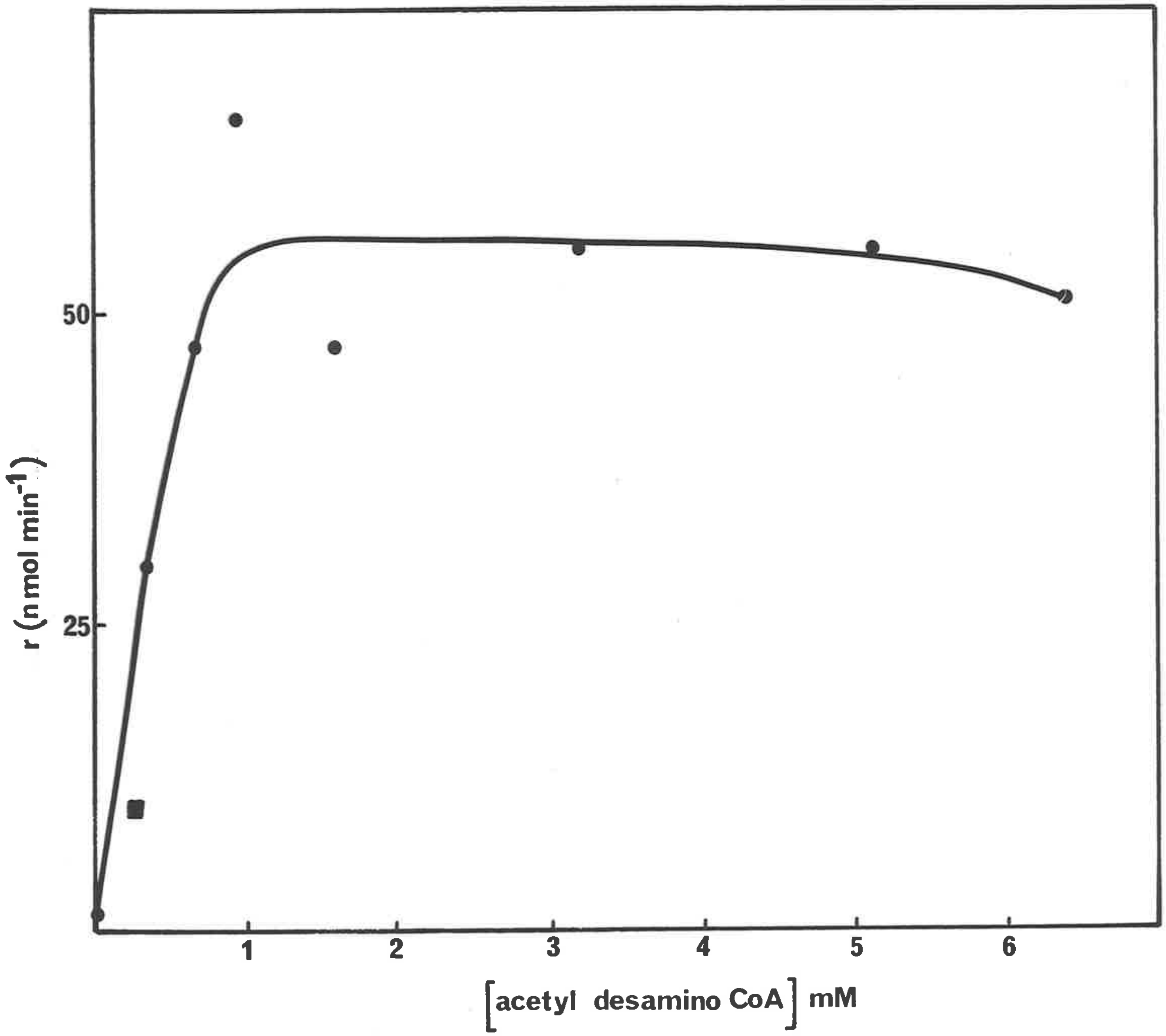

50
25
r (n mol min^{-1})
1
2
3
4
5
6
[acetyl desamino CoA] mM

Fig. 4.17 The effect of CoASH on the overall reaction

Enzyme activity was determined, using the radiochemical assay as described in section 2.2.5b, in the presence of the concentrations of CoASH indicated (● - ●), in lieu of acetyl CoA. Enzyme activity was also determined in the presence of 0.25mM acetyl CoA (■).

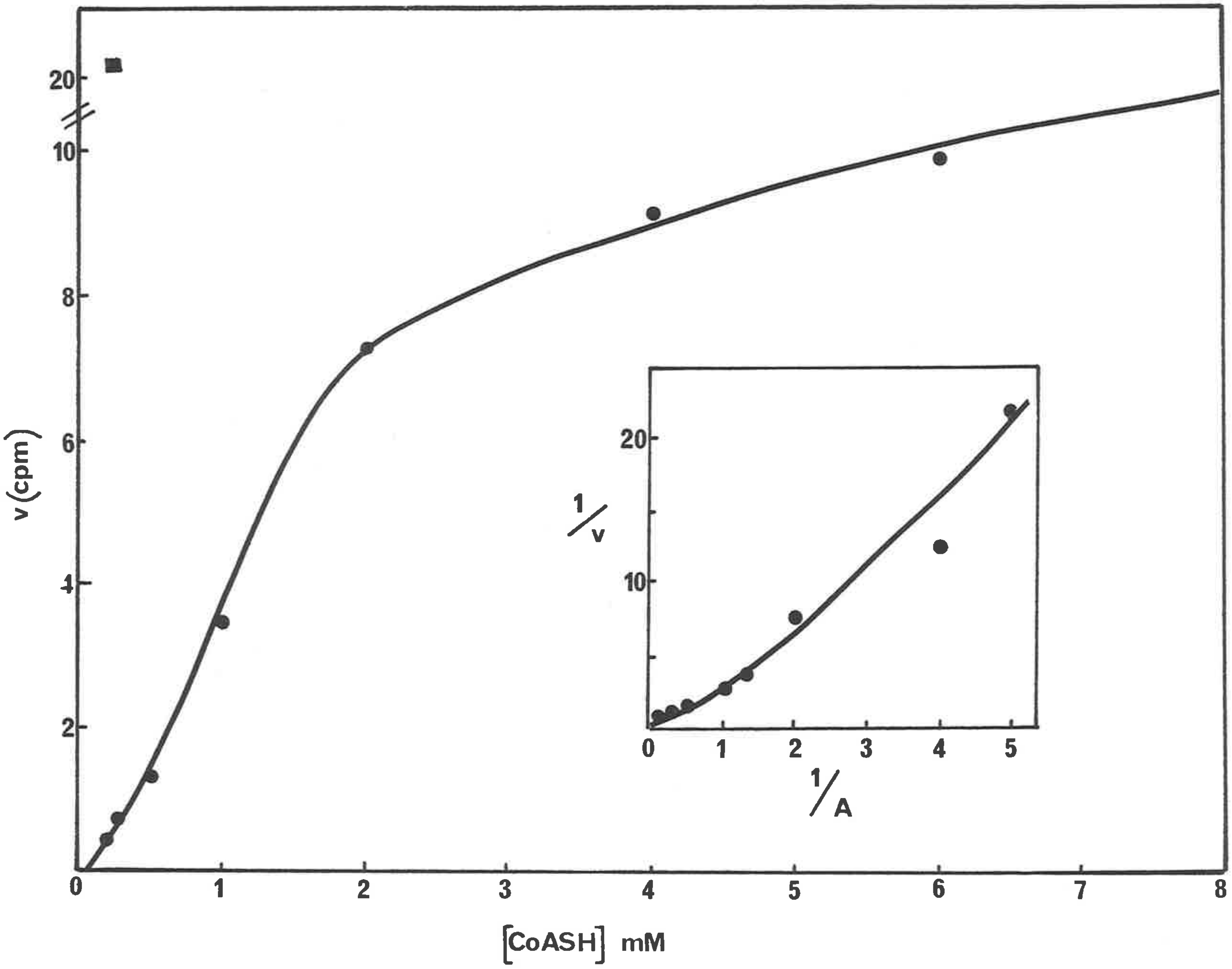

v (cpm)
20
10
8
6
4
2
0
1
2
3
4
5
6
7
8
[CoASH] mM
1/v
20
10
0
1
2
3
4
5
1/A

Fig. 4.18 The effect of acetyl-2'4'-phosphopantetheine on the overall reaction

Enzyme activity was determined using the radiochemical assay, as described in section 2.2.5b, in the presence of the concentrations of acetyl-2'4'-phosphopantetheine indicated (● - ●), in lieu of acetyl CoA. Enzyme activity was also determined in the presence of 0.25mM acetyl CoA.(■).

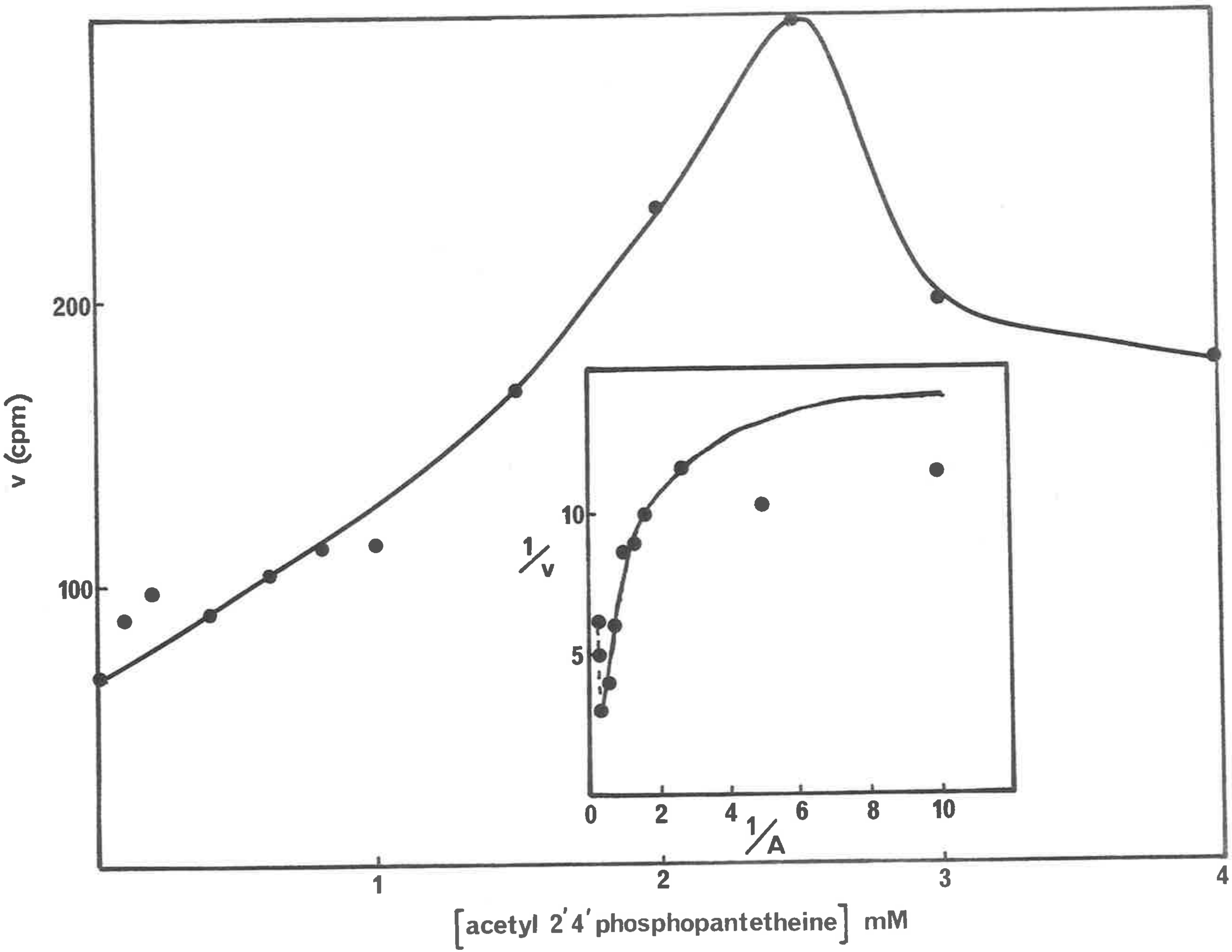

v (cpm)
200
100
1
2
3
4
[acetyl 2'4' phosphopantetheine] mM
1/v
10
5
0
2
4
6
8
10
1/A

Fig. 4.19 The effect of acetyldephospho CoA on the overall reaction

Enzyme activity was determined using the radiochemical assay, as described in section 2.2.5b, in the presence of the concentrations of acetyldephospho CoA indicated (● - ●), in lieu of acetyl CoA. Enzyme activity was also determined in the presence of 0.25mM acetyl CoA (■).

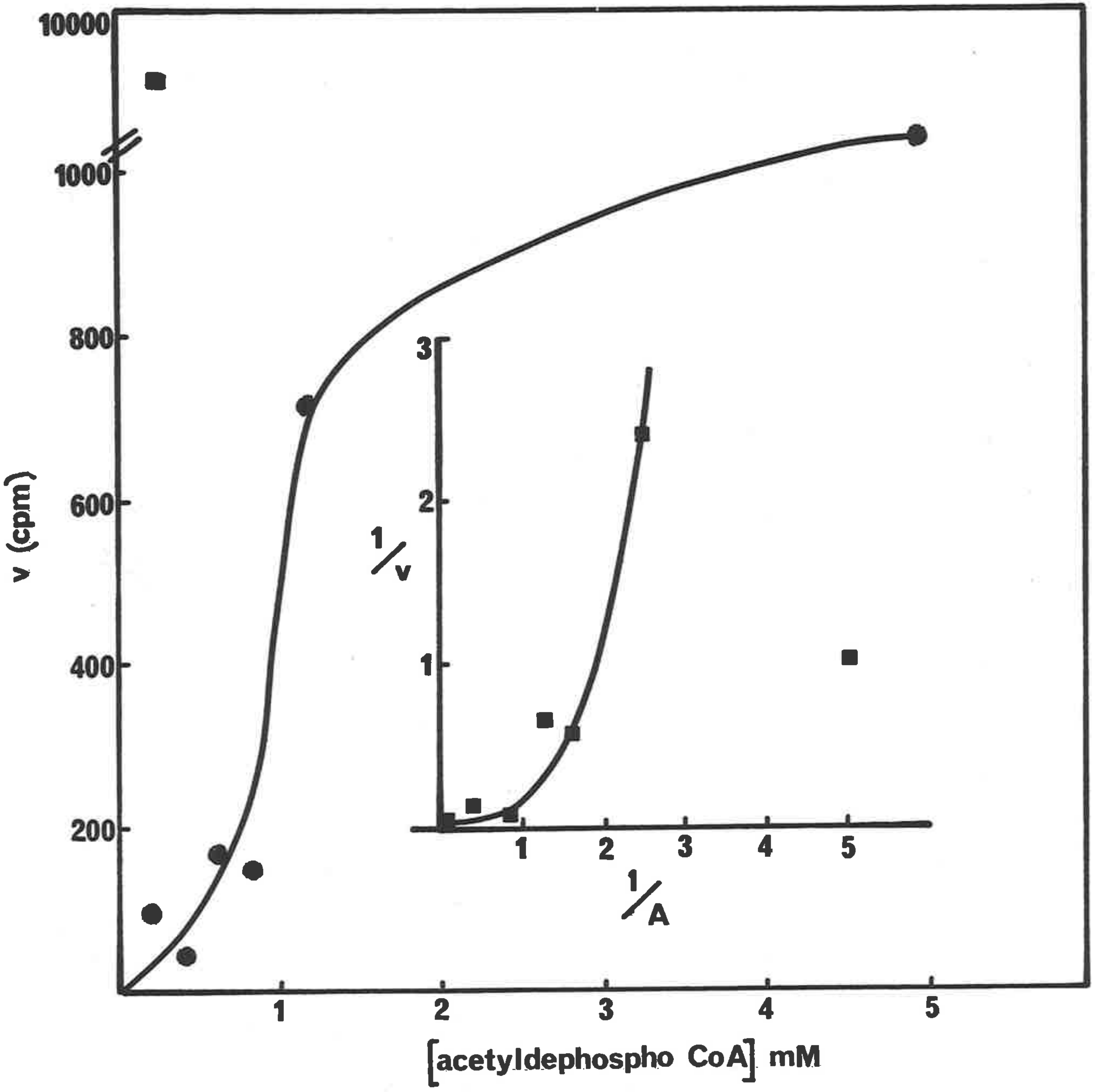

10000
1000
800
600
400
200
v (cpm)
1
2
3
4
5
[acetyldephospho CoA] mM
3
2
1
1/v
1
2
3
4
5
1/A

Fig. 4.20 The effect of acetyl-2'4'-phosphopantetheine, CoASH and acetyl CoA on the acetyl CoA - independent reaction

Acetyl CoA -independent enzyme activity was determined, as described in section 2.2.5b, in the presence of the concentrations of acetyl-2'4'-phosphopantetheine (□-□), CoASH (▼-▼) and acetyl CoA (●-●) indicated.

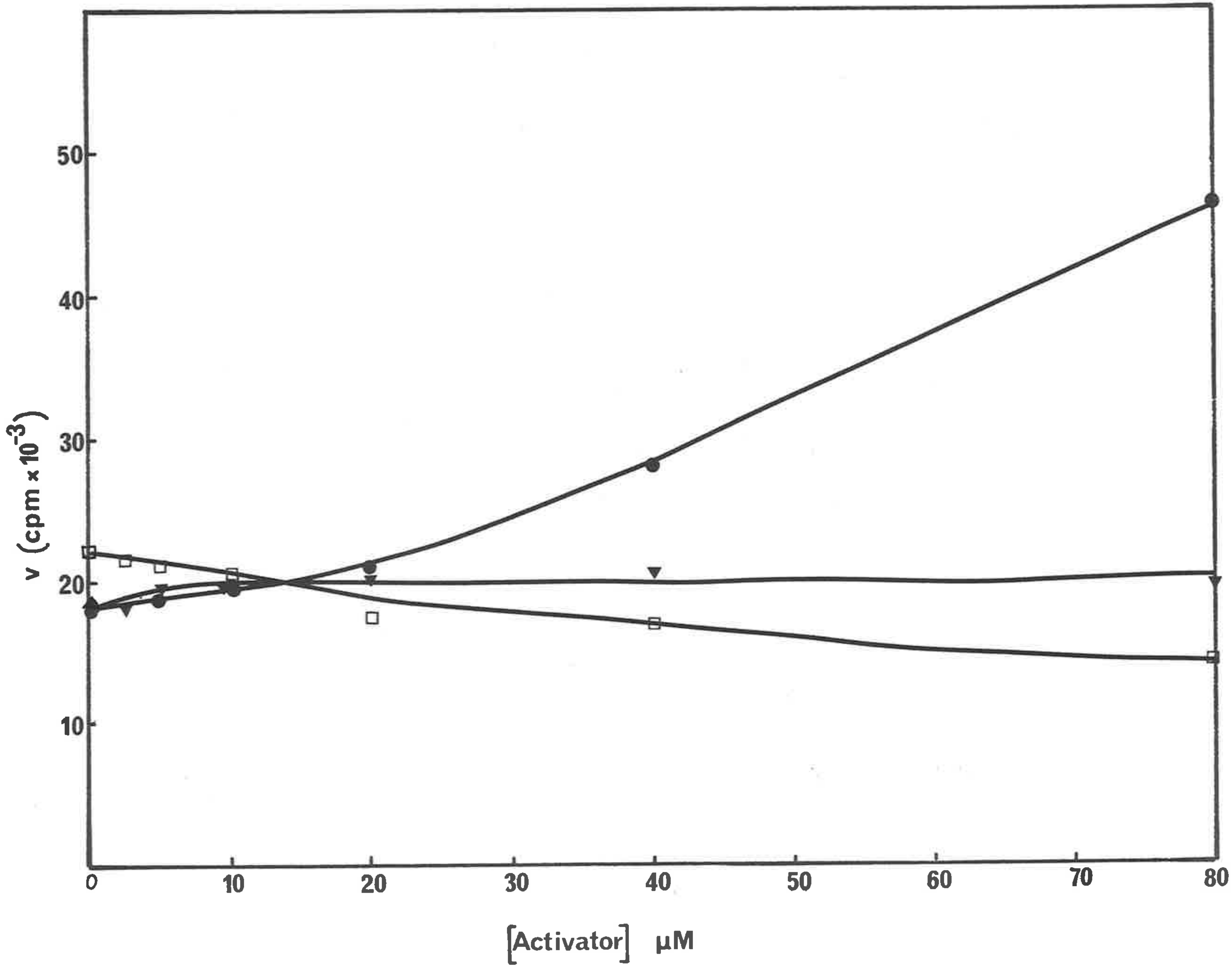

v (cpm × 10⁻³)
50
40
30
20
10
0
10
20
30
40
50
60
70
80
[Activator] µM

Fig. 4.21 The combined effect of acetyl-2'4'-phosphopantetheine and both CoASH and 3'5'-ADP, and acetyl CoA on the acetyl CoA -independent reaction

Acetyl CoA -independent enzyme activity was determined, as described in section 2.2.5b, in the presence of (10x) the concentrations of acetyl-2'4'-phosphopantetheine and CoASH (■ - ■), acetyl-2'4'-phosphopantetheine and 3'5' ADP (▼ - ▼) and in the presence of the concentrations of acetyl CoA (□ - □) indicated.

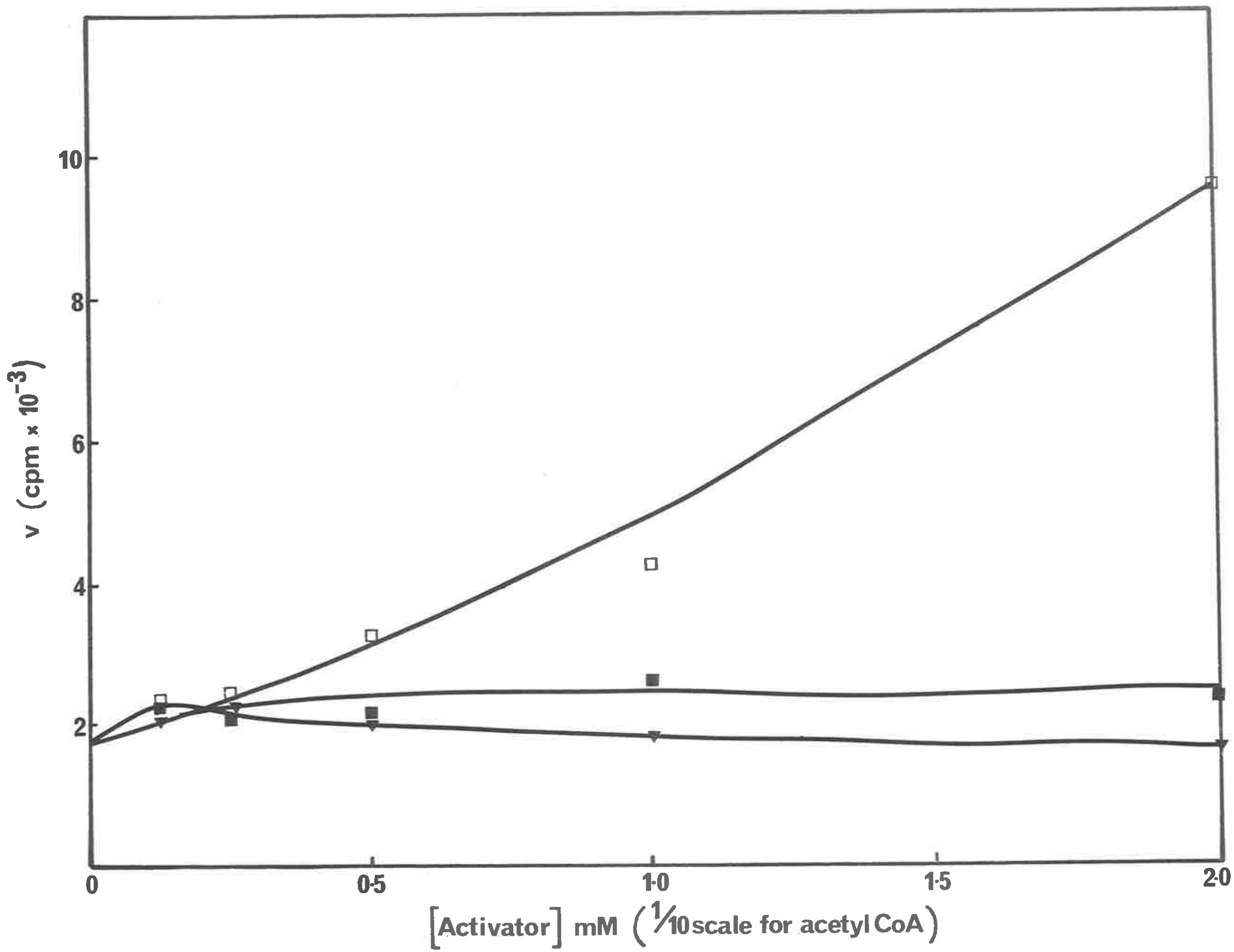
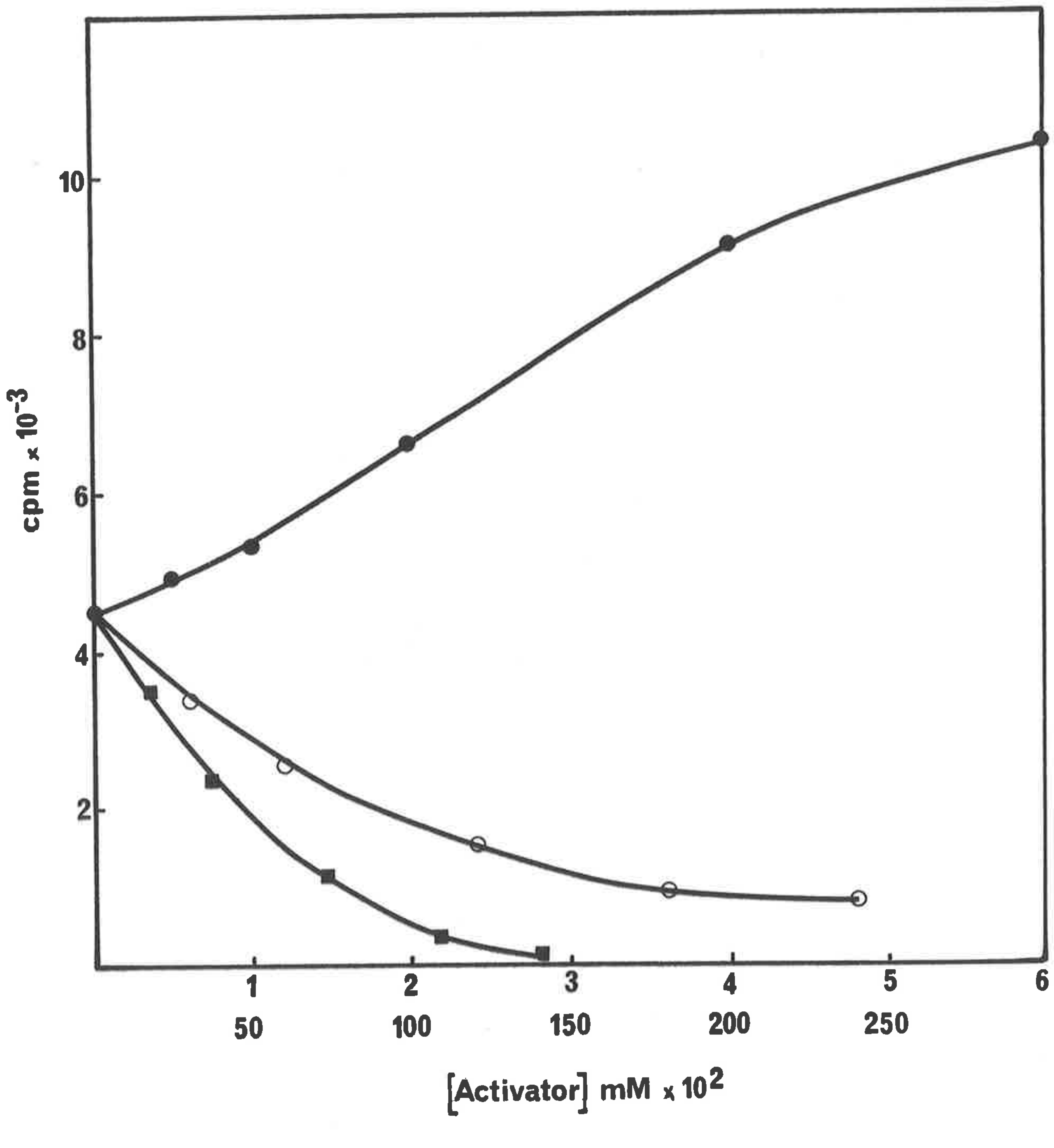

v (cpm × 10^{-3})
10
8
6
4
2
0
0·5
1·0
1·5
2·0
[Activator] mM (1/10 scale for acetyl CoA)

Fig. 4.22 The effect of acetyldephospho CoA, acetyldesamino CoA and acetyl CoA on the acetyl CoA -independent reaction.

Acetyl CoA -independent enzyme activity was determined, as described in section 2.2.5b, in the presence of the concentrations of acetyldephospho CoA (○ - ○), acetyldesamino CoA (■ - ■) and acetyl CoA (● - ●) indicated.


cpm x 10^{-3}
10
8
6
4
2
1
2
3
4
5
6
50
100
150
200
250
[Activator] mM x 10^2

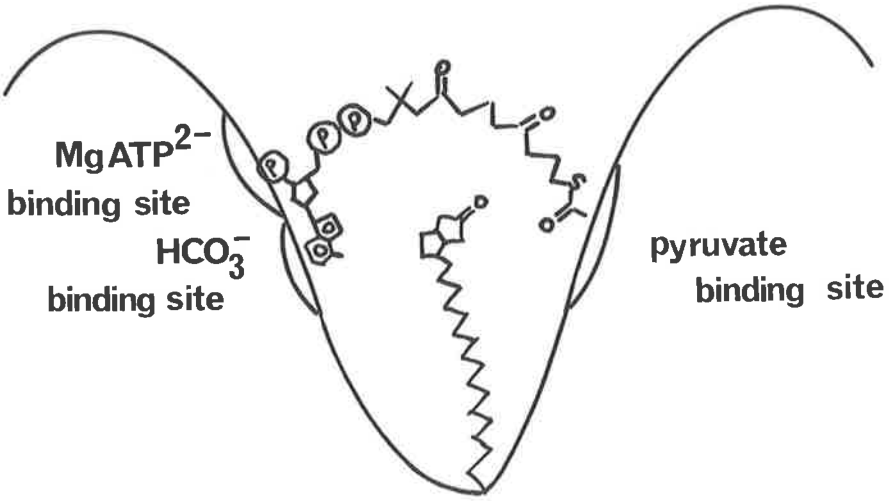

MgATP^{2-} binding site
HCO_3^- binding site
pyruvate binding site

Fig. 4.2 3

CHAPTER 5

DILUTION INACTIVATION

"the very point which appears to complicate a case is, when duly considered and scientifically handled, the one which is most likely to elucidate it."

A. Conan Doyle

5.1 STUDIES ON THE PROTECTION AFFORDED BY ACETYL CoA AGAINST INACTIVATION OF PYRUVATE CARBOXYLASE ON DILUTION

The results presented in the preceding chapter allowed the diverse roles of acetyl CoA, viz. the stimulation of both the first and second partial reactions of pyruvate carboxylase to be reconciled with the action of a single activator molecule. Yet another role of acetyl CoA was the protection against the inactivation of pyruvate carboxylase which occurs on dilution of the enzyme. The phenomenon and the protection against inactivation afforded by acetyl CoA were investigated in this laboratory using the enzyme from sheep kidney (Ashman *et al*., 1972). The inactivation phenomenon was previously regarded as a feature of inconvenience in handling the enzyme but has been used as a tool for the further investigation of the mode of action of acetyl CoA in this study.

One implication which could be drawn from the results presented in the preceding chapter was that since it was only the first partial reaction which exhibited dependence on the presence of an activator for activity and since the adenosine moiety of acetyl CoA stimulated only the first partial reaction, then the adenosine moiety could be acting by preventing inactivation of the first partial reaction site on dilution. This could imply that the effect of acetyl CoA on the first partial reaction is not activation in the absolute sense but relative activation by prevention of inactivation. The experiments presented in the following sections were designed to investigate the above possibility by using the inactivation of the enzyme on dilution as a tool for the further study of molecules which could replace acetyl CoA in its activation of pyruvate carboxylase. The approach adopted was to first characterise the inactivation phenomenon by investigating a selection of solute types for their ability to function in protecting the enzyme against inactivation in a similar manner to acetyl CoA.

Having established a basis for studying the behaviour of the enzyme the investigation was extended to the precise definition of both the site of acetyl CoA protection and the site of protein denaturation. For the latter study changes in the conformation of the polypeptide backbone were monitored using an optical spectropolarimeter from which measurements of the circular dichroism of enzyme samples were obtained.

5.2 METHODS

5.2.1 DILUTION INACTIVATION EXPERIMENTS

Dilution inactivation experiments were performed by transferring the enzyme from a concentrated solution in 0.01M potassium phosphate, pH 7.5 (unless otherwise indicated), into the same buffer solution containing the particular solute under investigation. Samples were taken at various time intervals and assayed immediately using the radiochemical assay procedure. The k_{inact} values were obtained from the reciprocal of $t_{0.5}$ values which were calculated from semilog plots of % residual activity plotted as a function of time.

5.2.2 CIRCULAR DICHROISM SPECTRA

Circular dichroism spectra were obtained using a Jasco J40-CS recording spectropolarimeter. All dilutions of the enzyme were made in 0.01M potassium phosphate buffer, pH 7.5. Molar ellipticities, $[\theta]$, were calculated from the observed optical rotations, ψ_{obs}, using the formula:-

$$[\theta] = \frac{\psi_{obs}}{lc'} \cdot \frac{MRW}{100}$$

where ψ_{obs} = optical rotation

l = path length

c' = solute concentration

MRW = mean residue weight (115)

5.3 THE PHENOMENON OF INACTIVATION OF THE ENZYME ON DILUTION

Investigations carried out previously in this laboratory, (Ashman *et al.*, 1972) on the characteristics of the inactivation phenomenon served as a basis for the studies to follow. The features of the phenomenon were

i) the log of the rate of inactivation was inversely proportional to the log of the enzyme concentration, and

ii) the presence of acetyl CoA protected the enzyme against dilution inactivation.

Two factors to be considered in explaining this behaviour are

i) that as discussed in section 1.1 pyruvate carboxylase could exist as an associating/dissociating system and would undergo dissociation to an inactive form at concentrations lower than 4 E.U./ml, and

ii) the change in enzyme concentration could permit change in solvent structuring resulting in perturbation of the solvent-protein interaction. This in turn could induce a change in protein conformation with concomitant loss of enzymic activity. Both of these possibilities could be tested experimentally.

5.3.1 THE EFFECT OF ACETYL CoA ON ENZYME ACTIVITY AFTER INACTIVATION BY DILUTION

An experiment was performed to determine whether the inactivation process was arrested after addition of aliquots from the dilute enzyme solution containing the solute being tested, into the radiochemical assay mixture which contained a sufficient level of acetyl CoA (0.25mM) to fully saturate the enzyme.

The results presented in Fig 5.1 show that, although acetyl CoA decreased the rate of inactivation of the enzyme, it did not completely arrest the process. It was decided that the rate of the inactivation observed in the presence of acetyl CoA would not present any complications in the interpretation of results since the effect was not significant over the 5 min. period of the radiochemical assay procedure. At worst the effect would result in larger observed k_{inact} values.

5.3.2 THE EFFECT OF BUFFER IONS AND pH

In order to establish optimum conditions for subsequent experiments, the effect of vaying both buffer ions and pH on the rate of inactivation of enzyme was tested. The results presented in Table 5.1 show that in general the inactivation rate is minimally dependent on pH but that the pH at which least effect on the inactivation process occurred was pH 7.5. For this reason, pH 7.5 was chosen for subsequent experiments.

The second effect considered was that of buffer ions and the results presented in Table 5.1 show that enzyme diluted into Tris buffer containing increasing amounts of an ionic solute underwent increasing rates of inactivation. The effect of ionic strength was not as marked in potassium phosphate buffer and had no effect at pH 7.5. The effect of buffers which contained bulky organic groups such as N-ethylmorpholine and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid was to reduce the rate of inactivation of the enzyme. The buffer which had the least effect on the rate of inactivation of the enzyme, 10mM potassium phosphate (pH 7.5) was chosen as the standard conditions for obtaining the control rate of inactivation in all subsequent experiments.

5.3.3 THE EFFECT OF TEMPERATURE

For any process which involves solvation effects or where viscosity is a contributing factor, the rates of diffusion of the interacting components will influence events at a molecular level.

Since diffusion controlled processes are temperature dependent, it was considered likely that the rate of inactivation of the enzyme would be sensitive to changes in temperature. The results of experiments to determine the effect of temperature on the rate of inactivation of the enzyme, which are presented in the form of an Arrhenius plot in Fig. 5.2, show a direct dependence of the inactivation rate of the enzyme upon temperature. The energy of activation for the inactivation process was calculated to be 66kjoule (or 15.9kcal) from the slope of the line of best fit drawn through the data points.

The pre-exponential factor, A, was obtained from the value of the intercept of the Arrhenius plot and was calculated to be 24.09. Using these parameters the value of $\Delta H^{\ddagger}$ was calculated to be 63,900 joule (15.3kcal) and the value of $\Delta S^{\ddagger}$ was calculated to be 49.7 entropy units. The positive value for the enthalpy of activation was consistent with an energetically unfavourable process and was of the same order as enthalpies of solvation calculated for some apolar solutes (Klotz, 1962).

The entropy value was positive and could therefore be interpreted as an indication of a decrease in the state of order of the system. This interpretation was consistent with a breakdown in the structuring of the solvent water molecules as being the initial process involved in the inactivation of the enzyme.

Further thermodynamic information might be obtained from an experiment to test the effect of acetyl CoA concentration on the rates of inactivation of the enzyme at different temperatures.

5.3.4 THE EFFECT OF ACETYL CoA IN THE PRESENCE AND ABSENCE OF Mg^{++}

In order to establish that the effect of acetyl CoA in protecting the enzyme against inactivation on dilution, was concentration dependent, the enzyme was diluted into buffer solutions containing varying concentrations of both Mg^{++} and acetyl CoA. The results presented in Table 5.2 show that the protection against inactivation by acetyl CoA is concentration dependent and that the presence of Mg^{++} lowers the concentration of acetyl CoA required to give 50% protection by a factor of 3. This can be compared with the factor of 5 which was obtained in this laboratory for the amount by which the K_a for acetyl CoA activation of the enzyme is lowered in the presence of Mg^{++} (Duc, 1977).

From Hill plots of the data in Table 5.2 which are presented in Fig. 5.3 and Fig 5.4 the K_a for acetyl CoA was calculated to be 9.6μM±.064 and the K_a for MgacetylCoA was calculated to be 3.45μM±0.01 which indicates in this case that Mg^{++} lowers the binding constant of acetyl CoA by a factor of 2.8. It should be noted that the values for the Hill coefficients were 1.3 ± 0.03 for Mg acetyl CoA and 2.2 ± 0.14 for acetyl CoA indicating a lack of cooperativity of binding of Mg acetyl CoA. This result can be compared with binding parameters obtained from the deacylation studies of Easterbrook-Smith *et al.*, (1978b) which indicated a lack of cooperativity of binding of acetyl CoA and the analogue studies in the preceding chapter which consistently gave concave downwards curving double reciprocal plots.

5.3.5 THE EFFECT OF APOLAR SOLUTES

In order to test for the possibility that the enzyme could be protected against inactivation after dilution into buffers containing apolar solutes, the effect of sucrose, glycerol and polyethylene glycol on the rate of inactivation of the enzyme was tested. The results presented in Table 5.3 show that increasing concentrations of each solute reduced the rate of inactivation of the enzyme. Complete protection against inactivation was observed with 50% sucrose and 2% polyethylene glycol. This result indicated that size was not a consideration in the explanation of the effectiveness of apolar solutes as protective agents.

5.3.6 THE EFFECT OF IONIC SOLUTES

The complement of protection by apolar solutes of the inactivation of enzyme after dilution would be enhancement of the inactivation rate by ionic solutes. The results of experiments to test this possibility, are presented in Table 5.4. The data show that increasing concentrations of both potassium chloride and ammonium chloride enhance the rate of inactivation. This result taken together with the effect of apolar solutes indicated that the inactivation process was related to the polarity of the dilution buffer.

One possible explanation for the effect of solute polarity is that perturbation of the solvation shell surrounding the enzyme would occur in the presence of ions. However, apolar solutes which have large solvation shells would act to increase the overall extent of H-bonding in the solvent structure and thus enhance protein solvation.

5.3.7 THE EFFECT OF D_2O

In order to test for the possibility that the extent of hydrogen bonding in the buffer affects the rate of inactivation of the enzyme after dilution, the effect of buffer prepared with D_2O in place of H_2O, was tested. It was expected that since solutes dissolved in D_2O are not solvated to the same extent as in water, then the inactivation rate would be reduced due to the lack of capacity of D_2O molecules to penetrate the protein structure. The results presented in Table 5.5 show that the rate of inactivation of the enzyme was reduced after dilution into buffer prepared in D_2O. It was noteworthy that even in 100% D_2O the inactivation process was not totally arrested indicating that D_2O was less efficient in inactivating the enzyme rather than that D_2O protected against the inactivation process.

5.3.8 THE EFFECT OF SOLUTES ON THE K_a, V and η_H VALUES FOR ACETYL CoA ACTIVATION OF THE ENZYME

In order to test whether solutes, which either enhance or protect against inactivation of the enzyme upon dilution, have an influence on the binding of acetyl CoA or its activation of the enzyme, the values of K_a, V and η_H for acetyl CoA in the overall reaction was determined in the presence of appropriate concentrations of the solutes of interest. The results presented in Table 5.6 show that while both the η_H value and the reaction velocity do not alter to any significant extent in the presence of the different solutes, the K_a value, however, was lowered in the presence of D_2O and sucrose and was raised in the presence of sodium chloride. These results indicate that under conditions where the rate of inactivation of the enzyme upon dilution is greatly reduced, the binding of acetyl CoA to the enzyme is strengthened and where the rate of inactivation is enhanced, the binding of acetyl CoA is weakened.

5.3.9 THE EFFECT OF ANALOGUES OF ACETYL CoA ON THE RATE OF INACTIVATION AFTER DILUTION

On the basis of the findings presented in the previous chapter, the effect of analogues of acetyl CoA on the rate of inactivation of the enzyme after dilution was expected to yield further information about the function of the moieties of acetyl CoA. The results of experiments to test the effect of analogues of acetyl CoA on the rate of inactivation, are presented in Table 5.7 and show that 3.0mM CoASH in the presence of Mg^{++} was as effective as 0.25mM acetyl CoA in the presence of Mg^{++}. Acetyl 2'4'-phosphopantetheine at 2.5mM enhanced the rate of inactivation while 1.8mM acetyldesamino CoA had little effect either in the presence or absence of Mg^{++}. These results suggested that only the adenosine moiety of acetyl CoA protected the enzyme against inactivation upon dilution. The lack of protection afforded by acetyldesamino CoA suggested that, like the activation of first partial reaction activity, the 6-amino group in the adenine ring is essential to the function of the adenosine moiety.

While the results from the preceding chapter indicated that the presence of the adenosine moiety of acetyl CoA was required to observe any activity in the first partial reaction, the above result suggests that in the presence of the adenosine moiety the enzyme is protected against inactivation upon dilution. Taken together these results suggest that the first partial reaction sub-site is the region of the enzyme which is inactivated upon dilution. Further investigation of the inactivation phenomenon was required to provide a physical basis for the above observations.

5.3.10 THE POLYPEPTIDE BACKBONE STRUCTURE OF PYRUVATE CARBOXYLASE AND THE EFFECT OF SOLUTES WHICH INFLUENCE THE RATE OF INACTIVATION UPON DILUTION

In the previous section the process by which the enzyme was inactivated upon dilution was attributed to hydration of the enzyme at a specific site or sites which resulted in loss of first partial reaction activity. A change in hydration of a protein structure would be expected to alter that structure and this has been shown to be the case in a study of protein solvation effects by Timasheff, (1970) using lysozyme. It was expected then that the inactivation of pyruvate carboxylase by hydration would be accompanied by a change in the conformation of the polypeptide backbone.

The technique of circular dichroism was therefore employed as a sensitive probe to detect any subtle conformational changes which might occur as a result of hydration of the enzyme. The spectra of the basic protein structures, viz. α-helix, β-sheet and random coil, have been well characterised (Gratzer and Cowburn, 1969) and some typical spectra are included for reference in Fig. 5.5.

(a) The effect of enzyme concentration

The results presented in Fig. 5.6 show that on lowering the enzyme concentration, some bands in the circular dichroism spectrum undergo a shift towards lower wavelengths. The appearance and increasing intensity of extrema at 206-207nm and 183-200nm on dilution of the enzyme indicated the occurrence of a dramatic conformational change. The negative extremum at 222nm did not change appreciably during the dilution and was the only band detectable at wavelengths below 250nm for the enzyme at or above concentrations of 4 E.U./ml.

(b) The effect of acetyl CoA

On the other hand, enzyme diluted to the same extent in the presence of 100 μM acetyl CoA (Fig.5.7), behaved quite differently. There was an unsymmetrical broadening of the 222nm extremum, complete absence of the 206-207nm extremum and a significant reduction in the 187-200nm positive extremum.

Since the spectrum of a 100 μM acetyl CoA solution exhibited a positive extremum at 200nm it was possible that some interference due to its interaction with the protein spectrum could have accounted for the observed effect. Therefore, a complementary experiment was performed where the enzyme was diluted to a constant concentration into buffer solutions containing varied concentrations of acetyl CoA. In a parallel experiment, the enzyme was diluted into the buffer solutions and allowed to equilibrate for 10 min. before the acetyl CoA was added. The circular dichroism spectra of both sets of dilutions were recorded, as shown in Figs. 5.8 and 5.9, and were not identical. It can be seen that the shape of the curves obtained in the former experiment (Fig. 5.7) could not be due to interference from the spectrum of acetyl CoA as shown in Fig. 5.8a. It is interesting to note that with increasing concentrations of acetyl CoA, the band shifted toward the longer wavelengths indicating a possible concentration dependent self-association of acetyl CoA.

(c) The effect of additives

The effect of other solutes, which were shown in a previous section to be capable of protecting the enzyme against inactivation by dilution, was examined with the exception of the most effective, viz. 2% polyethylene glycol. This compound was omitted since it contained a contaminant, (not removed by recrystallization), with a large positive extremum covering the wavelengths of interest. The effect of 50% glycerol (Fig 5.10) and of sucrose, (Fig 5.11), on the circular dichroism spectra of successively diluted enzyme was similar to that of acetyl CoA but varied in degree.

Another compound which was observed in previous sections to decrease the rate of inactivation of enzyme upon dilution was D_2O. The effect of D_2O on the spectra of the circular dichroism of successively diluted enzyme was investigated and there was no detectable difference from the spectra of enzyme diluted into buffer made with H_2O.

Obviously, it is difficult to relate the rate of inactivation upon dilution to a static spectral curve which reflects a situation existing at the end of any structural changes rather than the rate at which they occur. The result obtained with D_2O suggests, however, that the apolar solutes exert their effect largely on the degree of inactivation while D_2O exerts its effect on the rate of the process. This result is good circumstantial evidence that the inactivation phenomenon is related to the relative rate of hydration of a particular site in the enzyme in the presence of the different solutes. This is because D_2O would be expected to act merely as an efficient solvator due to its physical properties but the rate of hydration of any specific site would be much reduced in the presence of D_2O.

In order to completely characterise the effects of solutes on the circular dichroism spectra of pyruvate carboxylase, the effect of an ionic solute was investigated. The results, as presented in Fig. 5.12, show that when the enzyme was diluted to a constant concentration in buffer containing increasing amounts of potassium chloride, the formation of the negative extremum at 206-208nm and the positive extremum at 200-205nm was enhanced at a concentration of 10mM. At concentrations of potassium chloride above 10mM the negative extremum at 222nM was increased indicating that increased ionic strength produced a different conformational change from that obtained by dilution of the enzyme at a constant ionic strength.

The overall conclusion to be drawn from the circular dichroism spectra is that upon dilution, changes occur in the conformation of the protein. The extent of which of these changes is reduced in the presence of apolar aolutes or acetyl CoA and which is enhanced in the presence of 10mM potassium chloride. The changes which occur are consistent with the formation of α-helical structures since the circular dichroism spectra of α-helices exhibit negative extrema at 206-208nm and positive extrema at 190-200nm. (See Fig. 5.5). The complete absence of positive extrema in the spectrum of enzyme at 3.5 units/ml could be interpreted as the result of equal proportions of random coil and a combination of α-helix and β-sheet formations. Random coil formations have been shown to exhibit a negative extremum covering the wavelengths 190-205nm and this would exactly cancel the effect of positive extrema in the same wavelength range due to the α-helix and β-sheet formations to produce the observed effect.

5.3.11 THE EFFECT OF INACTIVATION UPON DILUTION ON THE ACTIVITY OF THE FIRST AND SECOND PARTIAL REACTIONS

The activity of enzyme which had been diluted was followed using the overall reaction, the ATP:orthophosphate exchange reaction and the pyruvate:oxaloacetate exchange reaction. The results presented in Table 5.8 show that the loss in activity of the ATP:orthophosphate exchange reaction was concomitant with the loss in overall activity. Controls using both the presence of acetyl CoA and high enzyme concentrations to prevent loss of activity showed greatly reduced rates of inactivation in both the overall and exchange reaction activities.

A similar procedure was adopted in order to test for the possibility that the second partial reaction was succeptible to inactivation by dilution of the enzyme. The results, presented in Table 5.8, show that loss in overall activity on dilution was not accompanied by a concurrent loss in activity of the pyruvate:oxaloacetate exchange reaction. These results clearly infer that loss of overall activity of the enzyme upon dilution is a result of the loss in activity of the first partial reaction. The requirement of the ATP:orthophosphate exchange reaction and the lack of requirement of the pyruvate:oxaloacetate exchange reaction for the presence of acetyl CoA can be explained on the basis of these results. Taken together with the results presented in section 5.3.7 these results suggest that the adenosine moiety of acetyl CoA acts to prevent inactivation of the first partial reaction on dilution of the enzyme.

The overall conclusion from the results presented in section 5.3 is that inactivation of the enzyme upon dilution is a hydration phenomenon and that the hydrophobic adenosine moiety of acetyl CoA protects the first partial reaction site of the enzyme by exclusion of water from the site, whereas apolar solutes protect by reducing the overall rate of hydration of the enzymes. The possibility that activation of the first partial reaction by acetyl CoA in the first partial reaction sub-site was to prevent inactivation upon dilution, was tested and the results are presented in the following section.

5.3.12 THE EFFECT OF PYRUVATE CONCENTRATION ON THE OVERALL ENZYME ACTIVITY IN THE PRESENCE OF SOLUTES WHICH PREVENT INACTIVATION ON DILUTION OF THE ENZYME

The working hypothesis that activation of the enzyme by acetyl CoA consisted of protection by the adenosine moiety against loss of first partial reaction activity and stimulation of pyruvate binding by the acetyl pantetheine moiety had strong supporting evidence from the analogue work presented in the previous chapter and from dilution studies presented in this chapter.

The prediction could therefore be made that in the presence of solutes which protected the enzyme against inactivation the overall activity of the enzyme could be induced to approach the levels obtained in the presence of acetyl CoA by increasing the level of pyruvate. The results of such an experiment as presented in Fig 5.13 show that in the presence of either 2% polyethylene glycol, 3.0mM CoASH or 250 μM acetyl CoA as the level of pyruvate increased, the reaction velocity increased and the double reciprocal plots were all concave downwards. The response in the presence of 2% polyethylene glycol was a constant 5% (approx.) of the response in the presence of 250 μM acetyl CoA at each level of pyruvate whereas the response in the presence of CoASH was approx. 33% of the response in the presence of acetyl CoA. This was further evidence that in the presence of solutes which acted to prevent inactivation of the enzyme upon dilution, enzyme activity was controlled by changes in pyruvate concentration. The differences in velocity, therefore, represented the relative tightness of binding of pyruvate in the presence of each solute. The hypothesis would allow the further prediction that in the presence of both a solute which protects against inactivation upon dilution and an analogue which stimulates only the binding of pyruvate, eg. acetyl-2'4'-phosphopantetheine, the enzyme velocity ought to approach that obtained in the presence of acetyl CoA. The results of such an experiment are presented in Table 5.9 and show that in the presence of either 3mM acetyl-2'4'-phosphopantetheine or 2% polyethylene glycol, the enzyme velocity was low but in their combined presence the velocity was more than their sum and approached the velocity obtained in the presence of acetyl CoA. This result meant that the effect of acetyl CoA on the activity of pyruvate carboxylase could be "reconstituted" by addition of solutes which elicited the two separate effects of protection against inactivation upon dilution and stimulation of pyruvate binding.

5.4 DISCUSSION

From the studies presented in chapter 4 it was possible to reconcile the stimulation of the first and second partial reactions with the action of a single molecule of acetyl CoA. The mechanism of stimulation of the first partial reaction by the adenosine moiety of acetyl CoA was further investigated and the data obtained was presented in this chapter. It was found that presence of the adenosine moiety protected the enzyme against inactivation upon dilution and that apolar solutes could replace adenosine in this function. It was further found by circular dichroism studies that the inactivation process was accompanied by major structural changes in the protein but that the presence of acetyl CoA or apolar solutes substantially reduced the extent of the changes. It was further found that only the first partial reaction lost activity upon dilution of the enzyme. Activation by acetyl CoA could be approximated by the addition of an apolar solute and acetyl-2'4'-phosphopantetheine indicating that the effects ascribed to the moieties at each end of the acetyl CoA molecule accounted for the overall activation effect.

In a study of protein solvation effects (Timasheff, 1970) it was observed that α-helices form in response to conditions where hydrogen bonding is disrupted. The effect involves the breaking of bonds which maintain the native conformation in favour of the formation of the much stronger hydrogen bonding found in α-helices. In the case of pyruvate carboxylase "free" water can be seen as the denaturant which is in equilibrium with the structured water comprising the solvation shell of the enzyme. Factors which disrupt this shell such as decrease in protein concentration, increase in ionic solute concentration or increase in temperature would enhance the amount of unstructured or "free" water which gains access to the protein (Klotz, 1962; Hammes, 1967). Factors which enhance the extent of the structured water such as apolar solutes would prevent the "free" water from entering the protein structure. Once inside the protein structure the "free" water presumably disrupts random coil structures in or near the active site which results in α-helix formation and subsequent loss in activity.

In a treatise on the hydrophobic influences on enzyme activity, Jencks, (1969) envisages the effect of apolar solutes as the limitation of diffusion caused by changes in the viscosity of the medium. This would help to explain the actions of the apolar solutes used since solutions of each were highly viscous. The effect of D_2O could therefore be seen as a reduction in the diffusion rate since the proton tunneling rate in D_2O is greatly reduced (Jencks, 1969).

The effect of acetyl CoA is seen as highly specific where the effect of apolar solutes was non-specific. The presence of saturating levels of acetyl CoA, while sufficient to prevent loss of activity upon dilution, did not fully prevent the changes in polypeptide backbone structure. This suggested that whilst acetyl CoA was having a specific protective effect at the active site of the enzyme, alteration in structure of parts of the enzyme not involved in the active site, in the presence of acetyl CoA, was not accompanied by loss of activity. The specific loss of activity could be envisaged as the entry of "free" water into the first partial reaction site which was limited in the presence of acetyl CoA. The acetyl CoA molecule could be acting to maintain a pre-existing hydrophobic environment within the first partial reaction site by blocking or "capping" the site as depicted in Fig. 4.23. This result is in conflict with the conventional view of the action of an allosteric molecule since such an activator would usually be expected to elicit a favourable conformational change in the enzyme. In this case, however, the allosteric effector acts to prevent an unfavourable change in the conformation of pyruvate carboxylase. This result is supported by work in this laboratory which failed to observe substantial changes in the conformation of pyruvate carboxylase, upon binding of acetyl CoA, by ANS fluorescence (Ashman, 1973).

The action of the acetylpantetheine group to stimulate pyruvate binding may involve only minor conformational changes since the evidence presented in the previous chapter taken together with the indications of proximity of binding from this chapter, indicates that the pyruvate and acetylpantetheine moiety binding sites may be quite close. The change in position of individual residues may be sufficient to account for the minor changes seen using ANS fluorescence measurements. Furthermore, the enhancement of ANS fluorescence in the presence of acetyl CoA as observed by Ashman (1973), is consistent with the hypothesis under discussion here, that acetyl CoA maintains a pre-existing hydrophobic environment within the protein. Estimates of the α-helical content of the protein samples were not attempted in this study although many methods for doing so have been published (Myer, 1970; Tinoco and Cantor, 1970; Baker and Isenberg, 1976; Greenfield *et al.*, 1967). However, Baker and Isenberg (1976), have questioned the validity of any estimates of β-sheet and random coil content of a protein from circular dichroism spectra. These considerations are overshadowed by the fact that in the enzyme samples which were close to 4 E.U./ml the α-helical content was hidden by the combined effects of a large spectral component due to β-sheet content and an apparently equal component due to random coil content. By comparison, circular dichroism studies on the structure of elastase (Visser and Blout, 1971) have shown that the known content of α-helix of 5% was undetectable from the spectra of the native protein.

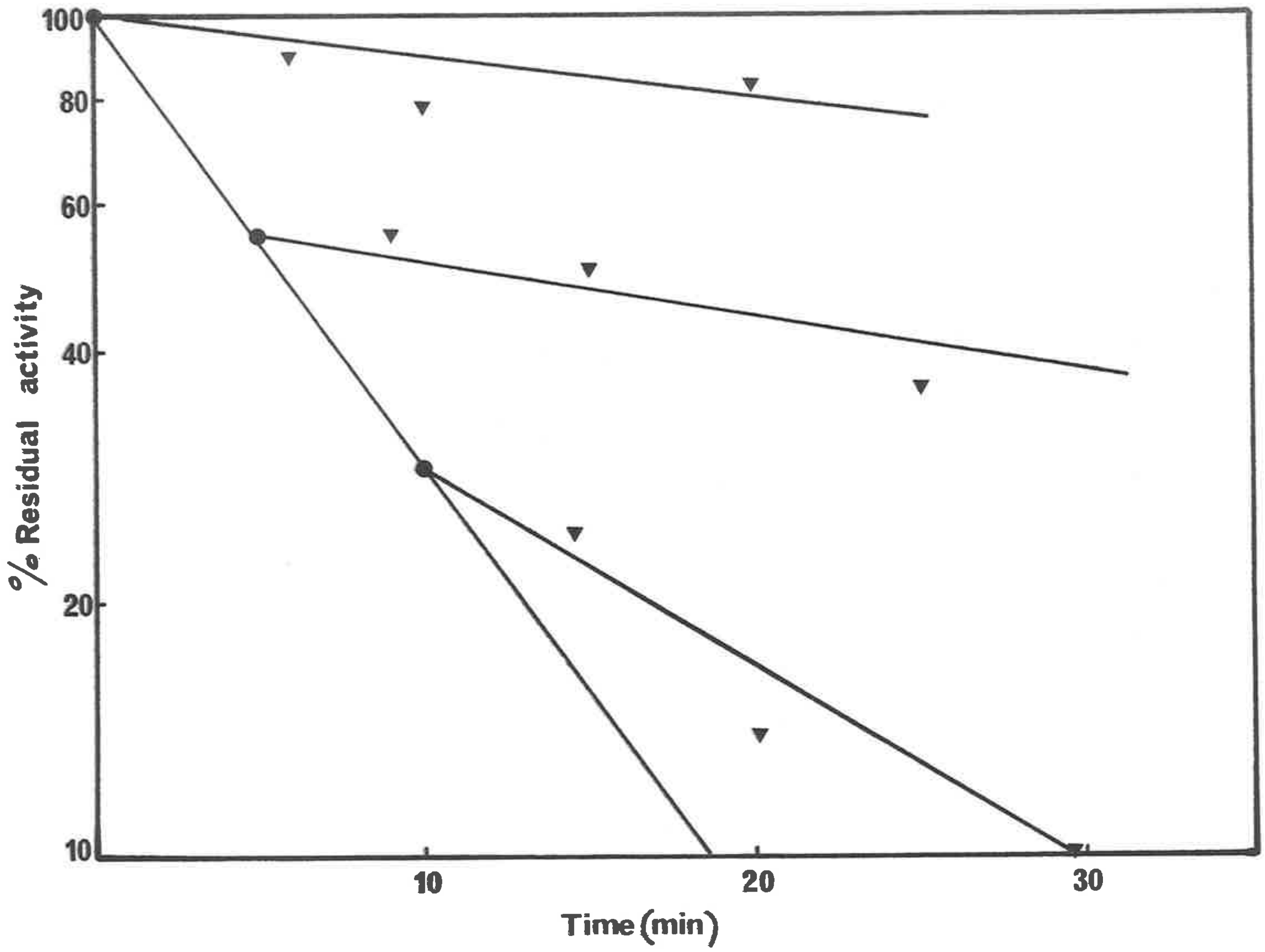
It is possible to surmise that a protein, such as pyruvate carboxylase, which resides within the mitochondrial matrix, where the water content is low, would not require rigid structural features such as α-helices to maintain the native conformation. Conversely extracellular proteases which survive a range of aqueous environments have a high content of disulphide bonds whereas intracellular enzymes and more particularly intramitochondrial enzymes, have little or no disulphide bonds. Pyruvate carboxylase has no disulphide bridges, although there are 11 cysteine residues in each 125,000 Dalton protomer.

It is interesting to speculate on the effect of K^+ which was observed in the circular dichroism spectra. The response obtained had some similar characteristics to the response obtained in the presence of acetyl CoA in that the negative extremum at 222nm was enlarged with increase in the concentration of either additive. This phenomenon suggests that the way in which both K^+ and acetyl CoA activate the enzyme may be similar and that the effect of acetyl CoA on the activation of the enzyme by monovalent cations (as outlined in Section 1.1) may therefore be explained.

It may be that the enhancement of the β-sheet structure by ionic strength is the result of a critical degree of exposure of the active site, which enhances activity and beyond which inactivation by over-exposure occurs.

Fig 5.1 The effect of acetyl CoA on enzyme activity after inactivation by dilution

Enzyme was diluted to a final concentration of 0.2 E.U./ml. in 0.01M potassium phosphate buffer pH 7.5. Aliquots (80 µl) were withdrawn at various times for determination of residual enzyme activity in the radiochemical assay (section 2.2.5b) (●-●). Aliquots, (0.33 ml) were also withdrawn at the same times and added to acetyl CoA to a final concentration of 0.25mM. Aliquots (80 µl) were withdrawn at various times for determination of residual enzyme activity as described above (▼-▼).


100
80
60
40
20
10
% Residual activity
10
20
30
Time (min)

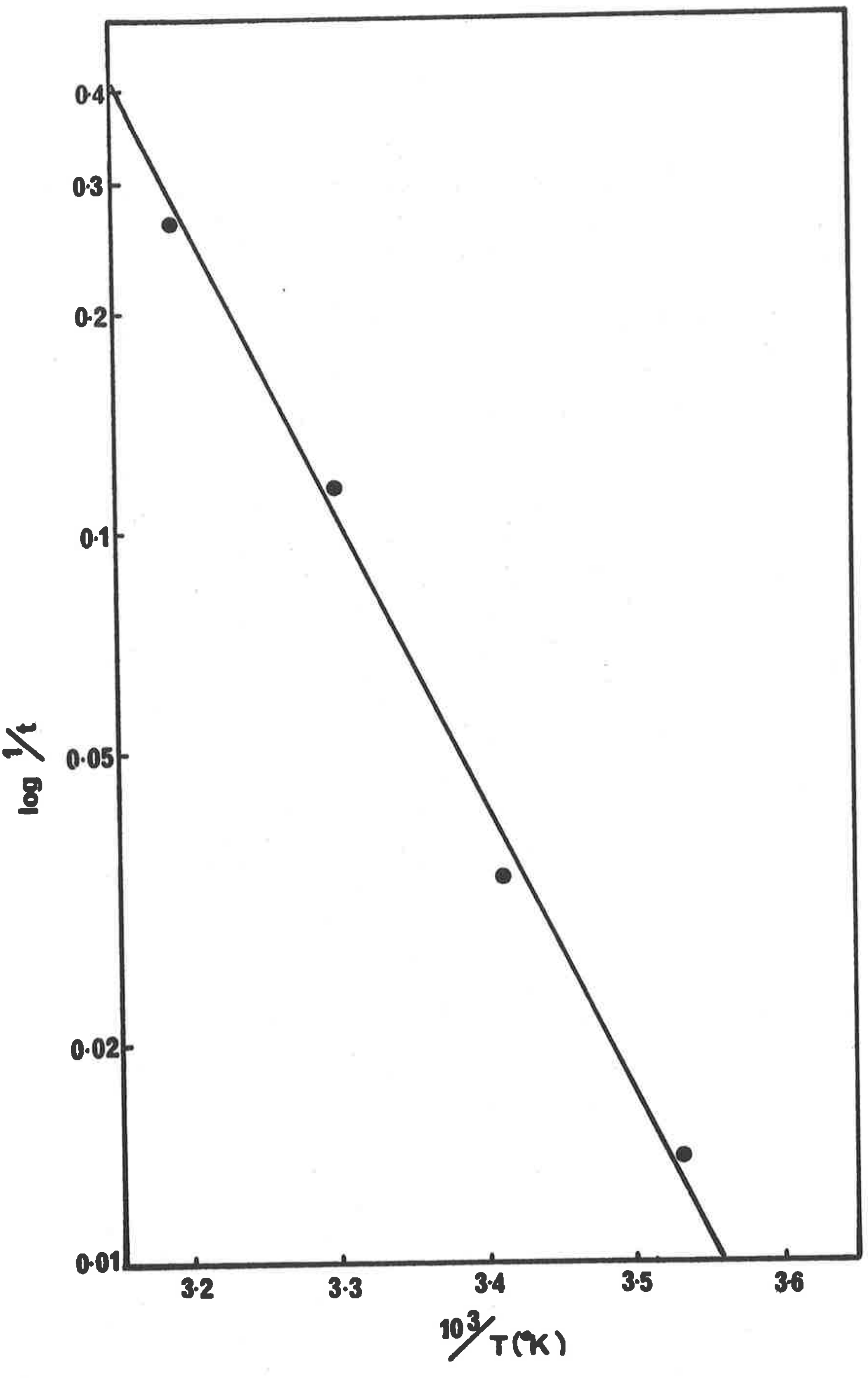
	[Cation] mM	[Anion] mM	pH	k_{inact} (min^{-1})
(a)	K^+	Phosphate^{3-} 10	7.0	.192
	K^+	Phosphate^{3-} 50	7.0	.132
	K^+	Phosphate^{3-} 10	7.5	.244
	K^+	Phosphate^{3-} 50	7.5	.238
	K^+	Phosphate^{3-} 10	8.0	.238
	K^+	Phosphate^{3-} 50	8.0	.137
	Tris$^+$ 10	Chloride$^-$	7.5	.137
	Tris$^+$ 50	Chloride$^-$	7.5	.50
(b)	K^+	Phosphate^{3-} 10	7.5	.263
	NEM$^+$ 10	Chloride$^-$	7.5	.182
	Na^+	HEPES$^-$ 10	7.5	.160
	NEM$^+$ 10	HEPES$^-$	7.5	.167

Table 5.1 The effect of buffer ions and pH on the rate of inactivation of pyruvate carboxylase upon dilution

Enzyme was diluted to a final concentration of 0.2 E.U./ml. in the presence of the concentrations of each buffer indicated. Aliquots (80 µl) were withdrawn at various times for the immediate determination of residual activity in the radiochemical assay, as described in section 2.2.5b, in the presence of 0.25mM acetyl CoA. The results in parts (a) and (b) of the table were from different experiments.

Fig. 5.2 An Arrhenius plot of the effect of temperature on the rate of inactivation of the enzyme after dilution

Enzyme was diluted to a final concentration of 0.2 E.U./ml. in 0.01M potassium phosphate buffer, pH 7.5, held at the temperatures indicated. Aliquots (80 µl) were withdrawn at various times and residual enzyme activity was determined, in the radiochemical assay as described in section 2.2.5b, in the presence of 0.25mM acetyl CoA.


0·4
0·3
0·2
0·1
0·05
0·02
0·01
log 1/t
3·2
3·3
3·4
3·5
3·6
10³/T(°K)

[acetyl CoA] (mM)	k_{inact} (min^{-1})
0	0
25	0.132
50	0.10
75	0.054
100	0.035
125	0.019

[Mgacetyl CoA] (mM)	k_{inact} (min^{-1})
0	0.069
2	0.046
4	0.032
8	0.0174
10	0.0149

Table 5.2 The effect of acetyl CoA in the presence and absence of Mg^{++} on the rate of inactivation of pyruvate carboxylase after dilution

Enzyme was diluted to a final concentration of 0.2 E.U./ml. in 0.01M potassium phosphate buffer pH 7.5 in the presence of the concentrations of each additive indicated. Aliquots (80 µl) were withdrawn at various times for the immediate determination of residual activity in the radiochemical assay, as described in section 2.2.5b, in the presence of 0.25mM acetyl CoA.

Fig. 5.3 Hill plot of the effect of acetyl CoA on the rate of inactivation of the enzyme after dilution

The k_{inact} data from Table 5.2 was used for this plot.

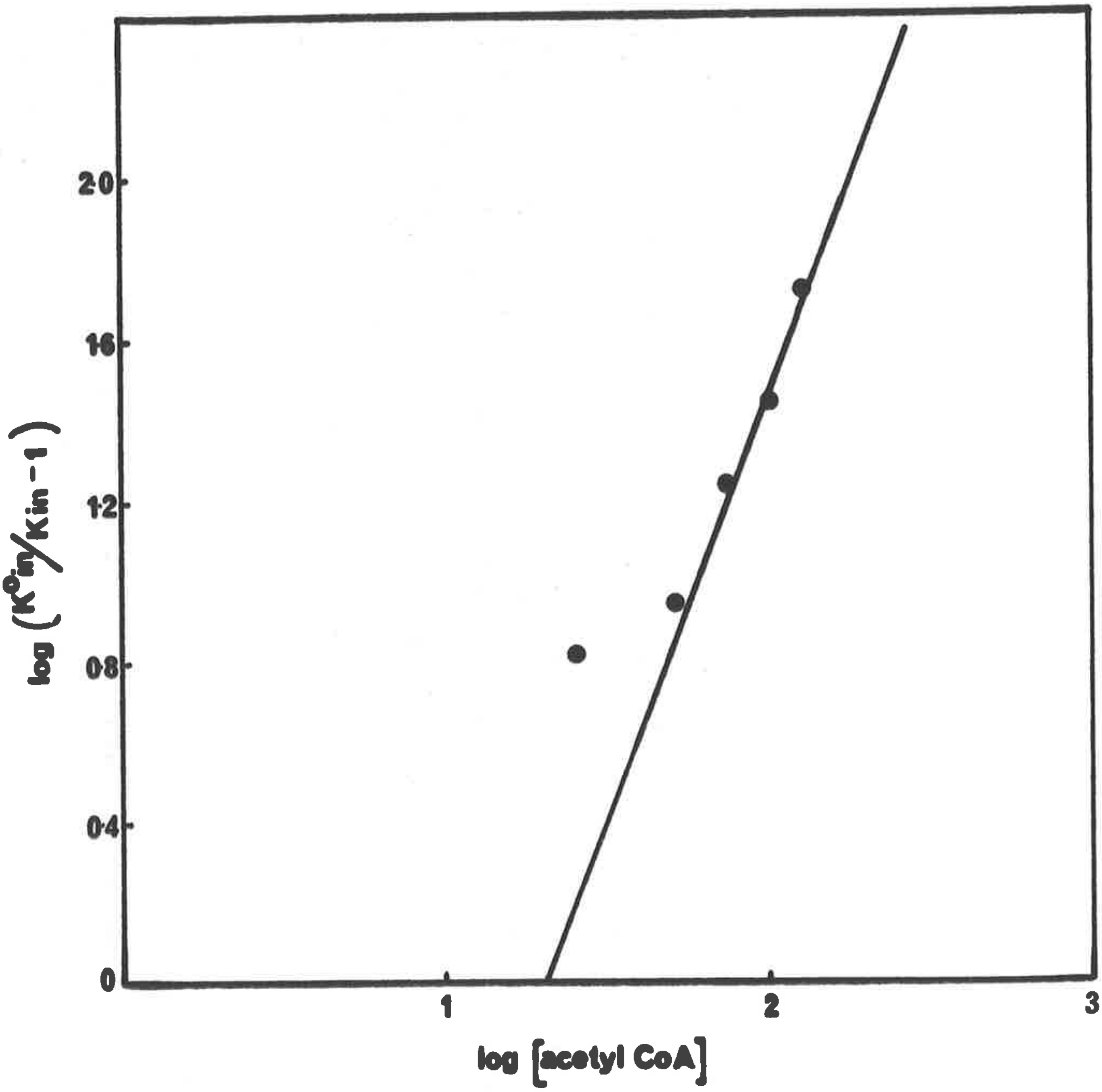
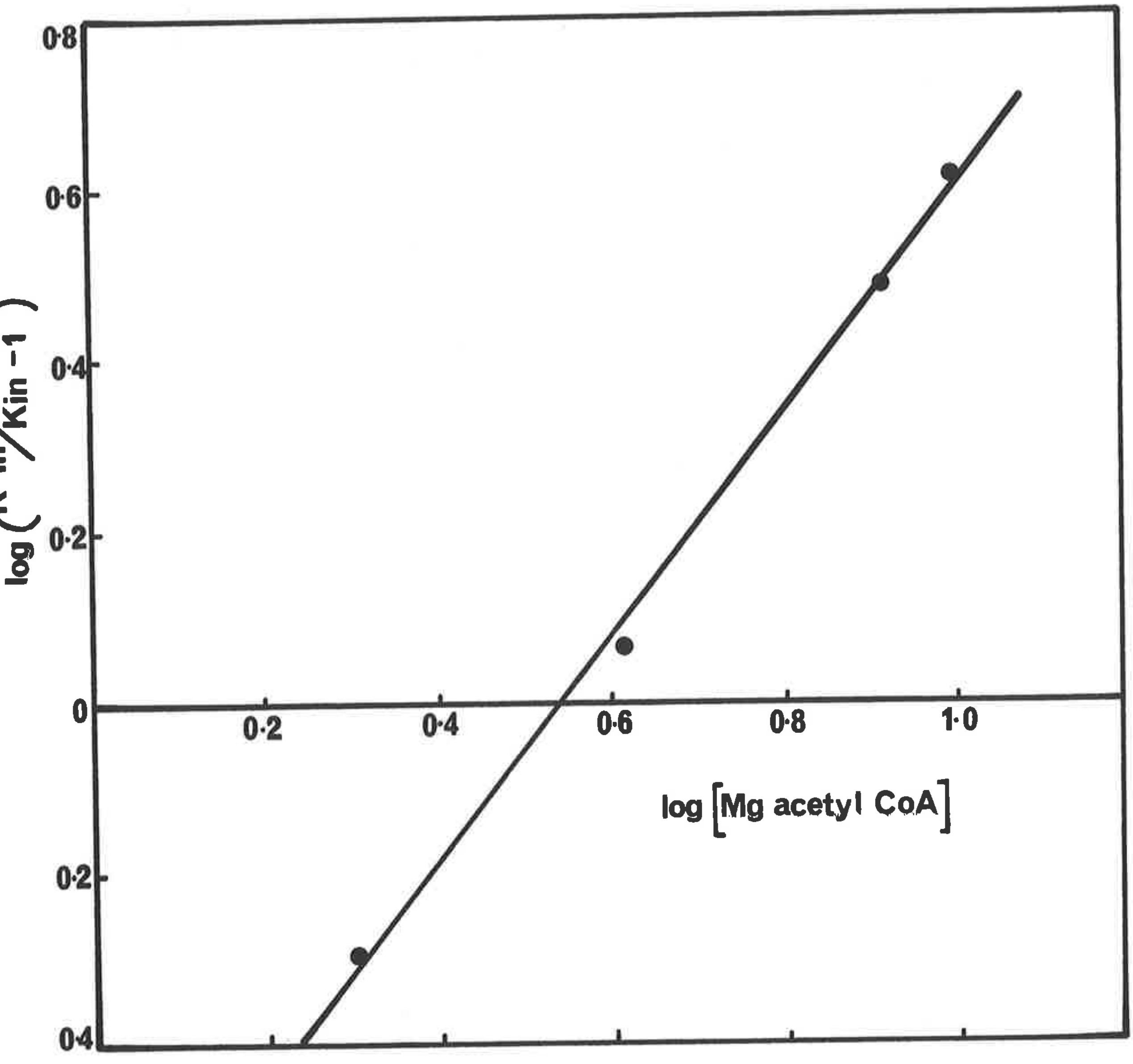

2·0
1·6
1·2
0·8
0·4
0
1
2
3
$\log\left(K^{o}_{in}/K_{in} - 1\right)$
log [acetyl CoA]

Fig. 5.4 Hill plot of the effect of Mg acetyl CoA on the rate of inactivation of the enzyme after dilution

The k_{inact} data from Table 5.2 was used for this plot.


0·8
0·6
0·4
0·2
0
0·2
0·4
$\log\left({}^{K^{o}_{in}}/_{K_{in}} - 1\right)$
0·2
0·4
0·6
0·8
1·0
log [Mg acetyl CoA]

solute	% $^w/_v$	k_{inact} (min^{-1})
glycerol	0	0.178
	5	0.105
	10	0.066
	20	0.05
	30	0.043
sucrose	0	0.2
	5	0.178
	10	0.04
	50	0
PEG	0	1.0
	0.1	0.42
	0.5	0.14
	2.0	0

Table 5.3 The effect of apolar solutes on the rate of inactivation of enzyme after dilution

Experimental details as for Table 5.2.

solute	mM	k_{inact} (min^{-1})
KCl	0	0.115
	1	0.133
	5	0.189
	10	0.222
	50	0.769
NH_4Cl	0	0.074
	1	0.104
	10	0.152
	50	0.143

Table 5.4 The effect of ionic solutes on the rate of inactivation of enzyme after dilution

Experimental details as for Table 5.2.

buffer solvent % D_2O	k_{inact} (min^{-1})
0	0.32
10	0.071
50	0.043
100	0.043

Table 5.5 The effect of D_2O on the rate of inactivation of the enzyme upon dilution

Enzyme was diluted to a final concentration of 0.2 E.U./ml. in 0.01M potassium phosphate buffer pH 7.5 made with the proportions of D_2O in water indicated. Enzyme activity was determined as described in the legend of Table 5.2.

Additive	$^{\eta}H$ (acetyl CoA)	V(cpm)	Ka (μM)
0	1.717 ± 0.048	13057 ± 118	17.3 ± 0.29
48.5mM NaCl	1.817 ± 0.154	13203 ± 439	24.5 ± 1.4
48.5% D_2O	1.60 ± 0.184	14017 ± 405	10.1 ± 0.68
24.3% sucrose	1.96 ± 0.31	11283 ± 469	15.7 ± 0.12

Table 5.6 The effect of sucrose, D_2O and NaCl on the $^{\eta}H$, V and K_a values for acetyl CoA

Initial velocities were determined in the radiochemical assay, as described in section 2.2.5b, in the presence of varying concentrations of acetyl CoA and the concentrations of each additive indicated. K_a, V and $^{\eta}H$ values were determined by fitting the initial velocity data to the empirical Hill equation using the nonlinear regression program developed by Vaughn *et al.*, (1976).

acetyl CoA or analogue	(mM)	k_{inact} (min^{-1})
acetyl CoA	0.25	0.018
Mg^{2+} acetyl CoA	0.25	0
acetyl 2'4'-phosphopantetheine	2.5	0.67
Mg^{2+} + acetyl 2'4'-phosphopantetheine	2.5	0.67
CoASH	3.0	0.018
Mg^{2+} + CoASH	3.0	0
acetyldesamino CoA	1.8	0.20
Mg^{2+} + acetyldesamino CoA	1.8	0.38
0	0	0.22
Mg^{2+}	5.0	0.15

Table 5.7 The effect of analogues of acetyl CoA in the presence and absence of Mg on the rate of inactivation of enzyme after dilution

Experimental details as for Table 5.2.

Fig. 5.5 Standard circular dichroism spectra of the three structural types found in polypeptides (from Gratzer and Cowburn, 1969).

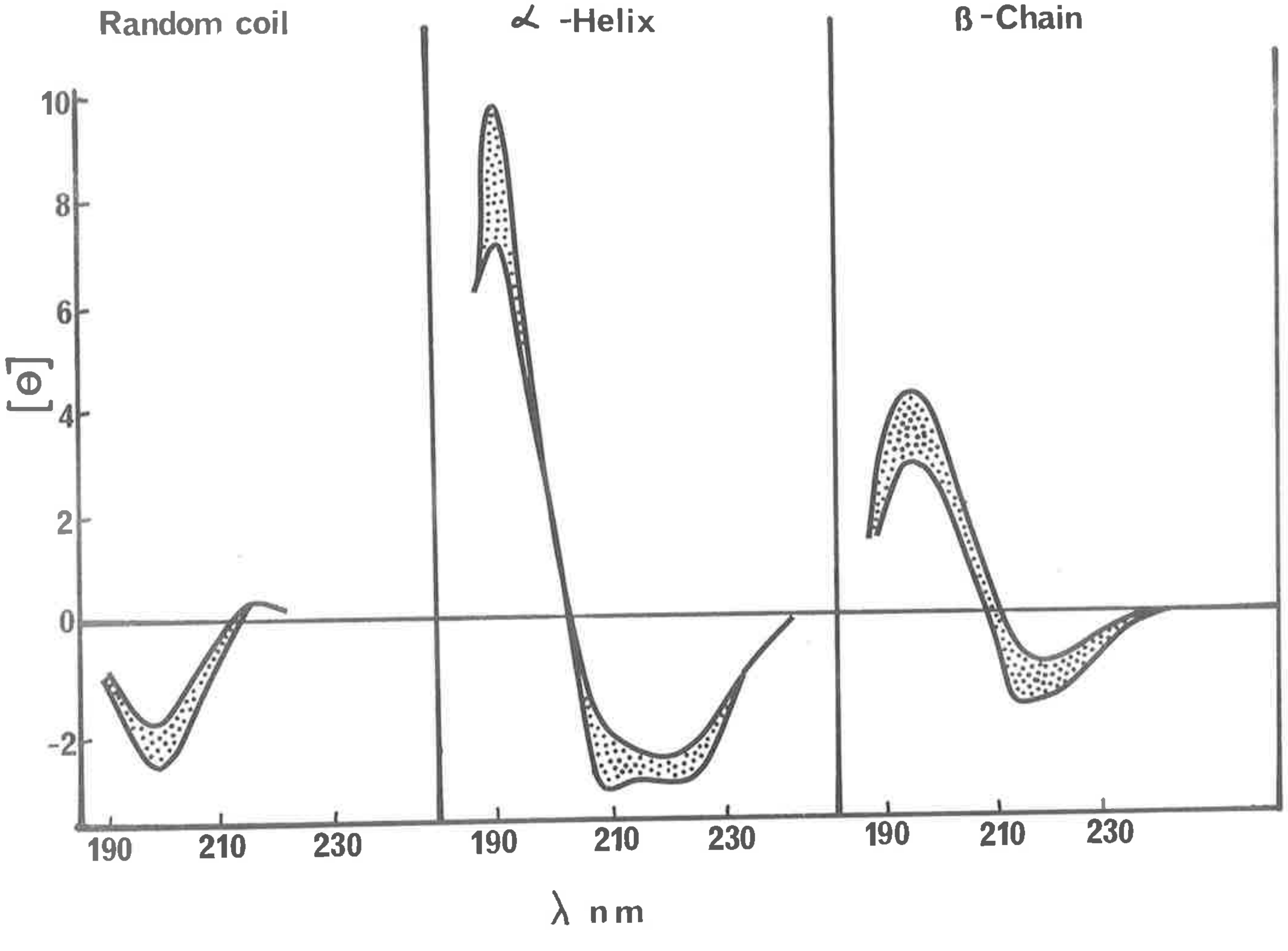

Random coil
α -Helix
ß -Chain
[θ]
10
8
6
4
2
0
-2
190
210
230
190
210
230
190
210
230
λ nm

Fig. 5.6 Circular dichroism spectra of pyruvate carboxylase at different concentrations

Spectra were recorded of enzyme which had been diluted to the following concentrations in 0.01M potassium phosphate buffer, pH 7.5:- 3.5 E.U./ml. (●-●), 1.7 E.U./ml. (▼-▼), 0.85 E.U./ml (□-□), 0.425 E.U./ml. (△-△).

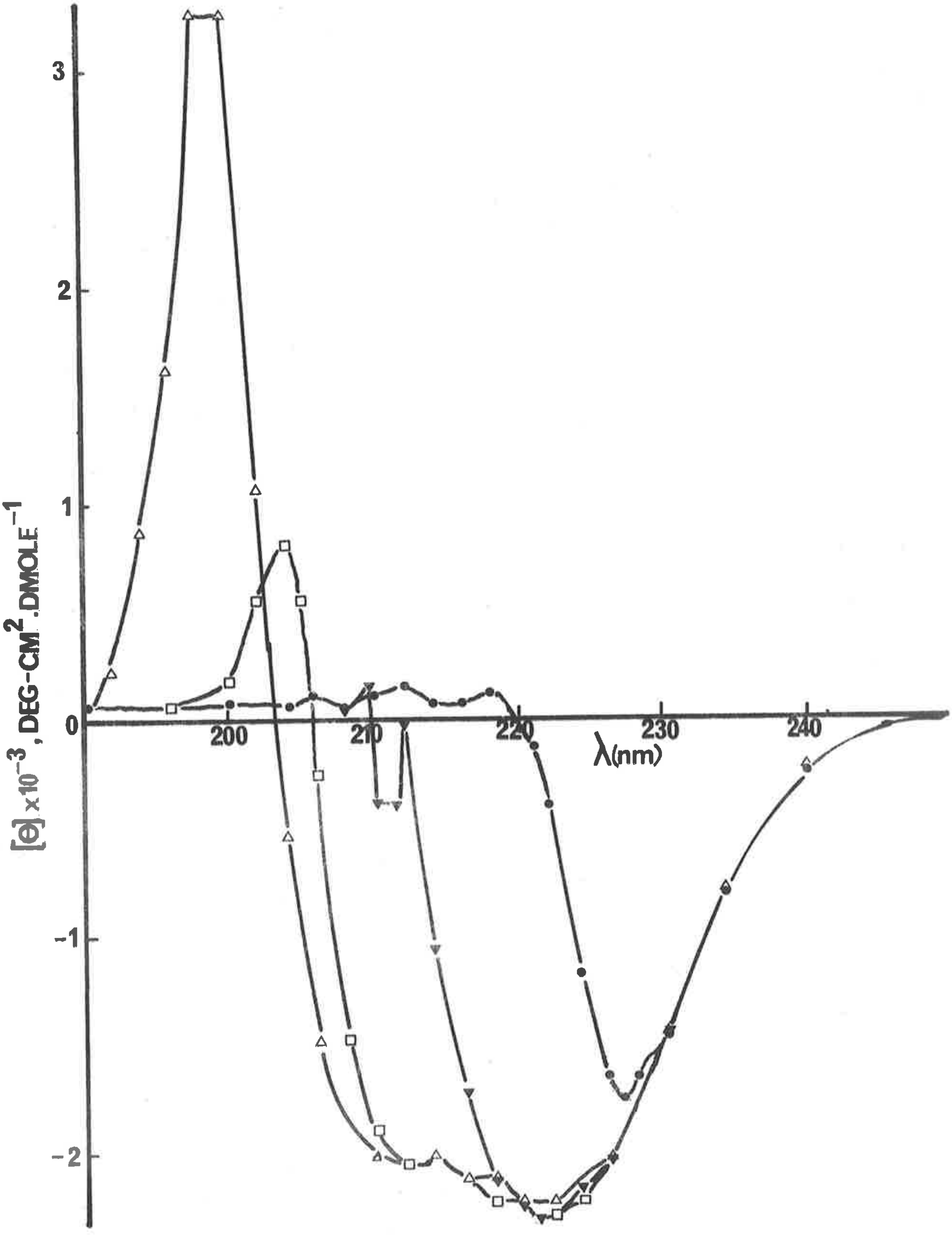

3
2
1
0
-1
-2
200
210
220
230
240
λ(nm)
[θ] x 10⁻³, DEG-CM², DMOLE⁻¹

Fig 5.7 Circular dichroism spectra of pyruvate carboxylase at different concentrations in the presence of acetyl CoA

Spectra were recorded of enzyme which had been diluted in the presence of 0.1mM acetyl CoA to the following concentrations in 0.01M potassium phosphate buffer, pH 7.5:- 0.1mM acetyl CoA alone (■-■), + 1.7 E.U./ml. (▼-▼), + 0.85 E.U./ml. (□-□), + 0.43 E.U./ml (△-△). The overlay is a reproduction of Fig. 5.6.

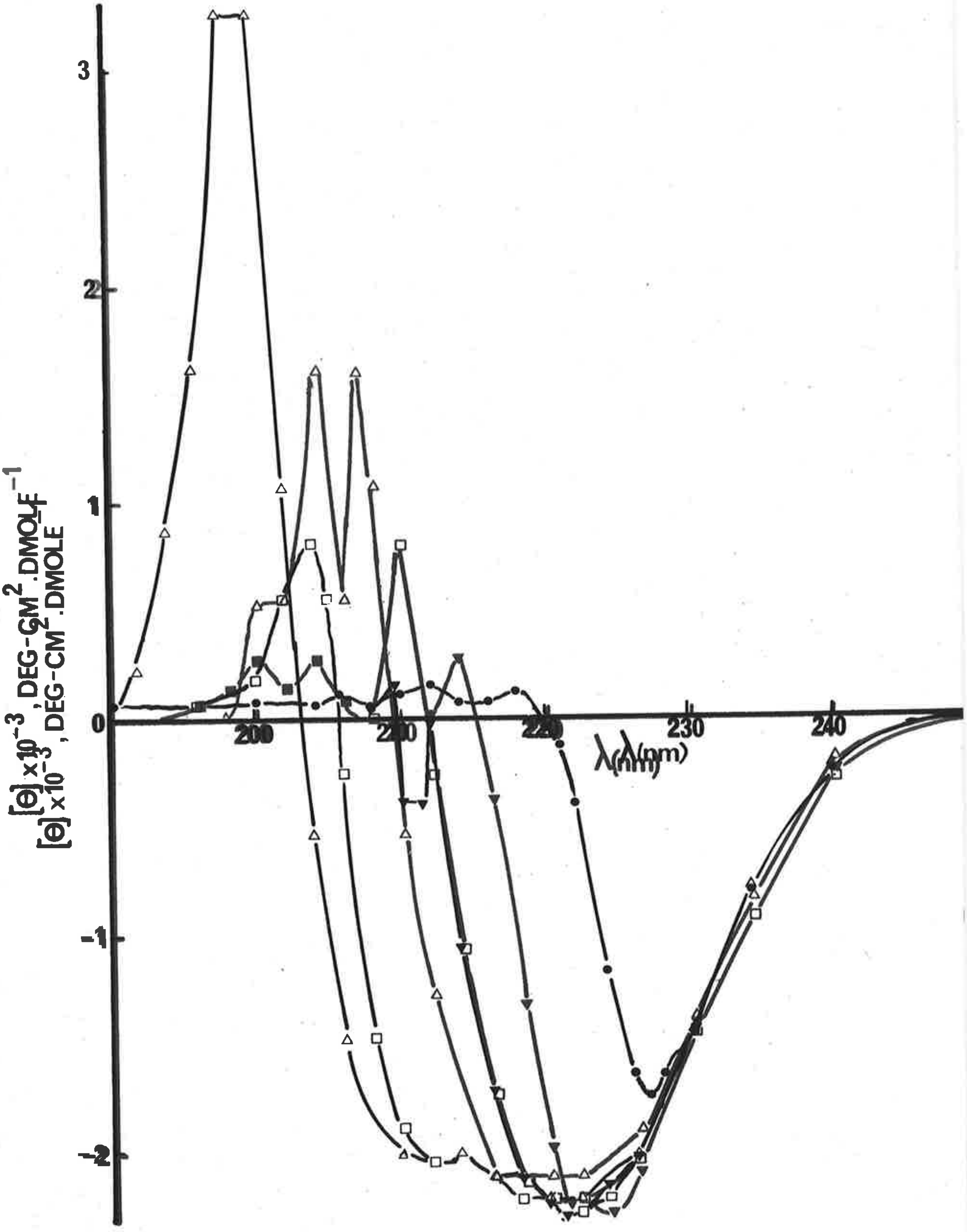

[θ] x10^-3, DEG-CM^2.DMOLE^-1
3
2
1
0
-1
-2
200
210
220
230
240
λ(nm)

Fig. 5.8 Circular dichroism spectra of pyruvate carboxylase in the presence of different concentrations of acetyl CoA

Spectra were recorded of enzyme at a final concentration of 0.5 E.U./ml. in 0.01M potassium phosphate buffer, pH 7.5, in the presence of the following concentrations of acetyl CoA:- 0 (o - o), 10 µM (▫ - ▫), 50 µM (▿ - ▿), 100 µM (▲ - ▲) and 200 µM (● - ●). The acetyl CoA spectra have been subtracted and are presented in Fig. 5.8a.

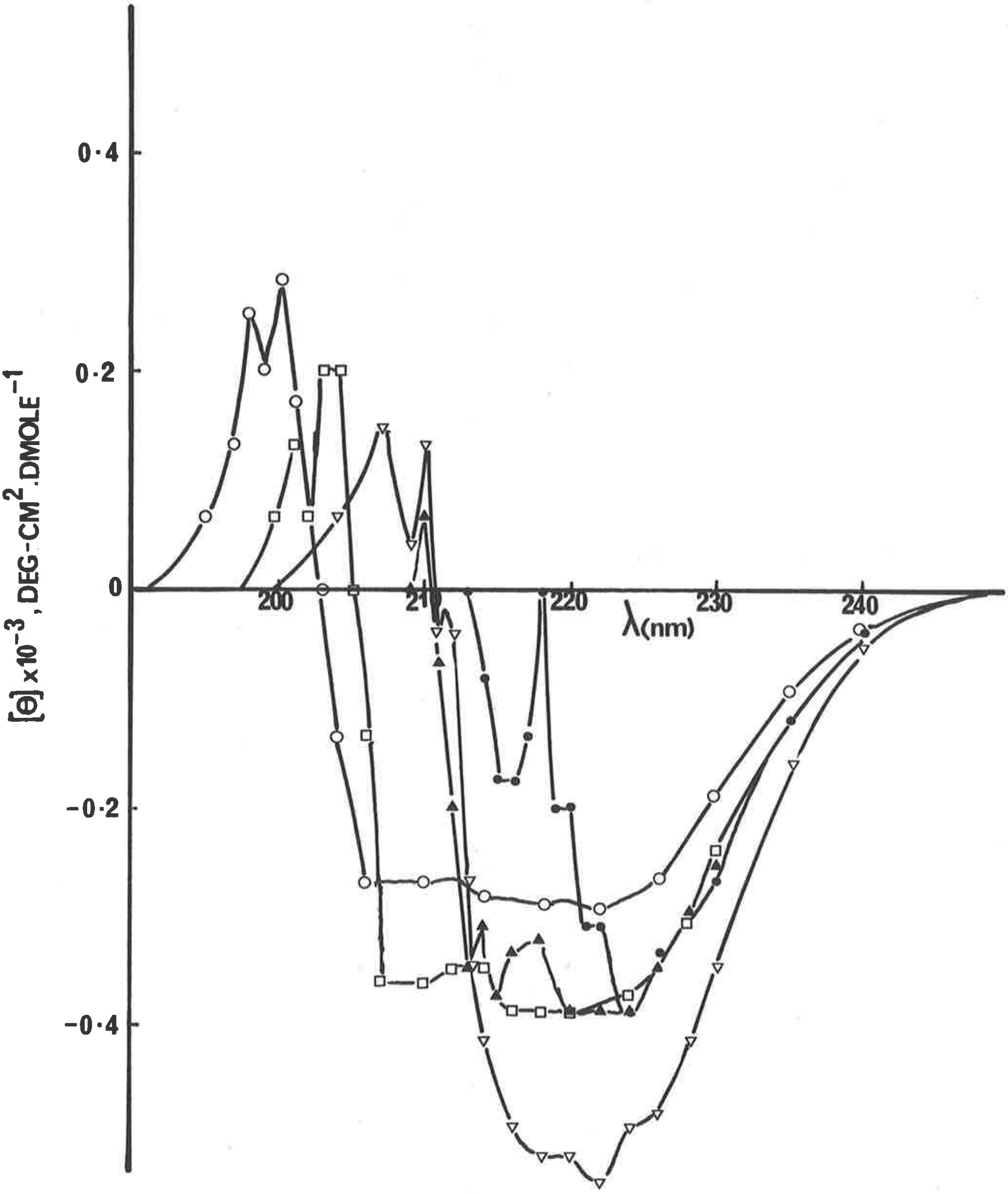
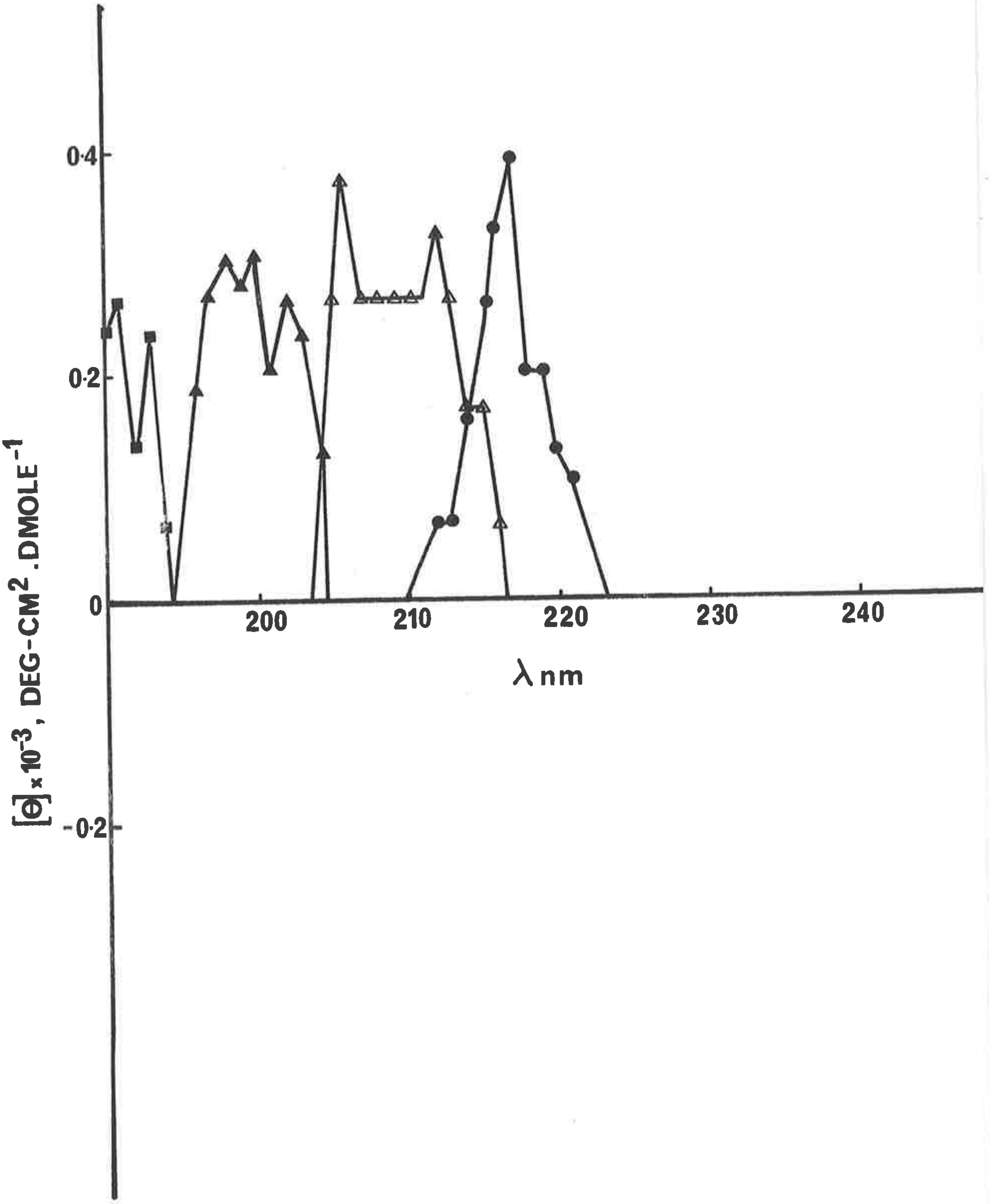

0·4
0·2
0
-0·2
-0·4
[θ] x 10^{-3}, DEG-CM^2.DMOLE^{-1}
200
210
220
230
240
λ(nm)

Fig. 5.8a Circular dichroism spectra of acetyl CoA at different concentrations

Spectra were recorded of acetyl CoA at the following final concentrations, 10 µM (■ - ■), 50 µM (▲-▲), 100 µM (△-△) and 200 µM (●-●) in 0.01M potassium phosphate buffer, pH 7.5.



0·4
0·2
0
-0·2
[θ] x 10^{-3}, DEG-CM^2.DMOLE^{-1}
200
210
220
230
240
λ nm

Fig. 5.9 The circular dichroism spectra of diluted enzyme to which has been added different concentrations of acetyl CoA

Enzyme was diluted to 0.5 E.U./ml. in 0.01M potassium phosphate buffer and allowed to remain at room temperature for 10 min. The following concentrations of acetyl CoA were then added before the spectra were recorded:- 0 (o - o), 10 μM (□ - □), 50 μM (▽ - ▽), 100 μM (▲ - ▲) and 200 μM (● - ●). The acetyl CoA spectra have been subtracted. The overlay is a reproduction of Fig. 5.8.

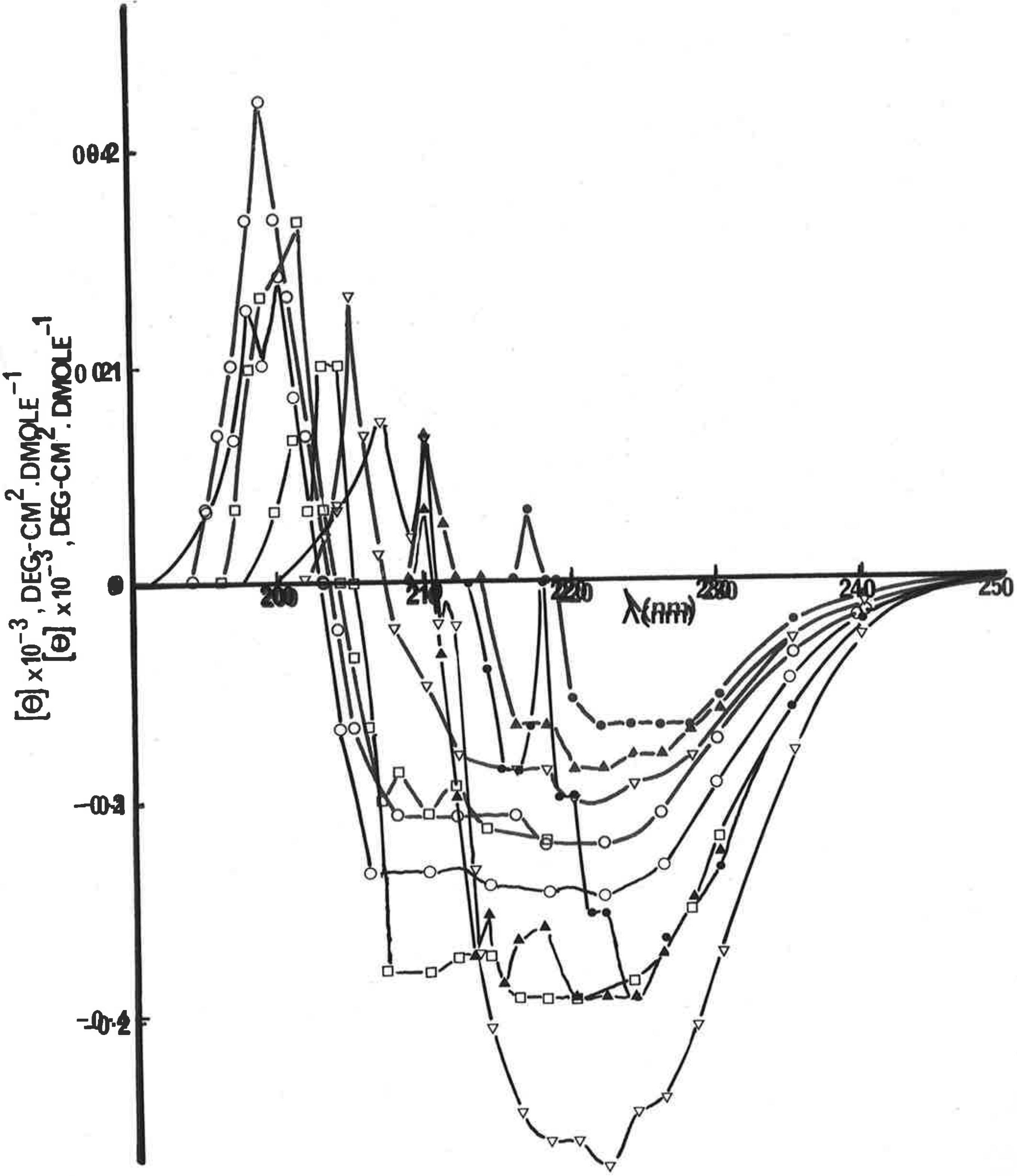

[θ] x 10^{-3}, DEG-CM^2.DMOLE^{-1}
[θ] x 10^{-3}, DEG-CM^2.DMOLE^{-1}
0·4
0·2
0·2
0·1
0
-0·1
-0·2
-0·2
-0·4
200
210
220
230
240
250
λ(nm)

Fig 5.10 Circular dichroism spectra of pyruvate carboxylase in the presence of 50% glycerol

Spectra were recorded of enzyme diluted to concentrations of 0.375 E.U./ml. (● - ●), 0.75 E.U./ml. (▲ - ▲) and 1.5 E.U./ml. (□ - □) in the presence of 50% glycerol and in 0.01M potassium phosphate buffer pH 7.5. The overlay is enzyme diluted to the same concentrations in buffer made without glycerol added.

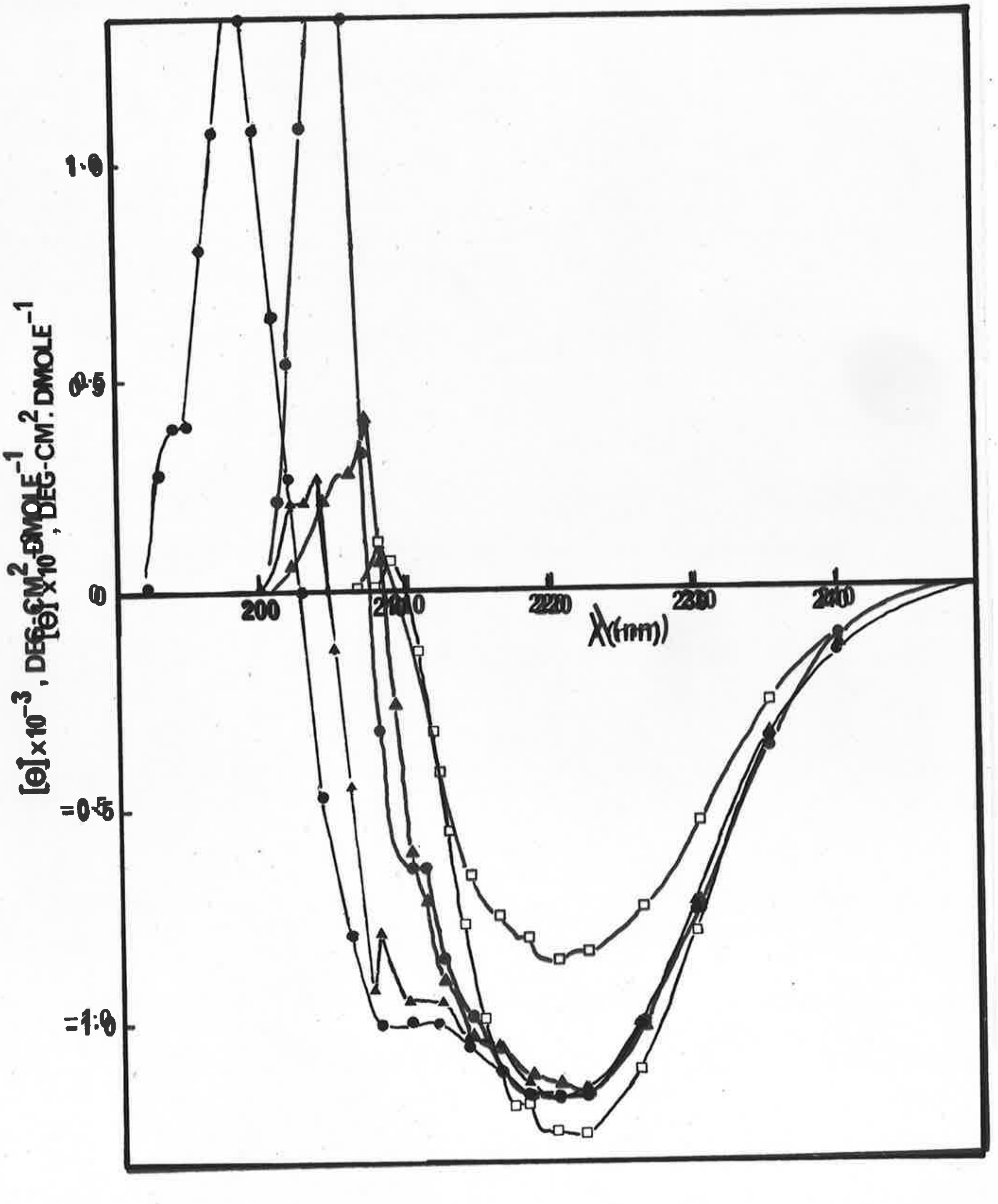

[θ] x 10⁻³, DEG-CM². DMOLE⁻¹
1·0
0·5
0
-0·5
-1·0
200
210
220
230
240
λ(nm)

Fig. 5.11 Circular dichroism spectra of pyruvate carboxylase in the presence of 50% sucrose

Spectra were recorded of enzyme diluted to the same concentrations as for Fig. 5.10, in the presence of 50% sucrose and in 0.01M potassium phosphate buffer. The overlay is the same as that for Fig. 5.10.

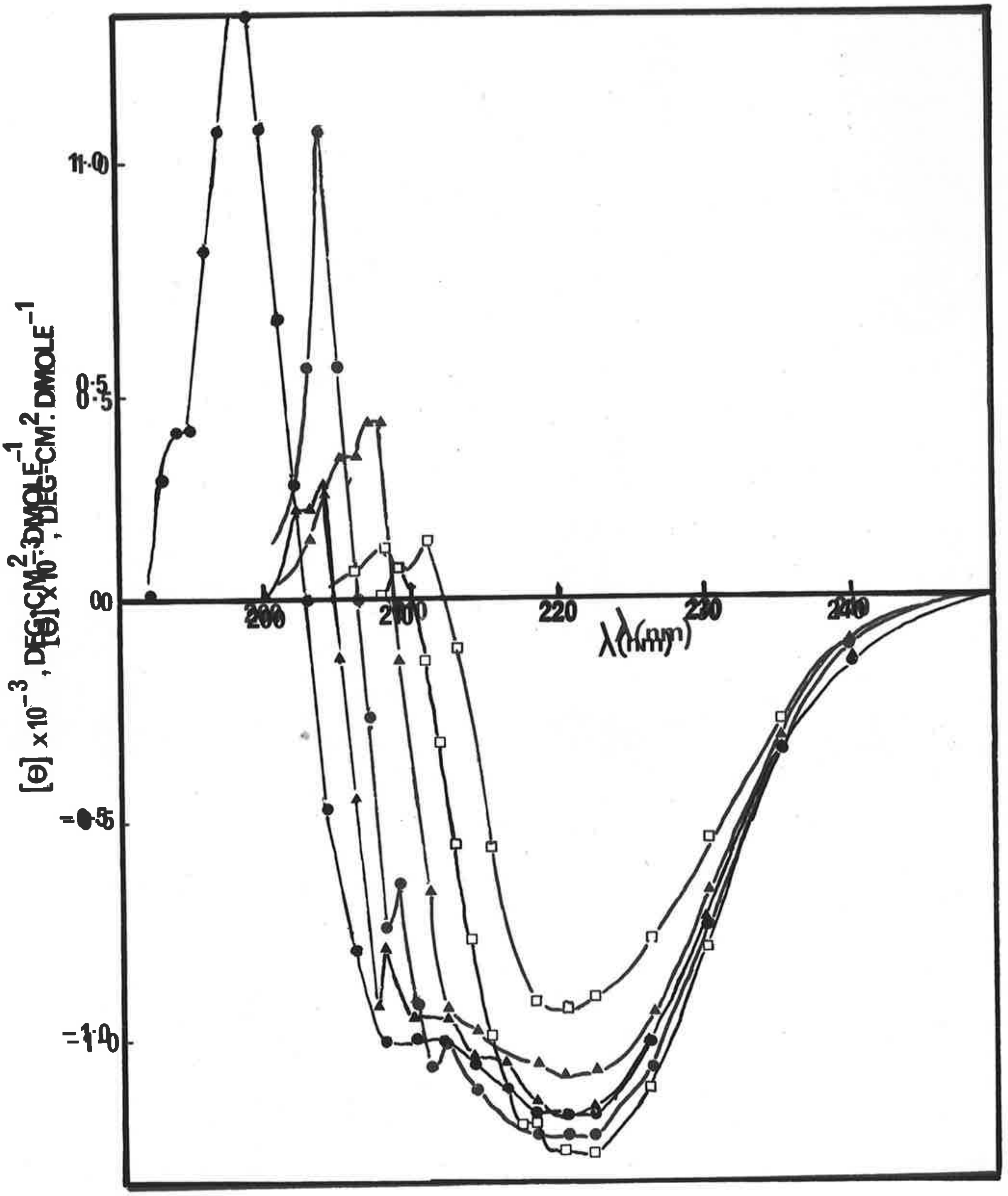

[Θ] x10^{-3}, DEG·CM^2·DMOLE^{-1}
1·0
0·5
0
-0·5
-1·0
200
210
220
230
240
λ (nm)

Fig 5.12 Circular dichroism spectra of enzyme in the presence of varying concentrations of KCl

Spectra were recorded of enzyme diluted to a final concentration of 0.375 E.U./ml in 0.01M potassium phosphate buffer and the following concentrations of KCl:- 0 (o - o), 10mM (■-■), 20mM (●-●), 50mM (▲-▲).

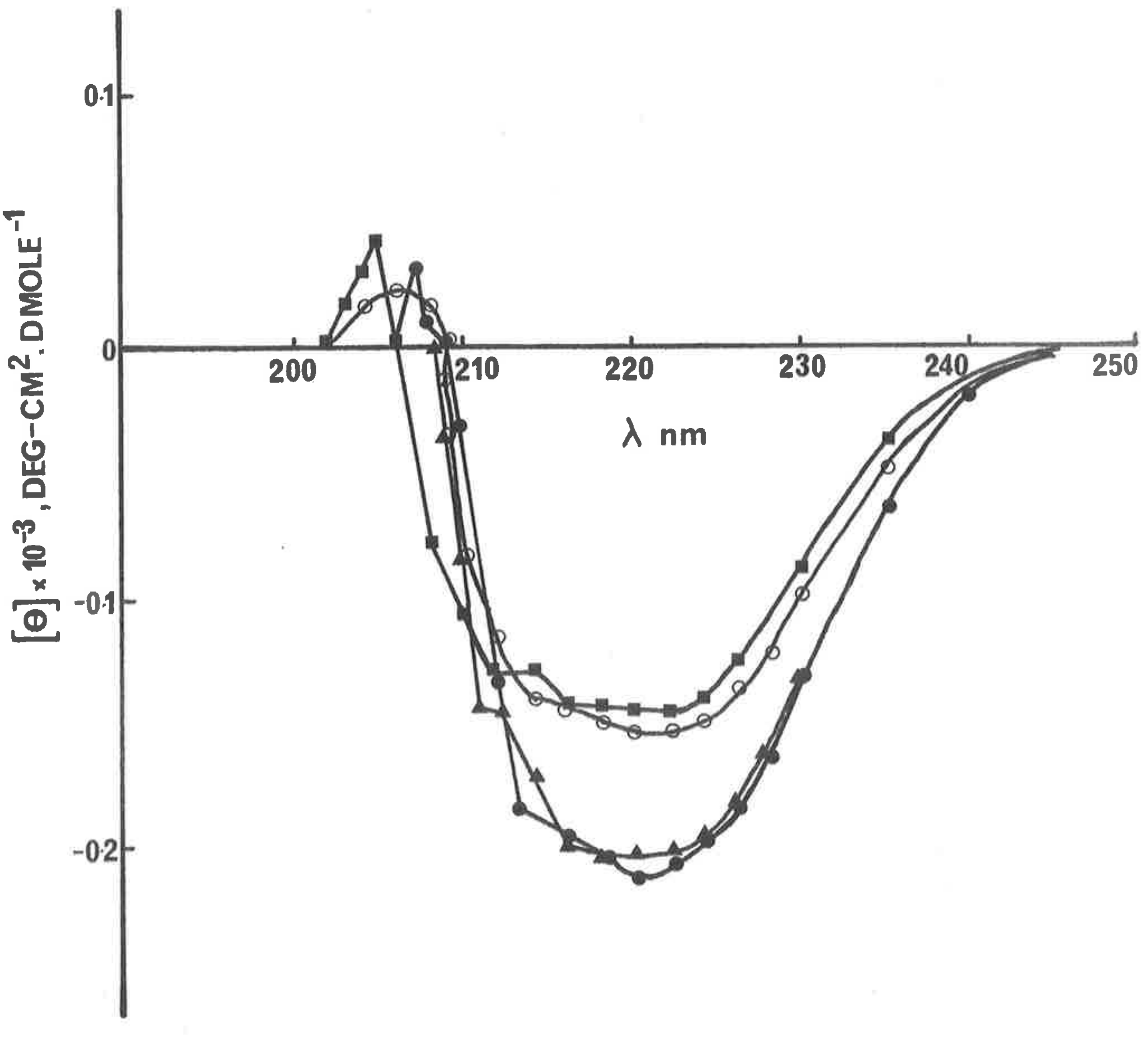

[θ] × 10⁻³, DEG-CM², DMOLE⁻¹
0·1
0
-0·1
-0·2
200
210
220
230
240
250
λ nm

Table 5.8 The effect of inactivation after dilution of pyruvate carboxylase on the activity of the first and second partial reactions

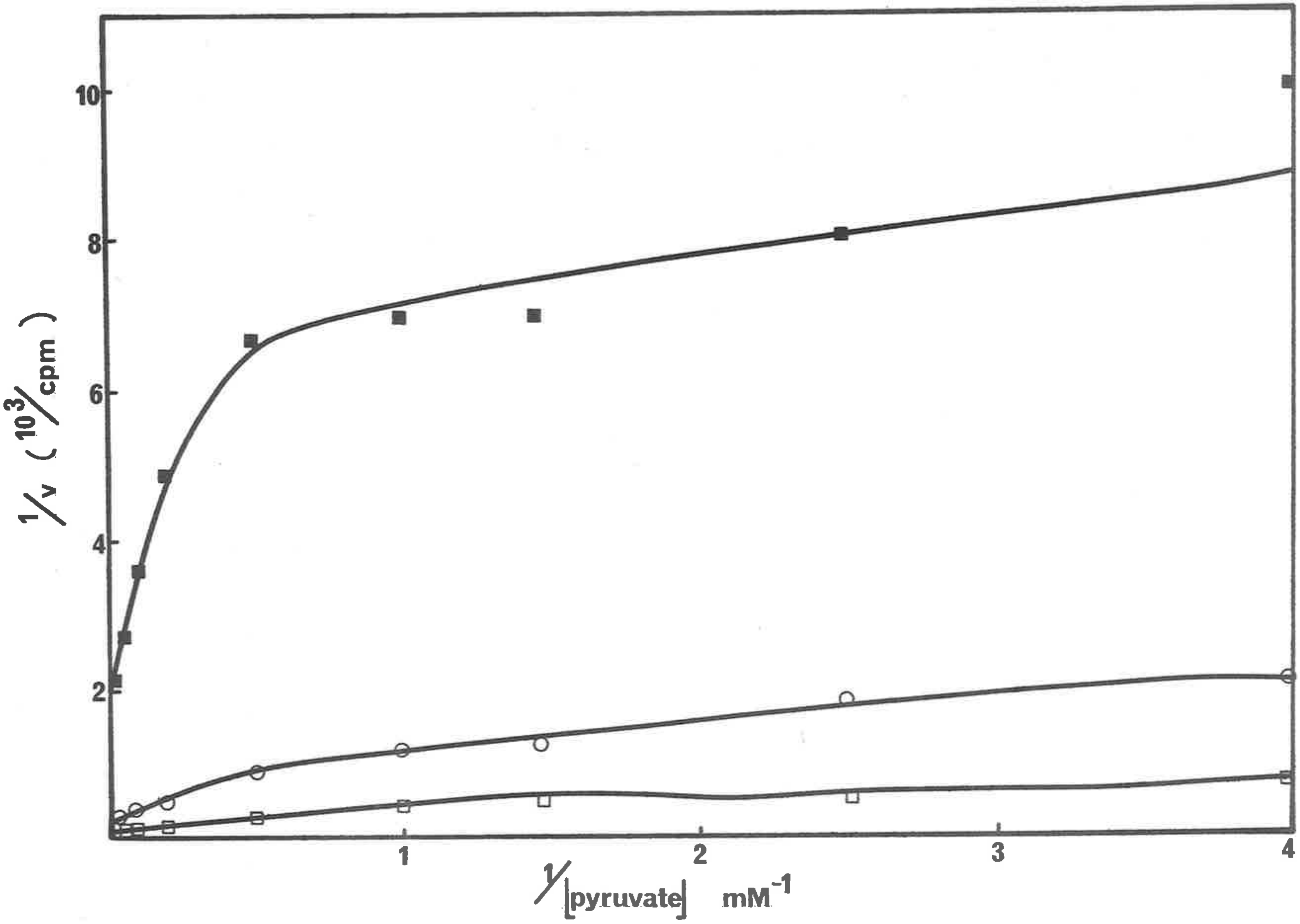
Enzyme was diluted to the final concentrations shown in 0.01M potassium phosphate buffer, pH 7.5, and aliquots (80 µl) were withdrawn at various times for the immediate determination of residual overall activity in the radiochemical assay, section 2.2.5b, in the presence of 0.25mM acetyl CoA. Aliquots (0.45 ml) were also withdrawn at various times for the immediate determination of pyruvate:oxaloacetate exchange activity, as described in section 2.2.5d, in the presence of 0.25mM acetyl CoA.

In similar experiments residual overall activity of 80 µl aliquots and ATP:orthophosphate exchange activity, as described in section 2.2.5c, of 0.45 ml. aliquots were determined in the presence of 0.25mM acetyl CoA.

Dilution:	Enzyme (0.44 E.U./ml)	k_{inact} (min^{-1})
	Overall activity	0.0128
	ATP:Pi exchange	0.022
	Enzyme (0.3 E.U./ml)	
	Overall activity	0.0526
	Pyr:OAA exchange	0
Control A:	0.44 E.U./ml. + acetyl CoA (250 µM)	
	Overall activity	0.0048
	ATP:Pi exchange	0.0056
	0.3 E.U./ml. + acetyl CoA (250 µM)	
	Overall activity	0
	Pyr:OAA exchange	0
Control B:	10 E.U./ml.	
	Overall activity	0
	Pyr:OAA exchange	0
	ATP:Pi exchange	0

Fig. 5.13 The effect of acetyl CoA, CoASH and 2% polyethylene glycol on the response of pyruvate carboxylase to varying concentrations of pyruvate

Enzyme activity was determined in the radiochemical assay, as described in section 2.2.5b, in the presence of varying concentrations of pyruvate and the following final concentrations of acetyl CoA, 0.25mM (□ - □); CoASH, 3mM (o - o) and polyethylene glycol, 2% (■ - ■) in lieu of a fixed level of 0.25mM acetyl CoA.


1/v (10³/cpm)
10
8
6
4
2
1
2
3
4
1/[pyruvate] mM⁻¹

Additive	(mM)	cpm	%
acetyl CoA	0.25	1641	100
acetyl-2'4'-phosphopantetheine	3.0	273	16.7
polyethylene glycol	2%	240	14.6
acetyl-2'4'-phosphopantetheine	3.0	1589	96.8
+ polyethylene glycol	2%		

Table 5.9 The combined effect of acetyl-2'4'-phosphopantetheine and polyethylene glycol on the overall activity

Enzyme activity was determined in the radiochemical assay, as described in section 2.2.5b, in the presence of the concentrations of each additive indicated.

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

The results presented in this thesis are part of an investigation in this laboratory into methods for studying the control of the activity of large molecular weight enzymes. The main emphasis in the overall approach has been firstly to define the system in precise kinetic terms. Subsequently, chemical modification followed by peptide sequencing and physical techniques can be directed in the light of the kinetically defined system.

The recent discoveries in this laboratory into the nature and mechanism of the allosteric activation by acetyl CoA have revealed that the previously accepted premise of cooperative binding was incorrect for this enzyme. Furthermore the dogma of 100% efficiency in enzyme catalysed reactions has been challenged in these studies with the finding that under certain conditions pyruvate carboxylase releases up to three times more orthophosphate than oxaloacetate (Easterbrook-Smith, *et al.*, 1976). Yet another challenge to the widely held view viz. that allosteric effectors necessarily promote conformational changes in enzymes, are the findings presented in this thesis that show a major effect of acetyl CoA is to prevent an unfavourable conformational change from occurring under adverse conditions.

Another feature of the multi-pronged attack on the secrets of pyruvate carboxylase has been the steady accumulation of specifically modified peptides from the active site of the enzyme. With the development of 3-chloroacetonyl-SCoA, as described in chapter 3, the modification of a peptide in the binding site of each substrate or effector of the enzyme is within reach.

The analogue studies presented in chapter 4 of this thesis have allowed the precise roles of acetyl CoA to be defined on a molecular basis. The alignment of the molecule in the active site will allow a more informed use of affinity labelling methods in future studies.

6.2 THE MODE OF ACTION OF ACETYL CoA

The findings presented in this thesis contribute directly to the understanding of the mode of action of acetyl CoA. On the basis of kinetic studies the emphasis had shifted from the exclusive consideration of the first partial reaction (Scrutton and Utter, 1967) to include the second partial reaction (Ashman, *et al.*, 1972). These latter authors showed that acetyl CoA increased by a factor of 3 the rate of the pyruvate:oxaloacetate exchange reaction. The subsequent studies of Easterbrook-Smith, *et al.*, (1978 b) defined in precise kinetic terms the importance of the lowering of the apparent K_m value of pyruvate to the overall enzyme mechanism.

The findings in this thesis enabled the enhancement of the binding of pyruvate to be assigned to the acetyl pantetheine moiety. The previous misconception of the role of acetyl CoA was able to be explained in terms of the susceptibility of the enzyme to a decrease in protein concentration and the protection of the enzyme against the inactivation by the presence of the adenosine moiety of acetyl CoA. The replacement of the protective action of the adenosine moiety by solutes which affect the hydration of the enzyme enabled the inactivation phenomenon to be extensively investigated and to be defined in terms of the physical effects of hydration on the tertiary structure of the polypeptide backbone of the enzyme. Having established that the formation of rigid α-helical structures in response to hydration of the ATP binding site was undesirable for the maintenance of enzymic activity it was then possible to define the role of the adenosine moiety as one of limiting the access of solvent water into the readily hydratable first partial reaction subsite. Once the enzyme was protected in this way, the acetyl pantetheine moiety was free to influence the binding of pyruvate and thus to increase the rate of the overall reaction.

6.3 THE ACTIVATION BY ACETYL PANTETHEINE

The remaining question which this study leads to is the molecular mechanism of the enhancement of pyruvate binding by the acetyl pantetheine moiety. One group which appears to be essential for this activation is the carbonyl group of the acetyl moiety. Evidence for this essentiality comes from the activation of the enzyme by the analogue ethyl-SCoA. The compound binds to the enzyme with a reduced affinity compared with acetyl CoA and has a value of 0.65mM compared with 50μM for acetyl CoA (or 10 - 15 μM for Mg acetyl CoA).

A role for the carbonyl group can be found if the other remaining enigma of pyruvate carboxylase action, viz. enzyme dependent deacylation of acetyl CoA, is used as the basis for the proposal of a model for the activation of pyruvate.

The model is based on several observations from the study of pyruvate carboxylase and is best described diagramatically (Fig. 6.1). The events which occur are as follows:-

(a) The active site complex (I) which consists of pyruvate and acetyl CoA aligned, as depicted, with each carbonyl group H-bonded to two sulphydryl residues.

(b) The abstraction of a proton from acetyl CoA allows the enol form of acetate in II to in turn abstract a proton from pyruvate to form the activated enol pyruvate species in III.

(c) The activated pyruvate species can either break down to reform keto pyruvate as in I and be released from the enzyme surface or

(d) it can attack the biotin CO_2 species to produce the product oxaloacetate as in IV.

The evidence for this model is as follows:-

(a) The existence of two sulphydryl residues is inferred from affinity labelling studies with 3-bromopyruvate in which a second sulphydryl residue was modified unless acetyl CoA was present. (Hudson, *et al.*, 1975). Conversely, affinity labelling studies with 3-chloroacetonyl-SCoA have revealed that a second site of modification occurs unless saturating levels of pyruvate are present. These findings suggest the existence of two sulphydryl residues which are close together and in the region of the pyruvate and acetyl pantetheine binding sites.

(b) The analogue studies suggest that the binding sites of the acetyl pantetheine moiety and pyruvate may be close together.

(c) Studies using analogues of pyruvate and oxaloacetate (Mildvan, *et al.*, 1966) indicated that the preferred tautomer of all the analogues for binding to the enzyme was the enol form. This point could provide a basis for the enhancement of the binding of pyruvate by acetyl CoA in the conversion of pyruvate to the enol form.

(d) The finding discussed in section 1.1.9 that 50% of the enzyme bound pyruvate is released before catalysis to products can occur can be explained by the equilibrium inherent in the above mechanism which would return at least 50% of the pyruvate to the keto form in the absence of biotinCO_2.

(e) The deacylase activity of the enzyme can be explained on the basis that the enol form of acetyl CoA would be very susceptible to hydrolysis. The deacylase rate is enhanced in the presence of pyruvate which is in accord with this model.

(f) The acetyl CoA-independent activity of the enzyme can occur when the Ygroup is used in place of enol acetyl CoA but at a reduced efficiency due to a possibly unfavourable steric arrangement. The model could be tested for by (i) Tritium transfer from ^3H-pyruvate into acetyl CoA and (ii) the effect of analogues of pyruvate and oxaloacetate on the rate of deacylation of acetyl CoA. Analogues which do not have removable protons should not accelerate the deacylation rate.

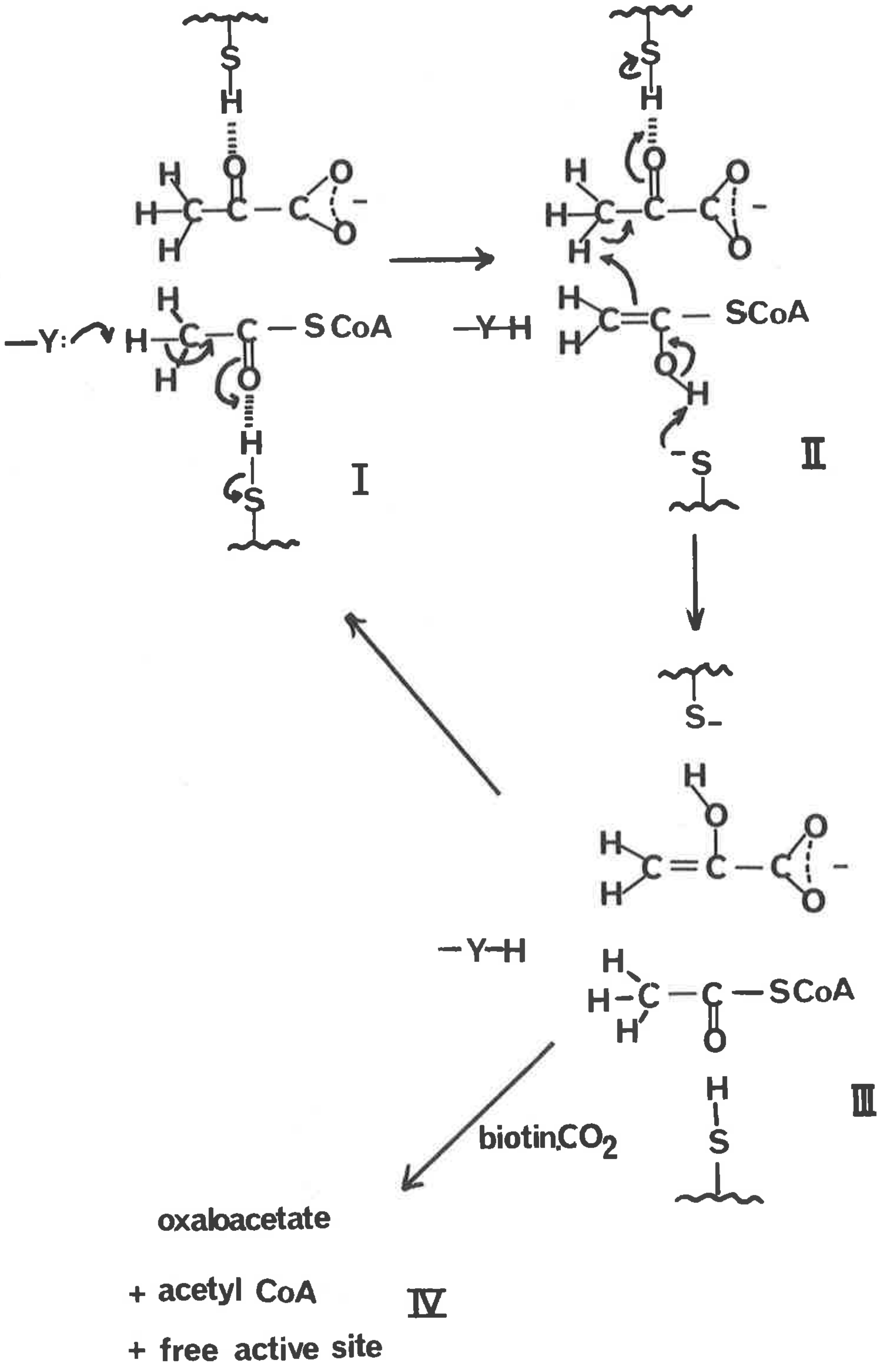

I
II
III
IV
—Y:
—Y–H
SCoA
biotin.CO2
oxaloacetate
+ acetyl CoA
+ free active site

Fig. 6.1

BIBLIOGRAPHY

Abiko, T., (1970) Meths. in Enzymol. 18A, 358-364.

Adler, A.J., Greenfield, N.J. and Fasman, G.D., (1973) Meths. in Enzymol. 28D, 675-735.

Anderson, R.A., Parrish, R.F. and Graves, D.J., (1973) Biochem. 12, 1901-1906.

Antonoff, R.S. and Ferguson, J.J., (1974) J. Biol. Chem 249, 3319-3321.

Ashman, L.K., Keech, D.B., Wallace, J.C. and Nielsen, J., (1972) J. Biol. Chem. 247. 5818-5824.

Ashman, L.K. and Keech, D.B., (1974) J. Biol. Chem. 250, 14-21.

Ashman, L.K., Wallace, J.C. and Keech, D.B., (1973) Biochem. Biophys. Res. Comm. 51, 924-931.

Ashman, L.K., (1973) Ph.D. thesis, Adelaide University.

Baddiley, J. and Thain, E.M., (1953) J. Chem. Soc., 903-906.

Baker, C.C. and Isenberg, I., (1976) Biochem. 15, 629-634.

Baker, B.R., (1967) "Design of Active Site Directed Irreversible Inhibitors", Wiley, New York.

Baillie, L.A., (1960) Inter, J. Applied Rad. and Isotopes. 8,1.

Bais, R. and Keech, D.B., (1972) J. Biol. Chem. 247, 3255-3261.

Barden, R.E. and Scrutton, M.C., (1974) J. Biol. Chem. 249, 4829-4838.

Barden, R.E., Fung, C-H., Utter, M.F. and Scrutton, M.C., (1971) J. Biol. Chem. 247, 1323-1333.

Barritt, G.J., Keech, D.B. and Ling, A.M., (1966) Biochem. Biophys. Res. Comm. 24, 476-481.

Bergmeyer, H.U., (1975) Z. Klin. Chem. Klin. Biochem. 13, 507-508.

Bloom, S.J. and Johnson, M.J., (1962) J. Biol. Chem. 237, 2718-2720.

Bousquet, W.F. and Christian, J.E., (1960) Anal. Chem. 32, 722-723.

Bridgeland, E.S., and Jones, K.M., (1967) Biochem J. 104, 9 - 10p.

Cazzulo, J.J. and Stoppani, A.O.M., (1967) Arch. Biochem. Biophys. 121, 596-608.

Chase, J.F.A. and Tubbs, P.K., (1969) Biochem. J. 111, 225-235.

Cheung, Y-F. and Walsh, C., (1976) Biochem. 15, 3749-3753.

Clark, M.G., Williams, J.F. and Blackmore, P.F., (1974) Cat. Rev-Sci. Eng. 9, 35-77.

Colombo, G., Marcus, F., (1974) Biochem. 13, 3085-3091.

Cuatrecasas, P., (1970) J. Biol. Chem. 245, 574-584.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M., (1969) Data for Biochemical Research ed.2. p.192. Oxford University Press.

Duc, J.A., (1977) Personal communication.

Dugal, B.S., (1976) Enzyme 21, 317-326.

Dwek, R.A., (1973) "Nuclear Magnetic Resonance in Biochemistry", Oxford University Press, England.

Easterbrook-Smith, S.B., Hudson, P.J., Goss, N.H., Keech, D.B. and Wallace, J.C., (1976) Arch. Biochem. Biophys. 176, 709-720.

Easterbrook-Smith, S.B., Wallace, J.C. and Keech, D.B., (1976) Eur. J. Biochem. 62, 125-130.

Easterbrook-Smith, S.B. (1977) Ph.D. thesis, University of Adelaide.

Easterbrook-Smith, S.B., Wallace, J.C. and Keech, D.B., (1978a) Biochem J. 169, in press.

Easterbrook-Smith, S.B., Campbell, A.J. Wallace, J.C. and Keech, D.B., (1978b) in preparation.

Ellman, G.L., (1959) Arch. Biochem. Biophys. 82, 70-77.

Epstein, J.E., Eisenthal, R.W.E. and Ess, R.J., (1955) Anal. Chem. 27, 1435-1439.

Fairbanks, G., Steck, T.L. and Wallach, D.F.H., (1971) Biochem. 10, 2606-2617.

Fasman, G.D., (1963) Methods in Enzymology. 6, 928-957.

Fee, J.A., Hegeman, G.D. and Kenyon, G.L., (1974) Biochem. 13, 2533-2538.

Frey, W.H. and Utter, M.F., (1977) J. Biol. Chem. 252, 51-56.

Fung, C-H., (1972) Ph.D. thesis, Case Western Reserve University, Cleveland, Ohio.

Fung, C-H. and Utter, M.F., (1970) F.E.B.S. Symposium. 19, p96.

Fung, C-H., Feldmann, R.J. and Mildvan, A.S., (1976) Biochem. 15, 75-84.

Fung, C-H., Gupta, R.K. and Mildvan, A.S., (1976) Biochem. 15, 85-92.

Glazer, A.M. Delange, R.J. and Sigman, D.S., (1975) "Chemical modification of Proteins". Elsvier, Holland.

Gratzer, W.B. and Cowburn, D.A., (1969) Nature. 222, 426-431.

Greenfield, N., Davidson, B., Fasman, G.D., (1967) Biochem. 6, 1630-1637.

Greenwell, P., Jewett, S.L., Stark, G.R., (1973) J. Biol. Chem. 248, 5994-6001.

Groman, E.V., Schultz, R.M. and Engel, L.L., (1975) J. Biol. Chem. 250, 5450-5454.

Gutte, B.,,(1977) J. Biol. Chem. 252, 663-670.

Hammes, G.G., Swann, J.C., (1967) Biochem. 6, 1591-1596.

Hansske, F., Sprinzl, M., Cramer, F., (1974) Bioorganic Chem. 3, 367-376.

Hill, E.J., Chou, T-H., Shih, M.C. and Park, J.H., (1975) J. Biol. Chem. 250, 1734-1740.

Hillel, Z. and Wu, C-W., (1976) Biochem. 15, 2105-2113.

Hudson, P.J., Keech, D.B. and Wallace, J.C., (1975) Biochem. Biophys. Res. Comm. 65, 213-219.

Jencks, W.P., (1969) "Catalysis in Chemistry and Enzymology" McGraw-Hill, N.Y. p.393-436.

Jorns, M.S. and Hersh, L.B., (1976) J. Biol. Chem. 251, 4872-4881.

Jornvall, H., Woenckhaus, C. and Jehnscher, G., (1975) Eur. J. Biochem. 53, 71-81.

Kaplan, N.O., (1956), Meths. Enzymol. 3, p873.

Klotz, I.M. (1962) Horizons in Biochem. (A. Szent-Györgyi dedicatory volume) p. 523-550.

Knowles, J.R. (1972) Accounts in Chem. Res. 5, 155-160.

Lau, E.P. Haley, B.E. and Barden, R.E., (1977a) Biochem. 16, 2581-2585.

Lau, E.P., Haley, B.E. and Barden, R.E., (1977b) Biochem. Biophys. Res. Comm. 76, 843-849.

Lee, C-H and Sarma, R.H., (1974) F.E.B.S. letters. 43, 271-276.

Lin, T-I. and Morales, M.F., (1977) Anal. Biochem. 77, 10-17.

Ling, A-M. and Keech, D.B., (1966) Enzymologia. 30, 367-380.

McClure, W.R., Lardy, H.A. and Kniefel, H.P. (1971a) J. Biol. Chem. 246, 3569-3578.

McClure, W.R., Lardy, H.A., Wagner, M. and Cleland, W.W., (1971b) J. Biol. Chem. 246, 3579-3583.

McClure, W.R., Lardy, H.A. and Cleland, W.W. (1971c) J. Biol. Chem. 246, 3584-3590.

Means, G.E. and Feeney, R.E. (1971) "Chemical Modification of Proteins", Holden Day, San Francisco.

Meloche, H.P., (1967) Biochem. 6, 2273-2280.

Mieyal, J.J., Blisard, K.S. and Siddiqui, U.A., (1976) Bioorganic Chem. 5, 263-273.

Mildvan, A.S., Scrutton, M.C., and Utter, M.F.,(1966) J. Biol. Chem. 240, 3714-3723.

Mildvan, A.S. and Scrutton, M.C., (1967) Biochem. 6, 2978-2994.

Moffatt, J.G. and Khorana, H.G., (1961) J. Amer. Chem. Soc. 83, 663-675.

Monod, J., Wyman, J., Changeux, J-P (1965) J. Mol. Biol. 12, 88-118.

Moss, J. and Lane, M.D. (1971) Adv. Enzymol. 35, 321-442.

Myer, Y.P., (1970) Res. Comm. Chem. Pathol. and Pharm. 1, 607-616.

Nakashima, K., Rudolph, F.B., Wakabayashi, T. and Lardy, H.A., (1975) J. Biol. Chem. 250, 331-336.

Ochoa, S. (1957) Prep., 5, 19-30.

Pal, P.K. Wechter, W.J. and Colman, R.F., (1975) Biochem. 14, 707-715.

Pincus, M, Thi, L.L. and Carty, R.P., (1975) Biochem. 14, 3653-3660.

Polakis, S.E., Guchhait, R.B., Zwergel, E.E., Lane, M.D., Cooper, T.G., (1974) J. Biol. Chem. 249, 6657-6667.

Porath, J., (1974) Meth. Enzymol. 34, B, p.13

Powell, J.T. and Brew, K., (1976) Biochem. 15, 3499-3505.

Priess, J. and Wood, E., (1964) J. Biol. Chem. 239, 3119-3126.

Quaroni, A. and Semenza, G., (1976) J. Biol. Chem. 251, 3250-3253.

Reed, G.H. and Scrutton, M.C., (1974) J. Biol. Chem. 249, 6156-6162.

Roskoski, R., (1974) Biochem. 13, 2295-2298.

Rylatt, D.B., 1976) Ph.D. thesis, University of Adelaide.

Rylatt, D.B., Keech, D.B. and Wallace, J.C., (1977) Arch. Biochem. Biophys. 183, 113-122.

Schlindler, M. and Sharon, N., (1976) J. Biol. Chem. 251, 4330-4335.

Schwartz, D.E., Gilham, P.T., (1972) J. Amer. Chem. Soc. 94, 8921-8922.

Scrutton, M.C. and Utter, M.F., (1965) J. Biol. Chem. 240, 3714-3723.

Scrutton, M.C., Keech, D.B. and Utter, M.F., (1965) J. Biol. Chem. 240, 574-581.

Scrutton, M.C. and Utter, M.F., (1967) J. Biol. Chem. 242, 1723-1735.

Scrutton, M.C. and Young, M.R., (1972) The enzymes. 6, 1-35.

Scrutton, M.C.,(1974) J. Biol. Chem. 249, 7057-7067.

Scrutton, M.C., Pearce, P.H. and Fatabene, F., (1977) Eur. J. Biochem. 76, 219-231.

Seubert, W. and Remberger, U., (1961) Biochem. Z. 334, 401-414.

Seufert, D., Herlemann, E-M., Albrecht, E. and Seubert, W., (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 459-478.

Shaw, E., (1970) Physiol. Revs. 50, 244-296.

Shepherd, G.B., Papadakis, N. and Hammes, G.G., (1976) Biochem. 15, 2888-2893.

Sigman, D.S. and Mooser, G. (1975) Ann. Rev. Biochem. 44, 889-931.

Simon, E.J. and Shemin, D., (1953) J. Amer. Chem. Soc. 75, 2520.

Singer, S.J. (1970) "Molecular Properties of Drug Receptors" A Ciba Foundation Symposium, Churchill, London.

Stewart, C.J., Thomas, J.A., Ball, W.J., and Aguire, A.R., (1968) J. Amer. Chem. Soc. 90, 5000-5004.

Timasheff, S.N. and Gorbunoff, M.J., (1967) Ann. Rev. Biochem. 36, 13-54.

Timasheff, S.N., (1970) Acc. Chem. Res., 3, 62-68.

Tinoco, I. and Cantor, C.R., (1970) Methods of Biochemical Analysis. 18, 81-204.

Toraya, T., Ushio, K., Fukui, S. and Hogenkamp, H.P.C., (1977) J. Biol. Chem. 252, 963-970.

Utter, M.F. and Keech, D.B., (1963) J. Biol. Chem. 238, 2603-2608.

Utter, M.F., Barden, R.E. and Taylor, B.L., (1975) Adv. Enzymol. 42, 1-72.

Vaughn, W.K., Neal, R.A. and Anderson, A.J., (1976) Comput. Biol. Med. 6, 1-7.

Visser, L. and Blout, E.R., (1971) Biochem. 10, 743-752.

Wahl, A.C. and Bonner, N.A., (1951) Radioactivity Applied to Chemistry, Wiley, New York.

Warren, G.B. and Tipton, K.F., (1974a) Biochem. J. 139, 311-320.

Warren,G.B. and Tipton, K.F., (1974b) Biochem. J. 139, 321-329.

Wolfenden, R., (1972) Acc. Chem. Res. 5, 10-18.

Wood, H.G., (1976) Trends Biochem. Sci. 1, 4-6.

Wood, H.G. and Zwolinski, G.K., (1976) Crit. Rev. Biochem. 4, 47-122.

Wood, H.G.and Barden, R.E., (1977) Ann. Rev. Biochem. 46, 385-413.

Yount, R.G., (1975) Adv. in Enzymol. 43, 1-56.

Ashman, L.K. and Keech, D.B. (1975) J. Biol. Chem. 250, 14-21

Barden, R.E., Fung, C-H, Utter, M.F. and Scrutton, M.C. (1972) J. Biol. Chem. 247, 1323-1333

Keech, D.B. and Farrant, R. (1968) Biochim.Biophys. Acta. 151, 493

Keech, D.B. and Utter, M.F. (1963) J. Biol. Chem. 238, 2609-2614

Utter, M.F. and Keech, D.B. (1960) J. Biol. Chem. 235, PC 17

Utter, M.F., Keech, D.B. and Scrutton, M.C. (1964) Adv. Enzyme Regulation 2, 49

Utter, M.F. and Keech, D.B. (1963) J. Biol. Chem., 238, 2603-2608

PAPERS PUBLISHED OR IN PREPARATION

1. Synthesis of 2-bromoacetyl-SCoA

   (with J.C. Wallace and D.B. Keech)

   Anal. Biochem. 72, 326-331 (1976)

2. Pyruvate Carboxylase : The mode of action of Acetyl CoA

   (with A.J. Campbell, J.C. Wallace and D.B. Keech)

   in preparation.