MECHANISTIC STUDIES ON ENZYME-CATALYSED DIMERIZATION REACTIONS

A Thesis submitted to the
DEPARTMENT OF BIOCHEMISTRY,
UNIVERSITY OF SOUTHAMPTON.

for the degree of

Doctor of Philosophy

by

PHILIP NORMAN BLANCHARD GIBBS, B.Sc.

JUNE 1984

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

BIOCHEMISTRY

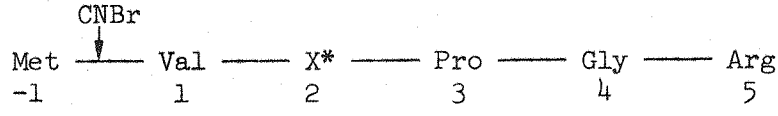
Doctor of Philosophy

MECHANISTIC STUDIES ON ENZYME-CATALYSED DIMERIZATION REACTION

by Philip Gibbs

5-Aminolevulinic acid dehydratase catalyses a dimerization reaction whereby the two identical substrate molecules, 5-aminolevulinic acid, are condensed together to yield porphobilinogen, the monopyrrolic precursor of porphyrin biosynthesis. A new procedure for the purification of the enzyme from human erythrocytes was developed and yielded 27.5 mg of pure enzyme protein in good recovery with a high specific activity (24.0 units/mg protein).

The human enzyme was treated with [4-^{14}C] 5-aminolevulinic acid and the resulting Schiff base complex was reduced with sodium borohydride. The modified protein was cleaved with cyanogen bromide and the peptide containing the ^{14}C label was isolated and sequenced. Similar studied were performed on the enzyme isolated from bovine liver. Comparison of the two active site sequences revealed a common hexapeptide which had the following structure where X* is amino acid-[4-^{14}C] 5-aminolevulinic acid adduct (probably ALA-lysine).



The metalloenzyme nature of human 5-aminolevulinic acid dehydratase was investigated and the findings confirmed the absolute requirement for zinc for catalytic activity. An extensive study of zinc binding to the enzyme using ^{65}zinc chloride revealed a single zinc binding site per subunit with a binding constant of 90 μM. Modification of enzyme sulphydryl groups by oxidation or alkylation prevented ^{65}zinc binding and demonstrated a close interrelationship between zinc binding and the status of reactive thiol groups in the enzyme. The involvement of sulphydryl groups in zinc binding was further investigated using 5,5'-dithiobis-(2-nitrobenzoic acid) by both conventional and stopped-flow spectrophotometry. Lead was shown to inhibit the human enzyme (K_i = 10 - 30 μM) and the relationship between ^{65}zinc binding, lead inhibition and the status of the thiol groups were determined. Fluorescence studies demonstrated that zinc binding stabilised the enzyme

in a catalytically active conformation, which was characterised by a positive fluorescence change, whilst interaction of the enzyme with lead resulted in a negative fluorescence change attributable to a catalytically inactive conformation. The data are consistent with the interaction of lead and zinc at different binding sites of the human dehydratase enzyme.

The mechanism of action of human 5-aminolevulinic acid dehydratase was investigated using a single-turnover technique, which enabled the order of addition of the two 5-aminolevulinic acid molecules to be determined. The single-enzyme-turnover technique involves the mixing of equimolar quantities of enzyme and [5-^{14}C] 5-aminolevulinic acid in a rapid mixing device followed by quenching with a large excess of unlabelled 5-aminolevulinic acid. Degradation of the product, porphobilinogen, revealed that most of the radioactivity was specifically located on the "propionic acid side" of the product (atoms 1, 2, 3, 8, 9 and 10). From these experiments the order of binding of the two substrate molecules during the enzyme reaction was shown to be at variance to that proposed in mechanisms in the literature. An alternative mechanism of action of 5-aminolevulinic acid dehydratase was proposed.

Related studies were carried out to investigate the mechanism of action of β-oxoacyl-CoA thiolase, another enzyme which catalyses the reaction between two identical substrate molecules, namely acetyl-CoA. Using a similar single-enzyme-turnover approach acetoacetyl-CoA, derived from [1-^{14}C] acetyl-CoA, was produced. Degradation of this product revealed that a larger proportion of the radioactivity was at C-3. Similar results were obtained when the [^{14}C] acetyl-enzyme complex, previously isolated by gel filtration, was reacted with unlabelled acetyl-CoA. From these data it was concluded that, of the two molecules of acetyl-CoA that were bound to the enzyme and converted into acetoacetyl-CoA, it was the one giving rise to C-3 and C-4 that was bound initially to the enzyme in the form of the acetyl-enzyme intermediate complex. The experiments provide the first direct evidence for the mechanism of action of β-oxoacyl-CoA thiolase which is consistent with the mechanism favoured in the literature.

ACKNOWLEDGEMENTS

I would like to thank Dr. Peter Jordan for his help, guidance and encouragement throughout the period of my research in Southampton. I also wish to thank Professor M. Akhtar, FRS for the provision of facilities and for many useful discussions.

I am also grateful for the following people for their gifts or help during this research:

Dr. Michael Gore for determining the amino acid compositions of the purified enzyme proteins and the active site peptides as well as for his help in using the stopped-flow apparatus and analysing and discussing the data obtained from these experiments.

Dr. Jas Seehra for 3-chlorolevulinic acid and 5-chlorolevulinic acid.

Dr. Paul Wyeth for ^{65}zinc chloride.

Dr. Ian Giles for the use of his computer program to analyse data by hyperbolic non-linear regression and for invaluable discussions.

Pete, Doug and Les in the Stores for all their help, especially with urgent orders.

Dr. Eric Mullen and Mr. Sarbjit Ner for demonstrating the techniques involved in the protein sequencing and Southampton General Hospital for the provision of time-expired blood.

Jackie Newman and Hazel Paul for their skilful and accurate typing! of this thesis in a very short time and the domestic and ancillary staff, too numerous to mention, for their help at various times.

Financial support from the S.E.R.C., M.R.C. and D.H.S.S. are gratefully acknowledged.

Finally, I would like to thank all my colleagues in the Department, especially Linda, the two Steve's, Alan, Amar, Chris, Vince and Mick, as well as the many friends I have made in Southampton, including Cath, Sue, Max, Martin, Andy, Tony (our Neanderthal friend), Manny, Richard, Chris, Mann and Steve.

Dedicated to my Parents,
Brother and Sister.

ABBREVIATIONS

ALA	5-Aminolevulinic acid
CoA	Coenzyme A
DABITC	4-N, N-Dimethylaminoazobenzene-4'-isothiocyanate
DABTH	4-N, N-Dimethylaminoazobenzene-4'-thiohydantoin
DABTZ	4-N, N-Dimethylaminoazobenzene-4'-thiazolinone
DEAE	Diethylaminoethyl
DNP	2, 4-Dinitrophenyl
EDTA	Ethylenediaminetetraacetic acid
FDNB	Fluorodinitrobenzene
HMG	β-Hydroxy-β-methylglutaryl
Hplc	High performance liquid chromatography
MMTS	Methanemethylthiosulphonate
$NaBH_4$	Sodium borohydride
NADH	Nicotinamide adenine dinucleotide (reduced form)
Nbs	Thionitrobenzoic acid
Nbs_2	5-5'-Dithiobis (2-nitrobenzoic acid)
NMR	Nuclear magnetic resonance
PBG	Porphobilinogen
PITC	Phenylisothiocyanate
PTH	Phenylthiohydantoin
SD	Standard deviation
SDS	Sodium dodecylsulphate
TFA	Trifluoroacetic acid
Tlc	Thin layer chromatography
Tris	Tris (hydroxymethyl) amino methane

DEFINITIONS

Native enzyme - The enzyme as isolated (see Section 2.2.2).

Holoenzyme - The enzyme which has its full complement of zinc (prepared as described in Sections 3.2.2 and 3.2.6).

Apoenzyme - The enzyme which has had essentially all its zinc removed (see Section 3.2.5).

CONTENTS

CHAPTER 2 (Cont.) Page No.

CHAPTER 2 (Cont.) Page No.

CHAPTER 3 (Cont.) Page No.

CHAPTER 1

METHODS FOR INVESTIGATING THE MECHANISM OF ACTION OF AN ENZYME-CATALYSED REACTION

CHAPTER 1

Methods for Investigating the Mechanism of Action of an Enzyme-Catalysed Reaction.

In an enzyme-catalysed reaction the conversion of substrate to product involves a highly organised interaction between the substrate and the enzyme which can be divided into two stages. First, there is the initial recognition and binding of the substrate molecule to the enzyme. This process involves non covalent chemical forces, such as hydrogen bonding, apolar (hydrophobic) bonding and ionic attraction, which are formed by specific interactions of the various groups of the substrate and the functional groups present on the side chains of the constituent amino acids located at the active site (Kollman, 1984; Page, 1984a). These specific interactions are responsible not only for determining the specificity of the enzyme for its substrate (Gutfreund, 1959; Popjak, 1970) but also for holding the substrate in the precise orientation (possibly by inducing strain) to permit the second stage, namely the chemical transformation of substrate into product. This second step can be achieved in a variety of ways and includes general acid catalysis, where a poor leaving group is protonated to make it a better leaving group, general base catalysis, where a base, which is too weak a nucleophile, assists the attack of water onto an electrophilic centre, and covalent catalysis, where a catalytically important group attacks the substrate to generate a stable covalently bound enzyme-intermediate complex which has a low activation energy profile and can subsequently react further to yield product (see Page (1984b) for further details).

The methods employed for investigating the mechanism of action of an enzyme-catalysed reaction must involve the elucidation of both the precise chemical nature of all the intermediates of the reaction as well as the detailed structure of the active site of the enzyme. The various methods employed to elucidate the catalytic mechanism of an enzyme-catalysed reaction are discussed in the following sections.

1.1 Enzyme Kinetics

Enzyme kinetics is a powerful experimental tool which can provide a wealth of information about the effects of enzyme, substrate and product concentrations, pH , temperature and other external influences on the enzyme activity. It is an important means for determining the order of addition of substrate(s) and release of product(s) and has been used to demonstrate the existence of transient enzyme intermediate complexes. Kinetics can also provide valuable information about cooperative changes during substrate binding. However, kinetics cannot be used to delineate the chemical nature of any intermediates in the catalytic cycle and cannot therefore be employed to determine the enzyme mechanism in terms of organic chemistry.

Kinetics can be conveniently divided into steady-state kinetics, in which the measurements are made after equilibrium (or steady-state) has been reached, and pre steady-state kinetics, in which the events prior to the attainment of the steady-state are ascertained. Pre steady-state kinetic studies depend as a rule on more specialised apparatus and in general it is the steady-state kinetic approach which has provided the majority of information about enzyme-catalysed reactions.

1.1.1 Steady-State Kinetics

The use of this approach originally stemmed from the studies of Brown (1902) who demonstrated that a stoichiometric complex was formed between enzyme and substrate which could subsequently break down to yield the product and regenerate the free enzyme, highlighting that the formation of the enzyme-substrate (or ES) complex was an essential initial step in enzyme catalysis. From this basis a general theory of enzyme action and kinetics was developed by Michaelis and Menten (1913) for single-substrate reactions which was subsequently extended by Briggs and Haldane (1925).

1.1.1.1 Single-Substrate Reactions

A simple mathematic treatment to account for the dependance of the initial rate of an enzyme-catalysed reaction (v) on the concentration of the substrate (s) was first devised by Henri and subsequently modified by Michaelis and Menten (1913). Consider the single-substrate reactions shown below (equation 1.1).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad \ldots.(1.1)$$

A plot of the initial rate of the enzyme reaction against substrate concentration yields a rectangular hyperbola (for exceptions see Koshland (1970), Engel (1977) and Tipton (1979)) which can be described by the following equation (1.2) known as the Michaelis-Menten equation.

$$v = \frac{V_{max}.s}{K_m + s} \qquad \ldots.(1.2)$$

This equation contains two constants, V_{max} and K_m. V_{max} is the maximum rate that can be obtained by the enzyme and occurs when the substrate concentration is sufficiently high for essentially all the enzyme to be present as the enzyme-substrate (ES) complex. Under these conditions the initial rate of reaction, v is equal to $k_3.e$ where e is the total enzyme concentration. The K_m is known as the Michaelis constant and if it is assumed to be equal to the substrate concentration the Michaelis-Menten equation (1.2) simplifies to:

$$v = \frac{V_{max}}{s/s+1} = \frac{V_{max}}{2}$$

Hence the K_m is defined as the substrate concentration which gives half the maximum rate of reaction. The Michaelis-Menten equation is based on the following assumptions. First, the concentration of enzyme (e) is very small compared to the concentration of substrate (s) so that the formation of ES does not significantly reduce s. Second, the concentration of the product is effectively zero. This is known as the initial rate assumption. This assumption implies that not only is P absent from the outset but also that the amount of P formed during the determination of the initial rate (v) is too small to give rise to a significant back reaction (or product inhibition). Third, although the product-releasing step (ES → E + P) is fast, it is slow compared to the back reaction which releases substrate and free enzyme and therefore it is assumed that E and ES are in equilibrium. From this third assumption it is clear that $k_3 << k_2$ (equation 1.1) and thus the Michaelis constant (K_m) can be simplified to k_2/k_1. This is equivalent to the dissociation constant (K_s) for the ES complex and is therefore

a measure of the affinity of the enzyme for its substrate.

The Briggs-Haldane theory differs from that of Michaelis-Menten with respect to this third assumption. Briggs and Haldane (1925) proposed that the ES complex was at equilibrium (steady-state) and that under these conditions the rate of formation of ES was equivalent to its rate of breakdown in any direction. The final form of the Briggs-Haldane equation is identical to that proposed by Michaelis and Menten (equation 1.2) but the value of K_m is no longer the same. In the Briggs-Haldane treatment the K_m is equal to $(k_2 + k_3)/k_1$, whereas in the Michaelis-Menten treatment the K_m is equal to K_s or k_2/k_1 (see Ainsworth (1977) for further details).

1.1.1.2 Measurement of Enzyme Activity.

Enzyme activity is measured by monitoring the substrate and/or product concentration in the assay mixture as a function of time to yield a progress curve. The initial rate of the enzyme reaction (v) is taken as the slope of the tangent to this progress curve. Experimentally, the initial rate of reaction is obtained either by a discontinuous or a continuous assay.

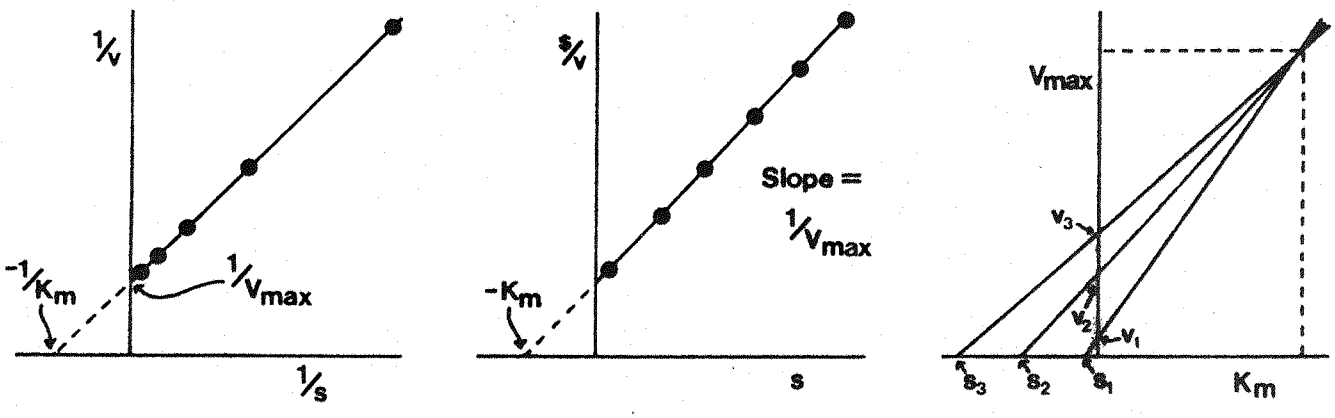
In a discontinuous assay, accurately measured aliquots are removed from the reaction mixture at timed intervals and the reaction terminated (e.g. by pH change, freezing or precipitation). The concentration of the substrate and/or product in the sample can then be determined. Examples of this type of assay include 5-aminolevulinic acid dehydratase (Mauzerall and Granick, 1956) and porphobilinogen deaminase (Berry, 1983).

In a continuous assay the changes in a physical property of the substrate or product can be monitored continuously without interfering with the assay mixture and consequently this type of assay is preferrable. Examples include β-oxoacyl-CoA thiolase (Middleton, 1974), alcohol dehydrogenase (Theorell and Chance, 1951) and citrate synthase (coupled assay with malate dehydrogenase; Spector, 1972).

1.1.1.3 Linear Plotting Techniques

A plot of the initial rate of an enzyme-catalysed reaction (v) against the substrate concentration (s) yields a rectangular hyperbola. A more convenient representation of the data is as 1/v against 1/s and is known as the Lineweaver-Burk plot (1934) (Scheme 1.1). The intercept of the ordinate is $1/V_{max}$ and the negative intercept on the absicca gives the reciprocal of the K_m. The major criticism of this treatment is that undue weight is given to the measurements made a low substrate concentrations and insufficient weight is given to the more accurate higher rates (Wilkinson, 1961; Dowd and Riggs, 1965). Therefore, various other linear plotting methods have been developed. They include the Eadie-Hofstee plot (v against v/s) (Eadie, 1942; Hofstee, 1952), the Hanes plot (s/v against s) and more recently the direct linear plot of Eisenthal and Cornish-Bowden (1974) (Scheme 1.1)

Scheme 1.1 Various Linear Plotting Techniques



Lineweaver-Burk Plot Hanes Plot Direct Linear Plot

The merits and defects of these various linear plotting techniques are discussed in detail by Markus et al. (1976).

1.1.1.4 Two-Substrate Reactions.

Most enzyme-catalysed reactions require two or more substrates and the kinetics of these reactions give a far greater insight into aspects of the mechanism of action with respect to the order of addition of substrate and release of product. Consider the two-substrate reaction shown below (equation 1.3).

$$A + B \rightleftharpoons P + Q \qquad \ldots.(1.3)$$

There are a large number of permutations as to the order of addition and release of both substrate and product and some examples are shown in Scheme 1.2. In a sequential mechanism (mechanisms 1, 4, 5, 6 and 7 in Scheme 1.2) reaction cannot occur until both substrates have bound to the enzyme to form a ternary complex (EAB). In the Theorell-Chance mechanism (mechanism 3) this complex is very short lived and is considered to be kinetically insignificant. However, this still leaves open the question as to the order of binding and release which can be either ordered or random. In a non-sequential mechanism (mechanism 2 in Scheme 1.2) reaction of the first substrate with the enzyme to release the first product generates an enzyme form (ε) which is chemically different from the free enzyme. Subsequent reaction with the second substrate molecule generates the second product. Therefore, the observation of ping-pong kinetics for an enzyme-catalysed reaction is the most useful of kinetic results because it directly suggests a chemical reaction mechanism (i.e. via a covalent intermediate).

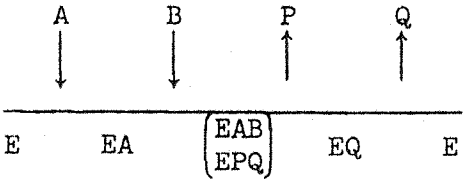
Using the steady-state assumptions, which were applied in Section 1.1.1.1, various mathematical equations can be derived to account for these possible mechanisms but are too detailed to be included in this account. Some of the rate equations are shown in Scheme 1.2. For further details you are referred to the reviews of Cleland (1967, 1970), Segel (1975), Engel (1977) and Cornish-Bowden (1979) .

1.1.1.5 Linear Plotting Procedures for Two-Substrate Reactions.

Linear plotting techniques enable one to determine the values of kinetic constants in the rate equations and, in many cases, the order of addition of substrates and release of products. For example, the overall rate equation for both the ordered bi-bi and Theorell-Chance mechanisms are identical (see mechanisms 1 and 3 in Scheme 1.2). By measuring the initial rate of reaction (v) for all possible combinations of a range of concentrations of both substrates one can construct a Lineweaver-Burk plot (Scheme 1.3a) of $1/v$ against $1/A$ for the different concentrations of B. By rearrangement of the overall rate equation (from Scheme 1.2) to the general form of a straight line ($y = mx + c$) (equation 1.4), it is clear that both the slope and intercept terms are inversely proportional to the concentration of B, and consequently

Scheme 1.2 Possible Mechanisms for the Order of Addition of Substrates and Release of Product in a Two-Substrate Reaction.

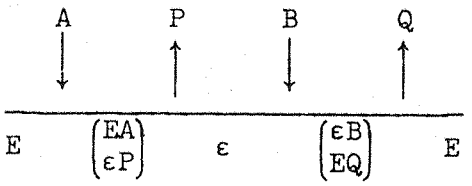
Mechanism 1. Ordered bi-bi.



$$v = \frac{V_{max}.AB}{AB+Ka.B+Kb.A+Kia.Kb}$$

e.g. NAD dehydrogenase

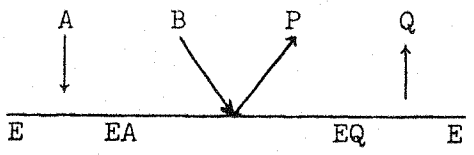
Mechanism 2. Bi-bi ping-pong.



$$v = \frac{V_{max}.AB}{Ka.B+Kb.B+AB}$$

e.g. Aspartate transaminase

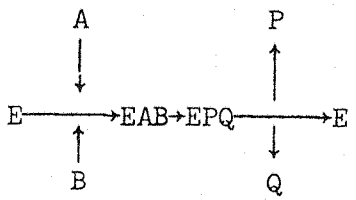
Mechanism 3. Theorell-Chance.



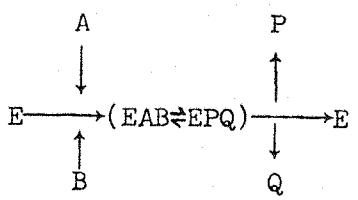
$$v = \frac{V_{max}.AB}{AB+Ka.B+Kb.B+Kia.Kb}$$

e.g. Choline acetyltransferase

Mechanism 4. Random bi-bi.



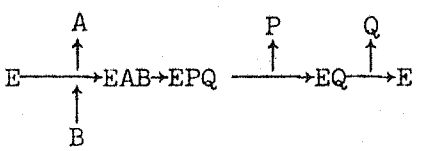
Mechanism 5. Rapid Equilibrium Random bi-bi.



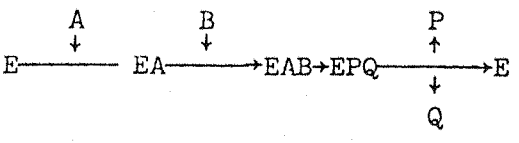
$$v = \frac{V_{max}}{\frac{Ka}{A} + \frac{Kb}{B} + \frac{Kia.Kb}{A} + 1}$$

e.g Creatine kinase

Mechanism 6. Random Addition, Ordered Release.



Mechanism 7. Ordered Addition, Random Release.



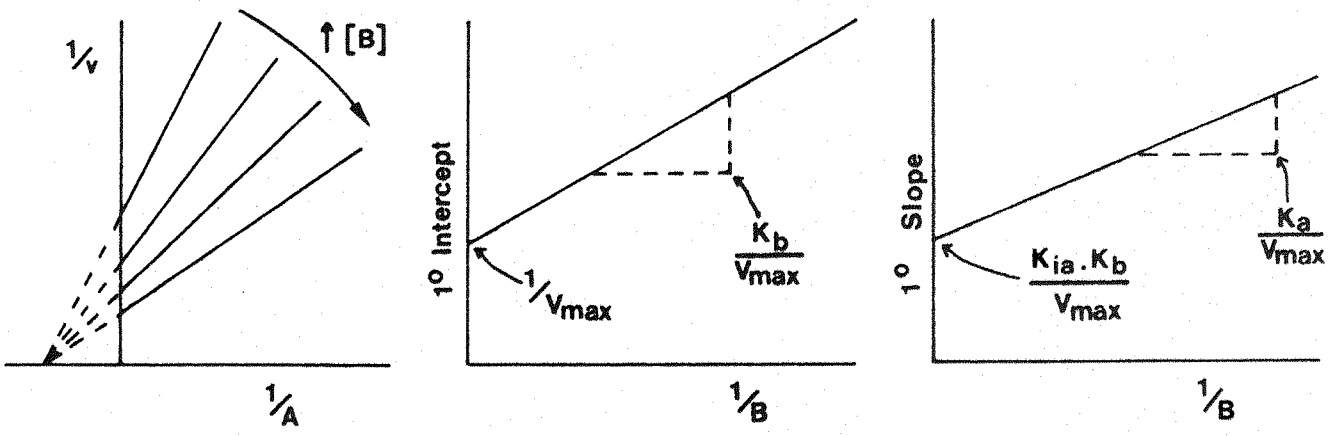
With mechanism 6 and 7 can also have rapid equilibrium (cf mechanism 5) which is mathematically less complicated.

the Lineweaver-Burk plot will yield a non-competitive pattern (Scheme 1.3a).

$$\frac{1}{v} = \left(\frac{Ka}{V_{max}} + \frac{Kia.Kb}{V_{max}.B}\right) \cdot \frac{1}{A} + \left(\frac{Kb}{V_{max}.B} + \frac{1}{V_{max}}\right) \qquad \ldots.(1.4)$$

After measuring the slope and ordinate intercept of these linear plots (one for each concentration of B), secondary plots of both the slope and intercept against 1/B will also be linear and will enable one to determine the kinetic constants (see Scheme 1.3).

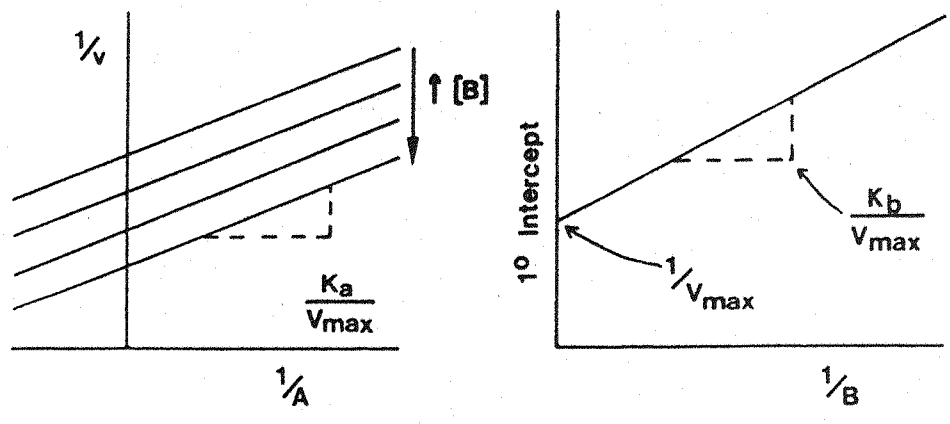
Scheme 1.3 Primary and Secondary Plots for Sequential Mechanisms.



(a) Primary Plot (b) Secondary Intercept Plot (c) Secondary Slope Plot

Alternatively if a 1/v against 1/A plot is carried out for a bi-bi ping-pong mechanism (mechanism 2 in Scheme 1.2) for a range of concentrations of B a series of parallel lines are produced (i.e. uncompetitive pattern). A secondary plot of the intercept against 1/B will yield the values for the kinetic constants (Scheme 1.4).

Scheme 1.4 Primary and Secondary Plots of a Non-Sequential Mechanism.



(a) Primary Plot (b) Secondary Intercept Plot

Therefore, in a sequential (ordered bi-bi or Theorell-Chance) mechanism there is a crossover point in the primary plots (Scheme 1.3) and contrasts with the non-sequential (bi-bi ping-pong) mechanism, in which the concentration of B does not affect the slope of the primary plot (Scheme 1.4). Unfortunately, however, the results from these types of experiments are often not clearcut as it may be difficult to distinguish between parallel lines and lines which intercept a long way from the ordinate axis. Product inhibition has proved successful in resolving between these mechanisms and is described below.

1.1.1.6 Product Inhibition Studies.

Product inhibition studies are of great importance for elucidating the order of binding of substrates and release of products in an enzyme-catalysed reaction. Experimentally, these studies are carried out by adding one of the products to the reaction mixture and measuring the inhibition of the initial rate of reaction. Inhibition is achieved by altering the distribution of the enzyme among the various complexes in the pathway and can be either competitive, uncompetitive or non-competitive depending on whether it affects the slope of the Lineweaver-Burk plot, the intercept, or both. From these studies a pattern of inhibition is constructed for each substrate with respect to non-saturating and saturating conditions of the other substrate. Typical results for different reaction courses are presented in Table 1.1 (Cleland, 1963a) and show how it is possible to distinguish between the ternary-complex (ordered bi-bi) mechanism, the enzyme substitution (bi-bi ping-pong or Theorell-Chance) mechanism and other random mechanisms (e.g. see Morris et al., 1971).

The use of a reversible inhibitor (P'), which is neither substrate nor product, can also help to distinguish between two similar mechanisms since, unlike the product, it is incapable of reacting further and specifically interacts with only one of the substrate binding sites to form a dead-end complex. This binding causes a very specific inhibition and has, for example, been used to verify the compulsory ordered addition of substrates to lactate dehydrogenase (Fromm, 1963) (see Cleland (1970, 1977) for further details).

Table 1.1 Product Inhibition Patterns for Two-Substrate Reactions.

Inhibitor Varied	VARY A		VARY B		Mechanism
	Non-Saturating B	Saturating B	Non-Saturating A	Saturating A	
P	NC	UC	NC	NC	Ordered bi-bi (Ternary complex)
P'	UC	UC	UC	UC	
Q	C	C	NC	O	
P	NC	O	C	C	Bi-bi ping-pong or Theorell-Chance
P'	UC	O	-	-	
Q	C	C	NC	O	
P or Q	C	O	C	O	Rapid equilibrium Random bi-bi
P or Q	NC	NC	NC	NC	Random bi-bi

NC = Non-competitive; UC = Uncompetitive; C = Competitive; O = No inhibition; P' = Substrate/Product analogue.

For further aspects of multisubstrate reactions the reader is referred to Cleland (1963a, b), McFarlane and Ainsworth (1972) and Tipton (1974).

1.1.2 Pre Steady-State Kinetics.

Although steady-state kinetics have proved very useful for elucidating the order of addition of substrate and release of product in enzyme-catalysed reactions the major limitation of such studies is that they can only be used to study the rate limiting step and therefore do not provide any information about the faster steps. In order to study the formation and decomposition of these other intermediates in the enzymic reaction it is necessary to perform experiments on a much faster time scale. The major techniques used to study pre steady-state kinetics are rapid mixing methods ($t \geq 0.5$ msec) and relaxation methods ($t \geq 1$ nsec).

1.1.2.1 Rapid Mixing Techniques

The first attempts to measure rates of fast reactions were made by Hartridge and Roughton (1923) using a continuous flow method; this was subsequently improved by the introduction of stopped flow analysis

(Chance, 1943). In this approach two syringes are simultaneously compressed to release 50-200 µl of reactants and then mechanically stopped. This stopping triggers off a recording device which, after a dead-time (∿ 0.5 msec), can measure the ageing of the solution for several minutes. Chance (1943) studied the reaction of horseradish peroxidase (syringe 1) and a mixture of the substrate, H_2O_2, and leuco-malachite green (syringe 2). On mixing he demonstrated a rapid increase in the absorbance at 400 nm (reflecting the formation of the ES complex) followed by a delayed increase at 610 nm, (reflecting the formation of oxidised malachite green). From these data, he concluded that a discernible intermediate was formed during the enzyme reaction (the ES complex) before the release of product. These findings confirm the initial proposal by Brown (1902) whereby the enzyme and substrate interact to form an ES complex (Michaelis complex) which subsequently reacts to yield product. Hartley and Kilby (1954) have used the stopped flow technique to demonstrate burst kinetics from a study of the chymotrypsin-catalysed hydrolysis of nitrophenylethyl carbonate. This method has proved successful for measuring the molarity and number of functionally active sites in an enzyme. Other variations, such as quenched flow (Eady et al., 1978) and pulsed quenched flow (Fersht and Jakes, 1975), are modifications of the stopped flow technique and have proved useful in the study of fast reactions.

1.1.2.2 Relaxation Kinetics.

Many reactions, including proton and electron transfer, are too fast to be measured using flow methods as the mixing of reagents cannot be done effectively in less than 0.5 msec (the dead time). Therefore, in order to study faster reactions it is necessary to use pre-mixed solutions which are at equilibrium and then perturb the system to alter the equilibrium. The system can then be studied as it proceeds to the new equilibrium, a process known as relaxation. This perturbation can be carried out in a variety of ways and includes temperature jump methods (Criddle et al., 1968), magnetic resonance perturbation (Sykes and Scott, 1972), flash photolysis (Hiromi, 1979) and pulse radiolysis (Frieden et al., 1974).

For further details of both flow and relaxation kinetics (including mathematical analysis of the data) you are referred to the reviews by Gutfreund (1971, 1972), Halford (1974) and Hiromi (1979).

1.2 Isotope Exchange Reactions

Isotope exchange reactions have proved to be of great importance in the elucidation of the mechanism of action of a large number of enzymes. In particular exchange reactions can provide valuable information about the order of substrate binding and product release and are used in conjunction with kinetics to distinguish between postulated mechanisms which cannot be resolved by kinetics alone (e.g. sequential mechanisms).

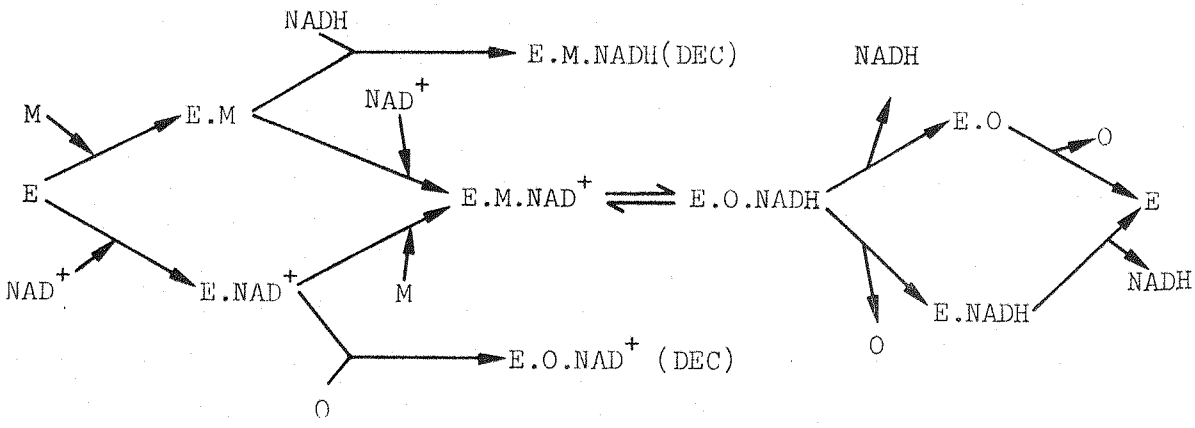
Two important considerations must be made when considering the results of exchange experiments. First, the reaction involving radioactive substrates must follow the same mechanism as the normal reaction and with the same rate constants. This assumption is generally true provided that when tritium (or deuterium) isotopes are used one ensures that the isotopically substituted atom is not directly involved in the mechanism. However, even in situations where an isotope effect is observed it is possible to make some correction (Rose, 1970) except when studying partial reactions. The second assumption is that the concentration of all radioactive species is so low that there is no perceptible effect on the concentrations of the unlabelled species.

Isotope exchange reactions are generally studied by allowing the reactants (substrates, products and enzyme) to come to chemical equilibrium and then to add a very low concentration of radioactive substrate or product (not sufficient to perturb the equilibrium). Subsequent sampling of the mixture at timed intervals, in conjunction with separation and analysis of the relative proportions of the label in the various components, enables one to determine the rate of exchange of the label. Information about the mechanism of an enzyme-catalysed reactions is gained by studying how the rate of exchange between one substrate-product pair varies with the concentration of the other substrate-product pair(s). The most widely applied use of isotopic exchange reactions has been for distinguishing between ordered and random bi-bi reaction mechanisms. Initial rate studies (Section 1.1) make no distinction between these mechanisms whilst product inhibition patterns (Section 1.1.1.6) are often either complicated by additional dead-end complexes or simply are not decisive. For example, a Theorell-Chance mechanism and a rapid equilibrium random mechanism with two dead-end complexes (EAP+EBQ) give identical initial rate and product inhibition patterns. In contrast, isotopic exchange reactions can readily distinguish between these two mechanisms since the random mechanism will show exchange with both products whilst the Theorell-Chance mechanism will

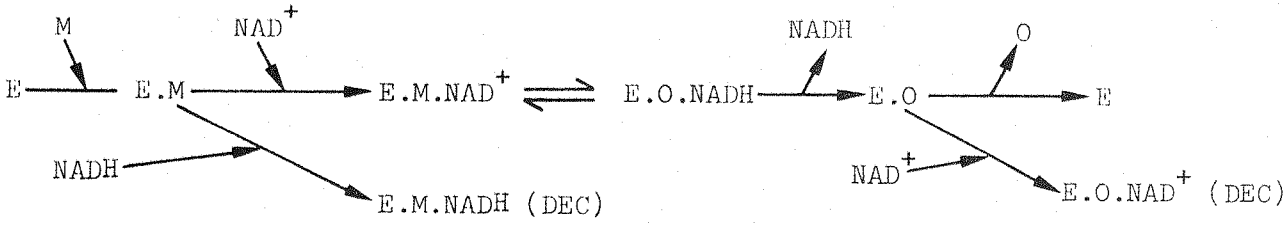
only show exchange of one product (in the absence of the other). In fact isotopic exchange is the best technique available for determining whether, in a random order mechanism, there is truly a rapid equilibrium or not. This is due to the fact that if the interconversion of the ternary complexes ($EAB \rightleftharpoons EPQ$ in Scheme 1.2) is the sole rate limiting step then all exchanges in either direction must proceed at the same rate for any given set of reactant concentrations.

The mechanism of action of pig heart malate dehydrogenase has been elucidated successfully using isotope exchange reactions. Previous studies using steady-state kinetics have revealed that the enzyme reaction is sequential i.e. that both the substrate molecules bind before any product is released. However this still leaves us with a choice between random-order or compulsory-order mechanisms of which there are three possibilities (shown below).

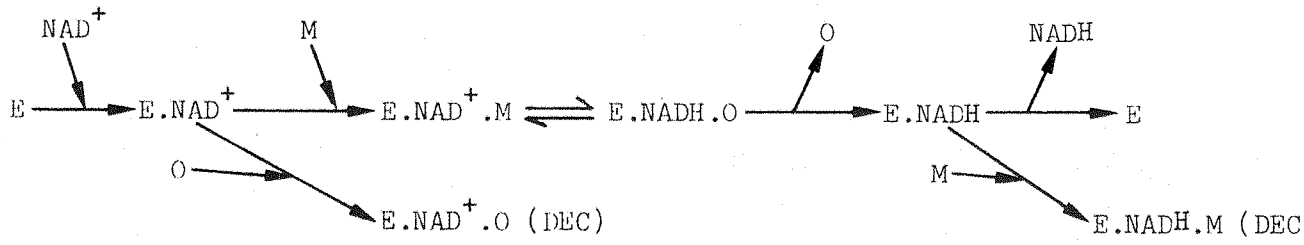
Mechanism 1. Random bi-bi.



Mechanism 2. Ordered bi-bi (Malate/oxaloacetate outer pair).



Mechanism 3. Ordered bi-bi (NAD^+/NADH outer pair).



(DEC = Dead-end complex)

In these three schemes the symbols M and O denote malate and oxaloacetate respectively. Silverstein and Sulebele (1969) demonstrated isotopic exchange between labelled NADH and NAD^+ using malate dehydrogenase and showed that on increasing the concentration of the other substrate pair, oxaloacetate and malate, this exchange was eliminated. In the converse experiment, when the exchange between labelled oxaloacetate and malate was measured in the presence of increasing concentrations of NAD^+ and NADH , they showed that the rate of exchange increased to a maximum plateau value. These data are consistent with Mechanism 3 in which NAD^+ and NADH are the outer substrate pair. The experimental observations are due to the fact that on increasing the malate and oxaloacetate concentration the enzyme is forced into the central complexes ($E.NAD^+.M$ and E.NADH.O) which would be reformed as rapidly as they dissociated via steps 4 and 8. Therefore, the concentration of $E.NAD^+$ and E.NADH would decrease and consequently the exchange between NAD^+ and NADH would also drop eventually reaching zero when the concentration of malate and oxaloacetate were sufficiently high. This result differs from that expected for a random-order mechanism (1) due to the fact that at a high concentration of malate and oxaloacetate the upper pathway (steps 2, 5 and 6) would predominate and consequently there would still be exchange between NAD^+ and NADH. Therefore as a rule of thumb in a compulsory-order mechanism the raising of the concentration of the inner substrate pair ultimately eliminates the exchange between the outer pair.

Silverstein and Sulebele (1969) also observed that both the exchange of NAD^+ into NADH and of malate into oxaloacetate were inhibited by increasing the concentration of malate and NADH in a constant ratio. This has been attributed to the formation of an E.NADH. Malate dead-end complex (Step 10) (Segel, 1975).

Another enzyme, choline acetyltransferase, was studied using steady-state kinetics but there was some conflict as to whether the double reciprocal $1/v$ against $1/s$ plots were parallel (ping-pong mechanism) (Schuberth, 1966) or convergent (sequential-ternary complex mechanism) (Potter et al., 1968). This conflict was neatly resolved by Morris and Grewaal (1971) who demonstrated that the isotopic exchange between [^{14}C] choline and acetylcholine was completely dependent on the presence of CoA and indicated that a sequential mechanism was operating. Further kinetic studies by Morris et al. (1971) led to the conclusion that choline acetyltransferase has an ordered mechanism of the Theorell-Chance type (Scheme 1.2).

Isotope exchange reactions have also proved useful for probing ping-pong mechanisms because isolated parts of the overall reaction sequence can be studied. In the case of a simple ping-pong bi-bi mechanism (Scheme 1.2) the exchange of A→P and P→A can be measured in the absence of B and Q (a partial reaction). The converse is also true, in which the exchange of B→Q and Q→B can be studied on their own. Doudoroff et al. (1947) used this technique to study sucrose phosphorylase in which glucose-1-phosphate (A) and fructose (B) are enzymically converted to phosphate (P) and sucrose (Q). They demonstrated that labelled phosphate (^{32}P) could be converted into glucose-1-phosphate in the absence of fructose and similarly that label from fructose could be incorporated into sucrose in the absence of phosphate. These data are consistent with a ping-pong bi-bi reaction mechanism as shown below.

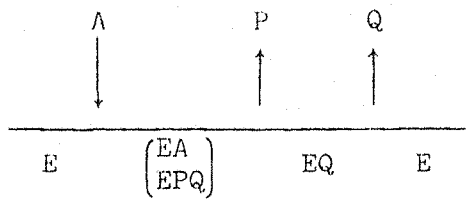
$$E + \text{glucose-1-phosphate}(A) \rightleftharpoons \text{glucosyl-E}(\varepsilon) + \text{phosphate}(P)$$

$$\text{glucosyl-E}(\varepsilon) + \text{fructose}(B) \rightleftharpoons E + \text{sucrose}(Q)$$

The covalent glucosyl-enzyme intermediate (ε) shown by ping-pong kinetics can be isolated and used to determine the chemical nature of the active site residue responsible for formation of the covalent bond. This is discussed in greater detail in the section on covalent catalysis (Section 1.4).

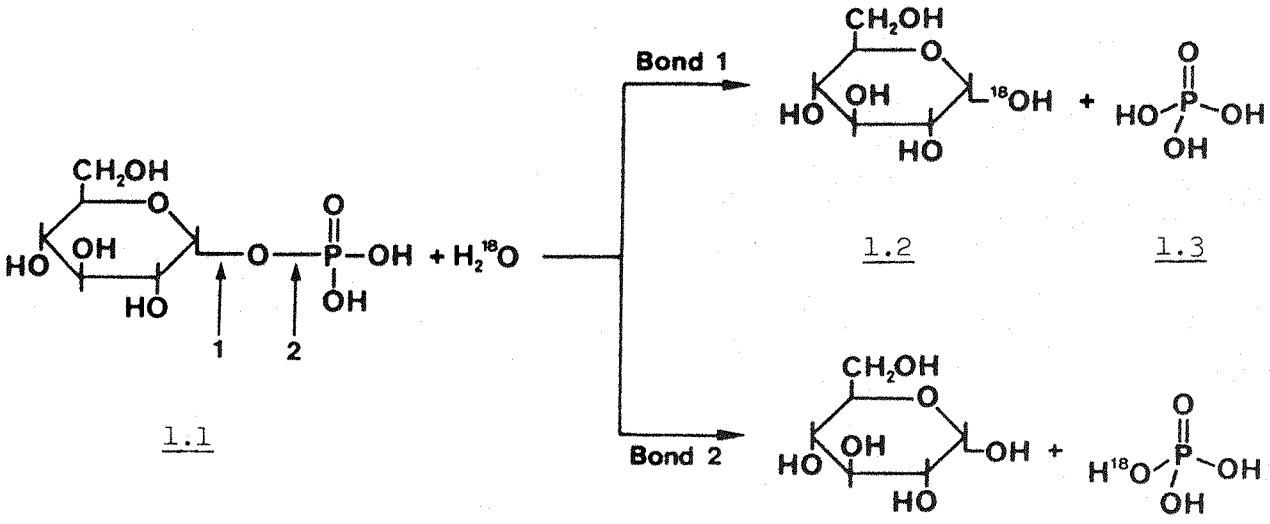
All the isotope exchange studies mentioned thus far have been carried out at chemical equilibrium. However, it is sometimes profitable to measure the exchange between product and substrate when a reaction is

proceeding. For example, consider the ordered uni-bi reaction:



The rate of isotopic transfer from the product, P , to the substrate A, will be reasonably fast compared to the rate of the chemical reaction in the forward direction, as long as there is a reasonable amount of the EQ complex present. On the other hand, the exchange of Q into substrate will be much slower and will be dependant on the presence of P. Consequently, one can use this exchange to determine the order of product release, whereby the product that shows exchange into substrate in the absence of the other is the first one released. However, if both products show exchange (when studied at concentrations greater or, equal to their K_i's) then the release of product is evidently random. This approach was used by Hass and Byrne (1960) with glucose-6-phosphatase to show the ordered release of glucose before phosphate.

Isotope exchange reactions have also proved useful for studying the precise position of bond cleavage. For example the enzyme-catalysed cleavage of glucose-1-phosphate (1.1) to glucose (1.2) and free phosphate (1.3) can occur either side of the bridge oxygen (bonds 1 and 2 in (1.1)). A study of the reaction in $H_2{}^{18}O$ will enable one to determine the location of the label in the product and hence which one of the two oxygen bridge bonds is broken. Studies have revealed that bond 1 is cleaved by phosphorylase whereas bond 2 is cleaved by alkaline phosphatase (Cohn, 1949).



1.1 1.2 1.3

More recently Gold and Osber (1972) have studied the exchange of ^{18}O from the bridge oxygen of glucose-1-phosphate in glycogen phosphorylase and demonstrated that cleavage of the phosphate (generating an oxocarbonium ion) preceeds the transfer to the glucosyl acceptor.

Isotope exchange reactions can also be used to provide information about the stereochemistry of an enzyme-catalysed reaction. For example, the study of the exchange of stereospecifically labelled NADH (at C-4 hydrogen) with various NAD-dependent dehydrogenases has demonstrated that there are two classes of these enzymes which have different mechanisms They are the A-side specific dehydrogenases, which selectively transfer the pro-R hydrogen at C-4 of NADH (e.g. D- and L-specific lactate dehydrogenase) and the B-side specific dehydrogenases which transfer only the pro-S hydrogen (e.g. cytochrome c and β-hydroxybutyryl-CoA dehydrogenase) (Foster, 1980). Similar studies on the pyridoxal-phosphate-dependant enzyme, 5-aminolevulinic acid synthetase, have demonstrated that the enzyme stereospecifically removes the 2-H_R atom in both the substrate, glycine and the product, ALA (Laghai, 1977).

The process whereby the substitution of one isotope for another in the substrate results in an actual change in the rate of the enzyme-catalysed reaction (kinetic isotope effect) is discussed in the following section (Section 1.3).

In summary, isotope exchange reactions have been used both to determine the order of substrate addition and product release to confirm or delineate between mechanisms based on kinetic studies and also for a more detailed determination of the precise chemical bond cleaved during the reaction. Derivations of the equations defining the rates of isotopic exchange as well as a more detailed analysis of isotope exchange are to be found in Boyer (1959), Cleland (1970, 1977), Segel (1975) and Rose (1979).

1.3 Kinetic Isotope Effects

In an enzyme-catalysed reaction the conversion of substrate to product has a profound effect on many of the chemical bonds; new bonds are formed, old bonds are broken and other bonds experience either transient or permanent changes in their state of hybridization. Many of these

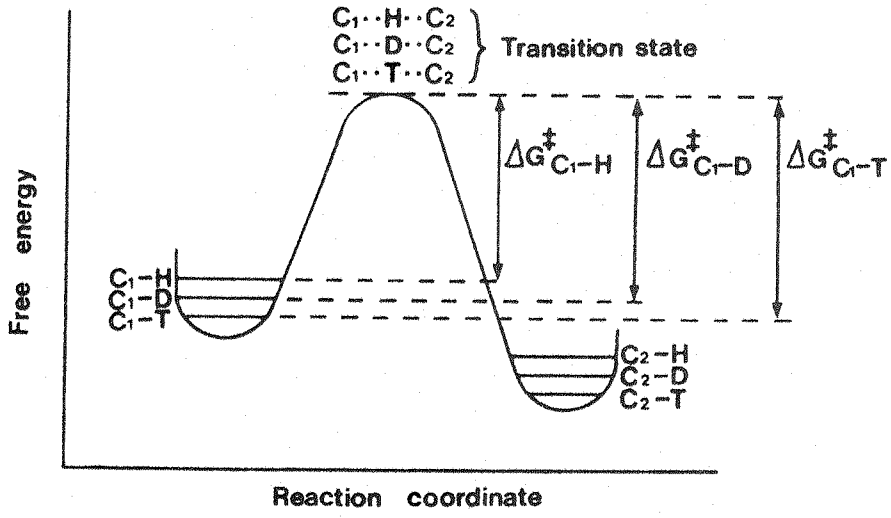
bonding changes can be studied by substituting one of the atoms of the substrate for one of its isotopes and determining the effect of this on the maximum rate of reaction (V_{max}). The effect is known as the kinetic isotope effect and can give an important insight into the atomic details of an enzyme-catalysed reaction. Consequently, the kinetic importance of a particular step in a complex enzyme reaction can be evaluated by comparing the magnitude of the observed isotope effect with those observed for model reactions. Also, kinetic isotope effects have proved useful for distinguishing between step-wise and concerted reactions and for elucidating the chemical nature of the intermediates. For the purposes of discussion isotope effects will be divided into three sections, primary, secondary and solvent isotope effects, and are described below.

1.3.1 Primary Kinetic Isotope Effects.

A primary hydrogen isotope effect results when a proton is abstracted in a rate determining step of an enzyme-catalysed reaction and is characterised by observing that a significant reduction in V_{max} occurs on substituting deuterium or tritium for the hydrogen atom of the cleaved bond. However, if no isotope effect is observed this does not necessarily mean that there is no hydrogen transfer, but may simply mean that it is not occurring in the rate determining step. A primary hydrogen isotope effect is observed as a consequence of the difference in the energy required to break the bond containing the isotopically substituted atom when compared to the unsubstituted atom. Therefore, it is energetically more favourable to cleave the unsubstituted bond than the bond containing deuterium or tritium. For example, consider the reaction whereby a proton is transferred from one carbon atom to another i.e. $C_1 — H + C_2 \rightarrow C_1 + C_2 — H$. The free energy profile of the carbon-hydrogen bond for this reaction is shown in Scheme 1.5. However, on substituting deuterium (D) or tritium (T) for this proton one can clearly see that the zero point energy for both the carbon-deuterium (C_1-D) and carbon-tritium (C_1-T) in the ground state are lower than that of the carbon-hydrogen (C_1-H) bond. This zero point energy is usually "frozen out" in the transition state so that the energy of the $C_1 \cdot\cdot H \cdot\cdot C_2$, $C_1 \cdot\cdot D \cdot\cdot C_2$ and $C_1 \cdot\cdot T \cdot\cdot C_2$ are identical (see Jencks (1969) and Cleland et al., (1977) for exceptions). Consequently the difference in the energy ($\Delta G^{\ddagger} C_1$-T) for activation of the C_1-T bond from the ground state to the transition state will be greater than $\Delta G^{\ddagger} C_1$-D, which in turn, will be greater than $\Delta G^{\ddagger} C_1$-H. Therefore, it is easier to

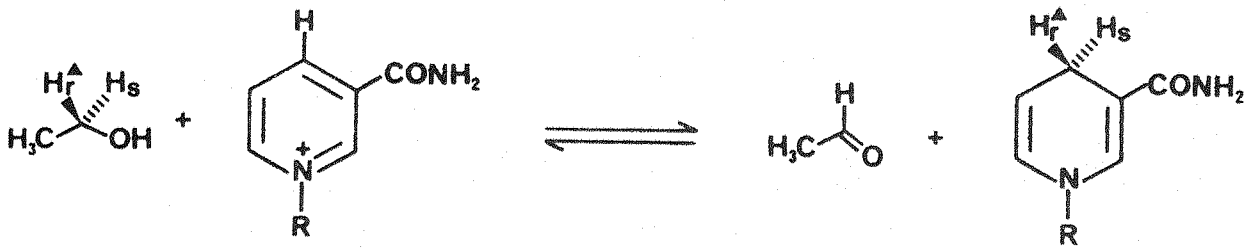
break the carbon-hydrogen bond and hence the rate of reaction for the unsubstituted substrate will be faster than for the substrate containing deuterium or tritium. In fact the substitution of deuterium for the hydrogen atom of the cleaved bond results in a seven fold decrease in the rate of reaction at 25°C (k_H/k_D value). In general, however, a primary hydrogen isotope effect of between 2 and 15 provides good evidence that the carbon-hydrogen bond is broken in the rate limiting step.

Scheme 1.5 Comparison of the Activation Energies ($\Delta G^{\ddagger}$) for Proton, Deuterium and Tritium Transfer Reactions between Carbon Atoms C_1 and C_2.



However, there is an important experimental distinction between deuterium and tritium kinetic isotope effects. With deuterium (a stable isotope) essentially all the substrate molecules will have hydrogen replaced by deuterium and therefore every molecule will experience the same reaction and thus the substitution will effect V_{max} directly. In contrast, however, tritium is an unstable isotope and is used only in trace quantities (1 molecule in 10^{10}). Therefore only those substrate molecules labelled with tritium will experience an isotope effect and there will be no detectable macroscopic rate reduction. Consequently a tritium isotope effect is determined by measuring the specific activities of both the substrate and product.

Primary hydrogen isotope effects have been used to study the enzyme alcohol dehydrogenase, which catalyses the transfer of the pro-R hydrogen at C-1 of ethanol (1.4) to NAD^+ (1.5) to yield acetaldehyde (1.6) and NADH(1.7) (Weber et al., 1966).



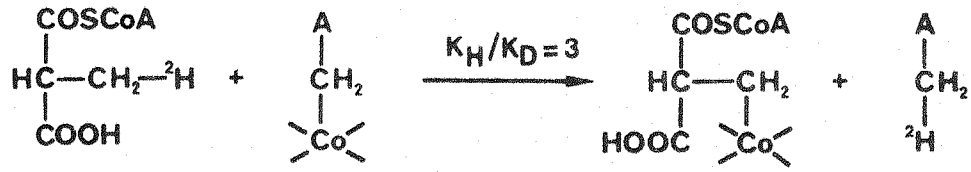
1.4 1.5 1.6 1.7

The substitution of deuterium for the pro-R hydrogen of ethanol catalysed by horse liver alcohol dehydrogenase gives a k_H/k_D value of 2.28, whilst in the reverse direction (using NADD) a k_H/k_D value of 3.11 is obtained (Mahler et al., 1962). Similarly using 1-^{3}H ethanol values of k_H/k_D = 3.2 - 3.8 have been observed (Palm, 1966) and suggests that the transfer of the pro-R hydrogen is a rate determining step in the enzyme-catalysed reaction.

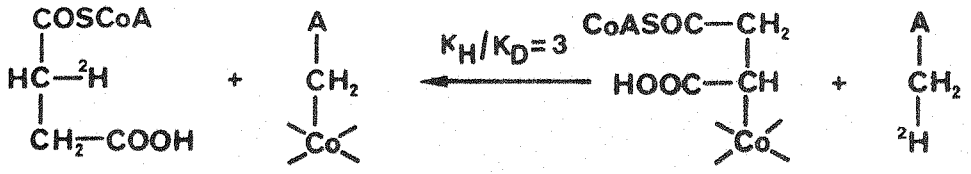
Another primary hydrogen isotope effect was observed for the coenzyme B_{12} catalysed isomerisation of methylmalonyl-CoA (1.8) to succiny-CoA (1.9) by the enzyme, methylmalonyl-CoA mutase. Miller and Richards (1969) demonstrated a k_H/k_D value of 3 on substituting deuterium for hydrogen in the methyl group of methylmalonyl-CoA. This result shows that the transfer of one of the methyl group hydrogens, probably to the C-5' position of the deoxyadenosine residue of coenzyme B_{12}, is the rate determining step. Interestingly, the transfer of the hydrogen atom from coenzyme B_{12} to C-3 of succinyl-CoA also exhibits an isotope effect (k_H/k_D = 3). Based on these and other results a mechanism of action for methylmalonyl-CoA mutase has been proposed (Miller and Richards, 1969) (Scheme 1.6).

So far only primary hydrogen isotope effects have been discussed since they are the simplest and give the largest isotope effects. However, heavy atom primary isotope effects have also proved useful for studying

Scheme 1.6 Mechanism of Action of Methylmalonyl-CoA Mutase



1.8



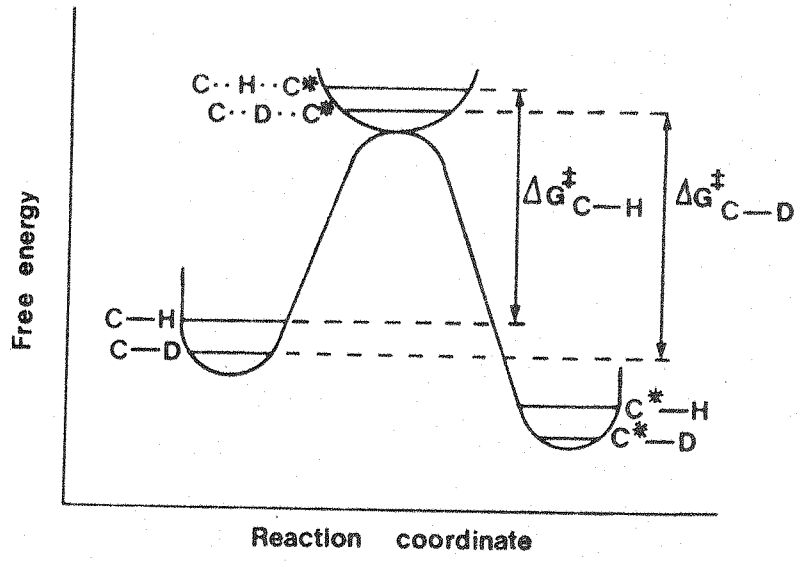
1.9

reaction mechanisms but their application is more restricted since the magnitude of the effect is much smaller compared to primary hydrogen isotope effects. Consequently, more sophisticated experimental techniques and methods of detection are required. Examples include carbon isotope effects of acetoacetate decarboxylase (O'Leary and Baughn, 1972) and oxaloacetate decarboxylase (Seltzer et al., 1959), nitrogen isotope effects (Papain; O'Leary et al., 1974) and oxygen isotope effects (chymotrypsin; Sawyer and Kirsch, 1975).

1.3.2 Secondary Kinetic Isotope Effects

A secondary kinetic isotope effect can be observed when the chemical bond of the isotopically substituted atom, though not directly involved in the reaction, experiences a change in its state of hybridization and consequently may react more slowly than the unsubstituted substrate. These secondary isotope effects are much smaller than the corresponding primary isotope effects and this is due to the fact that in the transition state there is still a substantial difference in the energy level of the substituted bond (C··D··C*) when compared to the unsubstituted bond (C··H··C*). (Scheme 1.7 compared with Scheme 1.5).

Scheme 1.7 Secondary Kinetic Isotope Effect



These secondary isotope effects have been particularly helpful in detecting carbonium ion intermediates in enzyme-catalysed reactions and is exemplified by the dehydration of malate catalysed by fumarase. Schmidt et al., (1969) demonstrated a k_H/k_D value of 1.09 when either the C-2 or C-3 proton of malate was substituted for by deuterium. These results are consistent with the formation of a carbonium ion intermediate at C-2 of malate when the hydroxyl group leaves. Similarly, the cleavage of the glycosidic bonds of polysaccharide derivates by lysozyme have been shown to give a secondary isotope effect of 1.11 (Dahlquist et al., 1969) over a wide range of pH which supports the occurrence of a carbonium ion intermediate in this reaction. In contrast, the hydrolysis of polysaccharide derivatives by β-galactosidase shows no secondary isotope effect (k_H/k_D = 1.01) suggesting that the enzyme effects hydrolysis by an SN-2 type mechanism (Richards, 1970).

1.3.3 Solvent Kinetic Isotope Effects

Solvent isotope effects have been studied in enzyme-catalysed reactions in which water is either a reactant or labels a catalytically important group of the enzyme. For example, significant solvent isotope effects have been observed for the hydrolysis of N-acetyl -L-tryptophanamide by chymotrypsin (k_{H_2O}/k_{D_2O} = 2.3) (Inagami et al., 1969) and suggests

that there is a kinetically important proton transfer reaction in the formation of the acyl-enzyme intermediate. This was later shown to be due to the charge relay system between Ser-195, His-57 and Asp-102 (Blow, 1976)(See Section 1.5.1 for further details).

Solvent isotope effects have also been used to demonstrate a step-wise conversion of dihydroxyacetone phosphate (DHAP) to glyceraldehyde-3-phosphate (G-3-P) by triose phosphate isomerase in which no step is fully rate determining (Albery and Knowles, 1976 and References therein).

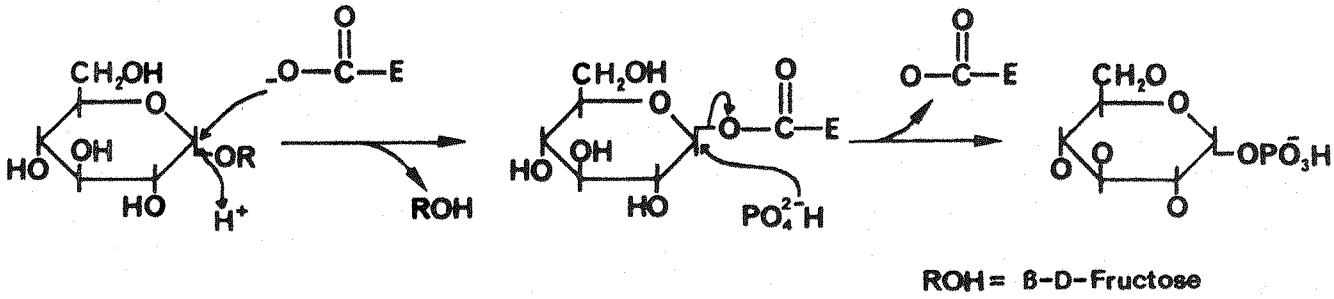
In summary, a study of the kinetic isotope effects of various enzyme-catalysed reactions have proved useful not only for determining the rate limiting step of the reaction but also for distinguishing between step-wise and concerted reactions and for elucidating the chemical nature of the intermediates. For further information and examples of the use of kinetic isotope effects for the study of enzyme-catalysed reactions you are referred to the book by Jencks (1969) and the reviews by Richards (1970), Rose (1970), Cleland et al. (1977) and Klinmann (1978). Also the review by Cleland (1977) looks at the combined use of enzyme kinetics (Section 1.1), isotope exchange (Section 1.2) and kinetic isotope effects for determining the chemical mechanisms of enzyme-catalysed reactions.

1.4 Methods Used to Demonstrate Covalent Catalysis.

To date over 450 enzymes have been shown to catalyse reactions via a covalent intermediate (Bell and Koshland, 1971; Spector, 1982) and they have been investigated using a wide variety of techniques. For example, the demonstration that an enzyme exhibits ping-pong kinetics (see Section 1.1 and Scheme 1.2) directly suggests that the reaction proceeds via a covalent enzyme-bound intermediate (e). This suggestion can be further corroborated by showing that the enzyme catalyses two partial reactions using isotopic exchange (see Section 1.2). However, not all enzymes which catalyse reactions via a covalent intermediate exhibit ping-pong kinetics. This is due to the fact that either the reaction involves only one substrate (e.g. glucose-6-phosphatase) or that the enzyme catalyses a homopolymerization reaction involving two or more identical substrate molecules (e.g. 5-aminolevulinic acid dehydratase, β-oxoacyl-CoA thiolase and porphobilinogen deaminase). Therefore, other techniques must be employed to demonstrate the formation of a covalent intermediate during the enzyme reaction and

generally involve the isolation of the enzyme-bound intermediate from reaction mixtures either in their native form (e.g. by gel filtration) or by trapping the intermediate by chemical reaction or denaturation. Subsequent analysis of the isolated intermediate to determine its chemical nature will give information about the transition state of the reaction and the catalytically important group at the active site which is responsible for the formation of the covalent intermediate. This information will give an important insight into the mechanism of action of these enzymes..

The first evidence for the participation of a covalent enzyme-substrate intermediate in enzyme-catalysed reactions was provided by Doudoroff and co-workers. They studied the enzyme sucrose phosphorylase, which catalyses the reversible transfer of an α-D-glucosyl group between D-fructose and phosphate. They were able to demonstrate exchange reactions (see Section 1.2 for further details) and suggested that a β-glucosyl-enzyme intermediate (1.10) was formed during the reaction (Doudoroff et al., 1947; Wolochow et al., 1949). Direct evidence for this intermediate was obtained by Silverstein et al. (1967) who found that one mole of ^{14}C glucose was incorporated per mole of sucrose phosphorylase on incubation with U ^{14}C-labelled sucrose. The reactive enzyme group involved in the formation of the covalent intermediate was later shown to be a carboxyl residue (DeToma and Abeles, 1970) (See reaction scheme below).

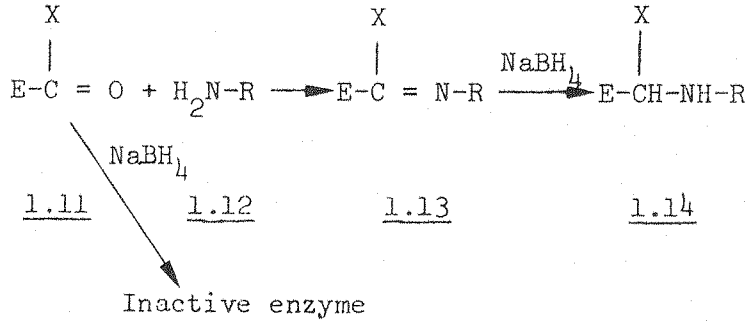


1.10

The formation of a Schiff base intermediate (1.13 or 1.17) between an amine (1.12 or 1.15) and a carbonyl compound (1.11 or 1.16) by a large number of enzymes has been shown to play one important role in their catalytic action. This is due to the fact that the Schiff base is readily protonated (generating an effective electron sink) which makes

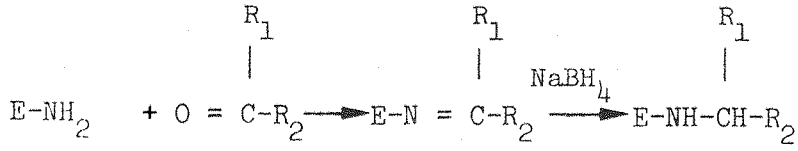
it highly reactive towards nucleophilic attack by the carbonyl group acceptor.

There are two categories of enzymes that operate via Schiff base mechanisms (Snell and di Mari, 1970). In category I, the carbonyl (or potential carbonyl) group (1.11) is present in the enzyme as part of a prosthetic group or coenzyme and include the numerous pyridoxal phosphate-dependant enzymes (e.g. transaminase) as well as those enzymes containing covalently bound pyruvate (histidine decarboxylase), α-ketobutyrate (urocanase) or dehydroalanine (phenylalanine ammonia lyase). The substrates are amines of various types (1.12).



1.11 1.12 1.13 1.14

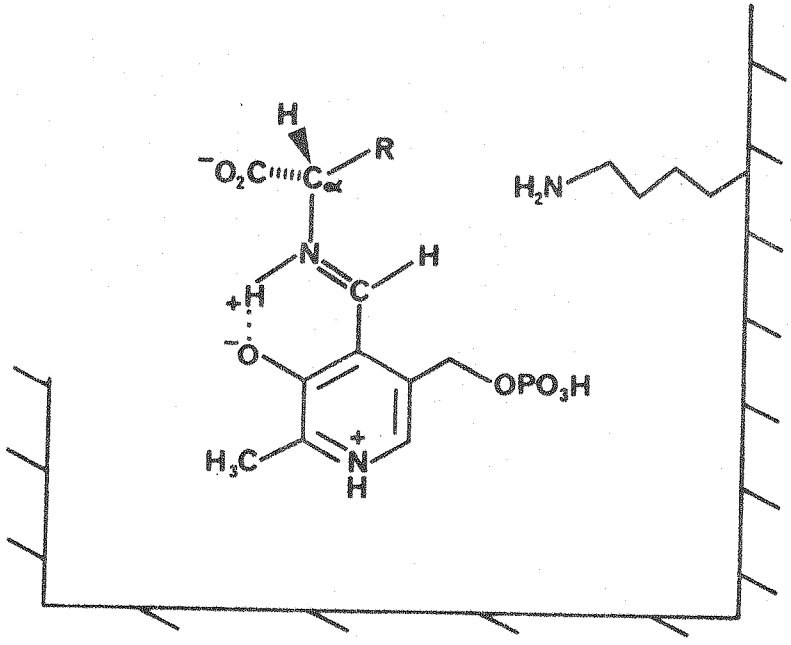
Enzymes in category II lack a carbonyl group, but contain an active site amino group (1.15) which reacts with a carbonyl-containing substrate (1.16). This category includes β-oxo acid decarboxylases, aldolases, transaldolases and 5-aminolevulinic acid dehydratase.



1.15 1.16 1.17 1.18

Initial evidence for the participation of a Schiff base intermediate in an enzyme-catalysed reaction can be provided by demonstrating that these enzymes are inactivated by $NaBH_4$ in the presence of substrate. Also, unlike the enzymes in category II, those enzymes in category I are also inactivated by $NaBH_4$ in the absence of substrate and also by various carbonyl reagents such as hydroxylamine and phenylhydrazine.

Further studies on the pyridoxal phosphate-dependent enzymes (category I) have shown that in the absence of substrate they generally exist as Schiff bases with the ε-amino group of an active site lysine residue (Snell and di Mari, 1970). Subsequent transaldimination with substrate yields a pyridoxal phosphate-substrate Schiff base (1.19) and a free lysine amino group.

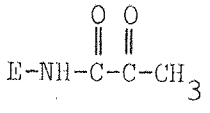


1.19

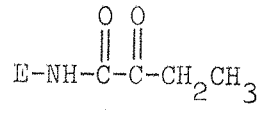
The aromatic nitrogen residue in this intermediate acts as an efficient electron sink and enables one of the three α-carbon bonds to be cleaved depending on the specificity of the enzyme. Examples include serinetranshydroxymethylase (Cα-R bond cleavage), aspartate transaminase and tryptophanase (C -H bond cleavage) and pyruvate decarboxylase (C $-CO_2H$ bond cleavage). These enzymes have also been studied using spectrophotometry since the chromophoric system of the free coenzyme absorbs at 390nm and is affected by binding to both the enzyme and substrate (see Snell and di Mari,(1970) and Akhtar et al. (1984) for further details).

The formation of a catalytically important Schiff base intermediate by enzymes which contain a carbonyl group as part of the enzyme structure rather than a property of the prosthetic group has been demonstrated by Snell and co-workers. They demonstrated that the bacterial enzyme, histidine decarboxylase, was inactivated completely by reduction with NaB^3H_4 and, after acid hydrolysis, they identified the labelled product as lactate (Riley and Snell, 1968). These data are consistent with the involvement of a catalytically important pyruvyl residue (1.20) which acts as an electron sink, generating a Schiff base on reaction with the amino group of histidine, and allowing subsequent decarboxylation to take place (Walsh, 1979). A pyruvyl group is also thought to be responsible for Schiff base formation in D-proline reductase (Hodgins and Abeles, 1969) and phosphatidyl serine decarboxylase (Satre and Kennedy, 1978).

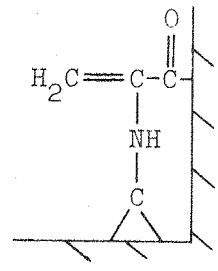
The isolation of labelled α-hydroxybutyric acid from acid hydrolysates of both serine-threonine dehydratase (Kapke and Davis, 1975) and urocanase (George and Phillips, 1970) upon reduction of these enzymes with NaB^3H_4 suggests that the carbonyl group at the active site is derived from α-ketobutyrate (1.21). Also a novel electrophilic centre, dehydroalanine (1.22) has been proposed as the catalytically important carbonyl group in phenylalanine ammonia lyase (Hanson and Havir, 1972; Al-Shammary, 1984). Both the α-ketobutyryl and dehydroalanyl groups, like the pyruvyl group, play an important role in stabilizing the transition state of the reaction in the form of a covalently bound Schiff base.



1.20



1.21



1.22

Enzymes in category II contain an active site amino group and have been investigated by reduction with $NaBH_4$ in the presence of substrate. For example, acetoacetate decarboxylase is inactivated by $NaBH_4$ in the presence of ^{14}C-labelled acetoacetate with the concommitant incorporation

of 1 mole of label per mole of enzyme (Fridovich and Westheimer, 1962). On acid hydrolysis a labelled adduct, N-ε-(^{14}C-isopropyl) lysine, was isolated demonstrating that the catalytic amino function was provided by the ε-amino group of an active site lysine residue (Warren et al., 1966). Based on these results a mechanism of action of acetoacetate decarboxylase was proposed in which the lysine residue reacts with the β-carbonyl group of the substrate to form a Schiff base. The protonation of the relatively basic nitrogen atom of the Schiff base, makes it a more efficient electron sink, which allows decarboxylation to take place. Finally, hydrolysis of the Schiff base yields acetone.

Another enzyme thought to contain an active site amino group is porphobilinogen deaminase. This enzyme catalyses a homopolymerization reaction in which four molecules of porphobilinogen (PBG) are condensed together to yield preuroporphyrinogen. Berry and Jordan (1981) demonstrated that on mixing stoichiometric equivalents of labelled PBG with enzyme (1, 2, 3 and 4 moles PBG/mole enzyme), followed by gel electrophoresis, four bands assayed for enzyme activity. These bands were shown to be due to free enzyme (E) and three distinct covalent intermediates ES_1, ES_2 and ES_3, demonstrating the step-wise conversion of PBG to preuroporphyrinogen. However, no ES_4 was found presumably due to its rapid conversion to product. The three covalently bound ES complexes were subsequently shown to be catalytically viable (Berry et al., 1981) and highlights the fact that it is possible to isolate stable and kinetically competent enzyme-bound intermediates. These intermediates have also been observed on studies with the human enzyme by Anderson and Desnick (1980). Other enzymes have been shown to contain a catalytically important lysine residue and include fructose-1, 6-diphosphate aldolase (Grazi et al., 1962), other aldolases (Rosen et al., 1965; Meloche et al., 1966; Rosso and Adams, 1967), transaldolase (Horecker et al., 1961), deoxyglucarate dehydratase (Jeffcoat et al., 1969) and pyruvate-aspartic semialdehyde condensing enzyme (Shedlarski and Gilvarg, 1970) (Hupe, 1984).

Studies on the enzyme-catalysed hydrolysis of p-nitrophenyl acetate by chymotrypsin has demonstrated that the enzyme is esterified to yield a covalently bound acyl-enzyme intermediate (Gutfreund and Hammond, 1959). This intermediate is formed by reaction of an active site serine residue

with the electrophilic centre of the substrate demonstrating the importance of this serine residue in the mechanism of action of chymotrypsin (see Section 1.5.1 for further details).

Acyl-enzyme intermediates have also been isolated from non-proteolytic enzymes and include many of the enzymes involved in the metabolism of thioesters. For example, on incubation of β-oxoacyl-CoA thiolase with (^{14}C) acetyl-CoA, followed by gel filtration, a radioactive acetyl-enzyme complex can be isolated demonstrating that a covalent intermediate is formed during the reaction. Subsequent chemical analysis of this intermediate has shown that the catalytic group responsible for reacting with the acetyl moiety is a cysteine residue (Gehring and Harris, 1968, 1970). Similar studies on the enzyme, hydroxymethyl glutaryl-CoA (HMG-CoA) synthase, have also demonstrated a catalytically important cysteine residue (Stewart and Rudney, 1966). Miziorki et al., (1975) further demonstrated that the acetyl-enzyme intermediate formed during the reaction was kinetically competent and went on to show that a second acyl-enzyme intermediate was formed on addition of acetoacetyl-CoA. This intermediate is subsequently hydrolysed to yield the product HMG-CoA (Miziorki and Lane, 1977).

The demonstration that a phosphoryl moeity is bound to the enzyme during the catalytic cycle of many phosphotransferase reactions suggests that this phosphoryl-enzyme intermediate is an integral part of the mechanism of action of these enzymes. For example, phosphoglucomutase catalyses the reversible transfer of a phosphoryl group between C-1 and C-6 of glucose and has been shown to produce a phosphoserine intermediate during the reaction (Kennedy and Koshland, 1957) whilst more recent studies have suggested the existence of a second phosphoryl binding site (a tyrosine residue) (Layne and Najjar, 1979). This observation helps to explain the extremely rapid exchange of the phosphoryl moeity between the C-1 and C-6 positions. Many other enzymes have been shown to catalyse reactions by the formation of a phosphoryl-enzyme intermediate and include alkaline phosphatase (phosphoserine intermediate; Schwartz, 1963), phosphoglycerate kinase (phosphoryl glutamic acid intermediate; Brevet et al., 1973) and glucose-6-phosphatase (phosphoryl histidine intermediate; Feldman and Butler, 1972).

In summary, the methods used for demonstrating the involvement of a covalent intermediate in an enzyme mechanism clearly relies on the isolation of one of the ES complexes (and demonstrating that it is kinetically competent and catalytically viable), or, if this is not possible, the use of exchange (Section 1.2) and kinetic (Section 1.1) techniques. For further information and examples of covalent intermediates in enzyme-catalysed reactions you are referred to the books by Jencks (1979) and Spector (1982).

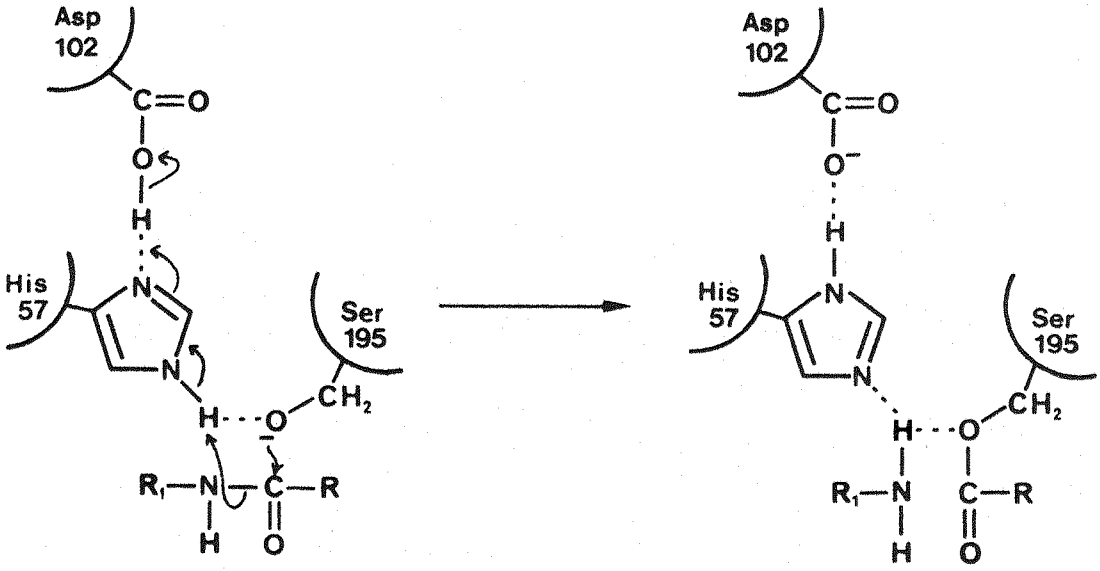
1.5 Active Site Modification

1.5.1 Active-Site-Directed Reagents

The covalent modification of catalytically important groups at the active site of an enzyme can be achieved by utilising a reagent with structural features similar to that of the substrate but which also contains a highly reactive group. The structural features of the enzyme active site should therefore direct the reagent to the vicinity of important catalytic groups and irreversible modification will occur. Subsequent analysis of the protein to identify the modified group can yield information about the topography of the active site and, in many cases, whether this group is catalytically important or not (Jakoby and Wilchek, 1977).

The nature of the catalytic groups at the active sites of both chymotrypsin and trypsin have been investigated by Shaw and co-workers using various chloromethyl ketone derivatives. Schoellmann and Shaw (1963) studied the reaction of chymotrypsin with N-tosyl-L-phenylalanylchloromethyl ketone (TPCK)(Scheme 1.8) and demonstrated that the inactivation of the enzyme was associated with the modification of the histidine residue (His-57). The competitive inhibitor, β-phenylpropionic acid, was shown to retard this inactivation suggesting that the modifiation was occurring at or very near the active site. Similarly, the chloromethyl ketone derivatives of both L-lysine (TLCK) and L-arginine (p-NO_2-ZACK) (Scheme 1.8) were found to inactivate trypsin , again, by modification of the active site histidine residue (His-46) (Shaw and Glover, 1970). The specificity of these active-site-directed reagents is highlighted by the lack of effect of TLCK on chymotrypsin and of either TPCK or the D-isomer of TLCK on trypsin (Shaw, 1970b).

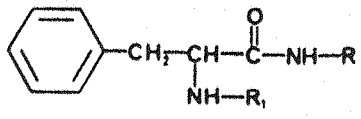
Further studies by Blow and co-workers (1969) using X-ray crystallography have demonstrated that Asp-102, His-57 and Ser-195 are closely associated in a charge relay system at the active site of chymotrypsin. This system is thought to make the oxygen atom of the serine residue a powerful nucleophile which is capable of attacking the carbon atom of the carbonyl group in a substrate to form the tetrahedral intermediate (Blow, 1969, 1976). A similar arrangement is found in trypsin and subtilisin (Shaw, 1970a).



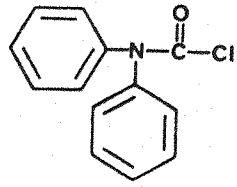
The fact that His-57 rather than the enzyme nucleophile Ser-195 reacted with TPCK in chymotrypsin highlights (retrospectively) the difficulty in designing a reagent whose electrophilic group can interact precisely with the primary enzyme nucleophile. This fact is exemplified by treatment of trypsin with p-guanidinophenylacyl bromide, an active-site-directed reagent which has its reactive group nearer to the guanidino function than in p-NO_2-ZACK (Scheme 1.8) and results in the modification of the active site serine residue (Ser-183) (Schroeder and Shaw, 1971). Similar work by Erlanger and co-workers (1966a) have shown that the inactivation of chymotrypsin by diphenylcarbamyl chloride (Scheme 1.8) is associated with the modification of Ser-195 and provides further evidence for the importance of this serine residue in the catalytic mechanism.

Scheme 1.8 Structures of Substrates and Active-Site-Directed Reagents Used to Study Catalytically Important Groups in Chymotrypsin and Trypsin.

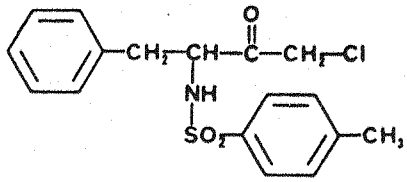
Chymotrypsin



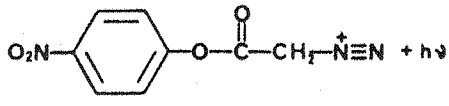
L-Phenylalanyl (Substrate)



Diphenylcarbamyl chloride

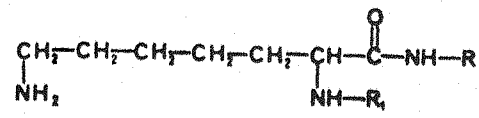


N-Tosyl-L-phenylalanyl-chloromethyl ketone

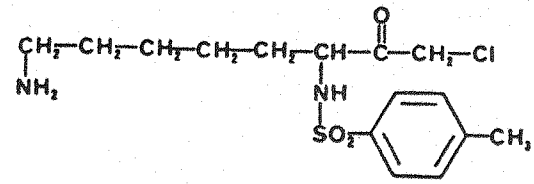


p-Nitrophenyldiazoacetate

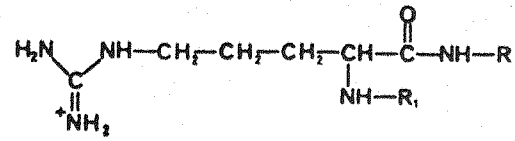
Trypsin



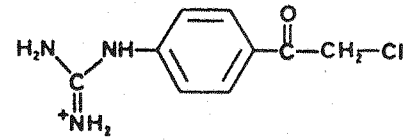
L-Lysyl (Substrate)



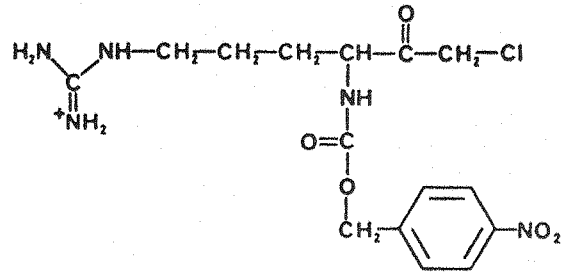
N-Tosyl-L-lysylchloromethyl ketone



L-Arginyl (Substrate)



p-Guanidinophenylacyl bromide



p-Nitrobenzyl carbonylarginyl chloromethyl ketone.

Photoaffinity labelling has also been used successfully to modify catalytically important groups at the active site of enzymes. For example, the reactive carbene, generated from ^{14}C-labelled p-nitrophenyl diazoacetate (Scheme 1.8) by irradiation at wavelengths > 320 nm, was shown to be incorporated stoichiometrically into chymotrypsin to yield a diazoacetylated enzyme (Shafer et al., 1966). Subsequent analysis of the protein demonstrated that the products formed included O-carboxymethyl serine, 1-carboxymethyl histidine and O-carboxymethyl tyrosine. The first two products represent derivatives of the active site residues, Ser-195 and His-57, whilst the third product is formed by an intermolecular reaction of the photochemically generated carbene generated at the active site of one molecule of α-chymotrypsin with Tyr-146 of another (Hexter and Westheimer, 1971). Tyr-146, together with Ala-149, are thought to be involved in the intermolecular interactions of α-chymotrypsin and are therefore not catalytically important, showing that care has to be taken in interpretation of data from what should be a highly specific reagent.

The covalent labelling of active site groups can also be achieved using suicide inhibitors, sometimes known as K_{cat} inhibitors. These reagents are sufficiently similar to the substrate to enable their participation through one or more stages in the catalytic action of the enzyme to generate a reactive species which can subsequently modify a catalytically important adjacent group. For example the enzyme, aspartate transaminase, which catalyses the conversion of L-aspartate to oxaloacetate while pyruvate is transformed to L-alanine, has been studied by Rando (1974) using 2-amino-3-butenoate (vinylglycine). The results suggest that this substrate analogue undergoes transamination to a conjugated β, γ-unsaturated ketimine which subsequently reacts with the active site lysine residue to yield an inactive alkylated enzyme (see Walsh, 1979 for further details). Another example is the reaction of 3, 4-dihydro-3, 4-dibromomethyl coumarin with α-chymotrypsin. Vilkas (1978) demonstrated that the inhibitor was hydrolysed by the active site serine residue (Ser-195) to generate a reactive p-hydroxybenzyl bromide group (still bound to Ser-195) which subsequently alkylates the neighbouring His-57 to form a permanent covalent bridge between the two catalytically important residues.

This section has concentrated mainly on the use of active-site-directed reagents in the study of catalytic groups at the active site of two serine

proteases, chymotrypsin and trypsin. The roles of these groups in the mechanism of action of both enzymes are discussed in greater detail by Sigler et al., (1968), Blow (1969, 1976), Hess (1970) and Shaw (1970b). For more information about other studies using active-site-directed reagents you are referred to the reviews by Shaw (1970b) and Jakoby and Wilchek (1977).

1.5.2 Group-Specific Reagents

The covalent modification of a specific functional group at the active site of an enzyme by a group-specific reagent can be used to investigate the importance of the particular group in the catalytic mechanism. Unfortunately, the term "group-specific" is rather misleading as it implies that the reagent will only modify a specific functional group. This is far from true since the microenvironment imposed by the structure of the native protein often drastically alters the behaviour of the functional groups towards the reagent. For example, fluorodinitrobenzene (FDNB) is usually considered to be an amino-group reagent, which is typified by the modification of the catalytically important Lys-41 in ribonuclease (Carty and Hirs, 1968). However, FDNB has also been shown to react preferentially with a sulphydryl group in glyceraldehyde-3-phosphate dehydrogenase (Shaltiel and Soria, 1969) and a histidine group in carbonic anhydrase (Henkart and Dorner, 1971). Therefore, when studying group-specific modifications it is very important to select carefully the reagent and reaction conditions and then subsequently determine precisely which group is modified. Also data from these type of studies must be cautiously interpreted since the inactivation of an enzyme by a group-specific reagent may not necessarily be due to modification of a catalytically important group at the active site but may be due indirectly to an accompanying structural change, the modification of residues in allosteric or effector sites, or even due to steric effects.

Two techniques have proved useful for determining whether modification of a catalytically important group has taken place or not. First, the demonstration that the inactivation by a group-specific reagent is prevented by the presence of substrate, product or related compounds suggests that the modification is occurring at or very near the active site and that the functional group is likely to be catalytically important. Second, differential labelling can be used to modify non-specific residues outside

Table 1.2 The Modification of Functional Groups at the Active Sites of Various Enzymes by Group-Specific Reagents.

Amino Acid Modified	Reagent Used	Enzyme Studied (Group Modified)	Reference
LYSINE	Acetic anhydride	Acetoacetate decarboxylase	O'Leary and Westheimer (1968)
	FDNB	Ribonuclease A (Lys-41)	Carty and Hirs (1968)
	Dansyl chloride	Rennin	Hill and Laing (1967)
	N-(N'-acetyl-4-sulphamoylphenyl) maleimide	Glutamate dehydrogenase	Hollbrook and Jeckel (1969)
ARGININE	Butanedione	Phosphoglycerate mutase	Rose (1980)
	Phenylglyoxal	Ribonuclease (Arg-39 and 85)	Takahashi (1968)
	1, 2-Cyclohexanedione	Ribonuclease (Arg-39)	Patthy and Smith (1975)
ASPARTATE	p-Bromophenylacyl bromide	Pepsin	Erlanger et al., (1966b)
	Ethyldimethylaminopropyl carbodiimide	Chymotrypsin (Asp-102)	Carraway and Koshland (1972)
	N-Alkyl-5-phenylisoxazolium salts	Trypsin (Asp-177)	Feinstein et al., (1969)
	Triethyloxonium fluoroborate	Trypsin (Asp-177)	Nakayama et al., (1970)
CYSTEINE	Nbs_2	5-Aminolevulinic acid dehydratase	Seehra et al., (1981)
	Iodoacetate	Alcohol dehydrogenase	Rashed and Rabin (1968)
	α-Bromo-β-(5-imidazoyl) propionic acid	Papain (Cys-25)	Jolley and Yankeelov (1972)
HISTIDINE	Methylene blue/photoxidation	Alkaline phosphatase	Coleman and Gettins (1983)
	FDNB	Carbonic anhydrase	Henkart and Dorner (1971)
	Iodoacetate	Ribonuclease (His-119)	Crestfield et al., (1963)
	1, 3-Dibromoacetone	Papain (His-106 and Cys-25; Cross linked)	Husain and Lowe (1968)
TRYPTOPHAN	N-Bromosuccinimide	Lysozyme (Trp-62)	Spande et al., (1970)
	Formic acid	Trypsin	Coletti-Previero et al., (1969)
	Tetranitromethane	Papain	Morihara and Nagami (1969)
SERINE	Cyanate	Chymotrypsin (Ser-195)	Shaw et al., (1964)
	Diisopropylfluorophosphate	Acetylcholinesterase	Walsh (1979)
TYROSINE	Acetylimidazole	Carboxypeptidase A (Tyr-248)	Simpson et al., (1963)
	Tetranitromethane	Staphylococcal nuclease (Tyr-85)	Cuatrecasas et al., (1968)

the active site by reacting the enzyme initially with the group-specific reagent in the presence of an excess of the substrate (or a good competitive inhibitor). Removal of the substrate (inhibitor) and other reagents (usually by dialysis) and subsequent incubation in the presence of the radioactively labelled modifying agent results in the selective labelling of active site residues and can yield information about the catalytic importance of these groups (Singer, 1967).

The advantages of active-site-directed reagents over group-specific reagents is exemplified by the specific modification of a single histidine residue in trypsin by 0.5mM TLCK with a half-time of about 6 minutes at pH7. Conversely, modification of trypsin using 0.5M iodoacetamide under the same conditions produces six modified sites per mole of enzyme in 48 hours but has little effect on the activity.

However, despite the inherent limitations, group-specific reagents have been used successfully to demonstrate catalytically important groups at the active sites of many enzymes and some examples are summarised in Table 1.2. For further information on the subject you are referred to the reviews by Cohen (1968), Shaw (1970a), Stark (1970), Glazer (1976) and the book by Means and Feeney (1971).

1.6 Techniques for Studying Enzymes Which Require a Metal Ion.

More than a quarter of all known enzymes require the presence of a metal ion for full catalytic activity. Thus for a complete understanding of the mechanism of action of these enzymes it is essential to determine the role of the metal ion. Metal-requiring enzymes can be divided into two classes, metalloenzymes, where the metal ion is firmly associated with the protein, and metal-activated enzymes, where the metal ion readily dissociates from the enzyme and thus needs to be present in solution. Mildvan (1970) has classified the interaction of metal ion (M), enzyme (E) and substrate (S) in metal-activated enzymes as enzyme bridge (M-E-S), substrate bridge (E-S-M) or metal bridge (E-M-S or $E\begin{smallmatrix}M\\|\\S\end{smallmatrix}$) complexes. In metalloenzymes the substrate bridge complex is not formed due to the tight, stoichiometric binding of the metal ion to the enzyme in the absence of substrate.

Preliminary evidence for the involvement of a metal ion in the mechanism can be obtained by demonstrating that on adding a suitable chelating agent such as EDTA, 8-hydroxyquinoline and 1, 10-phenanthroline, the enzyme activity is decreased and is associated with the removal of the metal ion from the enzyme protein. Similarly, after removal of the chelating agent (e.g. by dialysis or dilution), the return of enzyme activity should be proportional to the metal ion concentration in the assay mixutre (unless the apoenzyme is unstable or susceptible to oxidation) (Kägi and Vallee,1960). In addition, the effect of metal ion substitution on the activity of the enzyme has proved useful for determining the specificity of the metal-binding site (Chlebowski and Coleman, 1976).

The nature of the metal ion bound to the enzyme can be determined by its characteristic absorption and emmision spectra using techniques of physical chemistry, such as atomic absorption spectroscopy and emmision spectroscopy. These techniques, together with equilibrium dialysis and isotopic substitution (e.g. ^{65}zinc substituted for unlabelled zinc) (Drum et al., 1967), have also been used to determine the precise stoichiometry of the interaction with the metal ion with the enzyme and, in many cases, the dissociation constant (K_D) for the metal ion.

These preliminary studies serve to demonstrate whether a specific metal ion is required for maximal catalytic activity and also to determine the stoichiometry and affinity of the metal ion binding. However, it is more difficult to resolve whether the metal ion is involved directly in the mechanism of action of the enzyme or is simply involved in the maintenance of the three-dimensional structure of the protein. To this end a variety of specific studies can be carried out to delineate the role of the metal ion in enzyme-catalysed reactions; in fact the properties of the metal ion itself can be used for probing its structural environment. These techniques include visible spectroscopy, magnetic resonance (NMR and ESR), resonance Raman spectroscopy, optical rotatory dispersion and circular dichroism. Also, in cases where the intrinsic metal ion displays poor probe characteristics it is often possible to substitute it for a better metal ion probe. For example, the substitution of cobalt for zinc in carbonic anhydrase has enabled the geometry of the metal-binding site to be investigated. The visible spectrum of the cobalt-substituted

enzyme shows distinct splitting of some of the d-d bands and suggests that the metal ion occupies a highly distorted tetrahedral site (Hughes, 1972; Chlebowski and Coleman, 1976), a proposition which has subsequently been confirmed by X-ray studies (Liljas et al., 1972). Studies by Taylor and Coleman (1973) on the copper-substituted enzyme using ESR have shown that under certain conditions, such as in the presence of the anionic inhibitor cyanide, the metal ion becomes five-coordinate. In addition, a study of the effect of sulphonamide inhibitors on the visible absorption spectrum of cobalt-substituted carbonic anhydrase has revealed that inhibition is achieved by binding to the metal ion, probably through coordination of the amino group nitrogen, and is associated with the displacement of water (or OH^-) from the fourth coordination position. Also pH studies (Lindskog, 1963, 1966) have demonstrated the presence of two forms of the enzyme which are in a protonation equilibrium. Lindskog has suggested that the ionizable group involved in this equilibrium is, in fact, the metal-bound water (or OH^-) molecule. However, the precise mechanism of action of carbonic anhydrase is, as yet unresolved although the most widely accepted model involves the attack of a zinc-coordinated hydroxide group on CO_2 (zinc-hydroxide mechanism) (Coleman,1967; Pocker and Sarkanen, 1978).

Paramagnetic metal ion substitution has also provided an effective probe for the study of creatine kinase, which catalyses the transfer of a phosphate group from ATP to creatine yielding phosphocreatine. The enzyme normally requires magnesium for maximum catalytic activity, but is active with manganese. Cohn and her colleagues have investigated the nature of the ternary complex formed between the enzyme, substrate and metal using ESR, in conjunction with the paramagnetic probe manganese, and demonstrated that there was no change in the ESR spectra of the binery Mn-ADP complex upon addition of enzyme (Cohn, 1970). These data are consistent with the formation of a substrate bridge (E-ADP-Mn) ternary complex. The geometry of the active site of Mn-creatine kinase has also been studied by using an iodoacetate derivative of a nitroxide spin label (N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl) iodoacetamide) which covalently modifies a catalytically important sulphydryl group at the active site. The effect of additon of both Mn-ADP and Mn-ATP on the ESR spectrum demonstrated that there was an interaction between the two paramagnetic probes and from the magnitude of interaction, the distance

between the maganese ion and the nitroxide moeity of the spin label could be estimated (E-ADP-Mn = 0.8-1.2nm; E-ATP-Mn = 1.3-1.8nm) (Mildvan, 1970). Similar conclusions have been drawn from a study of the proton relaxation rate of water, in Mn-creatine kinase using NMR (Mildvan, 1970).

Mildvan et al., (1967) have also studied another magnesium dependant enzyme, pyruvate kinase, using a variety of techniques. For example, they studied the fluorokinase raction of the Mn-substituted enzyme (a side reaction whereby fluoride is converted to fluorophosphate) using ^{19}F NMR and demonstrated the formation of a short-lived E-Mn-O_3PF complex. These findings are consistent with the formation of a metal bridge ternary complex. This proposal was further corroborated by demonstrating that the enhancement of the proton relaxation rate of water was drastically reduced when the binary E-Mn complex was reacted with either phosphoenolpyruvate (PEP) or fluorophosphate to yield the ternary complex. They also demonstrated that this reduction in rate enhancement was more pronounced when the phosphoryl donors (PEP and ATP) were present than when the corresponding phosphoryl acceptors (pyruvate and ADP) were present which suggested that the phosphoryl group transferred during the enzyme-catalysed reaction provided a ligand for the enzyme-bound metal ion. Therefore, as with creative kinase, the role of the metal ion is not only to hold the reacting groups in juxtaposition but also to promote the electrophilicity of the phosphorus by σ-electron withdrawal, thus promoting its attack and subsequent transfer. Studies on the phosphoenolpyruvate carboxylating enzymes, PEP carboxylase and PEP carboxykinase, have revealed a similar role for the metal ion (Mildvan, 1970).

The role of zinc ions in carboxypeptidase A, a proteolytic enzyme which catalyses the cleavage of C-terminal amino acids from peptide substrates, has also been investigated using magnetic resonance techniques. Shulman et al., (1966) studied the Mn-substituted enzyme using NMR and demonstrated that water was bound to the metal ion which was displaced on reaction with the inhibitor β-phenylpropionate, suggesting that the metal ion interacts with the carbonyl group of the inhibitor. More recently Kuo and Makinen (1982) used $H_2{}^{17}O$ broadening of the cobalt resonance in the Co-substituted enzyme to show that the metal ion binds to both water (or OH^-) and the carbonyl oxygen derived from the substrate. The topography of the active site of carboxypeptidase A has also been studied by resonance

Raman spectroscopy using diazotised arsanilic acid which generates an intramolecular coordination complex between the tyrosine residue and zinc (azo-Tyr-248-zinc complex) (Vallee and Riordan, 1978). From this and other evidence (see Lipscomb, 1983 for further details) the zinc ion is thought to promote catalysis by polarizing the carbonyl group of the substrate, making the carbonyl carbon more susceptible to nucleophilic attack, with Tyr-248 donating a proton to the NH group of the cleaved peptide bond. However, the precise details of the mechanism of action of carboxypeptidase A have not been fully elucidated, although the γ-carboxylate of Glu-270 is thought to be involved (Makinen et al., 1976, 1979; Breslow and Wernick, 1977; Walsh, 1979).

In summary, the role of metal ions in enzyme catalysis can be investigated using a variety of techniques either with the intrinsic metal ion or by substituting it with a metal ion which displays superior probe characteristics. Metal ions have been shown to play an important role in enzyme-catalysed reactions by holding the reactive groups in juxtaposition, hence promoting catalysis either by polarizing one of the reactive groups, making it more susceptible to attack, or by delivering OH^- to the nucleophilic centre, and also by stabilizing the resultant enzyme-bound intermediate. For further information and examples you are referred to the reviews by Mildvan,(1970), Chlebowski and Coleman (1976), Cohn and Reed (1982), Lipscomb (1983), Vallee and Galdes (1984) and the book by Hughes (1972).

1.7 Determination of the Three-Dimensional Structure of an Enzyme.

When studying the mechanism of action of an enzyme-catalysed reaction it is of paramount importance to elucidate the structural features of the active site which are responsible for the binding and subsequent chemical transformation of the substrate(s). One of the most powerful tools for investigating the precise orientation of these functional groups at the active site is X-ray crystallography. This technique, in combination with the knowledge of the primary structure (obtained either from protein or gene sequencing), has been used to study the three-dimensional structure of many enzymes, both in the presence and absence of substrates, products and related compounds,(Eisenberg, 1970; Matthews, 1977; Blake, 1979).

These structural details have been used both to confirm the presence of various functional groups which have been suggested as a result of other investigations (e.g. iodoacetate modification of a catalytically important His-119 in ribonuclease A; Section 1.5.2, Table 1.2). (Crestfield et al., 1963) as well as to propose catalytic functions for other groups at the active site whose nature had not been determined previously. For example, Roberts et al., (1969) investigated the structure of ribonuclease A using X-ray crystallography. They demonstrated that the binding of substrate to the enzyme was extremely specific and was due to the geometric constraints imposed by certain active site functional groups. They also showed that catalysis was promoted by the location of the histidine residues (His-12 and 119) in close juxtaposition to the appropriate groups of the substrate. These findings were in very close agreement with the mechanism of action of ribonuclease initially proposed by Rabin and co-workers solely on the basis of kinetic and spectrophotometric investigations into the interaction of the enzyme with substrates and inhibitors (Findlay et al., 1962 and References therein). Also studies by Phillips and co-workers on the mechanism of action of lysozyme using X-ray crystallography revealed that catalysis was achieved by the distortion of the enzyme bound substrate, a hexasaccharide of N-acetylglucosamine, which allowed subsequent hydrolysis to occur between the fourth and fifth residues from the non-reducing end (Blake et al., 1965; Johnson and Phillips, 1965; Phillips, 1966, 1967).

Other techniques which have been of particular use for determining the three-dimensional structure of an enzyme include ultraviolet and fluorescence spectroscopy, circular dichroism, optical rotatory dispersion (Timasheff, 1970; Cantor and Timasheff, 1982), NMR (Richards and Wyckoff, 1971; Metcalfe et al., 1974) and chemical modification (Cohen, 1970; Thomas, 1974) (Section 1.5).

1.8 Summary

In summary, the determination of the mechanism of action of an enzyme-catalysed reaction draws on a large number of different experimental techniques. The combination used for any one enzyme depends on the nature of the enzyme and the type of reaction catalysed. Also, factors such as the stability of the enzyme may play an important part in the selection of methods. However, in general, after purification of the enzyme, the initial studies usually involve the study of steady-state kinetics (Section 1.1.1) and exchange reactions (Section 1.2). Subsequent studies may involve a more detailed study of the various stages of the reaction (Sections 1.1.2 and 1.3) and also the demonstration and isolation of any covalently bound enzyme-substrate intermediates (Section 1.4). The nature of the catalytic groups involved in the conversion of substrate to product can be explored using inhibitors, inactivators and specific probes (Section 1.5). Also, the role of the metal ion in metal-requiring enzymes can be investigated (Section 1.6). Finally, the primary structure of the enzyme protein and its X-ray crystallographic pattern (Section 1.7) will lead to a model in which the various mechanistic conclusions from earlier experiments can be verified and/or modified. In this way, an overall understanding of the enzymic mechanism can be pieced together. The recent reviews on carbonic anhydrase (Pocker and Sarkanen, 1978; Lipscomb, 1983), fructose 1, 6-bisphosphatase (Benkovic and de Maine, 1982), serine hydroxymethyl transferase (Schirch, 1982) and creatine kinase (Kenyon and Reed, 1983) highlight the use of many different experimental techniques in the elucidation of the mechanism of action of an enzyme-catalysed reaction.

CHAPTER 2

AN INVESTIGATION INTO THE NATURE OF THE ACTIVE SITE OF MAMMALIAN 5-AMINOLEVULINIC ACID DEHYDRATASES

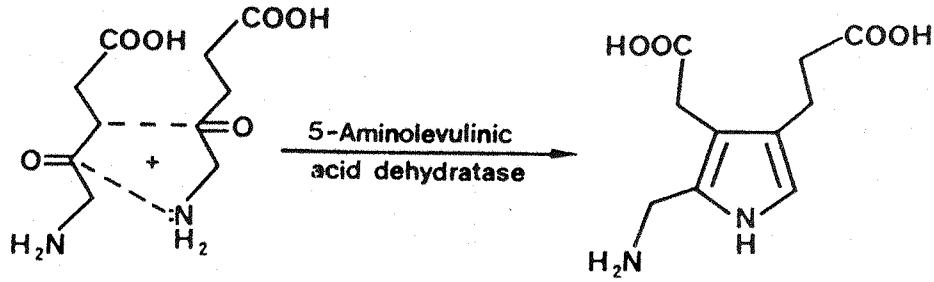
CHAPTER 2

An Investigation into the Nature of the Active Site of Mammalian 5-Aminolevulinic Acid Dehydratases

2.1 Introduction.

It was not long after the isolation and chemical characterization of porphobilinogen from the urine of patients with acute intermittent porphyria (Westall, 1952; Cookson et al., 1953) that its participation as an intermediate in porphyrin biosynthesis was demonstrated in chicken erythrocyte haemolysates (Falk et al., 1953) and in a purified system from duck erythrocytes (Schmid and Shemin, 1955). Porphobilinogen was subsequently shown to be biosynthesised from 5-aminolevulinic acid (ALA) in a haemolysed chicken erythrocyte system by two independant groups (Neuberger and Scott, 1953; Dresel and Falk, 1953). These results correlated well with the findings of Shemin and Russell (1953) who demonstrated that, like porphobilinogen, ALA was also incorporated into haem, though not as effectively as the former (Schmid and Shemin, 1955). Porphobilinogen was subsequently shown to be the monopyrrolic precursor of all porphyrins, chlorophylls and corrins (for reviews see Battersby and McDonald (1975) and Akhtar and Jordan (1978)).

5-Aminolevulinic acid dehydratase (porphobilinogen synthase; EC 4.2.1.24), the enzyme responsible for the conversion of ALA (2.1) into porphobilinogen (2.2), was first isolated and purified from bovine liver by Gibson et al., (1955).



2.1 2.1 2.2

5-Aminolevulinic acid dehydratase has subsequently been found to be ubiquitous in nature and has been isolated from a wide variety of sources. They include bovine liver (Batlle et al., 1967; Wilson et al., 1972; Gurba et al., 1972; Stella and Batlle, 1977; Seehra, 1980), mouse liver (Coleman, 1966; Doyle and Schimke, 1969), human erythrocytes (Granick and Mauzerall, 1958; Anderson and Desnick, 1979; Despaux et al., 1979; Bustos et al., 1980; Sassa and Kappas, 1983), other mammalian tissues (Yamada, 1972; Weissberg and Voytek, 1974; Stafforini et al., 1980; Zeitler et al., 1982), photosynthetic bacteria (Nandi et al., 1968; Nandi and Shemin, 1973), fungi (Komai and Neilands, 1969; Chandrika and Padmanaban, 1980) and plants (Nandi and Waygood, 1967; Shetty and Miller, 1969; Schneider, 1970; Liedgens et al., 1980).

Cheh and Neilands (1976) have classified the dehydratases derived from different sources into two classes, namely prokaryotic and plant 5-aminolevulinic acid dehydratases (termed "P" type) and eukaryotic 5-aminolevulinic acid dehydratases (termed "E" type). This distinction is based on the differences in the molecular and catalytic properties of the two groups and is similar to the Class I and Class II division of fructose-1, 6-diphosphate aldolases (Rutter, 1964). The basic differences between P type and E type dehydratases are briefly summarised below (see Shemin, 1972 and References therein). Firstly, although the molecular weights of the native dehydratase enzymes are all within the MR range of 260,000 - 324,000, the P type dehydratases are hexameric proteins of subunit molecular weight ≈ 47,500 - 50,000, whilst the E type dehydratases are octameric in nature (Subunit MR ≈ 31,000 - 35,000). Secondly, P type dehydratases require Mg^{++} (or Mn^{++}) (Schneider, 1970), monovalent ions (Nandi et al., 1968) or have no metal ion requirement (R. capsulata enzyme; Nandi and Shemin, 1973), whereas E type dehydratases require Zn^{++} for maximum catalytic activity (Cheh and Neilands, 1973; Tsukamoto et al., 1979; Bevan et al., 1980; see Chapter 3). Thirdly, the pH optima for the P type and E type dehydratases differ substantially (≈ 8.2 and ≈ 6.8 respectively) (Shemin, 1972, 1976).

Immunological studies using monoclonal antibodies raised against spinach (Schneider and Liedgens, 1981), mouse liver and human erythrocyte 5-aminolevulinic acid dehydratases (Chang et al., 1984) have revealed that the extent of cross-reactivity of these antibodies with the dehydratases from a wide variety of species were consistent with their evolutionary relationship.

One common feature of all dehydratases is their requirement for a thiol activator, which has been shown to be important for the maintenance of sulphydryl groups in the enzyme essential for catalytic activity (see Chapter 3 for further details). Barnard et al., (1977) investigated the inactivation of bovine 5-aminolevulinic acid dehydratase by the alkylating agents, iodoacetic acid and iodoacetamide, and demonstrated that inactivation only occurred only if the enzyme was activated initially by sulphydryl reducing agents. They further demonstrated that this inactivation was associated with the modification of two different cysteine residues in the primary structure. They went on to isolate and sequence labelled tryptic peptides derived from the enzyme inactivated by [2-^{14}C]-iodoacetic acid (▲) and [2-^{14}C]-iodoacetamide (●) respectively which had the following structures.

$$Cys^{▲}\text{-Glx-Pro-Gly-Ala-Val-Tyr-Arg}$$

and

$$Cys^{●}\text{-Tyr}$$

Protection against inactivation with iodoacetic acid and iodoacetamide by the product, porphobilinogen, suggested that these sulphydryl groups were present at or very near the active site of the bovine enzyme (Barnard et al., 1977). A more detailed investigation of the role of sulphydryl groups in human erythrocyte 5-aminolevulinic acid dehydratase is described in Chapter 3.

Mammalian 5-aminolevulinic acid dehydratases have a molecular weight of ≈ 285,000 and are composed of eight identical subunits of molecular weight 35,000 arranged into a cubic octameric structure with dihedral (D_4) symmetry (Wu et al., 1974b). The relationship between the quarternary structure of the bovine enzyme and its activity have been studied by two groups (Gurne et al., 1977; Batlle et al., 1978). They demonstrated the presence of active dimers and tetramers of the enzyme which could reassociate to yield a fully active octamer under certain conditions. The nature of the N-terminus of the bovine enzyme has recently been determined by Lingner and Kleinschmidt (1983) who isolated two N-terminal peptides of similar composition. They went on to show that one peptide was blocked, as N-acetyl methionine, whilst the other peptide contained free methionine (Met) as the N-terminal residue. They concluded that four of the eight subunits of the bovine dehydratase enzyme have free methionine as

the N-terminal amino acid whereas the other half are blocked (N-acetyl methionine). The primary structure of the N-terminus of bovine 5-aminolevulinic acid dehydratase was as follows.

Met-His-Pro-Gln-Ser-Val-Leu-His-Ser-Gly-Tyr-Phe-His-Pro-Leu-Leu-Arg-
Asn-Trp-Gln-Thr-Ala-Ala-Thr-Ser-Leu-Ser-Ala-Ser-Asn-Leu-Ile-Tyr-Pro-
Ile-Phe-Val-Thr-Asp-Val-Pro-Asp-Asp-Lys-

The topography of the active site of mammalian 5-aminolevulinic acid dehydratase has been investigated using both group-specific and active-site-directed reagents (see Section 1.5). Tsukamoto et al., (1975) demonstrated that photooxidation of the enzyme in the presence of methylene blue as a sensitiser led to a loss of activity which was associated with the modification of two histidine residues. Similar results were obtained using diethylpyrocarbonate, although full catalytic activity could be regained by treatment of the modified enzyme with hydroxylamine. These results cumulatively suggest that hisitidine residues either play an important role in the mechanism of action of the enzyme or that the conformational changes induced by the modifying agents are completely reversible. Various sulphydryl reagents, including the active-site-directed α-haloketoacids (Seehra and Jordan, 1981; see Section 3.1), have also been used to demonstrate the presence of catalytically important cysteine residues at or very near the active site of the bovine enzyme. Similarly, studies on the inactivation of 5-aminolevulinic acid dehydratase by $NaBH_4$ in the presence of the substrate, ALA, or the competitive inhibitor, levulinic acid (see Chapter 4), have suggested that the enzyme-catalysed reaction proceeds via a Schiff base intermediate (see Section 1.4) implicating the involvement of an amino group at the active site (Nandi and Shemin, 1968b; Doyle, 1971; Gurba et al., 1972; Wu et al., 1974a). Liedgens et al., (1983) in studies on the spinach enzyme have suggested that the amino function is provided by an arginine residue, but the evidence is based solely on the inactivation of the enzyme by butanedione, a modifying agent which is thought to be specific for arginine residues (Riordan et al., 1977). In contrast, however, Nandi (1978) has isolated a modified labelled amino acid from acid hydrolysates of the R.sphaeroides enzyme, which had been inactivated by $NaBH_4$ in the presence of $[4\text{-}^{14}C]$ ALA, and demonstrated that it was chromatographically and electrophoretically identical to chemically synthesised N-ε-[4-(5-aminovaleric acid)]lysine (ALA-lysine). From these results he concluded that the ε-amino group

of an active site lysine residue was responsible for substrate binding and the formation of a Schiff base intermediate (see Scheme 4.2).

No information is available about the nature of the amino acid sequence in the active site region of 5-aminolevulinic acid dehydratase. This Chapter describes the inactivation of both the bovine liver and human erythrocyte dehydratases by $NaBH_4$ in the presence of [4-^{14}C] ALA and the subsequent isolation and chemical characterisation of the labelled active site CNBr peptides.

2.2 Experimental.

2.2.1 Materials.

ALAHcl, dithioerythritol, insulin chain A (oxidised form) and phenylthiohydantoin (PTH) derivatives of standard amino acids were obtained from Sigma Chemical Co. Ltd., Kingston-upon-Thames, London. [4-^{14}C] ALA was obtained from either Amersham International, Bucks., or New England Nuclear, Southampton, Hants. DEAE Biogel A and Biogel A 0.5m were purchased from Bio-Rad Laboratories Ltd., Watford, Herts. Iodoacetic acid was obtained from Koch-Light Laboratories Ltd., Colnbrook. Acetonitrile (S grade), ethyl acetate (sequencing grade) and phenylisothiocyanate (PITC) were obtained from Rathburn Chemicals Ltd., Peeblesshire, Scotland. Schleicher and Schuell micropolyamide foils were from Anderman and Co., Ltd., E. Molesey, Surrey; Kieselgel cellulose 60 thin layer chromatography (tlc) plates (without fluorescent indicator) were from Merck, Darmstadt, Germany; Sequence grade trifluoroacetic acid (TFA) was obtained from Fluka products, through Fluorochem Ltd., Glossop, Derbyshire; Sephadex G-50 super-fine was from Pharmacia, Hounslow, Middlesex. Ammonium sulphate (especially low in heavy metals), 4-N, N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC), 4-dimethylaminobenzaldehyde (Analar), guanidine Hcl (Aristar), n-heptane (hplc grade) and all other basic laboratory reagents were obtained from B.D.H. Laboratories, Poole, Dorset.

2.2.2 Purification of 5-Aminolevulinic Acid Dehydratase from Human Erythrocytes

All purification procedures were carried out at 4°C and all buffers contained 20 mM 2-mercaptoethanol unless otherwise stated.

Preparation of Crude Extract

5 units of whole blood (2.4 litres) were centrifuged at 5,000 rpm (7,000 x g) for 15 minutes in a Sorvall RC-3B centrifuge (4 x 1 litre rotor). The plasma was removed by aspiration and the cells were washed by gentle stirring with an equal volume of 0.9% sodium chloride containing 1 mM 2-mercaptoethanol. The washing process was repeated twice.

The packed red cells (1.2 litres) were lysed by adding an equal volume of deionised water containing 40 mM 2-mercaptoethanol. The

mixture was then subjected to ultrasonic sonication in 800 ml portions for 30 seconds at low power and 30 seconds at high power using an MSE Ultrasonication apparatus (Soniprep 150) fitted with a 20 mm diameter probe. Cell debris was removed by centrifugation at 10,000 rpm (18,000 x g) for 45 minutes in an MSE 21 centrifuge (6 x 500 ml rotor) and 1M potassium phosphate buffer (pH 7.5) was added to the supernatant to give a final phosphate concentration of 10 mM.

Removal of Hemoglobin

The extract was carefully stirred into DEAE Biogel A (600 ml packed wet volume) previously equilibrated in 10 mM potassium phosphate buffer (pH 7.5). Stirring was continued for 90 minutes using a Gallenkamp overhead stirrer with a Z-shaped glass rod (at 25 rpm) after which time all the 5-aminolevulinic acid dehydratase had bound to the resin. The suspension was then centrifuged at 5,000 rpm (7,000 x g) for 5 minutes in a Sorvall RC-3B centrifuge (4 x 1 litre rotor) and the supernatant was discarded. The DEAE Biogel cake was washed four times in 3.5 litres of 10 mM potassium phosphate buffer (pH 7.5) followed by centrifugation in each case. Weakly bound proteins were removed from the DEAE Biogel by stirring the gel for 10 minutes in 3.5 litres of 10 mM potassium phosphate buffer (pH 7.5) containing 50 mM KCl. The suspension was centrifuged at 5,000 rpm (7,000 x g) for 5 minutes in a Sorvall RC-3B centrifuge (4 x 1 litre rotor) and the supernatant was discarded. This process was repeated three times. The enzyme was then eluted with four 500 ml portions of 10 mM potassium phosphate buffer (pH 7.5) containing 350 mM KCl or until all the enzyme activity had been recovered. The active enzyme fractions were pooled by precipitation with $(NH_4)_2SO_4$ (60% saturation) and the precipitate collected by centrifugation at 10,000 rpm (18,000 x g) for 25 minutes in an MSE 21 centrifuge (6 x 500 ml rotor). The pellet was resuspended in 0.1M potassium phosphate buffer (pH 6.8) containing 25 mM 2-mercaptoethanol to give a protein concentration of $\sim$30 mgs/ml (as determined by the optical density at 280 nm). This stage removes almost all the hemoglobin which greatly hinders the purification of most erythrocyte enzymes.

Heat Treatment

The enzyme solution was heated to 60°C in a 250 ml conical flask using a water bath held at 75°C. The temperature was maintained for 3 minutes after which the flask was cooled rapidly in an ice/salt/water mixture until the temperature was below 10°C. Denatured protein was removed by centrifugation at 20,000 rpm (50,000 x g) for 10 minutes in an MSE 21 centrifuge (8 x 50 ml rotor).

Ammonium Sulphate Fractionation

The supernatant was treated with $(NH_4)_2SO_4$ (30% saturation) and the precipitate was collected by centrifugation at 20,000 rpm (50,000 x g) for 10 minutes. The pellet was resuspended in 35 mls of 30 mM potassium phosphate buffer (pH 7.9) and the enzyme was dialysed overnight against 20 litres of the same buffer.

Chromatography on DEAE Biogel A

The dialysed enzyme was applied to a DEAE Biogel column (5.5 cm x 8 cm high) which had been equilibrated previously with 30 mM potassium phosphate buffer (pH 7.9) and the column was washed to remove all unbound protein. A linear gradient (600 mls; 0 to 0.4M KCl in 30 mM potassium phosphate buffer, pH 7.9) was applied to the column and the active fractions were pooled, precipitated with $(NH_4)_2SO_4$ (60% saturation) and the precipitate collected by centrifugation at 20,000 rpm (50,000 x g) for 10 min in an MSE 21 centrifuge (8 x 50 ml rotor). The pellet was resuspended in 20 mls of 50 mM potassium phosphate buffer (pH 6.8) and was dialysed against 20 litres of the same buffer overnight.

Chromatography on Hydroxylapatite

The dialysed enzyme was applied to a column of hydroxylapatite (5.5. cm x 8 cm high) previously equilibrated with 50 mM potassium phosphate buffer (pH 6.8) and the column was washed with 1 litre of the same buffer to remove unbound protein. A linear gradient (500 mls; 50 mM to 300 mM potassium phosphate buffer (pH 6.8)) was applied to the column and 10 ml fractions were collected. The active fractions were pooled and the enzyme was collected by precipitation with $(NH_4)_2SO_4$ (60% saturation) and centrifuged as above. The pellet was

resuspended in 3 mls of 60 mM potassium phosphate buffer (pH 6.8) containing 0.5M KCl.

Chromatography on Biogel A 0.5 m

The enzyme from the previous stage was applied to a Biogel A 0.5 m gel filtration column (2.8 cm x 1 metre) and the column was developed in the same buffer. Fractions with enzymic activity were pooled, precipitated with $(NH_4)_2SO_4$ (60% saturation), and the precipitate was collected by centrifugation at 20,000 rpm (50,000 x g) for 10 minutes in an MSE 21 centrifuge (8 x 50 ml rotor).

The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol and 0.1 mM zinc and was incubated at 37°C for 15 minutes to fully activate the enzyme. The enzyme was stored as an ammonium sulphate precipitate under nitrogen in a sealed screw top centrifuge tube at 4°C.

This method yields 27.5 mgs of pure enzyme protein (as determined by polyacrylamide gel electrophoresis) with 65% recovery of specific activity 24.0 units/mg protein in the presence of 0.1 mM zinc (18.8 U/mg in the absence of added zinc).

Note: Larger quantities of the enzyme have been isolated by processing 10 units of whole blood (5.3 litres) using the same method and 81 mg of purified enzyme protein was obtained with a specific activity of 22.1 U/mg in the presence of 0.1 mM zinc. The overall yield was 74%.

2.2.3 Isolation of 5-Aminolevulinic Acid Dehydratase from Bovine Liver.

The enzyme was purified from 4 kgs of bovine liver according to the method of Seehra (1980) to yield 308 mg of pure enzyme protein with 33% recovery of specific activity 19.0 units/mg. (1 unit of enzyme activity is defined as that required to produce 1 µmol of porphobilinogen per hour at 37°C).

2.2.4 Assay for Enzymic Activity.

The incubations contained potassium phosphate buffer, pH 6.8 (100 µmoles), dithioerythritol (10 µmoles), activated 5-aminolevulinic

acid dehydratase (max. 0.015 units) and 5-aminolevulinic acid (5 µmoles), neutralised with 0.1 M NaOH prior to use, in a final volume of 1 ml. The incubations were carried out at 37°C for 10 minutes and were terminated by the addition of a solution (1 ml) of trichloroacetic acid (10% w/v) containing mercuric chloride (0.1M). The solution was centrifuged and the supernatant was added to an equal volume of freshly prepared modified Ehrlich's reagent (4-dimethylaminobenzaldehyde (1g) in acetic acid (40 ml) and perchloric acid (60-62% w/v; 10 ml)). The coloured complex formed with porphobilinogen was measured spectrophotometrically (λ_{max} = 555 nm; ε_{555} = 6.02 x 10^4 M^{-1} cm^{-1}) (Mauzerall and Granick, 1956).

When the enzyme activity was assayed in the absence of thiol, the incubations were carried out as described above except that all buffers were saturated with nitrogen prior to use. Incubations were carried out in stoppered tubes for 2 minutes under an atmosphere of nitrogen with up to 0.05 units of enzyme.

2.2.5 Determination of the Purity of Human Erythrocyte 5-Aminolevulinic Acid Dehydratase.

(a) 5% Polyacrylamide Gel Electrophoresis.

5% Polyacrylamide cylinder gels (6.3 mm x 12 cm) were prepared by mixing 2.25 mls of solution A, 3 mls of solution B and 12.75 mls of water and degassing for 1 minute using a water pump. After the addition of ammonium persulphate (15 mg), the solution was poured into the gels and allowed to set. After the addition of a few crystals of sucrose and the tracking dye, bromophenol blue, the enzyme solution (5-100 µgs) was applied to the top of the gels and electrophoresis was performed at 5 mA/gel for 4 - 5 hours in a running buffer containing Tris (28.87 g/l; adjusted to pH 8.3 with 1N Hcl).

Solution A : Tris (363 g/l) + TEMED (4.6 ml/l) adjusted to pH 8.9 with 1N Hcl.

Solution B : Acrylamide (300 g/l) + Bis acrylamide (8.2 g/l).

Detection of 5-aminolevulinic acid dehydratase activity was accomplished by incubating the gel for 30 minutes at 37°C in 5 mls 0.1 M potassium phosphate buffer (pH 6.8) containing 20 mM 2-mercaptoethanol and 5 mM ALA. The porphobilinogen produced during

the incubation was visualised by the addition of 2.5 ml 0.1M mercuric chloride containing 10% w/v trichloroacetic acid, followed by 2.5 ml modified Ehrlich's reagent (Mauzerall and Granick, 1956).

(b) Isoelectric Focussing.

Isoelectric focussing of the purified dehydratase enzyme was performed on LKB Ampholine polyacrylamide gel plates (pH 3.5 - 9.5) (LKB 1804 - 101). An LKB 2117 Multiphor cooling plate was used as the support and the template maintained at 10°C by a Grant LB 50 refrigeration unit. White spirit was used as insulating fluid between cooling plate and template, and between template and the gel plate. The cathode electrode strip (soaked in 1M NaOH) and anode electrode strip (soaked in 1M phosphoric acid) were placed on the plate and the samples applied (≈15µl) on a sample application paper (5 x 10 mm). The samples were electrofocussed across the width of the plate. After 45 minutes the sample application pieces were removed and the electrophoresis continued; total running time was 1.5 hours. The pH gradient across the gel was measured with pH indicator paper, and after measurement, the zones were sharpened for 10 minutes. The power was supplied by an LKB 2103 power pack set at 15W. The voltage was limited to 1500 V and the current to 50 mA.

After focussing, the electrode strips were removed and the gel fixed for 1 hour in fixing solution (57.5g trichloroacetic acid and 17.25g sulphosalicylic acid in 500 ml distilled water). After fixing, the plate was washed for 5 minutes in destain (ethanol: acetic acid: water; 25:8:67, by vol) and stained for 10 minutes at 60°C in Kanacid blue R (0.46 g in 400 ml destain). The plate was then destained with several changes of destain for 24 hours. After this the plate was placed in preserving solution (40 ml glycerol in 400 ml destain) and dried.

2.2.6 Determination of the Molecular Weight of Human Erythrocyte δ-Aminolevulinic Acid Dehydratase.

The molecular weight of the native human enzyme was determined by applying 7 mg of enzyme (200 nmoles; 1ml) to a Biogel A 0.5m gel filtration column as described in Section 2.2.2. The elution

volume (V_e) of the enzyme was compared to those of proteins of known molecular weight.

The subunit molecular weight of the human dehydratase enzyme was determined by polyacrylamide gel electrophoresis (15% w/v acrylamide gels) under denaturing conditions (6M urea and 1% w/v SDS) according to the method of Weber and Osborn (1969). The relative mobility (Rf) of the enzyme was compared to the mobilities of standard proteins of known molecular weights.

The molecular weight standards used were: Urease (native MR 479,000), catalase (native MR 252,000), muscle aldolase (native MR 158,000), lactate dehydrogenase (native MR 140,000), bovine serum albumin (native MR 68,000), opsin (subunit MR 40,000), trypsin (subunit MR 23,300) and cytochrome c (subunit MR 11,700).

2.2.7 Purification of Solvents.

Highly purified solvents required for hplc and peptide sequencing were prepared as described below: Benzene was distilled twice using conventional distillation apparatus and the middle fraction (B.pt = 80.5°C) was collected each time. Butyl acetate was washed with dilute H_2SO_4 (0.1 M), dried over K_2CO_3, distilled from $KMnO_4$ (B.pt. = 124 - 125°C) and further dried with K_2CO_3. PITC was distilled under vacuum and water was double distilled using a glass distillation apparatus. Pyridine was distilled twice, first from calcium hydride (CaH_2) and then alone (B.pt. = 116°C). Methanol (technical grade) was distilled over $NaBH_4$. If the solvents were used for hplc they were filtered and degassed using a Sabata Micro Filter fitted with a multi-purpose PTFE filter (attached to a water pump) immediately prior to use.

2.2.8 Enzymic Synthesis of Porphobilinogen.

5-Aminolevulinic acid dehydratase (500 units) was incubated at 37°C for 15 minutes in 5 ml 0.1M potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol and added to 2 litres 10 mM potassium phosphate buffer containing 10 mM dithioerythritol (nitrogen saturated). ALA (1g), adjusted to pH 6.8 by the dropwise addition of 0.1 N NaOH,

was added and the mixture was incubated for 17 hours at 37°C in the dark under an atmosphere of nitrogen. A yield of 0.71g porphobilinogen was obtained as determined by reaction with modified Ehrlich's reagent (Mauzerall and Granick, 1956). The pH of the incubation solution was adjusted to 7.5 using 0.1N NaOH and slowly applied to a column (2 x 12 cm) of Dowex 1 x 8 (200 - 400 mesh) acetate. The column was washed with 1 litre of water, the porphobilinogen was eluted with 1M acetic acid and was isolated by freeze-drying. The porphobilinogen was further purified by isoelectric point recrystallization (in a minimum volume) from dilute ammonia by adjusting the pH to 5.5 with 0.1M acetic acid. The crystals were filtered and washed consecutively with ice-cold methanol and dry ether and were stored at -20°C under vacuum.

2.2.9 Modification of 5-Aminolevulinic Acid Dehydratase with $NaBH_4$ in the Presence of [4-^{14}C] ALA.

Initial experiments were carried out to determine optimal conditions for the incorporation of the ^{14}C-labelled ALA into the enzyme protein. In one experiment, stoichiometric equivalents of fully activated enzyme (see Section 3.2.2) and [4-^{14}C] ALA were mixed in 0.1M potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol at 0°C in a rapid mixing device so that the enzyme and substrate interacted for ≈ 100 msec before quenching the reaction into 1M $NaBH_4$. The pH of the reaction mixture was maintained at 6.8 for 30 minutes by the frequent additions of 1M acetic acid. Alternatively, the enzyme and labelled substrate (1 mole ALA/mole of subunits) were mixed at 0°C over a range of pH's (5, 6, 7, 7.9 and 8.7) in buffer containing 10 mM dithioerythritol and, after ≈ 10 seconds, 20 µl of 0.5M $NaBH_4$ was added followed by 20 µl of 0.5M acetic acid (see Gurba et al., 1972). These additions were repeated at 10 minute intervals for 30 minutes and the reaction was terminated by the addition of $(NH_4)_2SO_4$ (60% saturation). The precipated protein samples were collected by centrifugation at 21,000 rpm (53,290 x g) for 10 minutes in an MSE 21 centrifuge (24 x 14 ml rotor), dissolved in 0.1M potassium phosphate buffer (pH 6.8; 1ml) and dialysed exhaustively against the same buffer (5 x 5 litres). The protein concentration and radioactivity bound to the enzyme was determined as described in Sections 2.2.19 and 2.2.20.

From these data optimal conditions for the incorporation of labelled ALA into the enzyme protein were determined for the modification of large quantities of 5-aminolevulinic acid dehydratase and were as follows: The ammonium sulphate pellet of the dehydratase enzyme (33 mgs; 940 nmoles) was resuspended in 2 mls 0.1M potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol, incubated for 15 minutes at 37°C and dialysed overnight against 0.15M potassium phosphate buffer containing 10 mM dithioerythritol (3 x 250 mls) (pH 7.9). The enzyme solution was diluted to a final protein concentration of 10 mg/ml (ε_{280} = 28,570 $M^{-1}cm^{-1}$) using the same buffer and left at 0°C for 10 minutes. [4-^{14}C] ALA (50 µCi; 940 nmoles; 1 mole ^{14}C-ALA/mole subunits) was neutralised using 1N NaOH and was mixed with the enzyme at 0°C in the presence or absence of porphobilinogen (10 mM). After ≈ 10 seconds, 20 µls of 0.5M $NaBH_4$ was added followed by 20 µls of 0.5M acetic acid and the pH maintained at 7.9. This process was repeated three times at 10 minute intervals and, after a further 10 minutes, the reaction was terminated by the addition of 6 mls 0.15M potassium phosphate buffer (pH 7.9) containing 10 mM dithioerythritol. The enzyme was precipitated by the addition of $(NH_4)_2SO_4$ (60% saturation) and centrifuged as before. The supernatant, containing unbound "dihydro" ALA, was discarded and the enzyme pellet was resuspended in a saturated solution of $(NH_4)_2SO_4$ (10 ml) containing 20 mM 2-mercaptoethanol. The precipitate was collected as before and the washing procedure was repeated twice. The final enzyme pellet was resuspended in 2 mls 10 mM potassium phosphate buffer (pH 7.9) containing 20 mM 2-mercaptoethanol and dialysed against 2 x 5 litres of the same buffer overnight.

In order to ensure that all the available sites on the enzyme were modified an excess of unlabelled ALA (31.6 mgs; 188 µmoles; 200 moles ALA/mole subunits) was mixed with labelled enzyme at 0°C (pH 7.9) and treated sequentially with aliquots of $NaBH_4$ and acetic acid as before. The modified enzyme protein was precipitated using $(NH_4)_2SO_4$ (60% saturation), collected by centrifugation and dialysed extensively against 0.1M potassium phosphate buffer (pH 6.8) (5 x 5 litres) containing 20 mM 2-mercaptoethanol.

2.2.10 Carboxymethylation of 5-Aminolevulinic Acid Dehydratase.

The ^{14}C-labelled enzyme (33 mgs; 940 nmoles; 5-14 x 10^3 dpm/nmole) was resuspended in 0.1M potassium phosphate buffer (pH 6.8; 3.3 mls) containing 30 mM dithioerythritol (4.62 mg/ml), 0.3M $NaHCO_3$ (28 mg/ml), 7.33M guanidine Hcl (0.7 g/ml) and was incubated for 20 minutes at 37°C in the dark. The thiol groups were modified by the addition of 1M iodoacetic acid (186 mg/ml; 330 µl; adjusted to pH 7 with 1N NaOH) followed by incubation for 5 hours at 37°C in the dark and under nitrogen. The reaction was terminated by the addition of an equal volume of 0.1M potassium phosphate buffer (pH 6.8) containing 50 mM dithioerythritol. The enzyme was dialysed against 5 x 5 litres distilled water and freeze-dried.

2.2.11 Digestion of 5-Aminolevulinic Acid Dehydratase by Cyanogen Bromide (CNBr).

The modified enzyme (33 mgs; 940 nmoles) was dissolved in 3.3 mls of 70% (v/v) aqueous formic acid and stirred for 18 hours in the dark at room temperature with a 400-fold molar excess of CNBr (40 mgs; 377 µmoles). The reaction was terminated by the addition of distilled water (16.5 mls), stirred for 2 - 3 hours in the dark at room temperature and freeze-dried.

2.2.12 Separation of the CNBr Peptides by Sephadex G-50 Super-fine Gel Filtration.

The CNBr peptides were suspended in 3 mls of 9% (v/v) aqueous formic acid, applied to a Sephadex G-50 super-fine gel filtration column (2.8 cm x 1 metre) and developed at a flow rate of 0.25 ml/min. Fractions (2.5 ml) were collected and assayed for both protein concentration and radioactivity. The fractions containing radioactivity were pooled and freeze-dried.

2.2.13 Methods Used to Purify the Mammalian Dehydratase Active Site Peptides.

(a) High Performance Liquid Chromatography (hplc).

A Waters' Associates Liquid Chromatography System, fitted with a Model U6K injector, a Model 450 variable wavelength detector set at 230 nm and a Model 660 solvent programmer controlling both a Model 6000A

(pump A) and a M45 (pump B) solvent delivery system, was used for all separations. The active site peptides were initially purified on a Waters' C-18 μ-Bondapak reverse phase hplc column (3.9 mm x 30 cm) in the following solvent system:

Solvent A : 0.12% (v/v) trifluoroacetic acid in water.

Solvent B : 0.07% (v/v) trifluoroacetic acid in acetonitrile.

Initial separations were performed using a linear gradient (0 - 60% B) over a period of 20 minutes at a flow rate of 1.2 ml/min and subsequently modified in order to increase the resolution as detailed in the Results (Section 2.3.4).

(b) Thin Layer Electrophoresis.

The freeze-dried peptides (10 - 200 nmole) from the hplc column were dissolved in 0.1% (v/v) aqueous trifluoroacetic acid and applied to a Kieselgel cellulose 60 tlc plate (0.25 mm thick x 10 cm x 20 cm). The tlc plate was sprayed evenly with 0.1M ammonium bicarbonate (pH8) from a distance of about 1 metre using a Shandon spray gun and electro--phoresed for $\frac{1}{2}$ - 2 hours at 1 kV. The peptides were visualised by spraying a small area of the plate with ninhydrin (0.2% w/v in acetone). The radioactivity associated with the peptides was determined either by scanning the tlc plate using a Berthold tlc plate scanner or by scraping off 1 cm bands and mixing them in 1 ml methanol for 2 hours before measuring the radioactivity (see Section 2.2.19). The separation of amino acids by thin layer electrophoresis was identical to the method described above except that the amino acids (1 - 10 nmole) were dissolved in methanol (Smith, 1976).

2.2.14 Amino Acid Analysis of Peptides and Proteins.

Various peptide and protein samples (10 - 30 nmole) were placed in drawn test-tubes (1 x 6 cm) and 0.5 ml Hcl (6M) was added. Oxygen was removed from the sample by alternatively flushing the tube with N_2 and applying a vacuum (4 - 5 times). The sample was sealed under vacuum, hydrolysed for 18 hours at 110°C and the Hcl removed in vacuo. The residue was dissolved in 1 ml dilute Hcl (10 mM) and analysed on a Rank Hilger J 180 automated amino acid analyser in the presence of a norleucine internal standard. The amount of each amino acid was corrected to allow for the loss of water in the formation of the peptide

bond. When labelled peptides or proteins were analysed, fractions were collected at one minute intervals and the radioactivity in each fraction determined as described in Section 2.2.19.

2.2.15 Sequence Determination of Active Site Peptides.

The sequence analysis was carried out according to the method of Chang et al., (1978) as modified by Mullen et al., (1981). The peptide (5 - 20 nmole) was dissolved in 80 µl pyridine: water (1:1; v/v) in a pyrex vial (35 mm x 4.5 mm internal diameter) and mixed with a solution of DABITC in pyridine (40 µl; 1 mg of reagent dissolved in 180 µl pyridine). After flushing with N_2, the vial was stoppered and incubated for 30 minutes at 54°C in a heating block, then further treated with PITC (10 µl) for 30 minutes under the same conditions to allow the modification of amino groups remaining unreacted in the first coupling step. Excess reagents were removed by several washings (4 - 5) with benzene: pyridine (20:1; v/v; 300 µl each) followed by three more washes with ethyl acetate: heptane (1:2; v/v; 300 µl each). In each washing step the organic layer was separated from the aqueous layer (after thorough mixing using a Rotamixer) by centrifugation at 6,000 x g for 1 minute in an Anderman Eppendorf centrifuge (Model 5414). After removal of the organic layer with a drawn pasteur the walls of the vial were carefully washed with 150 µl of the same solvent mixture to remove any remaining reagent. This latter operation was performed without disturbing the aqueous layer hence obviating the requirement for further centrifugation. The aqueous phase was evaporated to dryness under vacuum in a heated block at about 50°C and the residue dissolved in 80 µl trifluoroacetic acid (TFA). The vial was flushed with N_2, stoppered and incubated at 54°C in the heating block for 12.5 minutes in order to cleave the modified N-terminal amino acid. The TFA was evaporated using a steady stream of N_2 and the residue taken up in a 20 µl water. The yellow coloured 4-N, N-dimethylamino azobenzene thiazolinone (DABTZ) and the colourless phenylthiohydantoin (PTH) derivatives were extracted with butyl acetate (2 x 150 µl) and the aqueous phase was evaporated to dryness in vacuo. This residue was again taken up in 80 µl pyridine: water (1:1; v/v) for the beginning of another cycle.

The butyl acetate extract was evaporated to dryness (in vacuo), dissolved in 30 µl TFA: water (1:1; v/v), stoppered under an atmosphere of N_2 and heated to 80°C for 12.5 minutes in a heating block. This procedure cyclised the thiazolinone (DABTZ) derivative to its corresponding 4-N, N-dimethylaminoazobenzene thiohydantoin (DABTH) derivative which could then be identified (see Section 2.2.16). The mixture was evaporated to dryness (in vacuo) and taken up in ethanol (10 µl).

Before sequencing any unknown peptides, however, insulin chain A (oxidised form) was always sequenced for at least six turns with an appropriate blank to ensure that all reagents were functioning and that the carry-over from one turn to the next was at an acceptable level.

2.2.16 Identification of DABTH-Derivatives by Two Dimensional tlc.

The DABTH-derivative was spotted in the left hand corner (≈ 3 mm from both edges) of a Schleicher and Schuell polyamide sheet (2.5 x 2.5 cm) and run in acetic acid: water (1:2; v/v) in the first dimension followed by toluene: hexane: acetic acid (4:2:1; by vol) in the second. The plates were developed in Hcl vapour to yield a red spot which was compared against standard DABTH-amino acid derivatives (see Chang et al., 1978) (Scheme 2.16).

Several precautions were taken to ensure that the unknown amino acid derivative was identified correctly. Firstly, the solvents used for the two dimensional tlc were made freshly each day and stored in opaque stoppered reagent bottles. Secondly, fresh solvent mixtures were used for each tlc run to ensure that its composition did not change due to evaporation of the solvents at different rates. Thirdly, the plates were placed vertically in each solvent mixture and finally, standard DABTH-amino acid derivatives were spotted on the back of the tlc plate to confirm that they co-chromatographed with the unknown derivative.

2.2.17 Preparation of DABTH-Derivatives of Standard Amino Acids.

The standard amino acid derivatives were freshly prepared for each sequencing run as follows: The amino acids (0.5 µmol) were dissolved in 100 µl of buffer containing triethylamine:water:acetone (2.4:47.6:50; by vol.), adjusted to pH 10.1 with 1M acetic acid.

After the addition of an equal volume of DABITC (100 µl; 28.2 mgs per ml of pyridine) the reaction vessel was flushed with N_2, stoppered and incubated at 50°C for 1 hour. Excess reagent was removed by washing the reaction mixture twice with 1 ml benzene:pyridine (20:1; v/v) and once with 1 ml ethyl acetate:heptane (1:2; v/v). The mixture containing the DABTZ derivative was evaporated to dryness under vacuum and the residue taken up in 40 µl TFA:water (1:1; v/v). The vial was stoppered under an atmosphere of N_2 and incubated for 12.5 minutes at 80°C. The standard DABTH derivatives were dried (in vacuo) and dissolved in 100 µl ethanol prior to use.

2.2.18 Resolution of PTH-Derivatives of Amino Acids by hplc.

The Waters' Associates Liquid Chromatography System was used essentially as described in Section 2.2.13 but with the following modifications. The Model 450 variable wavelength detector was set at 268 nm (not 230 nm) and pumps A and B were controlled by a Waters' Associates System Controller. The PTH-derivatives of standard amino acids were resolved by reverse phase chromatography on a Waters' C-18 µ-Bondapack hplc column (3.9 mm x 30 cm) using the following solvent system:

Solvent A: 10 mM Sodium acetate buffer (pH 4.16).

Solvent B: Redistilled methanol (technical grade).

The System Controller was programmed at a flow rate of 2 ml/min to produce a series of linear gradients over a period of 23 minutes as outlined below before returning to the initial conditions in order to equilibrate the column ready for the next sample. (A typical separation of standard PTH-derivatives is shown in Scheme 2.15).

Time After Injection (mins)	% B
0	14
4	22
10	22
14	48
23	50
27	14
32	14

The unknown PTH-derivatives (obtained from sequencing) were applied to the C-18 μ-Bondapak column under the same conditions and the elution profile compared to that of the standard derivatives. If the sample was radioactively labelled, fractions were collected at minute intervals and the radioactivity associated with each fraction was determined as described below.

2.2.19 Counting of Radioactive Samples.

All determinations were made using a Phillips P4700 Scintillation Spectrophotometer programmed to give a quench correction to dpm. Non-aqueous and aqueous samples up to 200 μl in volume were counted in 10 ml toluene:methanol (3:1; v/v) containing butyl PBD (5-(4-biphenyl) -2-(4-tert-butylphenyl)-1, 3, 4 oxadiazoline) (6 g/litre), whilst aqueous samples with volumes up to 2 ml were counted in the scintillation fluid "Tritoscint", composed of xylene (2 litres), synperionic NXP (1 litre), 2, 5-diphenyloxazole and 1, 4-bis-(4-methyl-5-phenyloxazol-2-yl) (1.5 g).

2.2.20 Protein Determinations

Protein concentrations were determined by the methods of Bradford (1976), Lowry et al., (1951) or by the measurement of the absorbance at 280 nm against the appropriate blank (ε_{280} = 28,570 M^{-1} cm^{-1} using a subunit molecular weight of 35,000). Standardization of these different methods was achieved by determining the absorbance of a known amount of the purified dehydratase enzyme after exhaustive dialysis against distilled water followed by lyophilisation.

2.3 Results and Discussion.

2.3.1 Purification and Some Properties of 5-Aminolevulinic Dehydratase from Human Erythrocytes.

A new method for the isolation of 5-aminolevulinic acid dehydratase from human erythrocytes has been developed, which for the purposes of clarity has been detailed in the Methods section (Section 2.2.2). Using this new technique 5-aminolevulinic acid dehydratase was purified ≈ 35,000 fold from 5 units of human erythrocytes to yield 27.5 mgs of enzyme protein with a recovery of 65%. The final specific activity was 24.0 units/mg when assayed in the presence of 0.1 mM zinc (18.8 units/mg in the absence of added zinc). A typical purification is shown in Table 2.1. Based on the specific activity (24.0 units/mg) a turnover number (K_{CAT}) of 14.0 moles of porphobilinogen per minute was calculated, assuming that all the enzyme subunits were catalytically viable.

This purification procedure (see Section 2.2.2) was far superior to the three-step purification of Despaux *et al.*, (1979) and Bustos *et al.*, (1980) (using affinity chromatography) and gave the highest specific activity reported for this enzyme. Also the overall yield of enzyme protein (27.5 mgs from 5 units of blood) was much better than that obtained by Anderson and Desnick (1979) (30 mgs from ≈ 18 units of blood) despite their claim that they had recovered 69% of the activity present in the erythrocyte lysate. Probably a more realistic yield was that obtained by Sassa and Kappas (1983) of 19% (6 mgs of protein from ≈ 5 units of blood) who used the method of Anderson and Desnick (1979) essentially as described. The advantage of the method presented in Section 2.2.2 appears to be due to the rapid removal (≈ 3 hours) of haemoglobin from the enzyme solution using a DEAE Biogel batch elution technique and is far more rapid than loading the erythrocyte lysate onto a column which takes ≈ 30 hours using the method of Anderson and Desnick (1979). Also, using the methodology of Anderson and Desnick it was found that some 20 - 25% of the enzyme did not bind to the DEAE column (data not presented).

Polyacrylamide gel electrophoresis of the purified erythrocyte enzyme was performed in the presence (Weber and Osborn, 1969) or absence of SDS (Sections 2.2.5 and 2.2.6) and clearly demonstrated that the enzymic activity was associated with a single protein band (Scheme 2.1). These results indicated that the enzyme was essentially

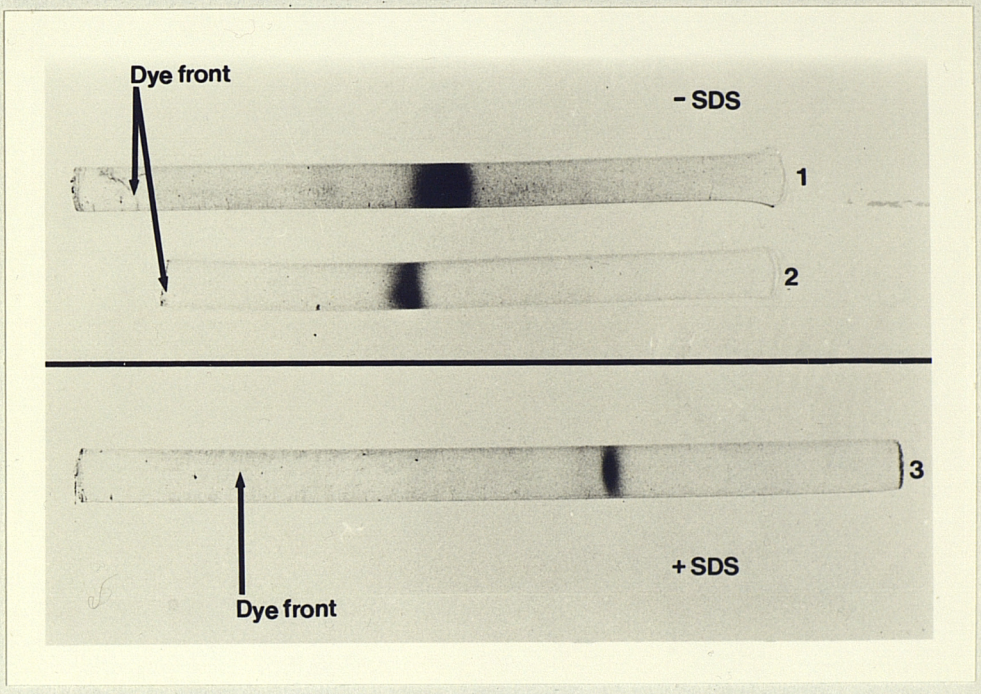
STAGE	(ml) VOLUME	(u) TOTAL UNITS	TOTAL PROTEIN	SPECIFIC ACTIVITY	(%) YIELD	PURIFICATION FACTOR
Whole Blood	2,360	N.D.	1.463 g	-	-	
Lysed + sonicated erythrocytes	2,290	578	996 g	58×10^{-5}	-	1.5
Batch DEAE Biogel (pH 7.5)	2,240	793	2.74 g	0.29	100	530
60°C Heat Treatment	175	666	795 mg	0.84	84	1,550
0 → 30% $(NH_4)_2SO_4$	36	633	382 mg	1.66	80	3,100
DEAE Biogel (pH 7.9)	97	605	151 mg	4.01	76	7,400
Hydroxyl Apatite (pH 6.8)	84	574	34.8 mg	16.5	72	30,300
Biogel A 0.5 m (- zinc) Gel filtration (+ 0.1 mM zinc)	14.7	517	27.5 mg	18.8	65	34,600
		660		24.0		44,200

1 Unit = 1 μmole Porphobilinogen produced/hour @ 37°C

Table 2.1 Purification of 5-Aminolevulinic Acid Dehydratase from Human Erythrocytes

Scheme 2.1 Polyacrylamide Gel Electrophoresis of Human Erythrocyte 5-Aminolevulinic Acid Dehydratase

Polyacrylamide gel electrophoresis of the human enzyme was performed in the presence (Weber and Osborn, 1969) or absence of SDS (Section 2.2.5). Enzymic activity was detected as described in Section 2.2.5 and was shown to be located in a single protein band. The gels were stained according to the method of Weber and Osborn (1969). Gels 1 and 2 were 5% non SDS polyacrylamide gels of the native (40 µg) and carboxymethylated (25 µg) enzymes respectively. Gel 3 was a 15% SDS gel of the denatured enzyme protein (20 µg).

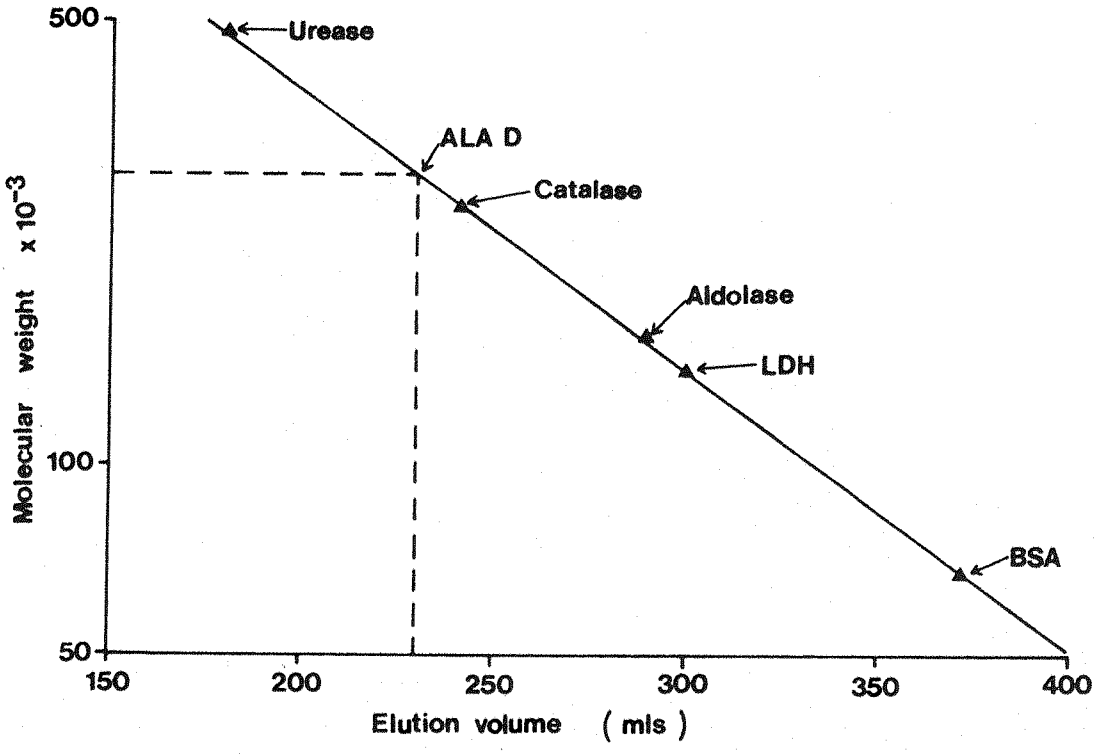


homogenous. Interestingly on carboxymethylation, the native enzyme electrophoresed as a more compact protein band when compared with that of the native enzyme. This has been attributed to the partial oxidation of the native enzyme during electrophoresis which results in a fairly broad protein band.

The enzyme was also assayed for possible minor contaminating activities from other enzymes of the haem biosynthetic pathway, such as 5-aminolevulinic acid synthetase (Laghai, 1977) and porphobilinogen deaminase (Berry, 1983), but no activity was detected even when incubations were carried out for 1 hour at 37°C with up to 100 µg protein. A single isoelectric point of 4.85 ± 0.20 (see Section 2.2.5) was obtained for the human enzyme and was in good agreement with the value of 4.9 obtained by Anderson and Desnick (1979).

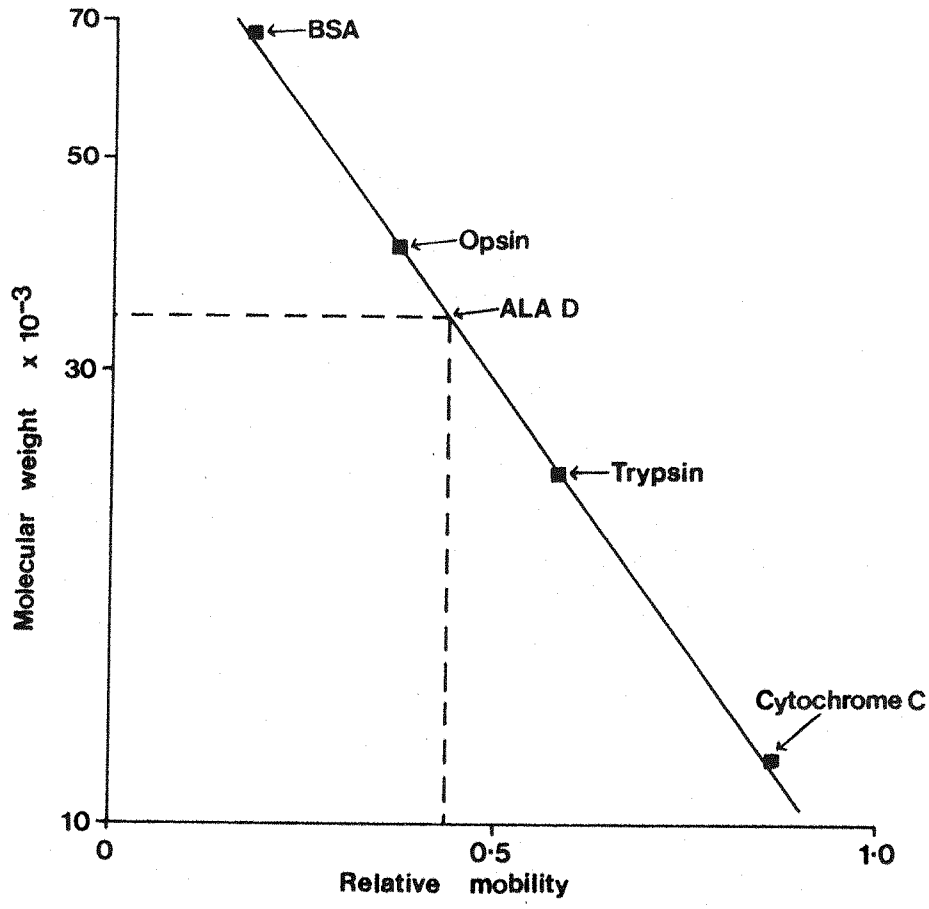
2.3.1.1 Molecular Properties of the Human Dehydratase Enzyme.

The molecular weight of the native enzyme was determined by comparing the elution volume (V_e) of the enzyme on a Biogel A 0.5m gel filtration column with the values obtained from proteins of known molecular weights (Section 2.2.6). A semilogarithmic plot of the elution volumes of the standard proteins produced a straight line (Scheme 2.2) and by extrapolating the value of the elution volume (230 ml) obtained for the human enzyme to the absicca a native molecular weight of 285,000 was obtained. Similarly, the subunit molecular weight of the enzyme was determined by comparing its relative mobility (R_f) on 15% polyacyrlamide gels containing 0.1% w/v SDS (Weber and Osborn, 1969) with those of standard protein solutions. From these data (presented in Scheme 2.3) a subunit molecular weight of 35,000 was obtained. These values for the human enzyme are in close agreement with data obtained by other groups (Despaux et al., 1979; Sassa and Kappas, 1983) but differ significantly from the values obtained by Anderson and Desnick (1979) (native MR 252,000; subunit MR 31,000). It therefore appears that, like the bovine enzyme (Wu et al., 1974b; Tsukamoto et al., 1975), the human enzyme is octameric in nature and is composed of eight identical subunits in a cubic octameric structure with dihedral (D_4) symmetry. This proposal has been corroborated by Despaux et al., (1979) who used electron microscopy to determine the quarternary structure of the human enzyme.



Scheme 2.2 Determination of the Molecular Weight of Native Human 5-Aminolevulinic Acid Dehydratase by Biogel A 0.5m Gel Filtration.

The elution volume of the human enzyme on a column of Biogel A 0.5m (2.8 cm x 1 metre) was compared to those of proteins of known molecular weights (see Section 2.2.6 for further details). A value of 285,000 was obtained.



Scheme 2.3 Determination of the Subunit Molecular Weight of the Human Dehydratase Enzyme Using SDS Electrophoresis.

The relative mobility of the dissociated enzyme on 15% polyacrylamide cylinder gels (6.3 mm x 12 cm) containing 0.1% w/v SDS was compared with molecular weight markers (see Section 2.2.6 for further details). From these data a value of 35,000 was calculated.

The amino acid composition of purified human 5-aminolevulinic acid dehydratase was determined as described in Section 2.2.14 and compared with the bovine liver enzyme (prepared as described in Section 2.2.3). The data (presented in Table 2.2; Columns 1 and 3 respectively) show that the composition of these two mammalian dehydratases were very similar (except for Glx, Ala and Val) and suggested that there may be a high degree of similarity between them. The amino acid compositions of other mammalian dehydratase preparations are also presented in Table 2.2 and again infer structural similarities between all mammalian dehydratases. However, the values obtained for the human enzyme by Anderson and Desnick (1979) (Column 2 in Table 2.2) were completely different and over half the amino acid residues were at variance to the proportions obtained for the other mammalian enzymes.

2.3.1.2 Catalytic Properties of the Human Dehydratase Enzyme.

Human 5-aminolevulinic acid dehydratase, in common with all mammalian dehydratases, requires the presence of both zinc (Tsukamoto et al., 1979; Bevan et al., 1980) and exogenous thiol (Shemin, 1972; Cheh and Neilands, 1976) for maximal catalytic activity and their roles have been studied in great detail (see Chapter 3). The pH optimum of the enzyme was also determined using a range of potassium phosphate buffers (0.1M; pH 5.6 - 7.7) and found to be 6.8 (Scheme 2.4). The $K_{m\ app}$ for the substrate, ALA, at pH 6.8 was 251.3 ± 8.1 μM as shown in Chapter 3 (Scheme 3.3).

2.3.2 Modification of the Active Sites of Mammalian 5-Aminolevulinic Acid Dehydratases.

Optimal conditions for the modification of the active sites of both the bovine and human enzymes were determined as described in Section 2.2.9 where equimolar quantities of enzyme and [4-^{14}C] ALA were mixed at 0°C at various pH's. If the enzyme and labelled substrate (1 mole ^{14}C ALA/mole subunit) were mixed in a rapid mixing device at pH 6.8 (see Chapters 4 and 5) and rapidly quenched into 1M $NaBH_4$ only ≈ 0.35 moles of ALA were incorporated per mole of enzyme subunits. However, if the enzyme and substrate were allowed to stand for 10 seconds at 0°C before the addition of $NaBH_4$ approximately 0.5 moles of ALA were incorporated/mole subunit and demonstrate that sufficient time must be allowed for the enzyme and substrate to interact and form

Amino Acid	Human Erythrocytes		Bovine Liver			Dog Liver	Mouse Liver
	This Thesis[a] (Section 2.2.2)	Anderson and[b] Desnick (1979)	This Thesis[a] (Section 2.2.3)	Wilson[b] et al., (1972)	Kreutzer et al., (1977)	Zeitler et al., (1982)	Doyle[b] (1971)
Asx, D,N	27.5	9.9	26.3	24.3	23-25	21.9	19.6
Thr, T	12.8	13.4	10.8	9.9	7	9.5	16.8
Ser, S	23.2	13.4	22.5	22.0	23-24	18.1	16.1
Glx,E,Q	38.7	34.8	34.1	32.7	29-31	28.1	36.4
Pro, P	16.7	22.9	16.9	22.9	22-23	20.1	14.0
Gly, G	25.3	23.9	26.1	23.1	22-24	21.9	25.2
Ala, A	26.7	46.7	35.8	35.7	36-37	37.1	33.6
Cys, C	8.5[c]	9.9	8.5[c]	9.8	6-7	3.0	7.0
Val, V	17.3	18.8	23.9	23.7	23-26	24.4	32.2
Met, M	9.4	9.9	8.2	8.4	7	4.9	7.7
Iso, I	9.6	6.0	8.7	8.9	9	8.5	7.7
Leu, L	31.0	35.8	27.9	33.6	34-35	33.7	33.6
Tyr, T	10.5	10.9	10.6	10.2	10	10.0	9.8
Phe, F	11.8	9.9	12.2	11.9	11-12	11.6	14.0
His, H	10.3	6.0	9.7	8.2	6-8	6.9	14.0
Trp, W	5.9	9.9	4.5	N.D.	1-2[d]	3.1[d]	7.0[d]
Lys, K	13.6	10.9	11.9	12.7	11-12	12.0	16.1
Arg, R	19.0	20.9	19.9	20.2	22	22.1	6.0
Total No. of Amino Acids	≈318	≈314	318-319	≈318	300-319	≈297	≈317

(a) Amino acid analysis was performed as described in Section 2.2.14. Values are average of at least 3 determinations from 2 different enzyme preparations.

(b) Values recalculated from paper based on a subunit molecular weight of 35,000 and corrected to allow for the displacement of water during the formation of the peptide bond.

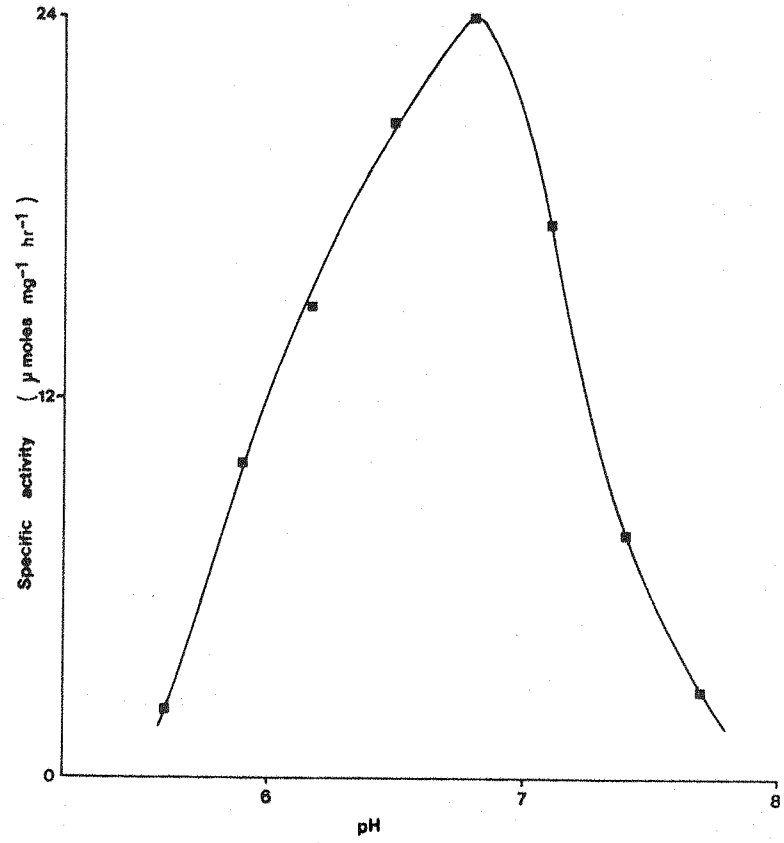
(c) Determined by reaction with Nbs_2 in the presence of SDS ($\varepsilon_{412} = 1.28 \times 10^4\ M^{-1}\ cm^{-1}$; pH 7.1).

(d) Determined spectrophotometrically.

Table 2.2 Comparison of the Subunit Amino Acid Composition of Various Mammalian Dehydratases.

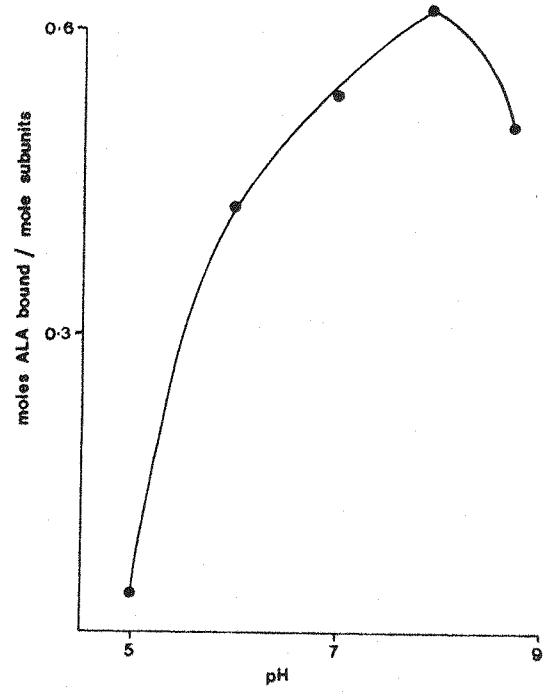
Scheme 2.4 Effect of pH on the Activity of the Human Dehydratase Enzyme.

The human enzyme was assayed at various pH's as described in Section 2.2.4.



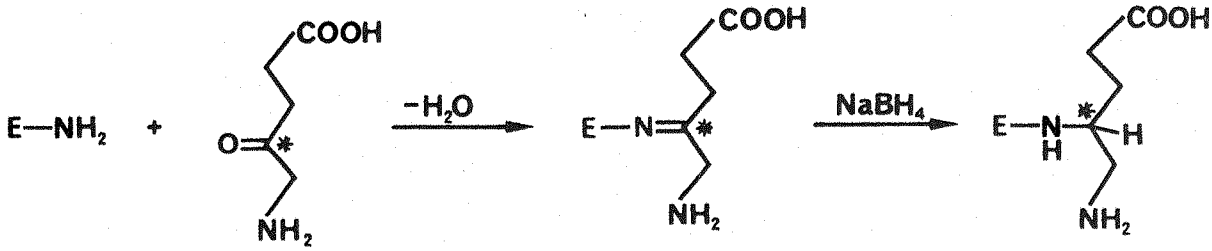
Scheme 2.5 Effect of pH on the Incorporation of [4-^{14}C] ALA into the Human Enzyme.

The enzyme was inactivated by $NaBH_4$ at various pH's in the presence of [4-^{14}C] ALA as described in Section 2.2.9.



the ES complex before quenching into the $NaBH_4$. The pH optimum for incorporation of labelled ALA into the enzyme was found to be 7.9 (Scheme 2.5). Interestingly, the modification of ≈ 2 subunits per octameric enzyme was shown to inactivate the enzyme completely.

Large amounts of modified enzyme were prepared by mixing equimolar concentrations of enzyme and [4-^{14}C] ALA at pH 7.9 (on ice) and the Schiff base intermediate (2.5) formed between the active site amino group of the enzyme (2.3) and the substrate (2.4) was stabilised by reduction with 0.5M $NaBH_4$ (25 µl) followed by 0.5M acetic acid (25 µl). This reduced enzyme-bound intermediate (2.6) was catalytically inactive and was subsequently used for the preparation of active site peptides.



2.3 2.4 2.5 2.6

The asterisk (*) indicates the position of the label from [4-^{14}C] ALA.

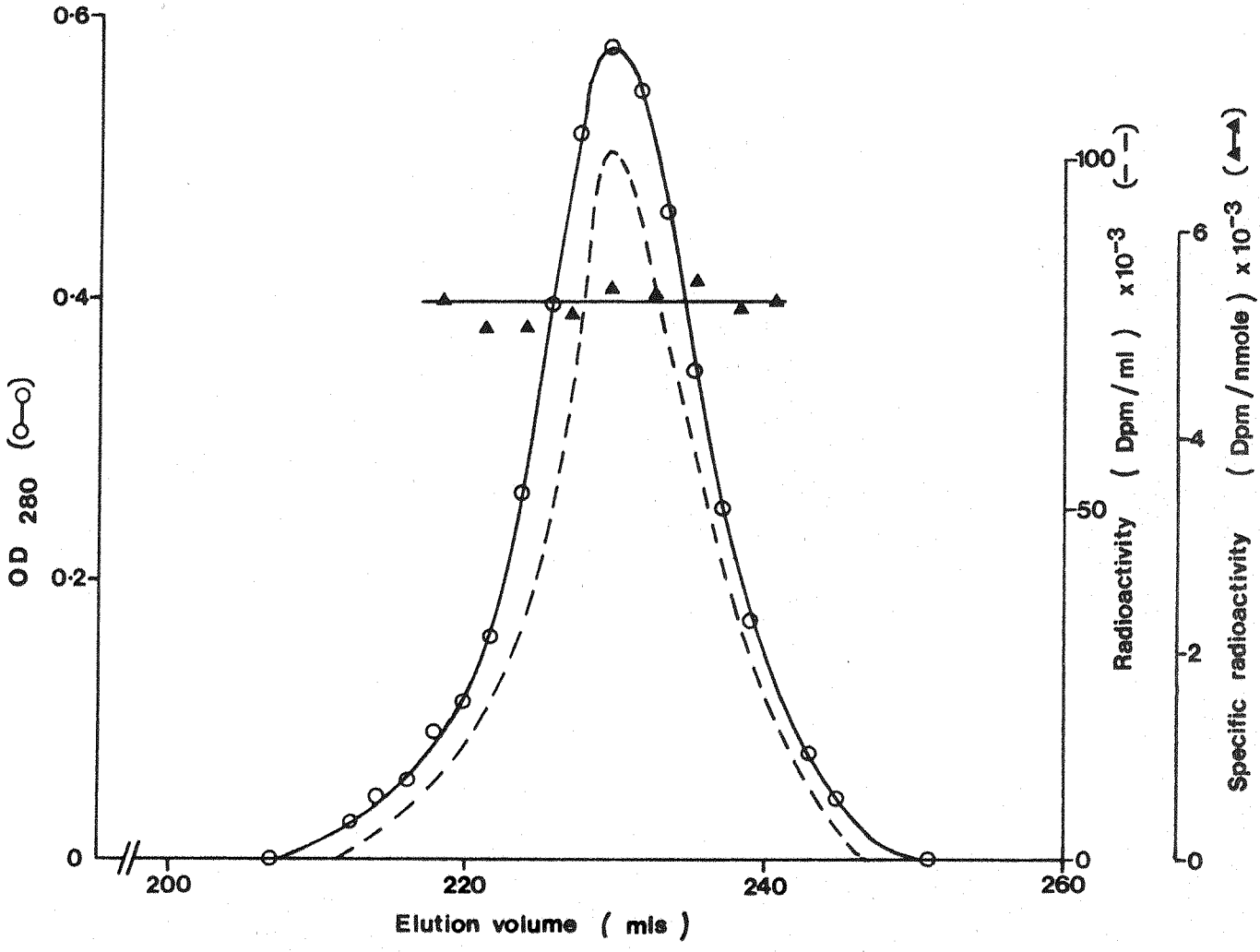
The modified dehydratases were exhaustively dialysed, modified with unlabelled ALA (Section 2.2.9) and after dilution with unlabelled modified enzyme, the specific radioactivities were determined. The values obtained were 13,370 dpm/n mole for the human enzyme and 5,070 dpm/nmole for the bovine enzyme and were equivalent to the incorporation of 1.023 and 1.06 moles ALA/mole subunit respectively.

The labelled modified enzymes were analysed in two ways. Firstly, the labelled enzymes were applied to a gel filtration column containing Biogel A 0.5m (Section 2.2.2) and developed at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and the protein concentration and the radioactivity associated with each fraction was determined to reveal a single protein peak of constant specific radioactivity. A typical profile for the labelled bovine enzyme is shown in Scheme 2.6. A similar profile was obtained for the human enzyme (data not presented). Secondly, the modified enzymes were analysed on 15% polyacrylamide gels in the presence of 0.1% w/v SDS when the only radioactive region of the gel (containing all the applied radioactivity) corresponding to the single protein band of molecular weight 35,000 (data not presented).

When equimolar quantities of enzyme and [4-^{14}C] ALA were mixed in the presence of 10 mM porphobilinogen on ice at pH 7.9 (Section 2.2.9) the enzyme was very significantly protected from inactivation by $NaBH_4$ (Scheme 2.7) suggesting that the modification was occurring at the active site of the enzyme.

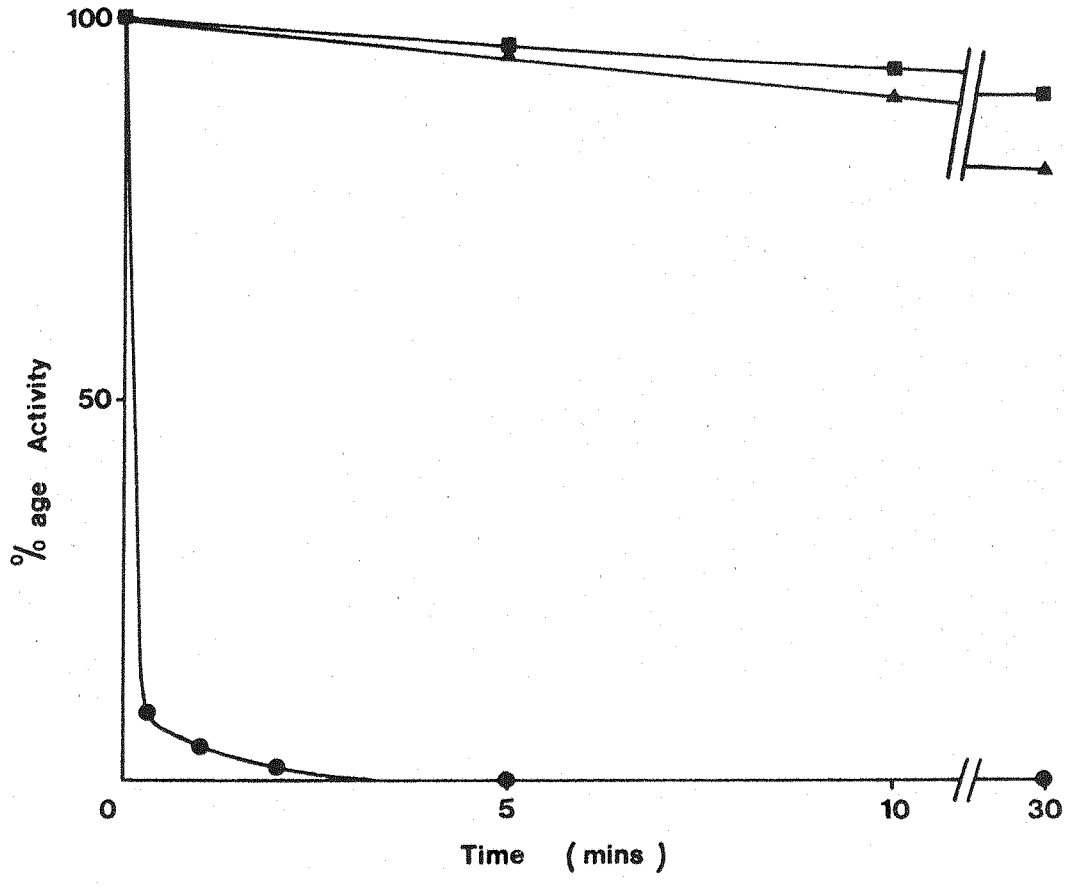
2.3.3. Preparation and Isolation of CNBr Peptides From Modified Bovine and Human 5-Aminolevulinic Acid Dehydratase.

Modified mammalian dehydratase (33 mg; 940 nmoles) was carboxymethylated (Section 2.2.10), digested with CNBr (Section 2.2.11) and applied to a Sephadex G-50 super-fine gel filtration column equilibrated with 9% v/v formic acid (Section 2.2.12). A typical profile for the separation of the human dehydratase peptides is shown in Scheme 2.8. A single radioactive peptide containing approximately 85% of the applied radioactivity was eluted from the column with an elution volume of 402 ml, corresponding to a molecular weight of 2,000 ± 300. The bovine dehydratase CNBr peptides showed a very similar elution profile (Scheme 2.9) which suggested that there was a high degree of similarity between the two mammalian dehydratases. The major radioactive peptide contained 41% of the applied radioactivity (bovine peptide A) and had an elution volume of about 279 ml, corresponding to a molecular weight of about 3,500. However, a second peptide (bovine peptide B) of molecular weight between 4,500 and 5,000 (V_e = 217 ml) contained 25% of the applied radioactivity. This second



Scheme 2.6 Elution Profile of ^{14}C-labelled 5-Aminolevulinic Acid Dehydratase from Biogel A 0.5m.

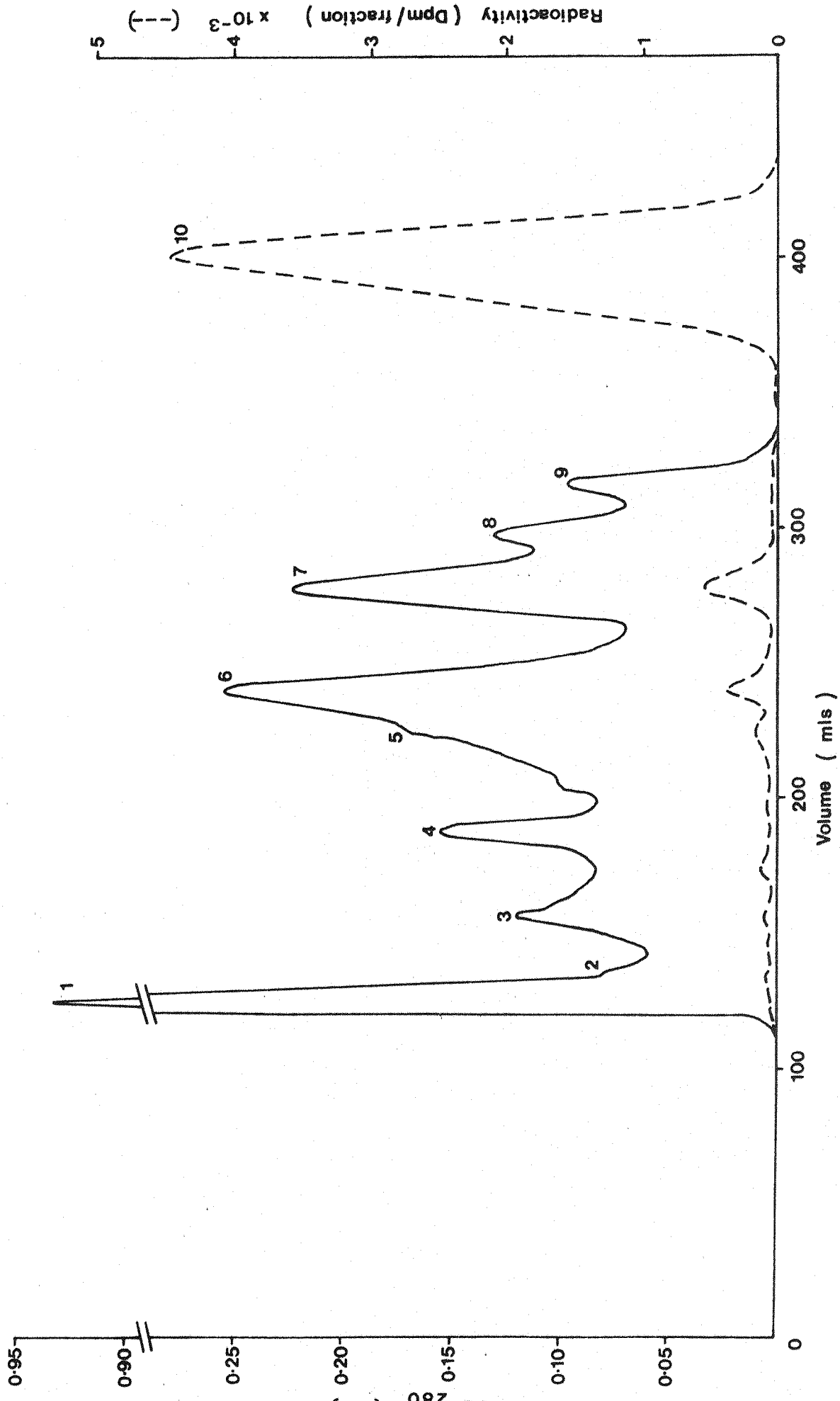
Modified bovine enzyme (prepared as described in Section 2.2.9; specific radioactivity 5,291 dpm/nmole) was applied to a gel filtration column (2.8 cm x 1 metre) containing Biogel A 0.5 m and developed in 10 mM potassium phosphate buffer (pH 6.8) containing 20 mM 2-mercaptoethanol at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and the protein concentration (Section 2.2.20) and radioactivity associated with each fraction (Section 2.2.19) was determined.



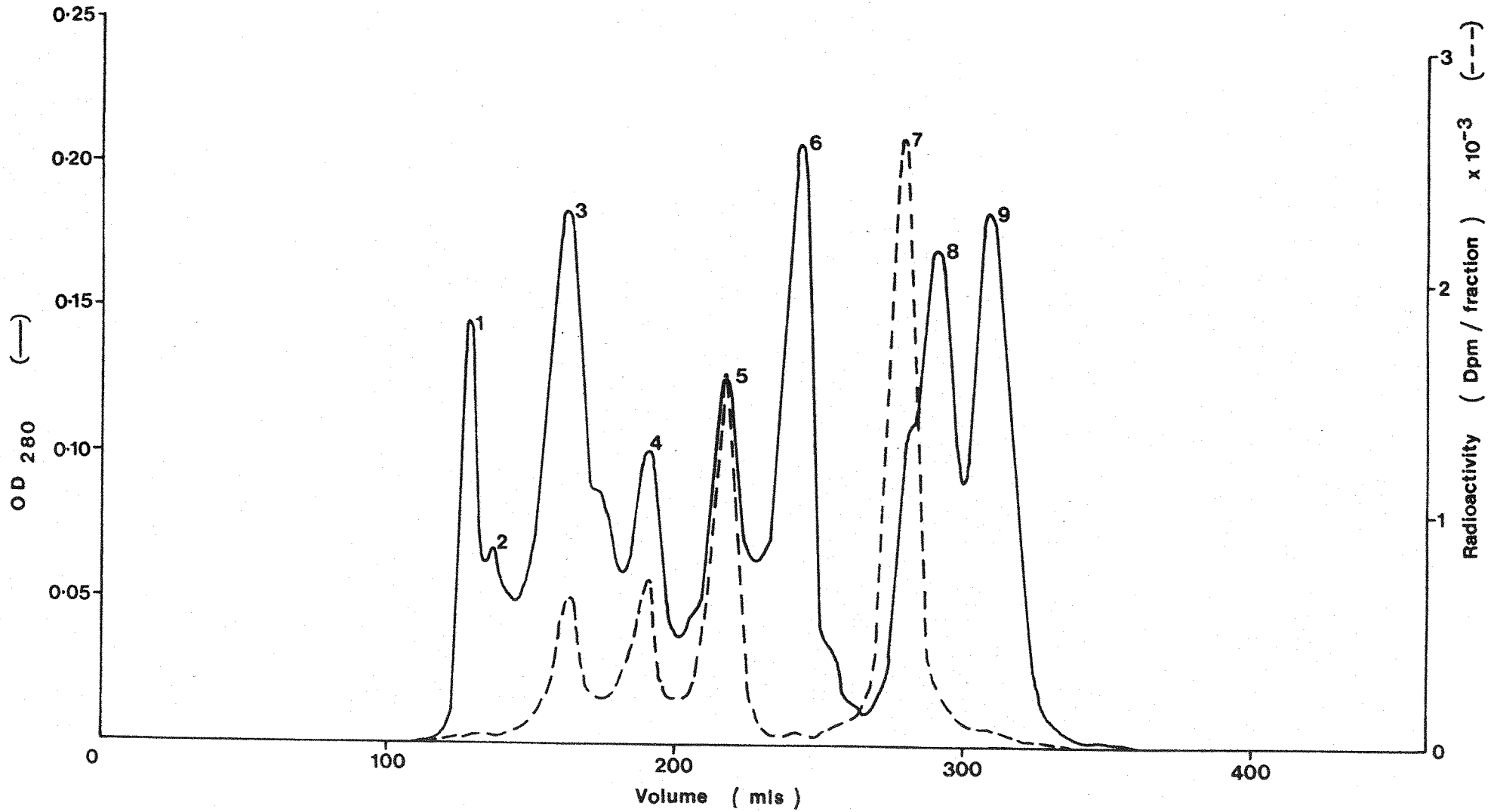
Scheme 2.7 Protection of 5-Aminolevulinic Acid Dehydratase from Inactivation by $NaBH_4$ in the Presence of [4-^{14}C] ALA by Porphobilinogen.

The human enzyme was mixed with a stoichiometric equivalent of [4-^{14}C] ALA on ice at pH 7.9 in the presence (▲——▲) or absence (●——●) of 10 mM porphobilinogen. After the addition of 0.5 M $NaBH_4$ (20 µl) followed by 0.5 M acetic acid (20 µl), aliquots were removed at timed intervals and the enzyme activity was determined (Section 2.2.4). In the control experiment (■——■) only $NaBH_4$ (followed by acetic acid) was added.

Scheme 2.8 Separation of Human Dehydratase CNBr Peptides by Sephadex G-50 Super-Fine Gel Filtration.



Scheme 2.9 Separation of Bovine Dehydratase CNBr Peptides by Sephadex G-50 Super-Fine Gel Filtration.

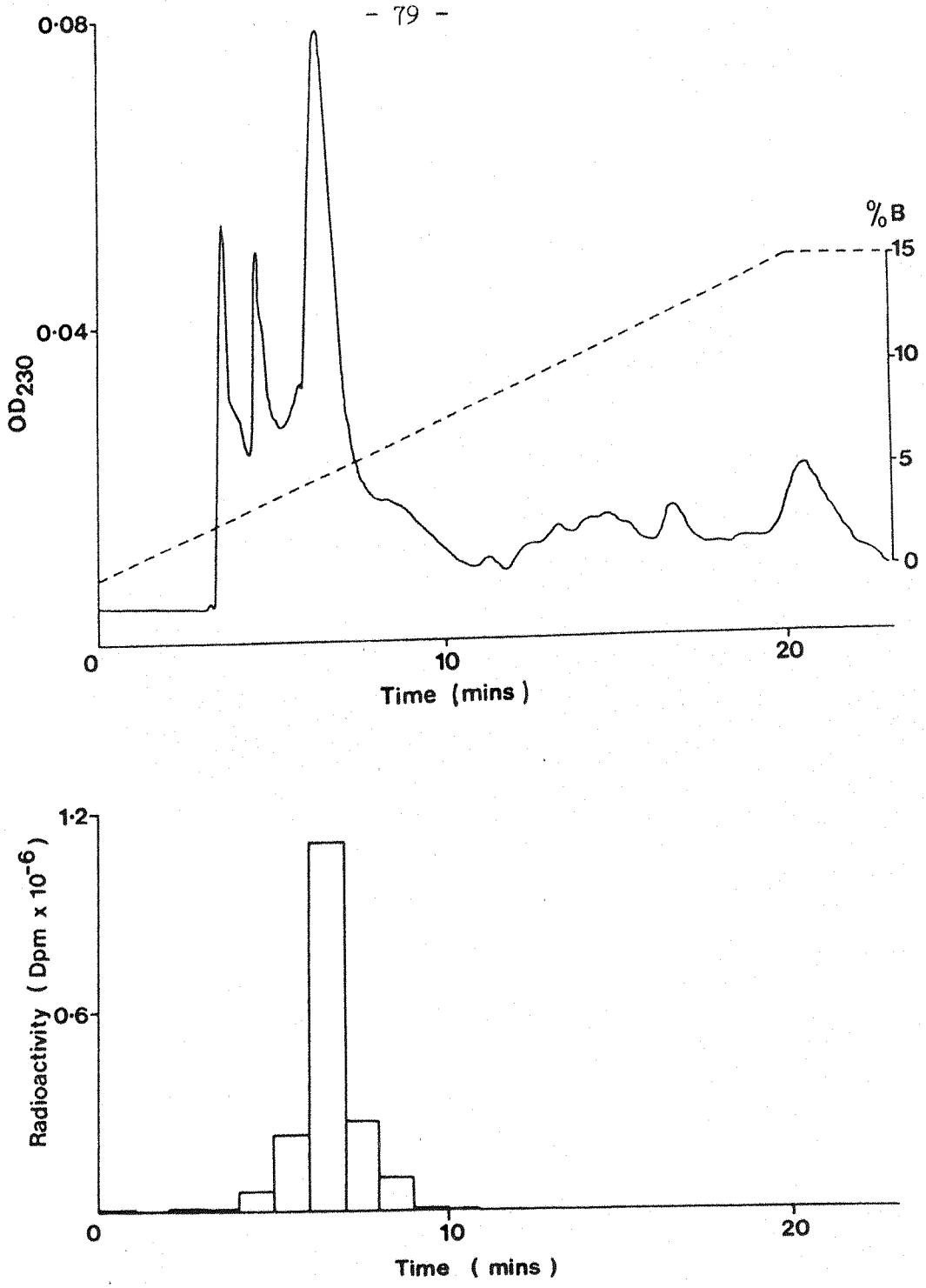


peak could have been due to a number of different factors. Firstly, the [4-^{14}C] ALA could have been incorporated into the two separate substrate binding sites of the bovine enzyme forming a Schiff base intermediate in both cases, which had subsequently been stabilised by reduction with $NaBH_4$. Consequently, the two labelled peptides produced on CNBr digestion would be different since they originated from different parts of the primary sequence. However, this possibility is thought unlikely since the modification of the bovine enzyme was performed with an equimolar concentration of [4-^{14}C] ALA as described in Section 2.2.9, conditions known to incorporate 0.89 moles ALA/mole subunit (Seehra, 1980). Secondly, the digestion of the modified enzyme by CNBr could have been incomplete either due to an insufficient amount of CNBr or duration of incubation for complete digestion to occur or, alternatively, that some of the methionine residues were modified, possibly during purification, and consequently were unreactive towards CNBr. Under both these circumstances there would be sequence homology between the two labelled peptides (A and B). The possibility of incomplete cleavage by CNBr was eliminated by redigestion of peptide B with CNBr for 48 hours and the subsequent demonstration that no further cleavage had occurred. Therefore, the possibility that certain methionine residues were unable to be modified by CNBr is thought to be most likely, but to confirm this pepetide B was also purified and sequenced.

2.3.4 Purification of the Labelled Dehydratase CNBr Peptides.

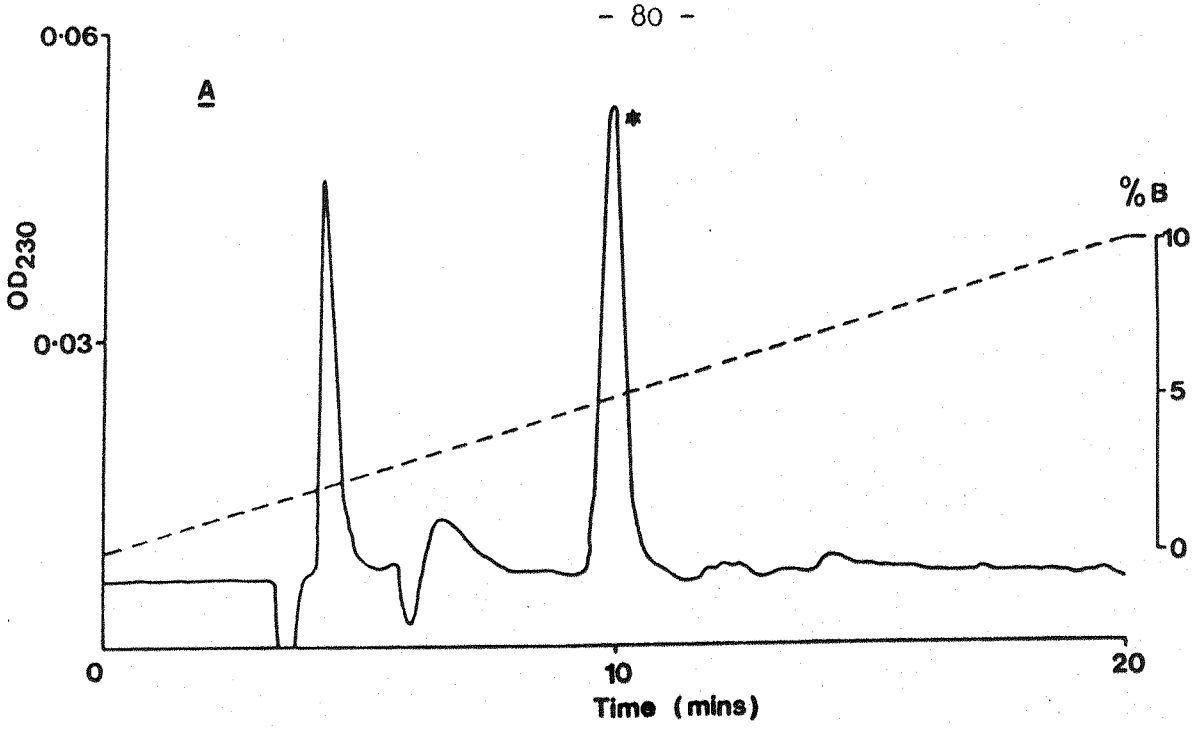
2.3.4.1 Human Active Site Peptide.

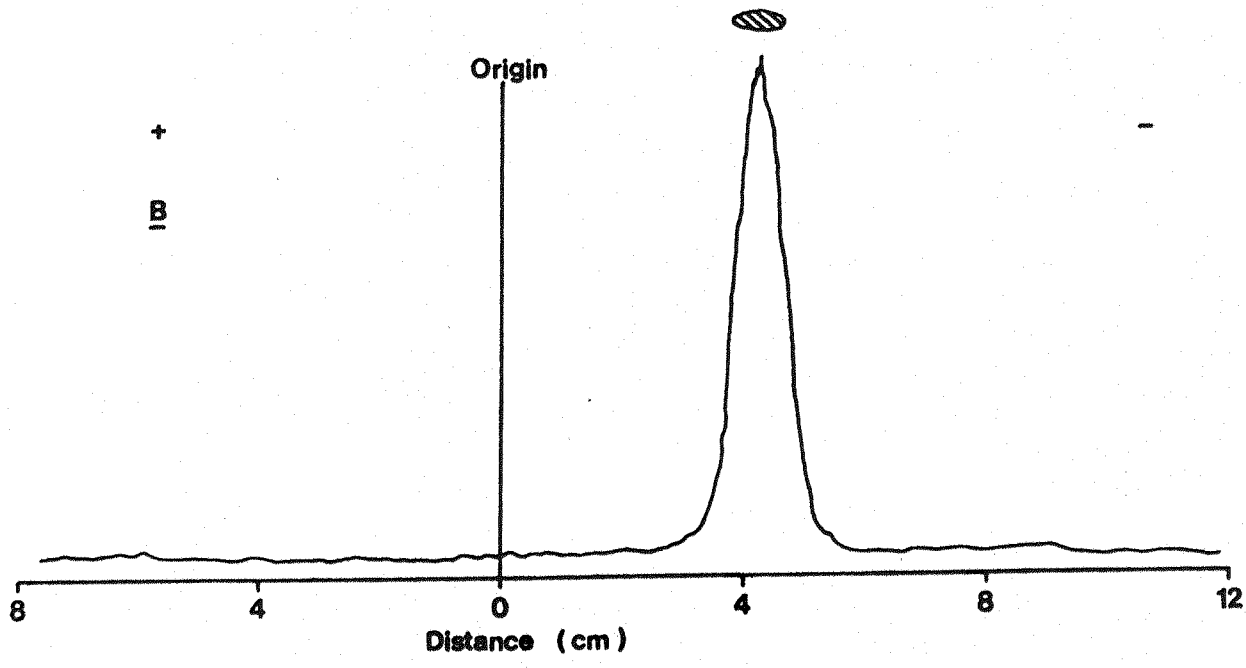
The human peptide (530 nmoles) was purified by hplc as described in Section 2.2.13 using a linear gradient of 0.07% v/v TFA in acetonitrile (0 → 15%) in 0.12% v/v aq TFA over a period of 20 minutes. The radioactivity was eluted in a single peak with a retention time of 6.4 minutes and was associated with an absorbance at 230 nm (the wavelength of absorption of the peptide bond) (Scheme 2.10). The labelled peptide was analysed by applying a sample (50 nmol; 668,000 dpm) to a shallower hplc gradient and yielded a single, sharp protein peak which contained all the applied radioactivity (Scheme 2.11a).



Scheme 2.10 Purification of the Labelled Human Dehydratase CNBr Peptide by Hplc.

The labelled human peptide (530 nmoles; 7.1 x 10^6 dpm) obtained from the Sephadex G-50 superfine gel filtration column (Scheme 2.8) was purified on a Waters' C-18 μ-Bondapak reverse phase hplc column using a 0 - 15% linear gradient of 0.07% v/v TFA in acetonitrile in 0.12% v/v aq. TFA over a period of 20 minutes. The peptide peaks were determined by measuring the absorbance at 230 nm (Section 2.2.13) and 1 minute fractions were collected. The radioactivity in each fraction was determined as described in Section 2.2.19.





Scheme 2.11 Further Purification of the Labelled Human Dehydratase CNBr Peptide.

(a) The labelled peptide was purified by hplc using a linear gradient from 0 → 10% 0.07% v/v TFA in acetonitrile in 0.12% v/v aq. TFA over a period of 20 minutes as described in Section 2.2.13. The symbol (*) represents the location of the radioactive peptide.

(b) Electrophoresis of the labelled peptide (3 x 130 nmole) was performed for 40 minutes at 1 kV as described in Section 2.2.13, followed by scanning using a Bertholt plate scanner (1 x 10^2 cps sensitivity).

The human CNBr peptide (3 x 130 nmole) was electrophoresed at 1 kV for 40 mins (pH 8.0) as described in Section 2.2.13. The peptide was located by spraying a small area of the plate with ninhydrin (0.2% w/v in acetone) and corresponded to the position of the radioactivity (Scheme 2.11b). This procedure yielded 260 nmoles of purified active site peptide from human erythrocyte 5-aminolevulinic acid dehydratase which was subsequently sequenced (see Section 2.3.5).

2.3.4.2 Bovine Peptide A.

Peptide A (370 nmole) was purified by hplc on a Waters' C-18 μ-Bondapak reverse phase column to yield a rather broad peak containing all the applied radioactivity (Scheme 2.12a). This peptide was further purified by thin layer electrophoresis at 1.5 kV for 2 hours to yield a single radioactive peak associated with the ninhydrin positive spot (135 nmole; 675 x 10^3 dpm) (Scheme 2.12b).

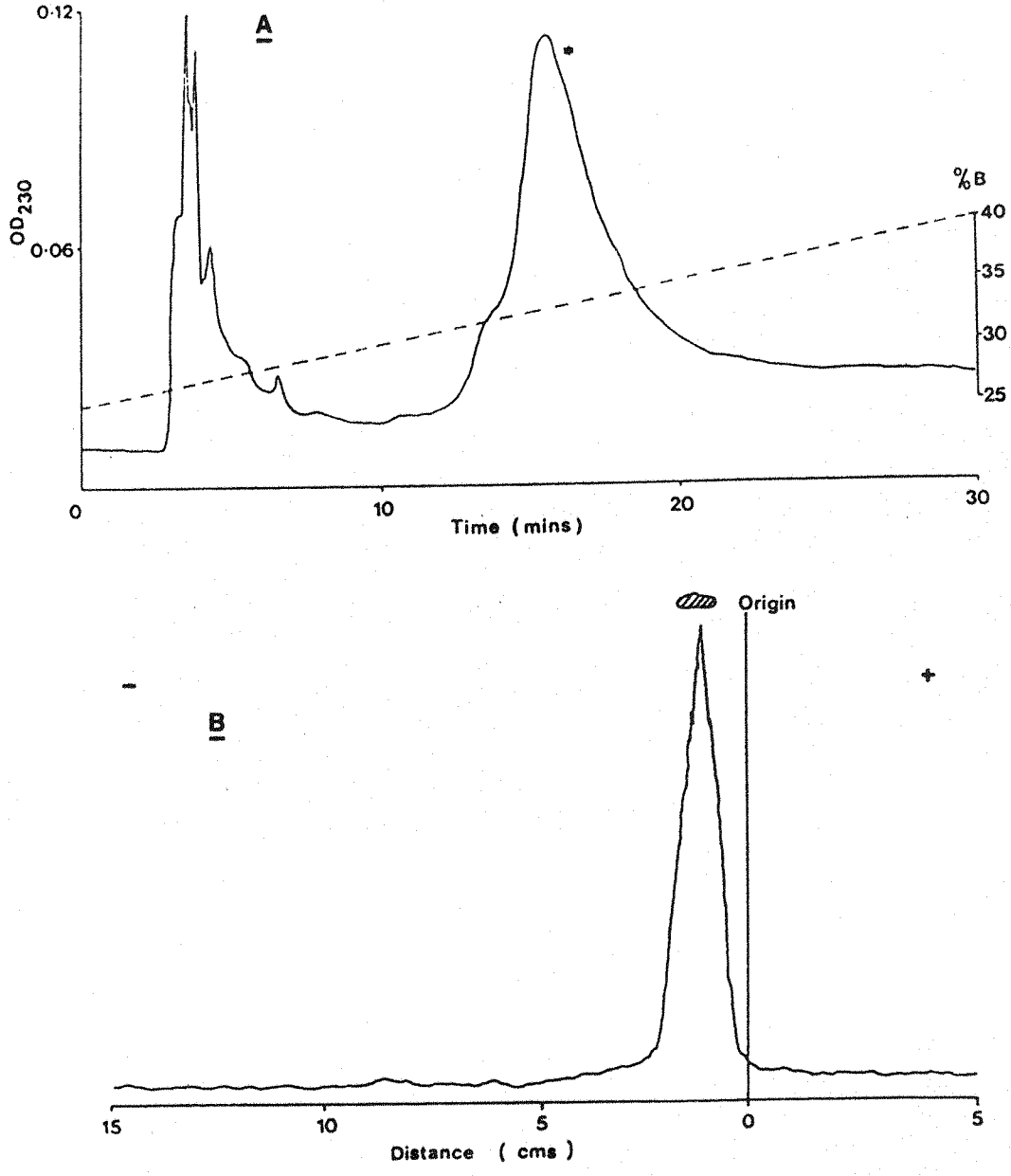
2.3.4.3 Bovine Peptide B.

Peptide B (225 nmole) was purified by hplc (30 → 70% solvent B; linear gradient; 20 mins) (see Section 2.2.13 for details of solvents) (Scheme 2.13a), followed by thin layer electrophoresis (1 kV for 90 mins; Scheme 2.13b) to yield ≈ 80 nmoles of peptide.

2.3.5 The Amino Acid Composition and Sequence of the ^{14}C-Labelled Human and Bovine Dehydratase Modified Active Site Peptides.

2.3.5.1 Human Dehydratase Active Site Peptide.

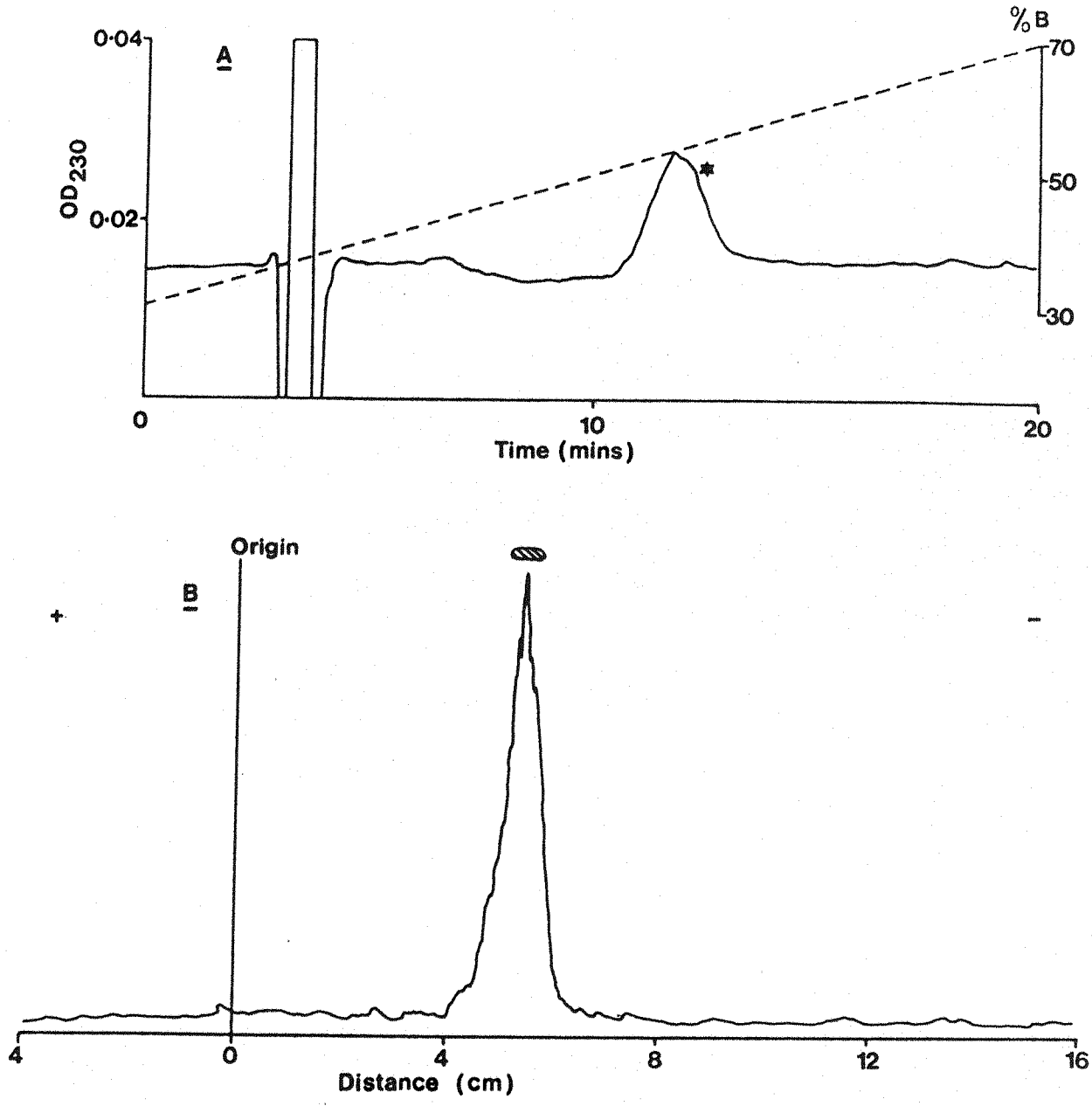
The amino acid composition of the human peptide was determined as described in Section 2.2.14 and is shown in Table 2.3. An initial sequencing run was carried out on 15 nmoles of the human peptide (195 x 10^3 dpm) as described in Section 2.2.15. However, it was discovered that the procedure used to remove excess reagents (DABITC and PITC), after modification of the N-terminal amino acid, resulted in a total loss of the labelled peptide by the start of the second turn. The hydantoin derivatives obtained in this sequence run were attributable to a minor peptide impurity (< 5%) present with the human active site peptide as a peptide free blank sequenced at the same time gave no derivatives when analysed either by hplc (Section 2.2.18) or by two-dimensional tlc (Section 2.2.16). The data for the minor impurity are presented in Table 2.4. Various washing methods were tried so as



Scheme 2.12 Purification of Bovine Peptide A.

(a) Bovine peptide A was purified by hplc using a linear gradient (25 → 40%) of 0.07% v/v TFA in acetonitrile in 0.12% v/v aq. TFA over a period of 30 minutes (see Section 2.2.13). The symbol (✱) shows the location of the radioactive peptide.

(b) The hplc purified peptideA was electrophoresed for 2 hours at 1.5 kV according to the method described in Section 2.2.13. The plate was scanned using a Bertholt plate scanner (3 x 10^3 cps sensitivity).



Scheme 2.13 Purification of Bovine Peptide B.

(a) Peptide B was purified on a Waters' C-18 μ-Bondapak hplc column using a linear 30 - 70% gradient of 0.12% v/v TFA in acetonitrile in 0.07% v/v aq. TFA (over 20 mins). The symbol (✱) locates the radioactive peptide.

(b) Electrophoresis was performed as described in Section 2.2.13 for 90 minutes at 1 kV. The scan sensitivity was 1 x 10^2 cps.

Table 2.3 The Amino Acid Composition of Human and Bovine (A) Active Site Peptides.

Amino Acid	Human Peptide	Bovine Peptide A
Homoserine/ Homoserine lactone [a]	1.00	1.00
Asx, D & N	1.63	2.17
Thr, T	0.22	0.52
Ser, S	0.22	1.06
Glx, E & Q	1.13	2.20
Pro, P	1.83	2.87
Gly, G	2.98	4.97
Ala, A	1.08	2.32
Cys, C	0.00	0.00
Val, V	2.12	3.30
Met, M	0.00[b]	0.00[b]
Iso, I	0.14[c]	0.00[c]
Leu, L	0.97	2.26
Tyr, Y	0.03	0.79
Phe, F	0.02	1.09
His, H	1.31	3.34
Trp, W	0.00	0.00
Lys, K	0.98	2.40
Arg, R	1.03	2.01
X*[d]	0.75	0.88
Total	17.00	31/32

(a) Produced at C-terminus of CNBr digested peptides (used as an internal standard).

(b) No methionine present (cleaved by CNBr).

(c) No isoleucine present. All DABTH-amino acids giving I/L spots (see Chang et al., 1978) must be leucine residues.

(d) Corresponds to the modified ^{14}C-labelled amino acid.

Table 2.4 The Amino Acid Sequence of the Human Dehydratase Active Site Peptide.

Radioactivity Present (dpm)					
Turn No.	Amino Acid Identified	In Butyl Acetate Extract	At Start of Turn	% age of Radioactivity at Start of Turn in Butyl Acetate Extract	Impurity Sequence
1	Val[a]	3,344	324,320	1.0	Ile/Leu
2	X*[a]	177,120	209,410	84.6	Gly
3	Pro[a]	16,653	30,960	53.8	Gly
4	Gly[c]	3,123	12,560	24.9	Val
5	Arg[cd]	452	11,744	3.8	Gly[d]
6	Pro/Arg[c]	279	8,614	3.2	-

(a) Identified by both hplc (see Scheme 2.15) and two dimensional tlc (see Scheme 2.16).

(b) Equivalent to 24.3 nmoles of the human peptide.

(c) Only sufficient hydantoin derivative to identify by two dimensional tlc.

(d) Arginine (Arg) and glycine (Gly) were both present in similar amounts at turn 5, but since glycine was present in the impurity sequence, arginine was deduced to be the amino acid residue present in the human peptide.

to minimise the losses of human peptide at each turn and the best procedure for the extraction of excess reagents was found to be three ethyl acetate:heptane (1:2; v/v) washes. However, despite limiting the number of extraction steps, losses of the human peptide at each stage was between 30 and 40%. Consequently, only the first few amino acids of the human peptide could be sequenced (as shown in Table 2.4) which had the following structure.

$$Val_{1}\text{-}X^{*}\text{-}Pro\text{-}Gly_{4}\text{-}Arg\text{-}Arg/Pro$$

X* corresponded to the amino acid at the active site of the human enzyme which was modified by $NaBH_4$ in the presence of [4-^{14}C] ALA and contained 85% of the total radioactivity from turn 2 (55% of the starting radioactivity; Table 2.4).

2.3.5.2 Bovine Active Site Peptide.

The sequence of both the bovine active site peptides (A and B) were identical (see Table 2.5) and unambiguously demonstrated that these peptides originated from the same part of the enzymes' primary structure. The incomplete cleavage of the enzyme by CNBr (yielding peptide B) was almost certainly due to the modification of some of the methionine residues which made them unreactive towards CNBr. The active site sequence of the bovine enzyme was as shown below (see Tables 2.3 and 2.5 for details of the amino acid composition and sequence).

$$Val_{1} - X^{*} - Pro - Gly_{4} - Arg - Pro - Tyr - Leu_{8} -$$

$$Asp - Leu - Val - Arg_{12} - Glu - Phe - Val - Asn_{16} -$$

Bovine Enzyme

The first five amino acids in the bovine active site sequence were identical to those obtained in the human active site peptide which is shown below.

$$Val_{1} - X^{*} - Pro - Gly_{4} - Arg - Arg/Pro$$

Human Enzyme

	Peptide A[a]		Peptide B[b]			Peptide A		Peptide B	
Turn No.	Amino Acid[c]	Radioactivity in Butyl Acetate (dpm)	Amino Acid	Radioactivity in Butyl Acetate (dpm)	Turn No.	Amino Acid	Radioactivity in Butyl Acetate (dpm)	Amino Acid	Radioactivity in Butyl Acetate (dpm)
1	Val	3,160	Val	320	9	Asp	294	Asp	306
2	X*	50,340[d]	X*	44,170[d]	10	Leu	363	Leu/Val	227
3	Pro	17,940	Pro	9,540	11	Val	244	Leu/Val	293[e]
4	Gly	3,852	Gly	2,480	12	Arg	476	-	
5	Arg	2,328	Arg	317	13	Glu	171	-	
6	Pro	780	Pro	283	14	Phe	323	-	
7	Trp/Tyr	313	Tyr	476	15	Val	354	-	
8	Leu	425	Leu	344	16	Asn	235[e]	-	

(a) Peptide A used = 18.5 nmol, 92,325 dpm

(b) Peptide B used = 12.6 nmol, 63,098 dpm

(c) Turns 1 - 10 (except turn 7; peptide A) confirmed by hplc (PTH-derivatives) and two dimensional tlc (DABTH amino acids). Turns 11 - 16 were only determined by two dimensional tlc.

(d) Modified ^{14}C-labelled amino acid (X*) containing 74% (A) and 85% (B) of total radioactivity.

(e) Radioactivity remaining in peptide at end of sequencing was 4,178 dpm (peptide B) and 1,056 dpm (peptide A).

Table 2.5 The Amino Acid Sequence of the Bovine Dehydratase Active Site (Peptides A and B).

2.3.6 Characterisation of the Modified ^{14}C-labelled Active Site Amino Acid (X*)

Amino acid analysis of the modified amino acid (X*) was performed as described in Section 2.2.14 from acid hydrolysates of the native enzymes and purified peptides. The results (presented in Scheme 2.14) revealed a single radioactive peak which was eluted between histidine and lysine and had a retention time of about 92 minutes (in all cases). Similarly, studies on the electrophoretic mobility of the modified residue from these acid hydrolysates by thin layer electrophoresis for 30 mins at 1 kV (Section 2.2.13) demonstrated a single radioactive peak with a relative mobility of 0.52 (towards the cathode). The mobility of this labelled amino acid was compared to those of standard amino acids and the data are summarised in Table 2.6.

DABTH - and PTH - derivatives of the modified amino acid were prepared as described in Section 2.2.15 and analysed by two dimensional tlc and hplc respectively. The results are presented in Schemes 2.15 and 2.16 and show a single radioactive peak containing essentially all of the applied radioactivity.

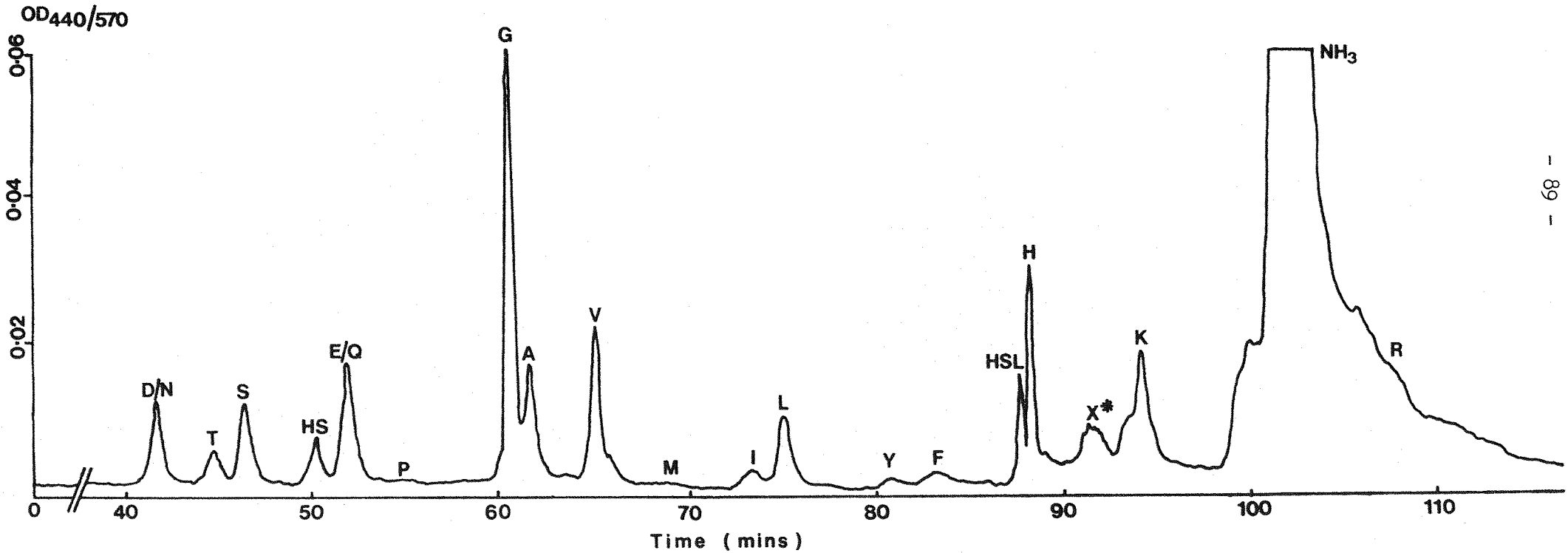
The results cumulatively demonstrate that a single labelled amino acid was obtained from the modification of 5-aminolevulinic acid dehydratase by $NaBH_4$ in the presence of [4-^{14}C] ALA (Section 2.2.9) which was different to standard amino acids. Unfortunately, attempts to synthesise ALA-lysine were unsuccessful and therefore it was not possible to compare its properties with that of the modified amino acid (X*). However, the properties of X* are similar to those of the ALA-lysine obtained from the *R.sphaeroides* enzyme by Nandi (1978) and therefore it appears likely that the active site amino group in mammalian 5-aminolevulinic acid dehydratase is the ε-NH_2 group of lysine.

2.4 Summary.

5-Aminolevulinic acid dehydratase was obtained from human erythrocytes (Section 2.2.2) by a new purification procedure to yield 27.5 mgs of pure enzyme protein with a recovery of 65% (Gibbs *et al.*, 1984). The final specific activity was 24.0 units/mg protein in the presence of 0.1 mM zinc ions and was the highest specific activity reported for

Scheme 2.14 Amino Acid Analysis of the Modified Active Site Residue (X*).

See Section 2.2.14 for experimental details. All abbreviations are one letter codes for amino acids except HS = Homoserine and HSL = Homoserine lactone. The modified amino acids (X*) contained essentially all the applied radioactivity.



Amino Acid	Relative Mobility (Rf)	Amino Acid	Relative Mobility (Rf)
X*	-0.53	Met,M	-0.80
Ala,A	-0.90	Phe,F	-0.47
Arg,R	-0.93	Pro,P	-0.52
Asp,D	+0.28	Ser,S	-0.45
Cys,C	-0.21	Thr,T	-0.84
Glu,E	-0.43	Trp,W	-0.48
Gly,G	-0.49	Tyr,Y	-0.68
His,H	-0.38	Val,V	-0.91
Ile,I	-0.37	DNP-A	+0.11
Leu,L	-0.48	DNP-R	+0.48
Lys,K	-1.00	DNP-G	-0.32

Table 2.6 Relative Mobilities of Amino Acids Using Thin Layer Electrophoresis.

Thin layer electrophoresis was performed for 30 minutes at 1kV (Section 2.2.13). The mobilities are expressed relative to that of lysine. The "+" and "-" signs represent mobility towards the anode and cathode respectively. DNP = Dinitrophenyl derivative.

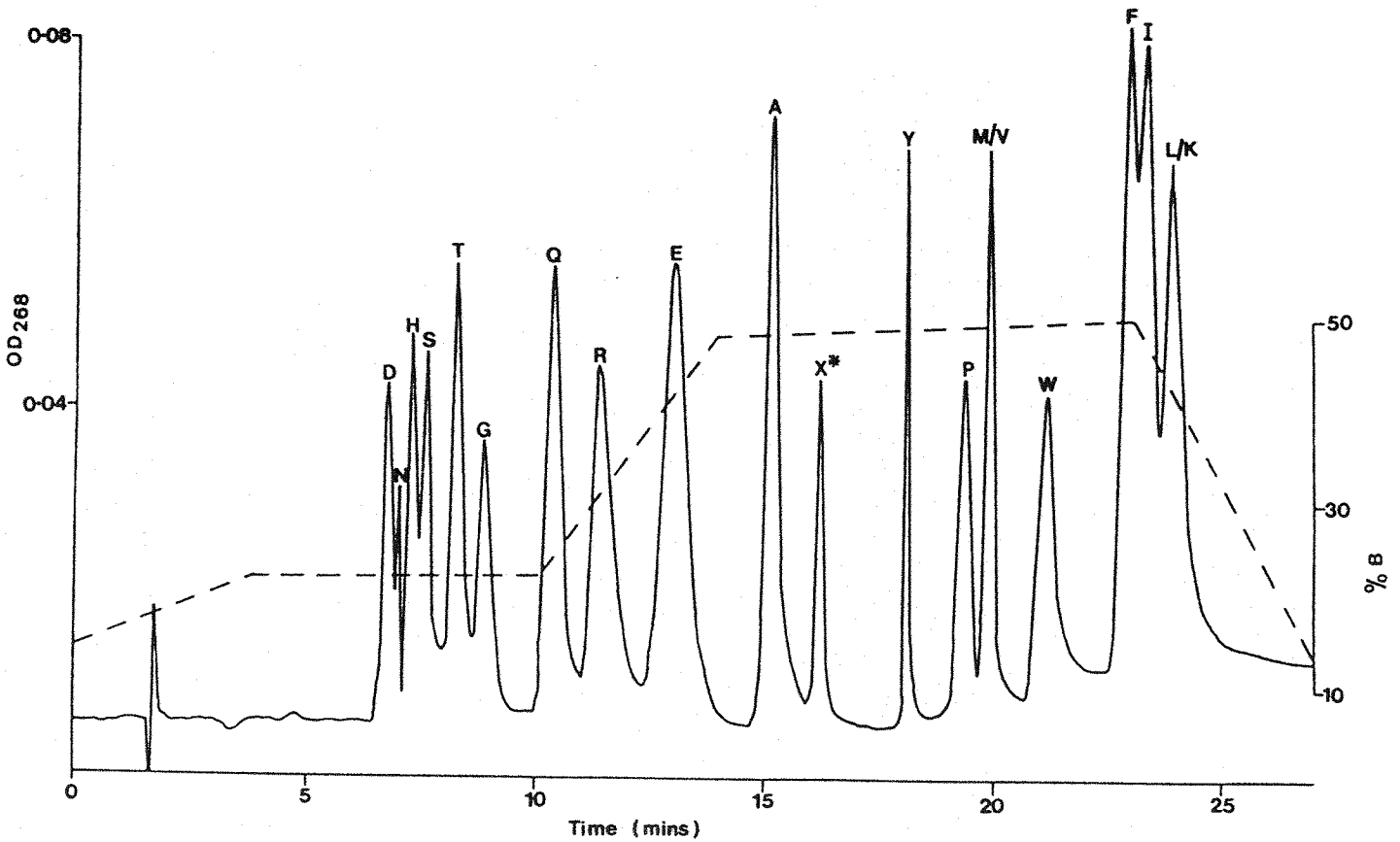
Scheme 2.15 Resolution of PTH-Amino Acids by HPLC

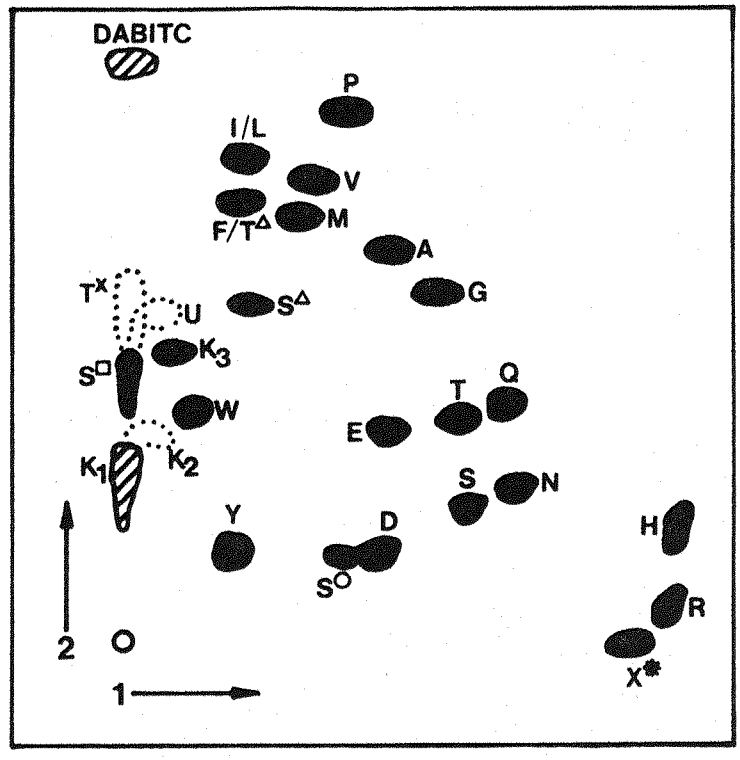
The PTH-derivatives were separated by hplc as described in Section 2.2.18. A typical separation is shown below.

(a) X* corresponds to the modified ^{14}C-labelled amino acid from turn 2 of the bovine and human dehydratase peptides and contained 95% of the applied radioactivity.

(b) Methionine and valine co-chromatograph on this system. However, since the peptides are derived from CNBr digestion there are no methionine residues present.

(c) Leucine and lysine co-chromatograph, but can be resolved by two dimensional tlc (Chang et al., 1978) (Scheme 2.16).





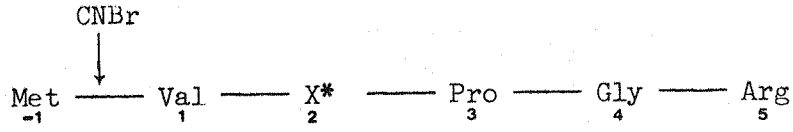
Scheme 2.16 Schematic Representation of the Resolution of DABTH-Amino Acids by Two Dimensional tlc. (Chang et al.,1978).

The solvents for the chromatographic separation are described in Section 2.2.16. The amino acids are identified by exposure to Hcl vapour. The colours of the derivatives are represented by solid areas (red), dotted areas (blue) and hatched areas (purple). U is the blue coloured thiourea formed by the coupling of PITC with hydrolysed DABITC. X* is the modified ^{14}C-labelled amino acid. Multiple spots are produced by lysine, serine and threonine (see Change et al., (1978) for details).

this enzyme. The enzyme was shown to have a native molecular weight of 285,000 and was composed of eight identical subunits (MR = 35,000). The amino acid composition of the human enzyme was compared to those of other mammalian dehydratases (see Table 2.2) and revealed that their compositions were very similar. These findings suggested that there was a high degree of structural similarity between mammalian dehydratases. A comparison of the amino acid compositions of the human and bovine enzymes revealed the presence of an additional methionine residue in the human enzyme (9.4 as opposed to 8.2; Table 2.3). This is consistent with the fact that on digestion with CNBr the human enzyme yielded one extra peptide when analysed by gel filtration on Sephadex G-50 superfine as compared to the bovine enzyme (see Schemes 2.8 and 2.9). The presence of this additional peptide in the human enzyme would be most simply explained if it originated from the active site region of the enzyme (located ≈ 16 amino acids from the labelled active site amino acid (X*) towards the C-terminus of the enzyme). If this explanation were true it would account for the difference in the molecular weights of the human and bovine active site peptides of ≈ 2,000 and 3,500 respectively. This suggestion is supported by the fact that all the amino acids in the human peptide are also present in the bovine peptide (see Table 2.3). The additional 15 - 16 amino acid residues present in the bovine active site peptide, which are not present in the human amino acid sequence, may be attributable to the fact that there was no methionine residue present ≈ 16 amino acids away from the modified active site amino acid (towards the C-terminus) in the bovine enzyme which was present in the human enzyme. However, this proposal is not based on solid evidence and further sequence information on both these mammalian enzymes is required before such a proposal can be corroborated.

Reaction of mammalian 5-aminolevulinic acid dehydratase with $NaBH_4$ in the presence of [4-^{14}C] ALA was shown to inactivate the enzyme and was associated with the incorporation of label into the enzyme protein. (up to a maximum of 1 mole ^{14}C ALA/mole subunit). This inactivation was due to the reduction of the Schiff base intermediate formed between the substrate, ALA, and an amino group (probably lysine) at the active site of the enzyme (see Sections 2.3.2 and 2.3.6) and consequently inactivation could be prevented by the presence of the product, porphobilinogen (Scheme 2.7). The modified enzyme was used to determine the nature of the active sites of both the bovine and human enzymes.

A comparison of the sequence of the active site peptides of both human erythrocyte and bovine liver (Tables 2.4 and 2.5 respectively) clearly demonstrated that the first five amino acids in both sequences were identical. Also, since both peptides originated from CNBr digestion of the native enzyme it can be inferred that the peptide valine is adjacent to a methionine residue in each case. This suggestion was further corroborated by the elucidation of the N-terminal sequence of the bovine enzyme by Lingner and Kleinschmidt (1983) (see Section 2.1) which showed that this valine residue was <u>not</u> the N-terminal amino acid of the native enzyme and consequently the valine present in the active site peptide was adjacent to a methionine residue. (The peptide bond between the valine and methionine residues was subsequently cleaved by treatment with CNBr). The common hexapeptide at the active site of the human and bovine enzymes was as follows:



The modified ^{14}C-labelled amino acid (X*) at the active site, responsible for the initial binding of substrate, has been characterised using a variety of techniques (see Sectin 2.3.6). However, the precise chemical nature of this residue was not determined.

Studies by Nandi (1978) have suggested that this active site group is the ε-amino group of a lysine residue in the case of the <u>R.sphaeroides</u> enzyme, but indirect evidence suggesting an arginine residue may also be found in the literature (Spinach enzyme; Liedgens <u>et al.</u>, 1983). The vital evidence to determine the nature of the modified residue (X*) involves the synthesis of ALA-lysine and subsequent comparison of the chromatographic and electrophoretic properties of both the ALA-lysine and the modified amino acid (X*).

An interesting feature of the common active site hexapeptide is the fact that proline is present, suggesting that the sequence is in a non helical structure. Also, a closer examination of the amino acid sequence of the bovine active site peptide revealed that most of the residues present are hydrophobic in nature (12/17) whilst only five of the residues are charged (including the active site amino group).

These data suggest that the peptide sequenced probably originates from the interior of the enzyme protein. The sequence homology between the bovine and human active site peptides may, in fact, be greater but it was not possible to sequence more than five turns of the human peptide due to the loss of peptide on extraction of excess reagents (PITC and DABITC) at the end of the coupling reaction. However, alternative methods of sequencing are available, particularly mass spectrometry (Shemyakin et al., 1970; Morris et al., 1971), which may be of use in further elucidating the primary structure of the human active site peptide. Alternatively, genetic techniques, involving the chemical synthesis of polydeoxynucleotide sequences based upon the amino acid sequence already known, could be used to obtain the 5-aminolevulinic acid dehydratase gene which could be subsequently used to determine the primary structure of the enzyme protein (Sanger et al., 1977).

CHAPTER 3

AN INVESTIGATION INTO THE INTERRELATIONSHIP BETWEEN ZINC, LEAD AND THE THIOL GROUPS IN HUMAN ERYTHROCYTE 5-AMINOLEVULINIC ACID DEHYDRATASE

CHAPTER 3

An Investigation into the Interrelationship Between Zinc, Lead and the Thiol Groups in Human Erythrocyte 5-Aminolevulinic Acid Dehydratase

3.1 Introduction

5-Aminolevulinic acid dehydratase (EC 4.2.1.24) is the second enzyme in the haem biosynthetic pathway and catalyses the condensation between two molecules of 5-aminolevulinic acid (ALA) to form the monopyrrole, porphobilinogen. The enzyme from human erythrocytes has been purified to homogeneity (Anderson and Desnick, 1979; Chapter 2 of this thesis) and has been shown to consist of eight identical subunits of MR 35,000 arranged into a cubic octameric structure with dihedral (D_4) symmetry (Despaux et al., 1979). The human enzyme, in common with all other dehydratases, regardless of source, is sensitive to oxygen and requires a high exogenous thiol concentration for the maintenance of full catalytic activity (Shemin, 1976). However, the activated enzyme (see Section 3.2.2) retains full catalytic activity in the absence of thiol provided that anaerobic conditions are maintained. The sensitivity of the bovine enzyme to inactivation by oxygen as well as by thiophilic reagents, such as p-chloromercuribenzoate (Gibson et al., 1955; Wilson et al., 1972), N-ethylmaleimide (Batlle et al., 1967), iodoacetic acid (Chaudhry et al., 1976; Jordan et al., 1976; Barnard et al., 1977), iodoacetamide (Batlle et al., 1967; Barnard et al., 1977) and various halolevulinic acids, including the active-site-directed 5-chlorolevulinic acid (Seehra and Jordan, 1981), has led to the suggestion that there are at least two SH groups at the active site of the enzyme which are important for catalytic activity.

A more detailed investigation into the nature of these SH groups in the bovine enzyme was carried out using Ellman's (1959) reagent, 5, 5'-dithiobis (2-nitrobenzoic acid) (Nbs_2), a thiophilic reagent which has been used to study many other cysteine-containing enzymes including glyceraldehyde-3-phosphate dehydrogenase (Wassarman and Majors, 1969) and succinic thiokinase (Nishimura et al., 1975). Studies of the reaction of bovine 5-aminolevulinic acid dehydratase with Nbs_2 revealed the existence of 4 reactive sulphydryl groups in the native enzyme, which increased to 8 - 9 groups after denaturation with

0.4% w/v SDS (Tsukamoto et al., 1979; Seehra et al., 1981). Preliminary studies by Gore et al., (1976) have shown that two SH groups (termed Groups I and II) per monomer of the native enzyme react very rapidly (<1 minute) with Nbs_2 and that one of these groups is alkylated by iodoacetic acid. These two groups have been analysed in greater detail using stopped-flow spectrophotometry (see Seehra et al., (1981) for further details). A third SH group (Group III) titrates over a period of 2 - 3 minutes and modification of this group causes irreversible inactivation of the enzyme. Seehra et al., (1981) have proposed that this group plays an important role in maintaining a catalytically competent conformation. Finally, a fourth group (Group IV) titrates over a period of ≈ 30 minutes.

On exposure of the native enzyme to oxygen, there is a rapid loss of enzymic activity which is associated with the oxidation of the two highly reactive sulphydryl groups (I and II) to form a disulphide bridge (Tsukamoto et al., 1979; Seehra et al., 1981). Full catalytic activity can be restored upon reexposure of the enzyme to reducing conditions, provided that the slower reacting SH group (III) has not been modified. Studies by Barnard et al., (1977) have demonstrated that the highly reactive sulphydryl groups have a differential reactivity towards the alkylating agents, iodoacetic acid and iodoacetamide, and they went on to isolate and sequence two different tryptic peptides obtained by modifying the bovine enzyme with the two alkylating agents (see Section 2.1 for further details).

The first indication of a metal requirement for mammalian 5-aminolevulinic acid dehydratase was reported by Gibson et al., (1955) who demonstrated that the bovine enzyme was inhibited by treatment with the chelating agent, EDTA. Gurba et al., (1972) and Wilson et al., (1972) also demonstrated inhibition of enzymic activity using other chelating reagents, such as O-phenanthroline and 8-hydroxyquinoline, which suggested the presence of a divalent metal ion in 5-aminolevulinic acid dehydratase. The demonstration that zinc was able to completely restore activity to the EDTA-inhibited enzyme (Wilson et al., 1972; Cheh and Neilands, 1973, 1976; Thomasino et al., 1977) suggested that zinc was the metal ion present in the enzyme and this was subsequently confirmed using atomic absorption spectroscopy which revealed the

presence of 1 - 2 g atoms zinc/mole of the octameric enzyme as isolated. Reconstitution experiments, however, suggested that the actual stoichiometry may be higher (Gurba et al., 1972; Cheh and Neilands, 1973, 1976; Bevan et al., 1980).

Investigations by Abdulla and Haeger-Aronsen (1971) showed a significant activation of human 5-aminolevulinic acid dehydratase in erythrocyte lysates upon addition of zinc (in vitro) and this was subsequently confirmed by numerous reports in the literature (Finelli et al., 1975; Border et al., 1976a, b; Meredith et al., 1977; Davis and Avram, 1978, 1980; Tomokuni, 1979; Trevisan et al., 1980). Similarly, zinc has been shown both to stimulate enzyme activity of red cell haemolysates in vivo in man (Abdulla and Svensson, 1979; Meredith and Moore, 1980) and in rats (Abdulla et al., 1976), and also to be essential for the de novo synthesis of the enzyme (Komai and Neilands, 1968; Finelli et al., 1974). Studies on the isolated enzyme have demonstrated a similar dependance on zinc for maximal catalytic activity (Wilson et al., 1972; Cheh and Neilands, 1973; Despaux et al., 1977; Anderson and Desnick, 1979). Consequently detailed studies on the binding of zinc to bovine 5-aminolevulinic acid dehydratase has been investigated by several groups (Gurba et al., 1972; Cheh and Neilands, 1973; Tsukamoto et al., 1979; Bevan et al., 1980). However, despite these reports there is some uncertainty about the number of zinc atoms bound to the mammalian enzyme and whether zinc is indeed involved in the catalytic mechanism (Cheh and Neilands, 1976; Batlle and Stella, 1978; Tsukamoto et al., 1980).

Cadmium has been shown to substitute for zinc in mammalian dehydratase and restore full catalytic activity, whereas cobalt, manganese and other divalent metal ions are ineffective (Cheh and Neilands, 1976; Bevan et al., 1980). Furthermore cadmium substitution has been shown to generate an absorption band centred at 235 nm and a positive circular dichroism (CD) band below 260 nm (Cheh and Neilands, 1976); properties indicative of a cadmium-mercaptide complex (Kagi and Vallee, 1961). Also, the binding of zinc to the bovine apoenzyme depends on the presence of the two highly reactive sulphydryl groups (I and II) because on oxidation or chemcial modification of these groups the apoenzyme is no longer able to bind zinc (Tsukamoto et al., 1979; Bevan et al., 1980); Jaffe et al., 1984). These results cumulatively

suggest that zinc binds to one or more free sulphydryl groups thus accounting for the requirement of a thiol activator for maximal catalytic activity (since thiol maintains the sulphydryl groups in the reduced form).

Numerous groups have reported that lead potently inhibits mammalian 5-aminolevulinic acid dehydratase activity in red cell haemolysates in vivo and in vitro (De Bruin, 1968; Haeger-Aronsen et al., 1971, 1974; Hernberg and Nikkanen, 1972; Roels et al., 1974; Meredith and Moore, 1978; Hellmut and Beyersmann, 1983). This inhibition of enzymic activity was used as a very specific and sensitive indicator of the levels of plasma lead, before more direct micromethods for determining plasma lead levels were available. In all cases there is a good negative correlation between $\log_{10}$ of the dehydratase activity and the blood levels (Pb-B) with correlation values (r) of between -0.5 and -0.9 (Hernberg and Nikkanen, 1970; Haeger-Aronsen et al., 1971, 1974; Lauwerys et al., 1973; Baloh, 1974; Howard, 1978; Mauras and Allain, 1979). Lead has also been shown to inhibit purified preparations of mammalian 5-aminolevulinic acid dehydratase (Gibson et al., 1955; Despaux et al., 1977; Anderson and Desnick, 1979) and it has been assumed that the inhibition is due to the interactions of lead with one or more of the crucial SH groups present in the enzyme (Vallee and Ulmer, 1972).

In this connection, lead has also been shown to produce a marked depression in the activities of many other enzymes with sensitive SH groups, including two other enzymes in the haem biosynthetic pathway, ferrochelatase and coproporphyrinogen oxidase, as well as ATPase, carbonic anhydrase and fructose-1, 6-bisphosphate (Vallee and Ulmer, 1972; McColl and Goldberg, 1980).

Recent studies using a radioimmunoassay technique have demonstrated that lead treatment results in an increased synthesis of 5-aminolevulinic acid dehydratase in bone marrow cells of rats (Fujita et al., 1981; Kajimoto et al., 1983) and humans (Fujita et al., 1982). It has been suggested that this increased synthesis is an attempt by the body to counteract lead inhibition but the precise mechanism has yet to be resolved. Other studies have revealed that zinc completely reverses

the lead-induced inhibition of 5-aminolevulinic acid dehydratase both *in vitro* (Finelli *et al.*, 1975; Haeger-Aronsen *et al.*, 1976; Meredith *et al.*, 1977; Davis and Avram, 1978; Tomokuni, 1979).

Ethanol (Moore *et al.*, 1971; Abdulla *et al.*, 1976; Wigfield *et al.*, 1982) and various metal ions, including manganese, copper, tin, mercury and aluminium, have been reported by many groups to effect the activity of erythrocyte 5-aminolevulinic acid dehydratase (Wada *et al.*, 1972; Meredith *et al.*, 1974; Thompson *et al.*, 1976; Border *et al.*, 1976b; Davis and Avram, 1980; Trevisan *et al.*, 1980) but the precise mechanism(s) by which these effects are achieved has not been determined.

In this Chapter, a *detailed* evaluation of the role of zinc, thiol and various metal ions, particularly lead, have been carried out on the *purified* human erythrocyte enzyme (see Section 2.2.2). Binding of ^{65}zinc to the human apoenzyme was demonstrated and the precise stoichiometry of this incorporation was determined. The interrelationship between ^{65}zinc binding, lead-inhibition and the oxidation status of the SH groups in the purified enzyme were determined in order to elucidate not only the gross effects on the enzyme, but also to try to determine the detailed mechanism of the metal ion interaction and inhibition and the relationship between this inhibition and sulphydryl group function.

3.2 Experimental.

3.2.1 Materials.

5, 5'-Dithiobis-(2-nitrobenzoic acid) (Nbs_2) was obtained from the Sigma Chemical Co., London, U.K. Sephadex G-50 was obtained from Pharmacia. ^{65}zinc chloride (specific activity = 11.6 m Ci/mmole) was purchased from Amersham International, Amersham, U.K. All metal salts used were of AnalaR grade and were obtained from BDH Laboratories, Poole, Dorset, U.K. 3-chloro-and 5-chloro-levulinic acid were synthesised by Dr. J.S. Seehra. Human erythrocyte 5-aminolevulinic acid dehydratase was purified as described in Chapter 2. ALA and other materials were obtained from the same sources as described in Section 2.2.1.

3.2.2 Activation of Human 5-Aminolevulinic Acid Dehydratase.

In order to ensure that this oxygen sensitive enzyme was fully active, the dehydratase was always subjected to an "activation" procedure immediately prior to use involving incubation with an excess of reducing agent and exogenous zinc ions. 5-Aminolevulinic acid dehydratase was dissolved in 0.1M potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol and 100 µM zinc chloride and incubated at 37°C for 10 minutes. The enzyme, thus activated, was desalted, under an atmosphere of nitrogen, on a column of Sephadex G-50 equilibrated at 0°C in 0.1M potassium phosphate buffer and stored at 0°C under nitrogen until required.

3.2.3 Rapid Gel Filtration of Human 5-Aminolevulinic Acid Dehydratase Using Sephadex G-50 "Syringe" Columns.

The rapid separation of low molecular weight compounds from small volumes of the human dehydratase enzyme was achieved using a Sephadex G-50 "syringe" column. Sephadex G-50 fine (10 g) was suspended in 115 mls of either 0.1M potassium phosphate buffer (pH 6.8) or 0.1M Tris-Hcl buffer (pH 7.1) (both buffers were made using deionised water) and was stored at 4 °C under an atmosphere of nitrogen until required. Plastic disposable syringe barrels (1 ml; plunger removed) were plugged with non-absorbant cotton wool, filled with the desired Sephadex G-50 solution and centrifuged at full speed for 2 minutes in an MSE bench

centrifuge in order to remove excess buffer. The enzyme solution (up to 150 µl) was applied to the top of the column, followed by a 50 µl "flush" of the required buffer, and placed in a 10 cm x 9 mm acid treated test-tube under an atmosphere of nitrogen. The test-tube was sealed with Parafilm and centrifuged for 20 - 30 seconds at full speed in an MSE bench centrifuge. The resultant enzyme solution was free of the low molecular weight contaminants and was stable for many hours when stored at 4 °C under an atmosphere of nitrogen. This method has proved particularly useful for the rapid separation of human 5-aminolevulinic acid dehydratase from unbound ^{65}zinc and exogenous thiol. One additional advantage of this technique is the fact that the enzyme solution is diluted by as little as 30% as compared to 500 to 1000% using conventional gel filtration.

3.2.4 Assay for Enzymic Activity.

The activity of human 5-aminolevulinic acid dehydratase was determined essentially as described in Section 2.2.4. When the effect of inhibitors or metal ions were studied they were preincubated with the enzyme for 10 minutes at 37°C before the addition of the substrate ALA (0.05 - 5 mM). In assays where the interaction of zinc and lead were being investigated (Section 3.3.3) the enzyme solution was always added to the assay mixture containing both metals. Also, all assays were performed in duplicate.

3.2.5 Preparation of Apoenzyme.

At all stages precautions were taken to ensure minimal contamination by trace elements (see Thiers, 1957). The human 5-aminolevulinic acid dehydratase apoenzyme was prepared by a method similar to Tsukamoto *et al.*, (1979): Purified holoenzyme (2 - 3 mg) was incubated in 0.1M Tris-HCl buffer (pH 7.1; 200 µls) containing 10 µmoles EDTA and 2 µmoles dithioerythritol for 30 minutes at 37°C. Column chromatography of the incubation mixture under nitrogen using Sephadex G-50 equilibrated in 0.1M Tris HCl (pH 7.1) yielded the apoenzyme which contained between 0.07 and 0.09 zinc atoms per subunit of the enzyme (as determined by atomic absorption spectroscopy). The holoenzyme was obtained when 100 µM (final concentration) zinc chloride was added to the apoenzyme and incubated at 25°C for 2 minutes before use.

3.2.6 Incorporation of ^{65}Zinc into the Apoenzyme.

The apoenzyme (250 µg; 7.2 nmoles) was treated with increasing amounts of ^{65}zinc (55,000 dpm/nmole) and incubated under an atmosphere of nitrogen for 2 minutes at 4°C. The unbound zinc was separated from the ^{65} zinc holoenzyme by Sephadex G-50 gel filtration as described above. Fractions were assayed for enzyme activity and the protein concentration was determined (see Section 2.2.20). The amount of radioactivity associated with the fractions containing enzyme protein was determined in a Beckmann Biogamma II γ-radiation detector.

3.2.7 Exchange of Label in the ^{65}Zinc Holoenzyme by Divalent Cations.

The ^{65}zinc holoenzyme (250 µg; 7.2 nmoles) was incubated for timed intervals with various concentrations of divalent cations at 4°C under an atmosphere of nitrogen and the displaced ^{65}zinc was removed by Sephadex G-50 gel filtration. Enzyme activity, protein concentration and the amount of label remaining in the enzyme protein was determined as described above.

3.2.8 Alkylation of 5-Aminolevulinic Acid Dehydratase.

The apoenzyme (2 mg) was pretreated with various alkylating agents, including iodoacetic acid, 3-chlorolevulinic acid and the active-site-directed reagent,5-chlorolevulinic acid (Seehra and Jordan, 1981), under an atmosphere of nitrogen in Tris-Hcl buffer (pH 7.1) for 30 minutes at room temperature. When the effect of alkylation on ^{65}zinc binding was being investigated, the apoenzyme was separated from the alkylating agents by gel filtration on Sephadex G-50 immediately prior to determining the incorporation of ^{65}zinc into the modified enzyme. In a converse series of experiments, the ^{65}zinc holoenzyme was treated with the alkylating agents as described above and after removal of displaced label the amount of ^{65}zinc bound to the enzyme protein was determined.

3.2.9 Oxidation of the Enzyme.

The enzyme was incubated at 37°C in 0.1M potassium phosphate buffer (pH 6.8) and exposed to air (Scheme 3.15) or oxygen (Scheme 3.18) in open tubes of area 1.45 cm^2 which had a depth of 5 cm. Aliquots were removed at timed intervals and the protein concentration and enzymic activity determined after Sephadex G-50 gel filtration.

3.2.10 Spectrophotometric Determinations.

(a) Reaction of 5-Aminolevulinic Acid Dehydratase with Nbs_2 Using Conventional Spectrophotometry.

The human dehydratase apoenzyme (10 nmoles), prepared as described above, was mixed in a final volume of 1 ml with Nbs_2 (5 - 500 nmoles) in 100 µmoles Tris HCl buffer (pH 7.1) at 25°C in the presence or absence of 100 nmoles of zinc chloride. The absorbance of the liberated Nbs was measured at 412 nm using a Pye-Unicam SP 8400 spectrophotometer (E_{412} = 12,800 M^{-1} cm^{-1}) and followed until the reaction was complete (≈ 30 minutes). Similar reactions were performed in the presence or absence of 50 nmoles of lead chloride. This method was used for time courses of greater than 30 seconds.

(b) Reaction of 5-Aminolevulinic Acid Dehydratase with Nbs_2 Using Stopped-Flow Spectrophotometry.

For time-courses of the reaction between 5-aminolevulinic acid dehydratase and Nbs_2 from 0.25 to 20 seconds, a Hi-Tech Instruments stopped-flow spectrophotometer (Salisbury, U.K.) was used. The apparatus had a dead-time of approximately 1.8 msec with a pathlength of 2 mm. The change in absorbance of each reaction was monitored simultaneously on an oscilloscope and on a Biomac 1000 data handling device (London, U.K.) which stored and averaged four consecutive traces before displaying the resultant trace onto a chart recorder. The stopped-flow apparatus was fitted with two 4 ml syringes, one containing 10 µM enzyme in 0.1M Tris HCl (pH 7.1) at 25°C and the other containing 1 mM Nbs_2 in same buffer. The reaction of 5-aminolevulinic acid dehydratase with Nbs_2 was performed in the presence or absence of either zinc chloride (100 µM) or lead chloride (50 µM) (final concentrations).

(c) Protein Fluorescence Spectrophotometry.

The human dehydratase apoenzyme (180 µg; 5.14 nmoles) was dissolved in 200 µmoles potassium phosphate buffer (pH 6.8; 2 ml) containing 20 µmoles dithioerythritol and placed in a fluorimeter cell (1 cm x 1 cm). The fluorescence maximum of the apoenzyme was determined in a Perkin Elmer MPF3 fluorimeter thermostated at 21°C by excitation of the endogenous aromatic amino acids at 280 nm and scanning the emission wavelength from 290 nm to 370 nm at a scan rate of 15 nm/minute .

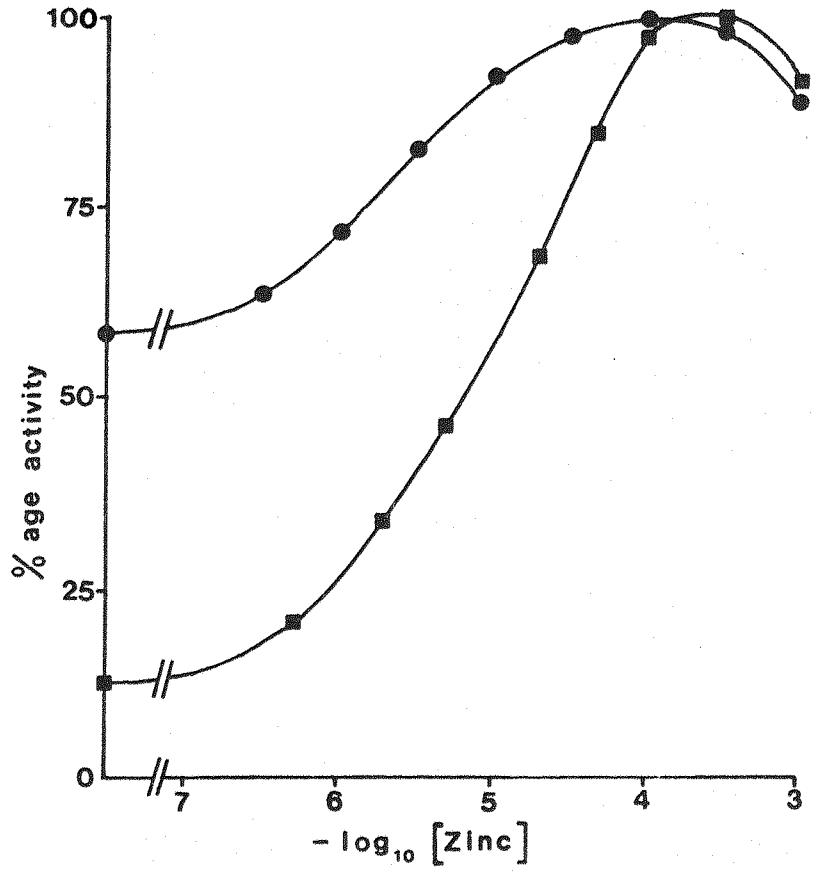
Small volumes (10 µl) of the divalent metal ions, zinc chloride (4 - 800 nmoles) and lead chloride (10 - 200 nmoles), were mixed with the apoenzyme and the fluorescence intensity and the wavelength of maximum emission were measured. The effect of the chelating agent EDTA (2 - 18 µmoles) on the fluorescence of both the apoenzyme and holoenzyme was also investigated.

3.3 Results.

3.3.1 Dependance on Zinc for the Catalytic Activity of Human 5-Aminolevulinic Acid Dehydratase.

The activity of the native human enzyme was measured with increasing amounts of zinc in the assay medium. Optimal stimulation of the enzyme occurred in the presence of 0.1 mM zinc (Scheme 3.1) when a specific activity of 24.0 units/mg was obtained (see Section 2.2.2). There was some variation in the specific activity of native 5-aminolevulinic acid dehydratase (ranging from 14 - 19 units/mg) obtained from different enzyme purifications when the enzyme was assayed in the absence of zinc. This variation is almost certainly attributable to the difference in the amount of zinc still associated with the enzyme after final purification. Therefore, in order to ensure that the enzyme contained its full complement of zinc, it was always subjected to an "activation" procedure with zinc and dithioerythritol as described in Section 3.2.2. This activated enzyme or holoenzyme was largely unaffected by the presence of zinc ions in the assay medium and had a specific activity of between 21 and 22 units/mg after excess free zinc had been removed by gel filtration.

To investigate the dependance on zinc for the catalytic activity of the enzyme in more detail, the apoenzyme (prepared as described in Section 3.2.5) was incubated in the presence of dithioerythritol (10 µmoles) with increasing amounts of zinc (0.5 nmoles - 10 µmoles) in a final volume of 1 ml. The results clearly show a concentration dependant activation of the apoenzyme by zinc (Scheme 3.1) with maximal stimulation occurring between 100 and 300 µM zinc. This dependance on zinc for the catalytic activity of human 5-aminolevulinic acid dehydratase was further investigated by incubating the apoenzyme under an atmosphere of nitrogen in the presence and absence of both zinc (100 nmoles) and dithioerythritol (10 µmoles) at 37°C in a final volume of 1 ml. The data (presented in Scheme 3.2) clearly shows that almost fully catalytic activity was maintained when the apoenzyme was incubated in the presence of zinc ions alone as was the case when the incubation was carried out in the presence of both zinc and dithioerythritol. Incubations of the apoenzyme with no addition or in the presence of dithioerythritol alone showed a much reduced activity, highlighting the requirement for zinc for full catalytic activity.

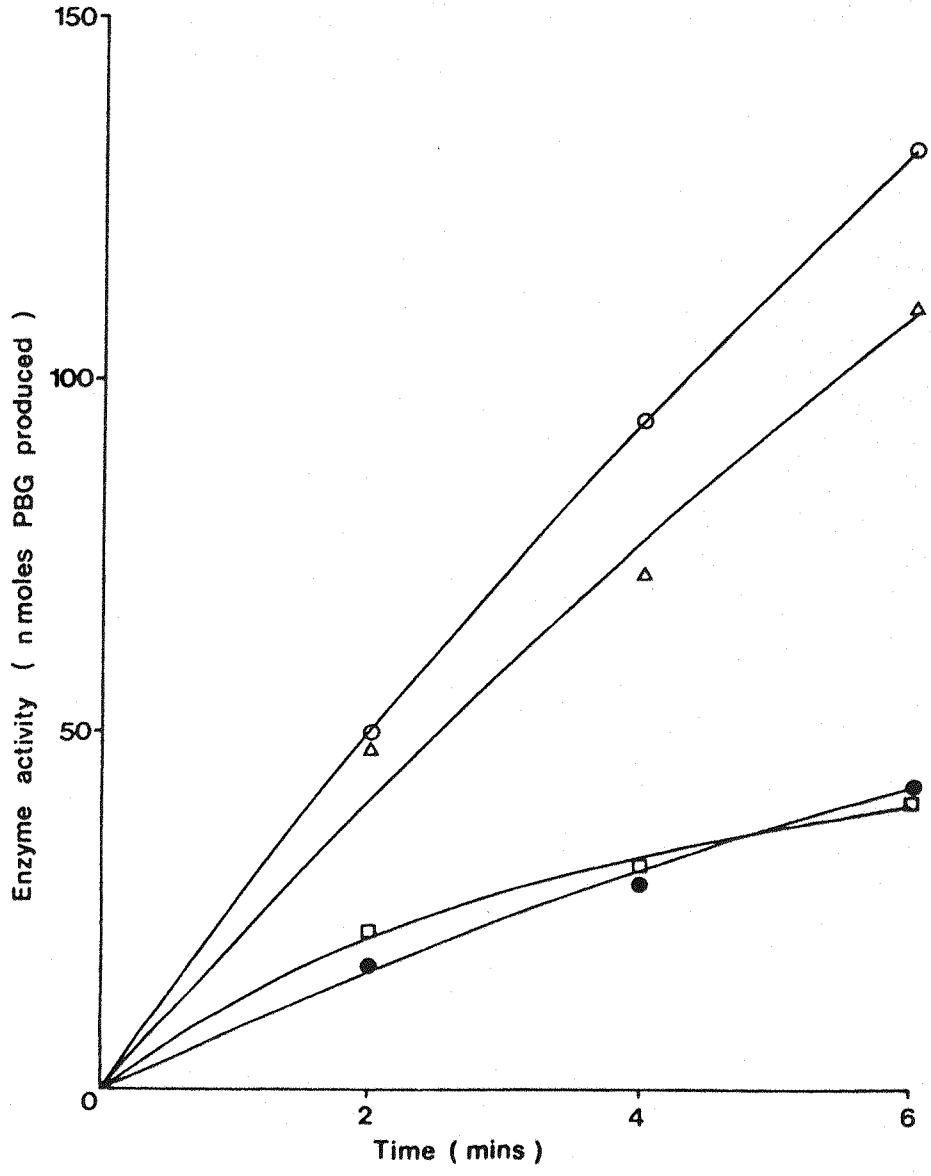


Scheme 3.1 The Effect of Zinc Ion Concentration on the Activity of Native and Apo-5-Aminolevulinic Acid Dehydratase.

Native (●——●) and apo- (■——■) 5-aminolevulinic acid dehydratase from human erythrocytes was preincubated for 10 minutes at 37°C in 100 µmoles potassium phosphate buffer (pH 6.8) containing 10 µmoles dithioerythritol and increasing amounts of zinc (0.5 nmoles - 1 µmole) in a final volume of 900 µl. After the addition of 100 µls of ALA (5 µmoles; neutralized with 0.1 N NaOH prior to use), incubations were carried out for 10 minutes and terminated by the addition of 1 ml of 10% w/v tricholoroacetic acid containing 0.1 M mercuric chloride. The porphobilinogen produced during the reaction was determined as described in Section 2.2.4. The data are presented in a graphical form as $\log_{10}$[zinc] versus the enzyme activity. (100% represents the maximum activity of the enzyme).

Scheme 3.2 Determination of the Enzyme Activity of the Human Dehydratase Apoenzyme Under Various Conditions.

The apoenzyme was prepared as described in Section 3.2.5 and was assayed under anaerobic conditions (N_2 atmosphere) at 37°C under the following conditions: No additives (□——□); 100 µM zinc chloride (△——△); 10 mM dithioerythritol (●——●); 100 µM zinc chloride plus 10 mM dithioerythritol (○——○). Aliquots were removed at timed intervals and the reaction terminated by the addition of an equal volume of 10% w/v trichloroacetic acid containing 0.1 M mercuric chloride. The porphobilinogen produced was determined spectrophotometrically after reaction with p-dimethylamino benzaldehyde (see Section 2.2.4).



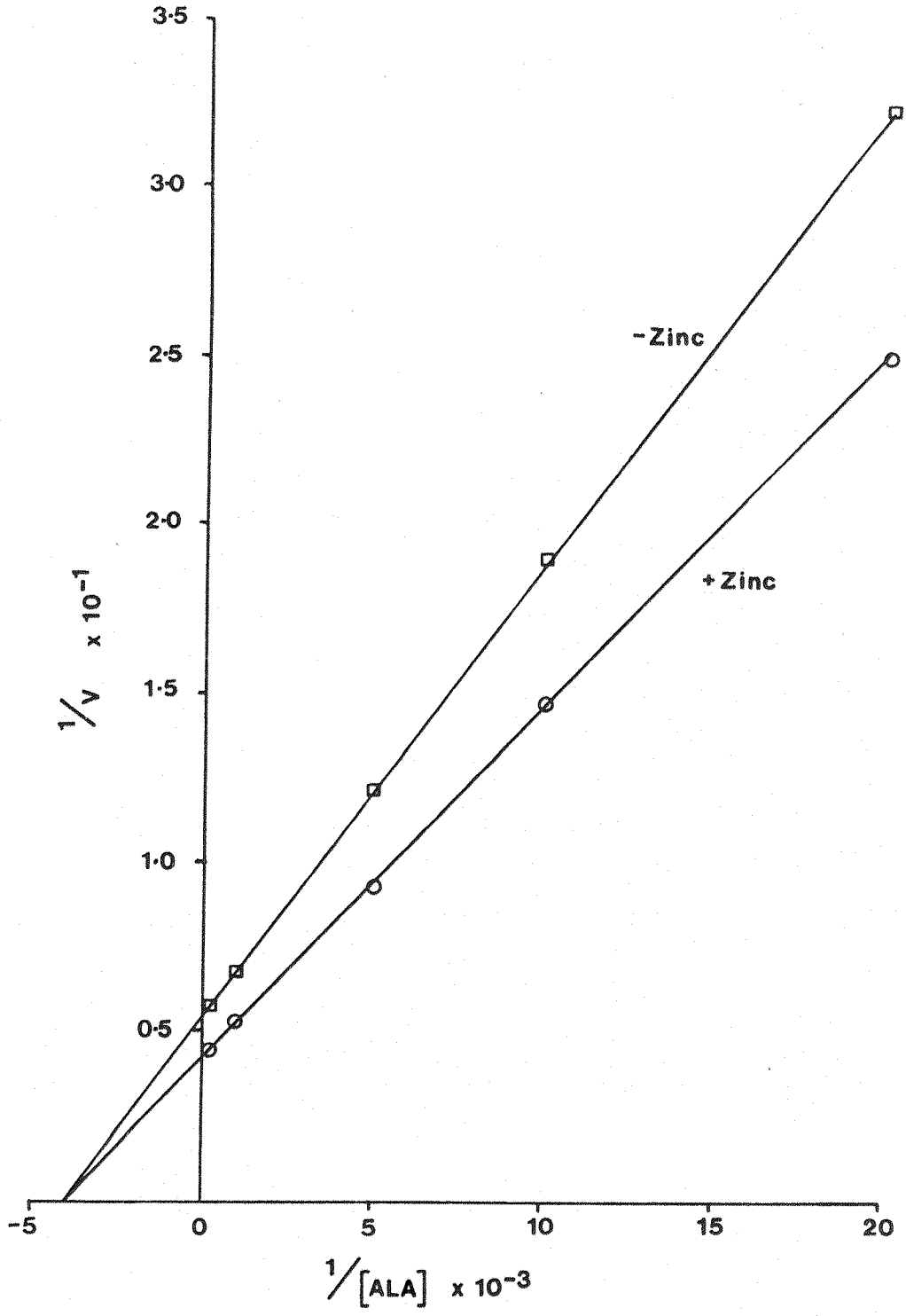
In fact the slight activation of the enzyme by dithioerythritol can be attributed to a small amount of zinc associated with this reagent (shown by atomic absorption spectroscopy).

Kinetic studies were performed on the native human dehydratase enzyme to determine the nature of the interaction of zinc with the enzyme. Incubations were carried out with increasing amounts of the substrate, ALA (50 nmoles - 5 μmoles), in the presence and absence of zinc ions (100 nmoles) for 10 minutes at 37°C in a final volume of 1 ml. The enzyme demonstrated Michaelis-Menten kinetics (see Section 1.1) and no cooperativity was observed. The two kinetic constants, K_m and V_{max}, were determined by hyperbolic non-linear regression analysis using a computer program. The results are most conveniently represented as a plot of $\frac{1}{v}$ against $\frac{1}{s}$ using the computer calculated intercepts (Lineweaver and Burk, 1934; Scheme 3.3). The data show that the affinity of the enzyme for ALA is unaffected by the presence of zinc (K_m = 0.251 ± 0.008 mM ± zinc; Table 3.1), whilst the maximum rate of the enzyme-catalysed reaction, V_{max}, is 18.78 ± 0.56 units/mg protein in the absence of zinc and increases to 23.90 ± 0.42 units/mg in the presence of 100 μM zinc.

3.3.2 The Effect of Various Metal Ions on the Activity of Native Human 5-Aminolevulinic Acid Dehydratase.

The effect of cadmium, a divalent cation which has many chemical properties similar to zinc (both belong to Group IIB of the periodic table) (Vallee and Ulmer, 1972), on the activity of native human 5-aminolevulinic acid dehydratase was investigated. The results are presented in Scheme 3.4 and clearly demonstrate that cadmium causes a slight, but significant, stimulation of enzyme activity at low concentrations which is similar to the effect produced by low concentrations of zinc ($\leq$ 3 μM). However, at higher concentrations of cadmium the enzyme activity is strongly inhibited ($K_i \sim$ 300 μM).

Lead has been shown to be a potent inhibitor of human 5-aminolevulinic acid dehydratase (De Bruin, 1968; Roels *et al.*, 1974; Davis and Avram, 1978, 1980). Therefore, studies of the effect of increasing amounts of lead on the activity of the native enzyme were determined under anaerobic conditions in the presence or absence of dithioerythritol (10 mM). The results clearly demonstrate a concentration dependant

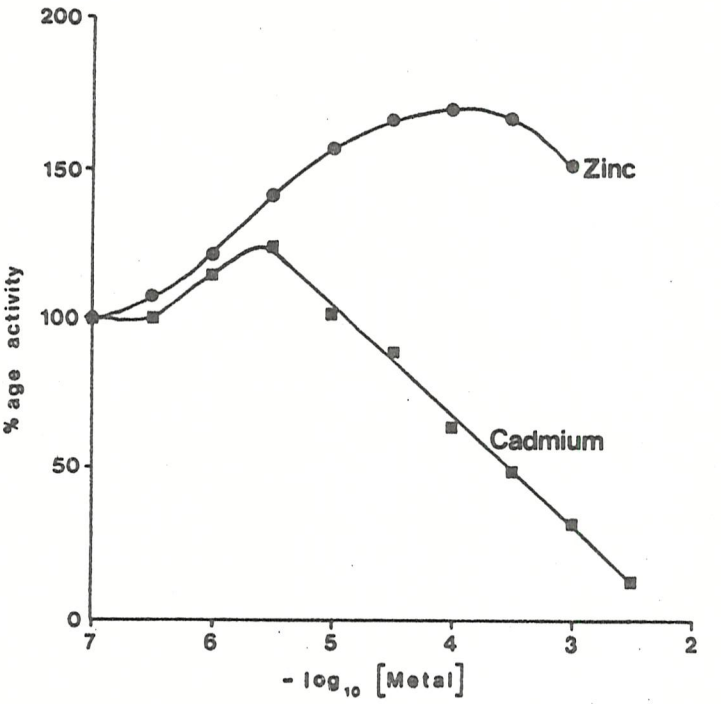


Scheme 3.3 Lineweaver-Burk Plot of the Effect of Zinc Ions on the Activity of Native Human 5-Aminolevulinic Acid Dehydratase.

The native enzyme (10.5 µg) was incubated with increasing concentrations of the substrate, ALA (50 nmoles - 5 µmoles) in the presence (100 nmoles) and absence of zinc chloride in a final volume of 1 ml for 10 minutes at 37°C. The concentration of porphobilinogen in the assay mixture was determined as described in Section 2.2.4. The plot of 1/v versus 1/s based on computer estimated intercepts obtained by hyperbolic non-linear regression analysis.

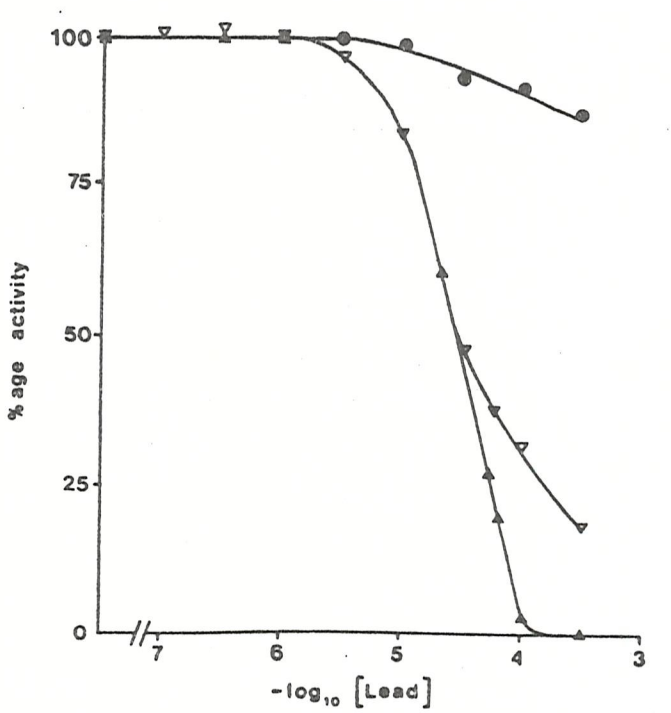
Scheme 3.4 The Effect of Cadmium and Zinc on the Activity of the Dehydratase Enzyme.

The activity of the native enzyme (10.5 µg) was determined as described in Section 2.2.4.



Scheme 3.5 Inactivation of the Native Dehydratase Enzyme by Lead and Protection by Zinc.

The activity of the native dehydratase enzyme was determined for increasing quantities of lead in the presence (▽——▽) and absence of 10 mM dithioerythritol (▲——▲)(see Section 2.2.4). The effect of the additional presence of zinc (100 µM) on lead inhibition was also determined (●——●).



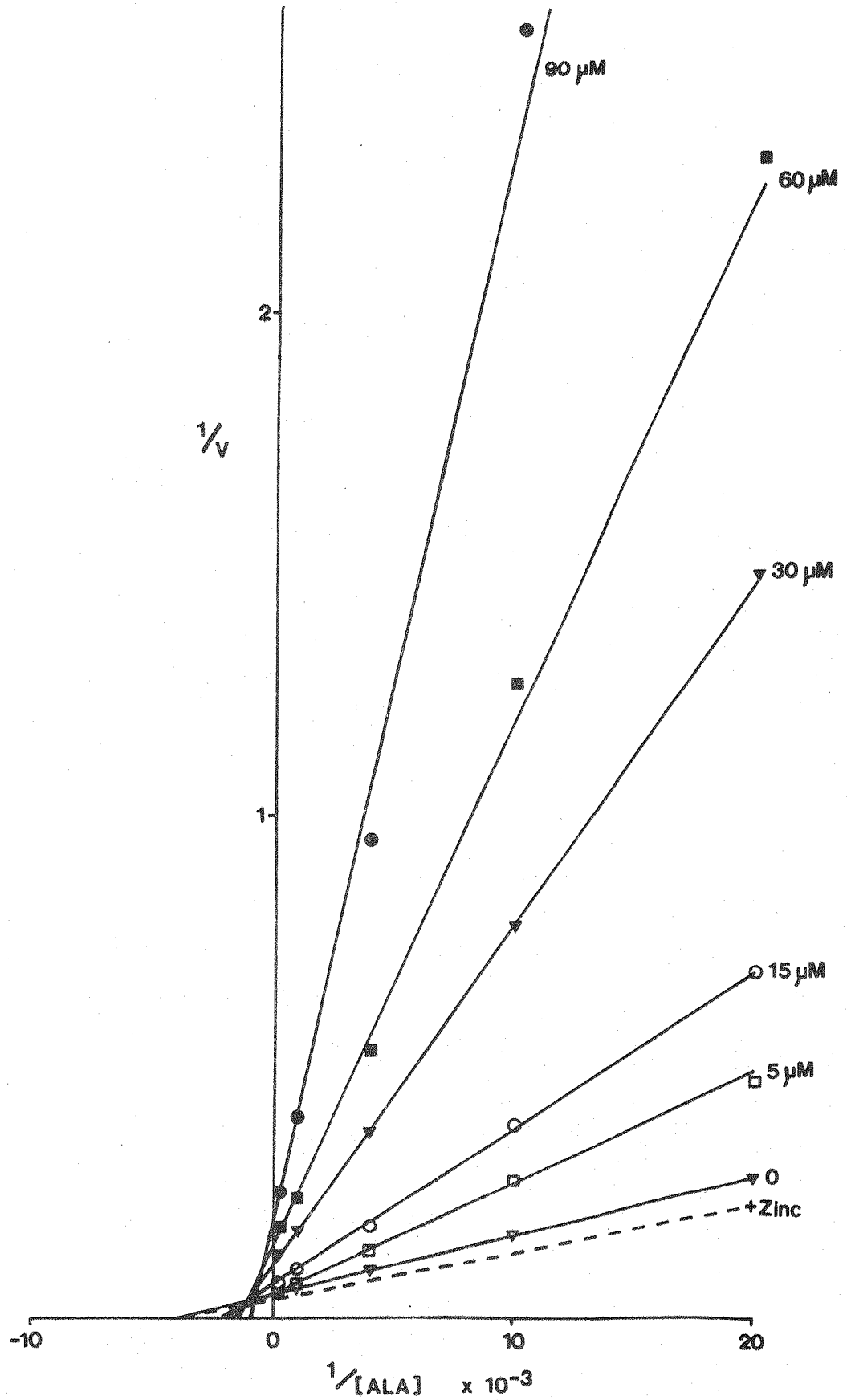
inactivation of the enzyme with a K_i of 28.1 ± 0.7 μM which was largely unaffected by the presence of dithioerythritol (Scheme 3.5). It was interesting to note that dithioerythritol protected the enzyme from inactivation by high concentrations of lead (≥ 60 μM) which is almost certainly due to the formation of a chelation complex between the lead and the exogenous thiol (lead-mercaptide complex). Kinetic analysis of the lead inhibition of the native dehydratase enzyme clearly showed that lead affected both the kinetic constants, K_m and V_{max} (Scheme 3.6, Table 3.1). These results are consistent with a non-competitive inhibition by lead.

Table 3.1 The Effect of Lead and Zinc on the Two Kinetic Constants, K_m and V_{max}, of Native Human 5-Aminolevulinic Acid Dehydratase.

Addition	K_m (μM ± SD)	V_{max} (μmoles/hr/mg protein ± SD)
100μ M Zinc	251.3 ± 8.1^{ns}	23.90 ± 0.42*
None	251.7 ± 7.3	18.78 ± 0.30
5 μM Lead	475.6 ± 21.7**	19.32 ± 0.56^{ns}
15 μM Lead	455.4 ± 21.9**	13.23 ± 0.40**
30 μM Lead	621.0 ± 39.4**	8.05 ± 0.35**
60 μM Lead	726.3 ± 96.5**	5.98 ± 0.55**
90 μM Lead	1043.0 ± 203.4**	4.38 ± 0.63**

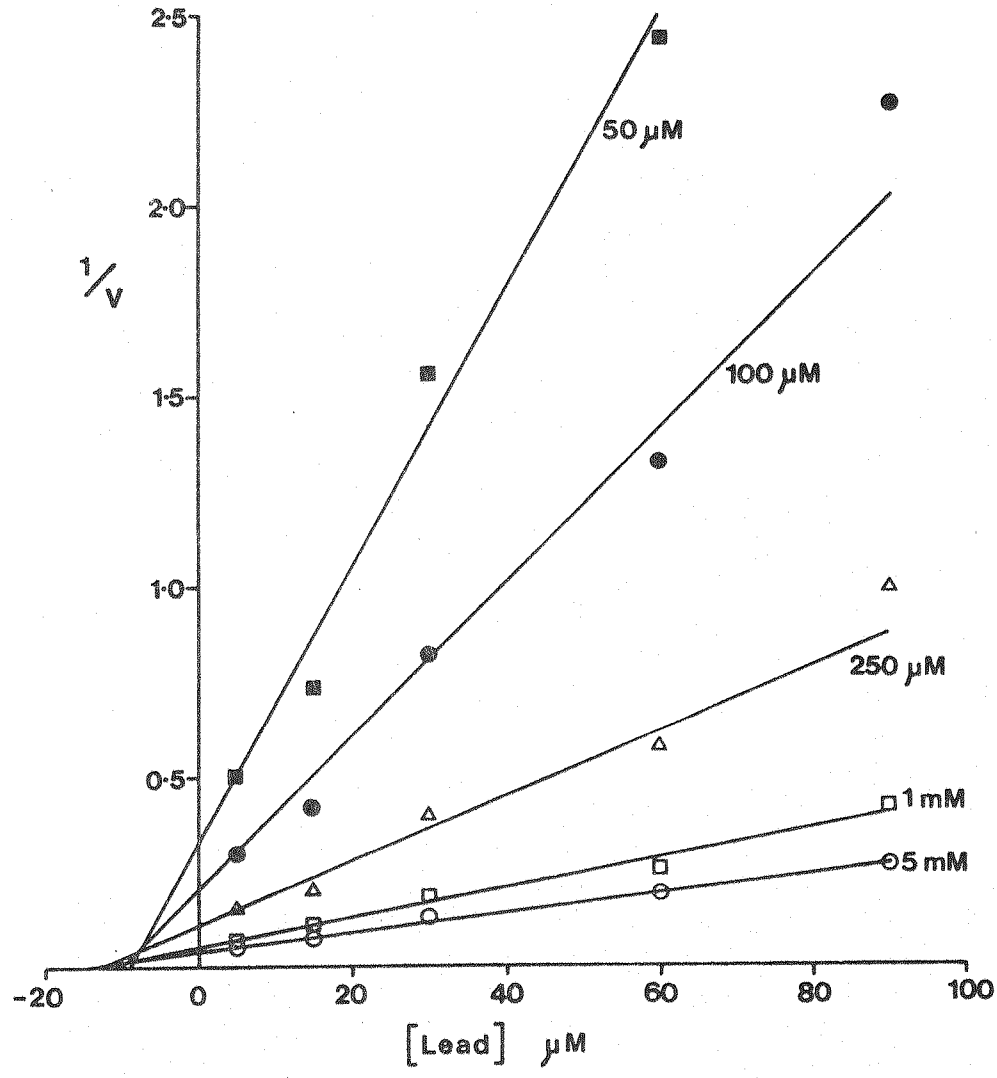
Statistical analysis by an unpaired"t-test".* = $p < 0.01$ ** = $p < 0.005$ ns = not significant.

These results were also analysed by hyperbolic non-linear regression according to the method of Dixon (1953) and revealed that the inhibition constant (K_i) for lead was dependant on the substrate concentration (Table 3.2). The data revealed that the presence of high substrate concentrations resulted in the requirement for a higher concentration of lead in order to produce the same degree of inhibition and suggested that the affinity of lead for the enzyme-substrate complex was lower than that for the free enzyme. The data are most conveniently presented as a plot of $\frac{1}{v}$ against the inhibitor concentration, i , for different substrate concentrations (Dixon plot; Scheme 3.7). A K_i app for lead of 9.7 μM was obtained and is in close agreement with the value of 28 μM which was determined previously (Scheme 3.5).



Scheme 3.6 Lineweaver-Burk Plot Representing the Inhibition of Native Human 5-Aminolevulinic Acid Dehydratase by Lead.

The effect of lead on the activity of the native enzyme (10.5 µg) was determined as described in Section 2.2.4. The graph of 1/v versus 1/s is based on computer estimated intercepts obtained by hyperbolic non-linear regression analysis. For each plot the concentration of lead is given.



Scheme 3.7 Dixon Plot (1/v Versus i) to Determine the Apparent Inhibition Constant (K_i app) for Lead.

The data from Scheme 3.6 was analysed by hyperbolic non-linear regression to yield the values for the intercepts on the ordinate axis and the absicca. For each plot the concentration of the substrate, ALA, is given.

Table 3.2 The Effect of Substrate Concentration on the K_i for Lead.

ALA (μM)	K_i (μM ± SD)
50	9.3 ± 3.3
100	10.6 ± 3.0
250	13.0 ± 3.3
1000	13.3 ± 1.8
5000	14.6 ± 1.9

Zinc has been reported to reverse the lead-induced inhibition of human 5-aminolevulinic acid dehydratase both in vivo and in vitro (Finelli et al., 1975; Haeger-Aronsen et al., 1976). Therefore the effect of increasing amounts of lead on the activity of the native enzyme was studied in the presence of 100 μM zinc (Scheme 3.5). The results clearly demonstrated that the inhibition of activity by lead was almost completely blocked by the presence of zinc. Additional experiments (Table 3.3), in which both lead and zinc were present, confirmed that zinc was very effective in protecting the enzyme from the inhibitory effect of lead.

Table 3.3 Interaction of Lead and Zinc. The Effect of the Presence of Varying Concentrations of Both Metal Ions on the Activity of the Human Enzyme.

	Zinc (μM)	Lead (μM)				
		0	10	30	100	300
APOENZYME	0	12.1	10.0	5.9	2.4	1.3
NATIVE ENZYME	0	75.3	63.3	35.7	24.1	13.5
	50	96.7	85.7	78.1	72.3	66.7
	100	100	99.5	93.6	92.2	87.8
	300	91.5	92.8	97.1	95.8	92.6

Analysis of the data in Table 3.3 by hyperbolic non-linear regression revealed that the dissociation constant (K_D) of lead from

the apoenzyme was 32.6 ± 8.6 μM. This value was almost identical to the K_D of lead from the native enzyme (29.2 ± 10.1 μM) and showed that the presence of zinc in the human enzyme had no effect on the dissociation constant for lead.

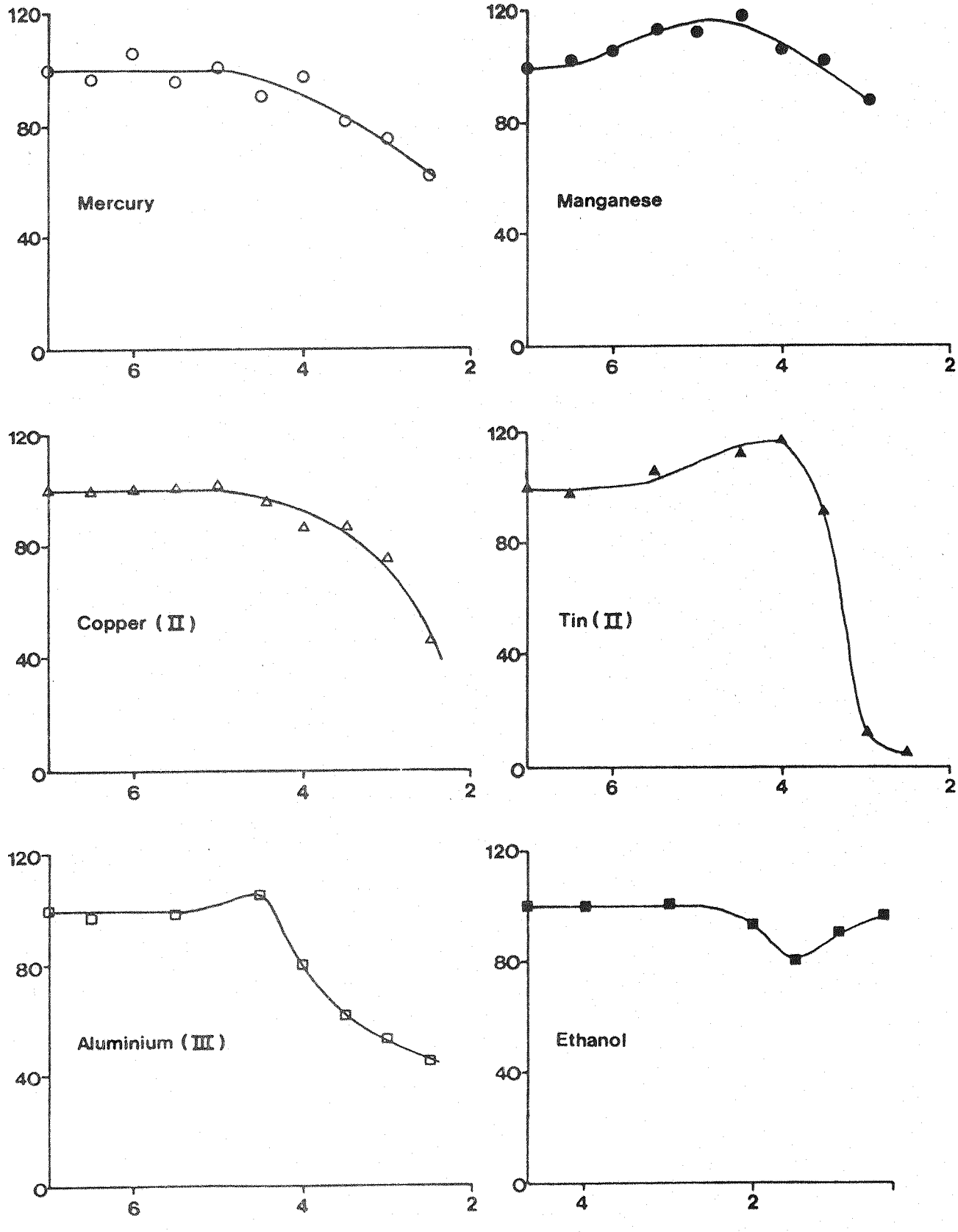
Various other metal ions, including aluminium, copper, tin, manganese, mercury and other heavy metals, have been shown to affect the activity of human 5-aminolevulinic acid dehydratase *in vitro* both with blood haemolysates (Border *et al.*, 1976; Davis and Avram, 1978, 1980; Trevisan *et al.*, 1980) and with the partially purified enzyme (Despaux *et al.*, 1977).

Ethanol has also been shown to affect dehydratase activity *in vivo* (Moore, 1972; Abdulla *et al.*, 1976; Wigfield *et al.*, 1982). Consequently the effect of these putative activators and inhibitors of human 5-aminolevulinic acid dehydratase were investigated *in vitro* using the highly purified enzyme (prepared as described in Section 2.2.2). The results are presented in Scheme 3.8 and clearly demonstrate that manganese (118% of native enzyme activity at 30 μM), aluminium (106% at 30 μM) and tin (117% at 100 μM) are all weak activators when compared to the intrinsic metal ion zinc (170% at 100 μM). Interestingly, aluminium and tin inhibited the enzyme activity at higher concentrations with K_i's of 0.48 mM and 1.6 mM respectively. Similarly copper ($K_i \sim 3$ mM) and mercury ($K_i \sim 4.3$ mM) are weak inhibitors of human dehydratase activity when compared to lead which has an inhibition constant of between 10 and 30 μM. On the other hand, ethanol did not have any substantial effect on the *in vitro* activity of the enzyme even when studied at high concentration (0.3M). Cumulatively these results suggest that the metal ions studied are either weak competitors for the lead and zinc binding site(s) or that they are acting at alternative low affinity binding sites and are therefore not physiologically significant.

The effect of various anions, including chloride, sulphate and acetate, on the activity of the native enzyme was investigated as a control to the studies on the metal salts above. None showed any significant effect on the enzyme activity.

Scheme 3.8 A Study of Various Putative Effectors of Native Human 5-Aminolevulinic Acid Dehydratase Activity.

The enzyme activity was determined as described in Section 3.2.4. The data points are means of at least duplicate assays, which varied by less than 5%. The x axis represents the negative $\log_{10}$ of the effector, whilst the y axis represents the % age activity compared to the activity of the native enzyme.



3.3.3 The Binding of ^{65}Zinc to the Human Dehydratase Apoenzyme.

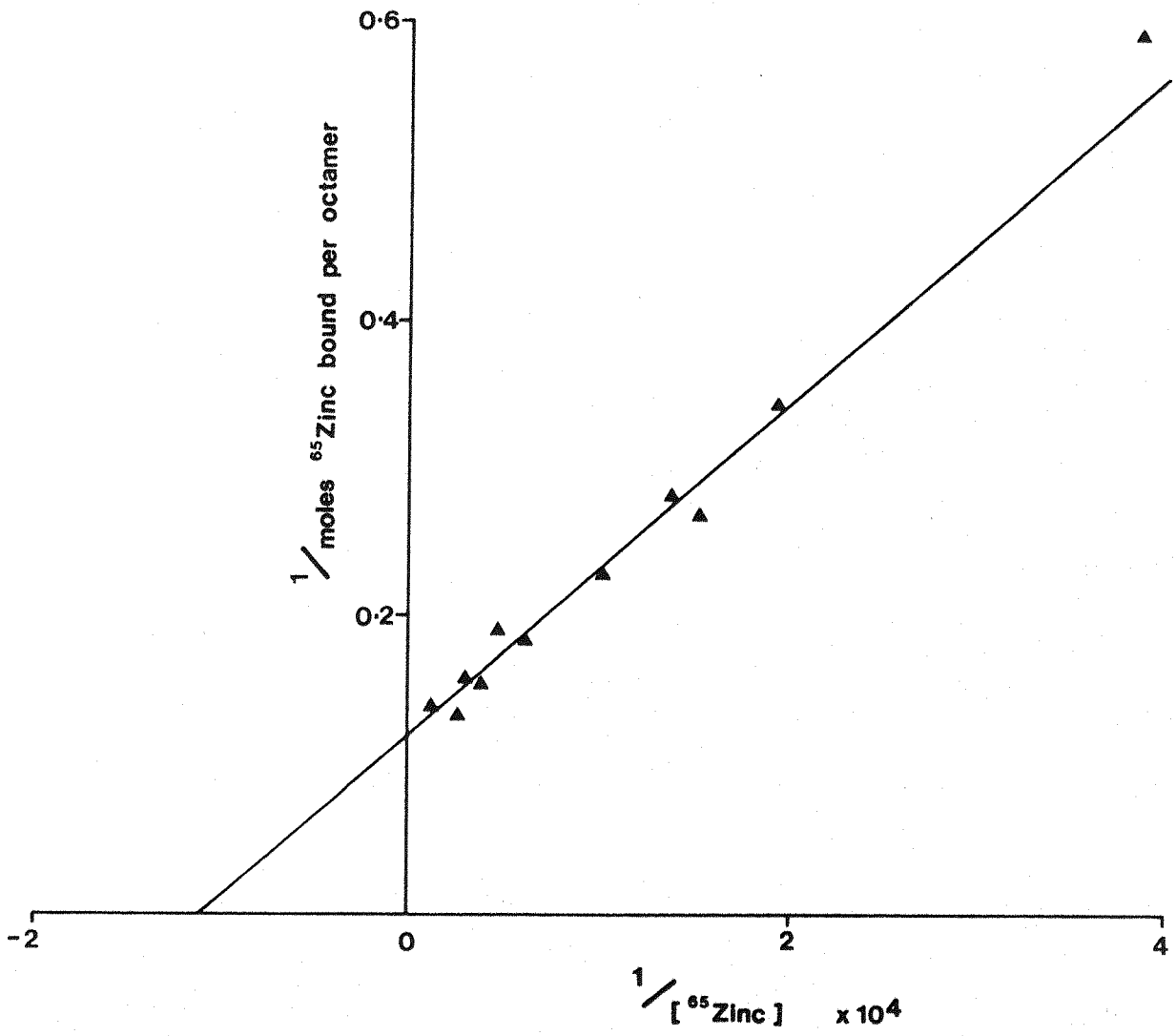
The human dehydratase apoenzyme was treated with increasing amounts of ^{65}zinc chloride (55,000 dpm/nmole) and the resulting holoenzyme was separated from unbound ^{65}zinc by gel filtration as described in Section 3.2.6. This ^{65}zinc labelled holoenzyme was stable under anaerobic conditions and repeated gel filtration failed to remove any significant amounts of the bound label. A typical saturation curve was found (Scheme 3.9) with a binding constant of 89.59 ± 8.15 μM. The number of moles of zinc bound per mole of subunit was 1.023 ± 0.035 assuming a subunit molecular weight of 35,000 (Section 2.3.1). Skatchaud analysis of the data revealed a single population of binding sites (data not presented).

The dependance on zinc for catalytic activity was further investigated by studying the relationship between the amount of ^{65}zinc bound to the human enzyme and its catalytic activity. The results are presented in Scheme 3.10 and show clearly that the activity of human 5-aminolevulinic acid dehydratase closely parallels the amount of ^{65}zinc bound. This further corroborates the evidence presented in Section 3.3.1 and demonstrates that zinc is an absolute requirement for the optimum catalytic activity of the human enzyme. The residual activity associated with the apoenzyme in the absence of zinc may be attributable to trace quantities of metal ions present in the enzyme and in solution which cannot be totally removed.

3.3.4 The Effect of Various Metal Ions on the ^{65}Zinc Holoenzyme Stability.

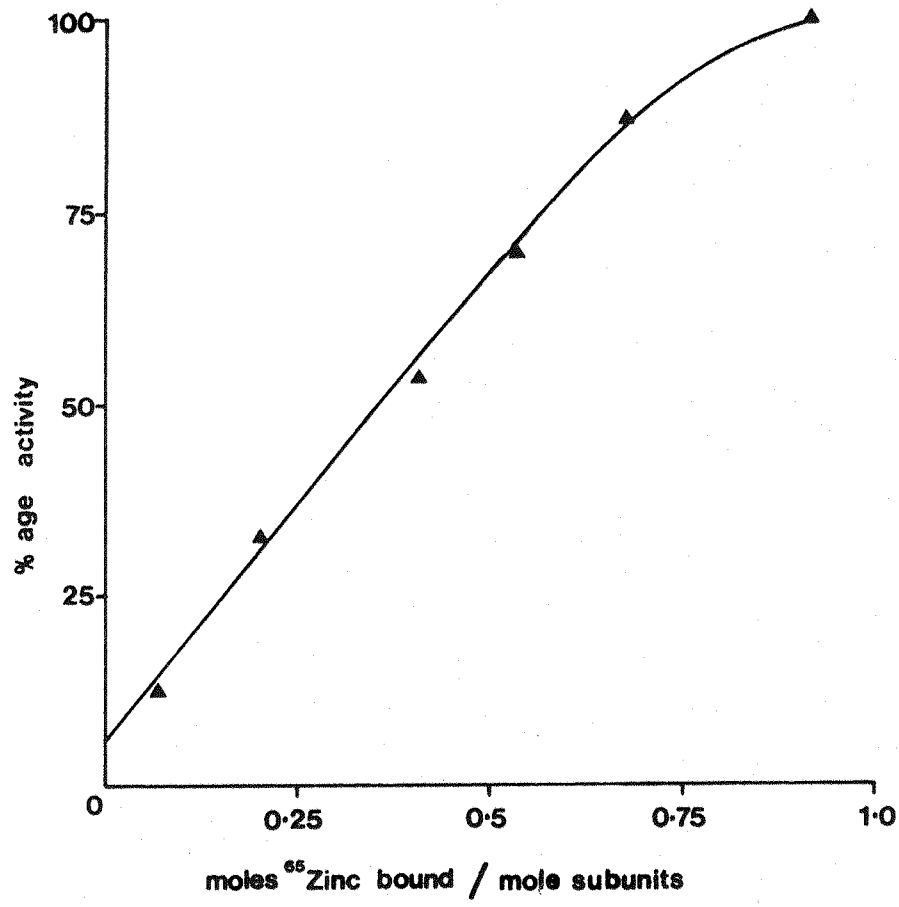
The time-course for the displacement of ^{65}zinc from the ^{65}zinc holoenzyme (prepared as described in Section 3.2.6) by unlabelled zinc and cadmium (final concentration 100 μM) was investigated under anaerobic conditions at 4°C by following the liberation of label into the medium. The results show that the exchange was relatively rapid, reaching equilibrium in less than 15 minutes (Scheme 3.11).

Treatment of the ^{65}zinc holoenzyme with increasing amounts of unlabelled zinc caused a displacement of ^{65}zinc showing that the zinc bound to the enzyme and in solution were freely exchangeable (Scheme 3.11). The concentration of unlabelled zinc that was required to displace



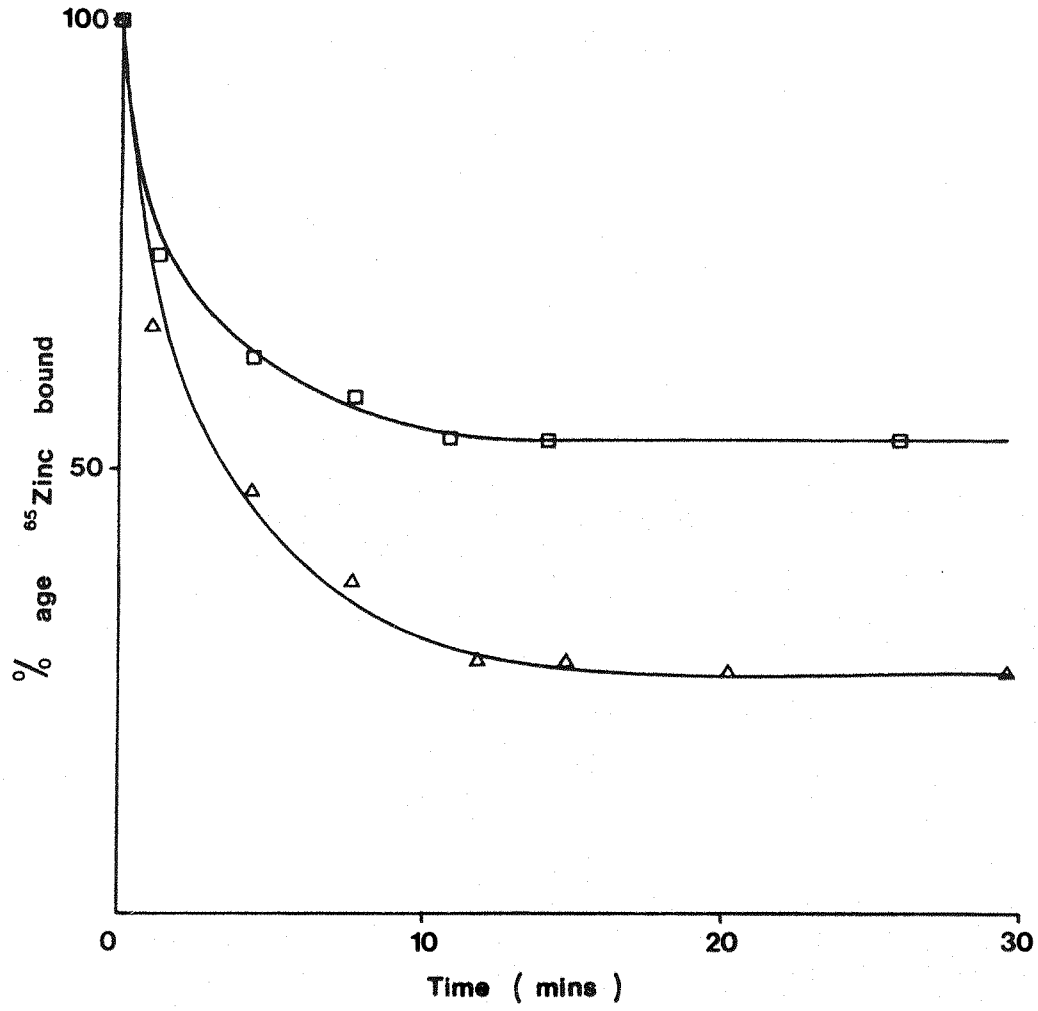
Scheme 3.9 Binding of ^{65}Zinc to the Human Dehydratase Apoenzyme.

The apoenzyme (prepared as described in Section 3.2.5) was treated with increasing quantities of ^{65}zinc chloride (55,000 dpm/nmole) in 0.1 M Tris Hcl buffer (pH 7.1) for 2 minutes at 4°C under anaerobic conditions. Unbound ^{65}zinc was removed by rapid Sephadex G-50 gel filtration as described in Section 3.2.3.



Scheme 3.10 Correlation Between the Amount of ^{65}Zinc Bound to the Human Enzyme and its Catalytic Activity.

The human dehydratase apoenzyme (5.75 nmoles) was incubated with increasing amounts of ^{65}zinc chloride in 0.1 M Tris-Hcl buffer (pH 7.1; 200 µl) under an atmosphere of nitrogen. After removal of unbound ^{65}zinc by rapid Sephadex G-50 gel filtration, the protein concentration (Section 2.2.20), enzyme activity (Section 3.2.4) and amount of ^{65} zinc bound to the enzyme was determined.



Scheme 3.11 Time-Course for Displacement of ^{65}Zinc from the Labelled Holoenzyme by Unlabelled Zinc and Cadmium.

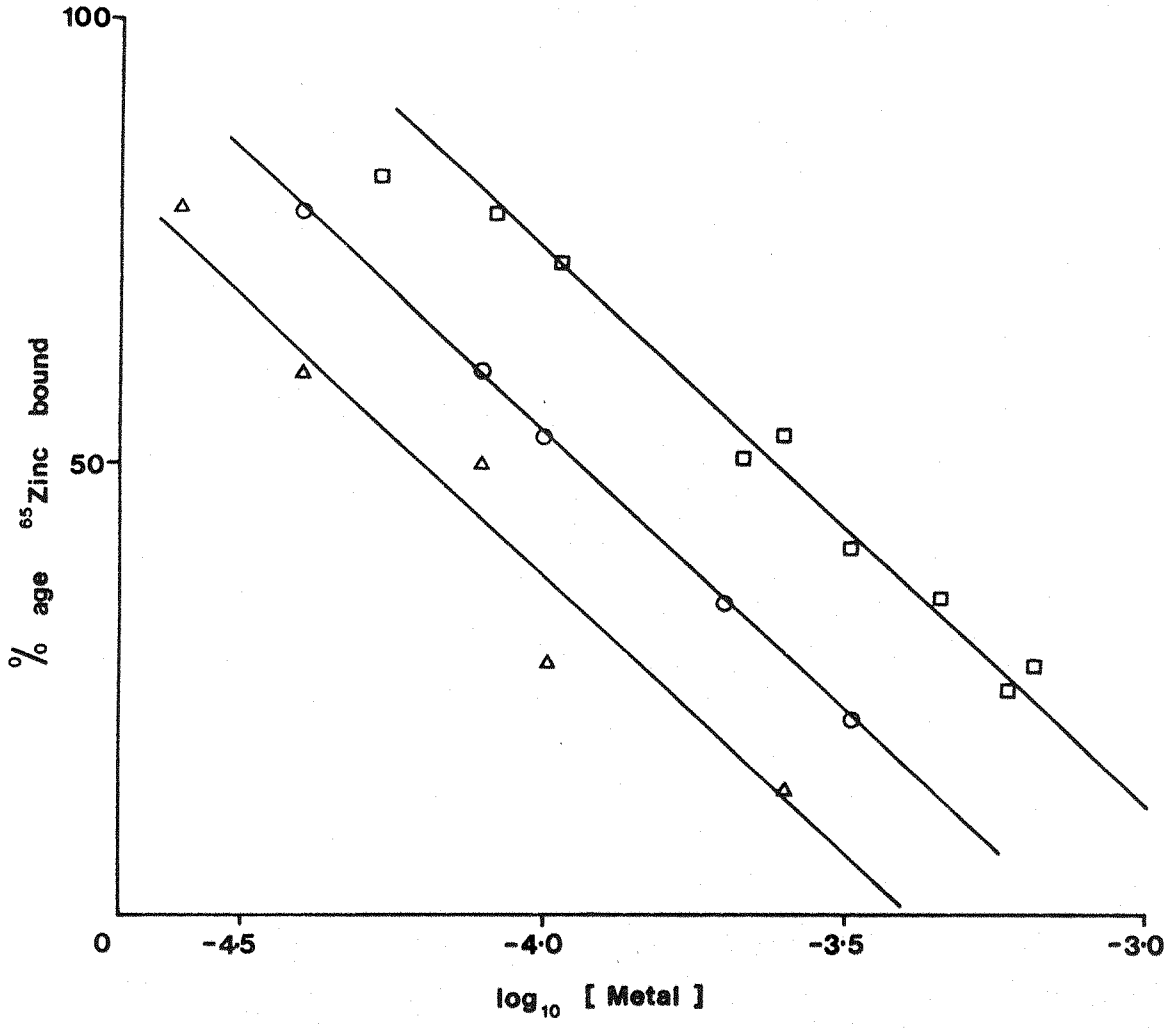
The apoenzyme (2 mgs) in Tris - Hcl buffer (0.1 M; pH 7.1; 200 µl) was incubated at $4^{\circ}C$, under anaerobic conditions, with either zinc (20 nmoles; □——□) or cadmium (20 nmoles; △——△). Aliquots were removed at timed intervals and the displaced ^{65}zinc was removed by gel filtration. The enzyme activity (Section 3.2.4) protein concentration (Section 2.2.20) and the amount of label remaining in the protein (Section 3.2.6) were subsequently determined.

half the label from the ^{65}zinc holoenzyme (IC_{50} value; Scheme 3.12) was found to be 100 μM, which is in very close agreement with the binding constant for zinc (90 μM; Scheme 3.9).

Cadmium has been shown to activate the native dehydratase enzyme at low concentrations ($\leq$ 3 μM; Scheme 3.4) and therefore the effect of various amounts of cadmium on the ^{65}zinc bound to the holoenzyme was investigated. The data are presented in Scheme 3.12 and showed that cadmium was slightly more effective than zinc itself in displacing ^{65}zinc from the labelled holoenzyme (IC_{50} = 60 μM). Similarly, when the ^{65}zinc holoenzyme was exposed to different concentrations of lead there was displacement of labelled zinc. However, lead was less effective (IC_{50} = 240 μM) in exchanging the ^{65}zinc than either cadmium or unlabelled zinc and suggests that the inhibition of human 5-aminolevulinic acid dehydratase by lead (K_i app = 10 - 30 μM) may not be related to the complete displacement of ^{65}zinc from all the subunits of the holoenzyme (IC_{50} = 240 μM). This suggestion was further corroborated by the demonstration that on incubating the ^{65}zinc holoenzyme with increasing amounts of lead in the absence of dithioerythritol almost total loss of activity was observed (96% loss at 103 μM) under conditions where only 25 - 30% of the ^{65}zinc had been displaced from the labelled enzyme (Scheme 3.13). These results suggest either that lead is affecting the enzyme activity by binding to a site distinct from the zinc binding site, or that the lead is exerting its effect at the zinc binding site whereby the displacement of a small percentage of the label (possibly from only one or two of the zinc binding sites in the octameric protein) causes a conformational change in the other subunits with concomitant loss of activity. It is interesting that magnesium, which is neither an activator nor an inhibitor of human 5-aminolevulinic acid dehydratase, was unable to cause any loss of label from the ^{65}zinc holoenzyme, as expected.

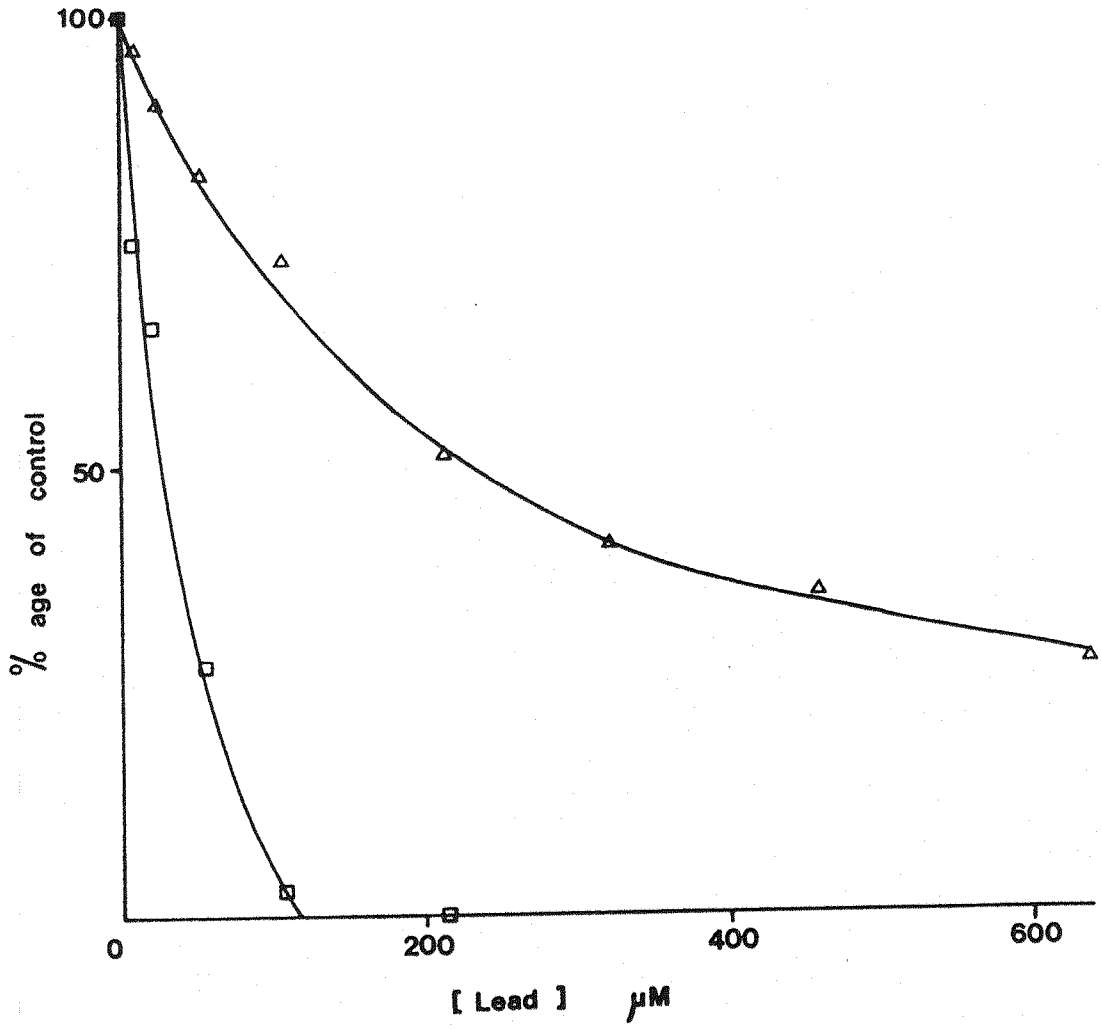
3.3.5 Other Influences on the ^{65}Zinc Binding to the Human Enzyme.

The chelating agent, EDTA, can bind to divalent metal ions such as zinc and has been shown to inhibit the activity of 5-aminolevulinic acid dehydratase potently (Gibson et al., 1955; Wilson et al., 1972, Cheh and Neilands, 1976). Consequently, the effect of 30 mM EDTA on both the ^{65}zinc binding to the holoenzyme and the enzyme activity was



Scheme 3.12 Displacement of ^{65}Zinc from the ^{65}Zinc Labelled Holoenzyme of Human 5-Aminolevulinic Acid Dehydratase by Various Divalent Metal Ions.

The ^{65}zinc holoenzyme (prepared as described in Section 3.2.6) was incubated for 20 minutes at 4°C under anaerobic conditions with increasing amounts of zinc (o——o), cadmium (Δ——Δ) and lead (□——□). The displaced ^{65}zinc was removed by Sephadex G-50 gel filtration.



Scheme 3.13 The Effect of Lead Concentration on Both the Activity and ^{65}Zinc Binding of Human 5-Aminolevulinic Acid Dehydratase

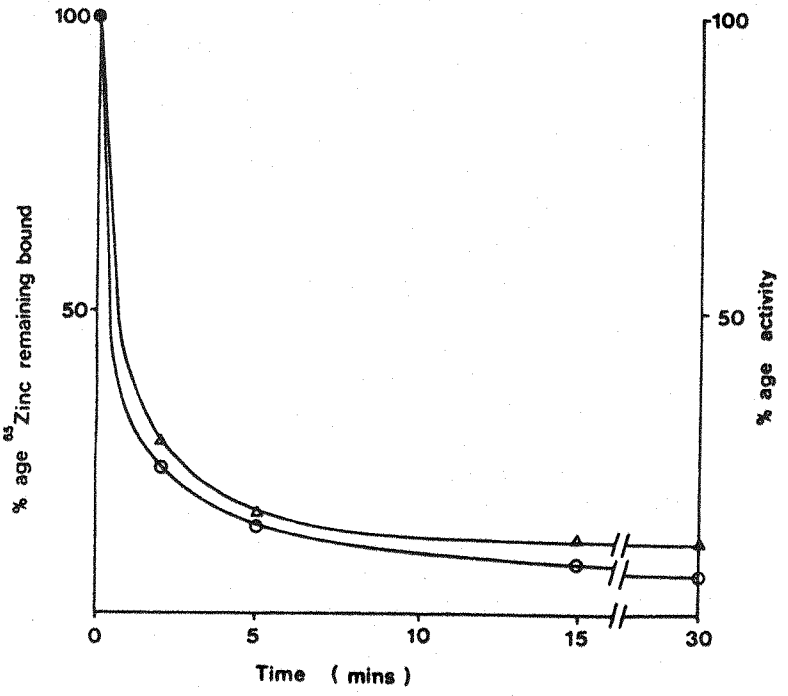
The ^{65}zinc holoenzyme (prepared as described in Section 3.2.6) was treated with increasing quantities of lead in 0.1 M Tris-Hcl buffer (pH 7.1) for 20 minutes at 4°C. Displaced zinc was removed by column chromatography using Sephadex G-50 and both the enzyme activity (□——□) and the amount of ^{65}zinc bound to the protein (△——△) were determined.

investigated. The data are presented in Scheme 3.14 and clearly demonstrate a time-dependant displacement of 94% of the label from the ^{65}zinc holoenzyme which is associated with an almost complete loss of activity (88%). These results, therefore, suggest that the inactivation of human dehydratase by EDTA is due to the removal of zinc from the enzyme protein. In fact, in this study conditions were used which were very similar to those employed to produce the apoenzyme (see Section 3.2.5) and demonstrated that this methodology yielded a zinc "free" apoenzyme of low activity. Atomic absorption spectroscopy of the apoenzyme has demonstrated that between 0.07 and 0.09 gram atoms of zinc are bound per subunit, which, together with the ^{65}zinc binding studies (Scheme 3.9), strongly suggest that there is a single zinc binding site per subunit which is important for the catalytic activity of the enzyme.

The substitution of cadmium for zinc in the human dehydratase enzyme has been shown to result in changes in the absorption and circular dichroism spectra (Cheh and Neilands, 1976). These changes are similar to those found for metallothionen and model complexes (Kägi and Vallee, 1961) and suggests that the metal ion is binding to a sulphydryl residue. To investigate this possibility, the effect of alkylating agents such as iodoacetic acid, 3-chlorolevulinic acid and the active-site-directed inhibitor, 5-chlorolevulinic acid (Seehra and Jordan, 1981), on both the binding of ^{65}zinc to the apoenzyme and the displacement of label from the ^{65}zinc holoenzyme were determined. The results clearly demonstrated that pretreatment of the apoenzyme with these reagents strongly inhibited the ^{65}zinc binding to the protein (Table 3.4). In fact, most of the ^{65}zinc was displaced from the labelled holoenzyme when it was inactivated by the same alkylating reagents. Similarly, heat treatment of the enzyme protein caused inactivation and was associated with the loss of ^{65}zinc and over 90% of ^{65}zinc binding capacity (Table 3.4). These results collectively demonstrate that alkylation of the essential sulphydryl residues causes both a displacement of zinc from the holoenzyme and a loss of zinc binding capacity thus providing further evidence for the role of sulphydryl groups in zinc binding. These results are in good agreement with the preliminary findings of Tsukamoto *et al.*, (1979), Bevan *et al.*, (1980) and Jaffe *et al.*, (1984).

Scheme 3.14 Time-Course of the Effect of EDTA (30 mM) on the ^{65}Zinc Binding and Activity of Human Dehydratase.

The ^{65}zinc holoenzyme (3 mg) was incubated with 9.1 µmoles EDTA in 0.1 M Tris-Hcl buffer (pH 7.1; 300 µl) containing 3 µmoles dithioerythritol at 37°C. Aliquots were removed at timed intervals and, after removal of displaced ^{65}zinc by Sephadex G-50 gel filtration, the amount of ^{65}zinc bound to the enzyme (△——△) and its activity (○——○) were determined.



Scheme 3.15 The Effect of Oxygen on the Enzyme Activity and ^{65}Zinc Bound to the Labelled Holoenzyme.

The ^{65}zinc holoenzyme was incubated at 37°C in the presence of oxygen (Section 3.2.9). Aliquots were removed at timed intervals and the protein was separated from unbound ^{65}zinc using Sephadex G-50 columns. The enzymatic activity (□——□) and the zinc content (△——△) in the protein fractions were determined.

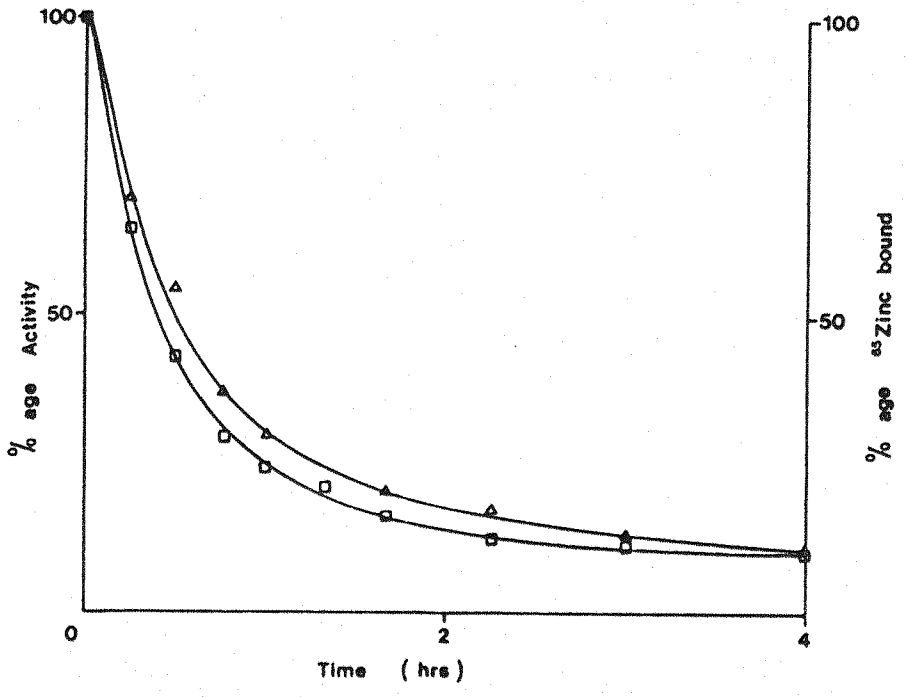


Table 3.4 Effect of Various Thiol Modifiers on the ^{65}Zinc Holoenzyme.

The apoenzyme (2 mg) was treated with various alkylating agents under an atmosphere of nitrogen for 30 minutes at room temperature. The modified protein was isolated by Sephadex G-50 gel filtration and the incorporation of ^{65}zinc was determined. Alternatively, the various alkylating agents were incubated with the ^{65}zinc holoenzyme for 30 minutes at room temperature and the displaced ^{65}zinc removed by gel filtration using Sephadex G-50.

Treatment	% ^{65}Zinc Remaining After Treatment of ^{65}Zinc Holoenzyme	% ^{65}Zinc Binding To Apoenzyme After Treatment
Nothing (under N_2)	100	100
Iodoacetic Acid (20 mM)	9.5	29.9
3-Chlorolevulinic Acid (50 mM)	16.2	15.6
5-Chlorolevulinic Acid (25 mM)	33.1	28.9
Boiling (20 mins)	3.9	11.0

Further information regarding the important role of sulphydryl groups for zinc binding was obtained by investigating the relationship between oxidation of the enzyme and zinc binding. It is well known that all dehydratases, regardless of source, require the presence of high concentrations of thiol in incubations to prevent oxidation of the thiol groups and attendant inactivation (Shemin, 1976). Consequently, when the ^{65}zinc holoenzyme was exposed to oxygen at 37°C there was a loss of enzymic activity which was closely paralleled by the displacement of ^{65}zinc from the enzyme protein (Scheme 3.15). Full catalytic activity was restored upon addition of dithioerythritol (5 mm final concentration) to the enzyme and was associated with the return of ^{65}zinc binding capacity. These findings further highlight the importance of the availability of free sulphydryl groups for the binding of the zinc ion.

3.3.6 Effect of Thiols and Nbs_2 on the Binding of ^{65}Zinc to the Holoenzyme.

When the ^{65}zinc holoenzyme was treated with increasing quantities of dithioerythritol (1 - 50 mM) under anaerobic conditions for 30 minutes at 4°C, very little ^{65}zinc was displaced from the holoenzyme (except at the highest concentration) showing that the ^{65}zinc was

not freely exchangeable with exogenous thiol. However, treatment of the labelled holoenzyme with Nbs_2 released bound ^{65}zinc in a time-dependant fashion at a rate similar to the rate of liberation of Nbs by the enzyme (Scheme 3.16). These results further corroborate the findings described above and suggests that there is a close interaction between the essential sulphydryl residues of the enzyme and its intrinsic metal ion.

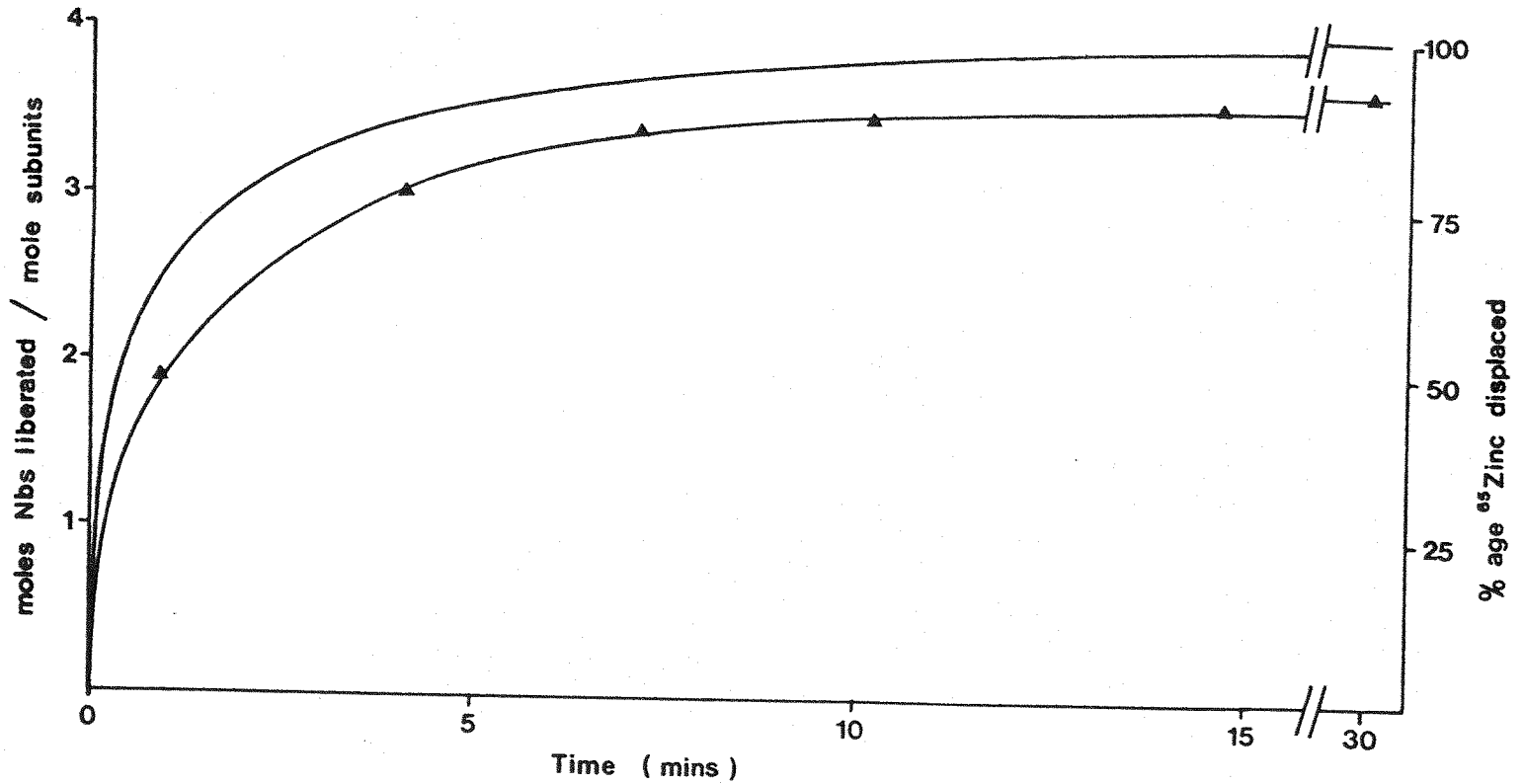
3.3.7 Reaction of Human 5-Aminolevulinic Acid Dehydratase with Nbs_2.

In order to obtain a fuller understanding of the nature of thiol groups in human dehydratase with a view to the future characterisation of the group or groups interacting with zinc a detailed investigation with the thiophilic reagent Nbs_2 was carried out. When the native human apoenzyme was treated with a 50-fold molar excess of Nbs_2 at 25^{o}C in the presence or absence of metal ions, four moles of Nbs were released into solution per mole of enzyme subunit (MR = 35 000) suggesting that four sulphydryl groups are available for reaction with Nbs_2. The reaction was complete within 30 minutes and the progress curves obtained using a conventional spectrometer are shown in Scheme 3.17. The progress curve for the reaction of the apoenzyme in the absence of added zinc chloride shows the rapid liberation of two moles of Nbs per mole of enzyme (within 10 seconds) implying the presence of two highly reactive sulphydryl groups (termed groups I and II). A third group titrated over a period of 4 to 5 minutes (group III), followed by the slow reaction of the final group (group IV) which took about 30 minutes to react completely with the Nbs_2. Groups I and II reacted far too rapidly for their rate constants to be determined from the above data; however, the rate constants for groups III and IV were determined as 1.63×10^3 M^{-1} min^{-1} and 2.76×10^2 M^{-1} min^{-1} respectively.

The reaction rate of the reduce apoenzyme in the presence of 100 µM zinc chloride (Scheme 3.17) was significantly different. This difference appeared to be confined to the reaction of the two fastest sulphydryl groups (groups I and II), whereas the rate of reaction of groups III ($k = 1.43 \times 10^3$ M^{-1} min^{-1}) and IV ($k = 2.5 \times 10^2$ M^{-1} min^{-1}) were largely unaffected by the presence of the zinc ions. The interaction of zinc with the enzyme appeared to be almost instantaneous since the protection afforded by zinc was virtually identical either when the

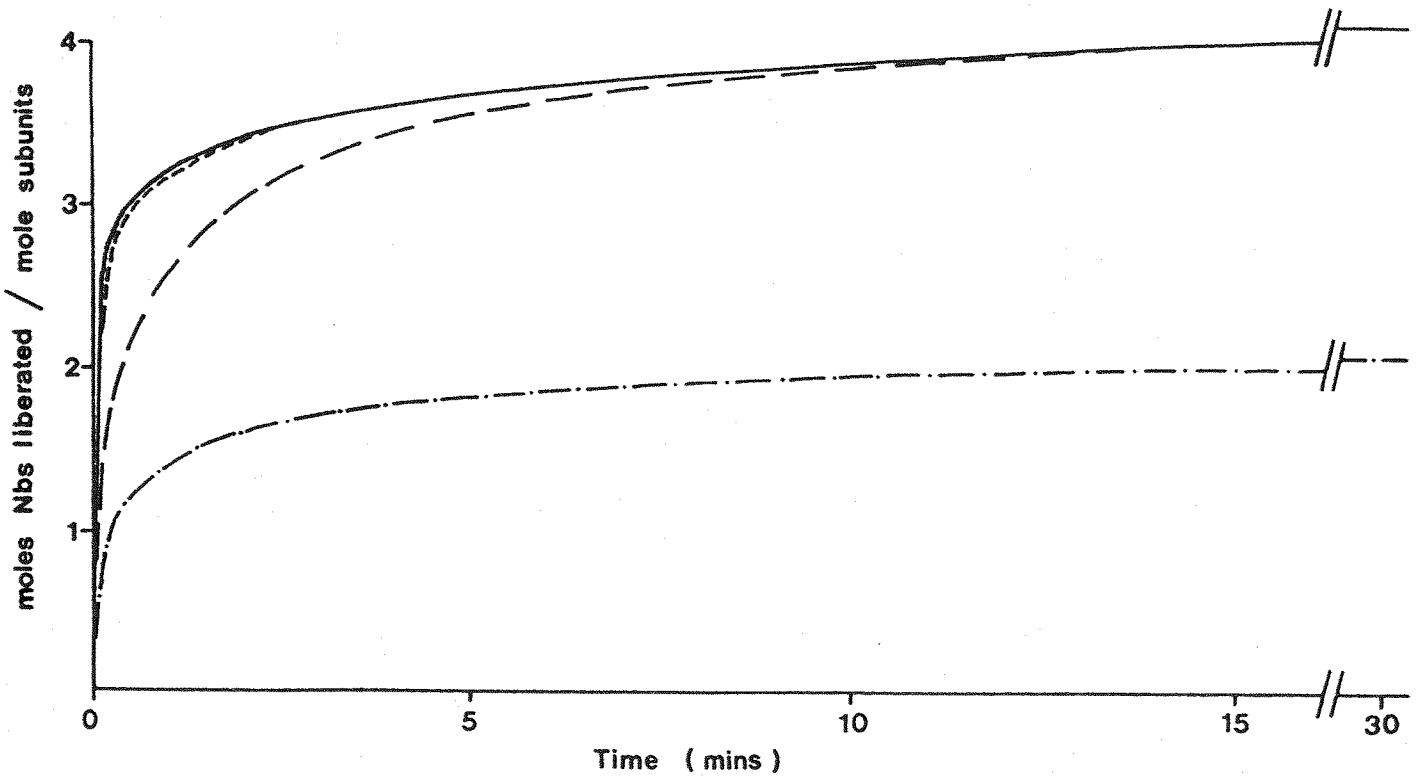
Scheme 3.16 The Effect of Modification of Sulphydryl Groups by Nbs_2 on the ^{65}Zinc Holoenzyme

The ^{65}zinc holoenzyme (10 nmoles) was incubated with a 50-fold excess of Nbs_2 in 1 ml of 0.1 M Tris-Hcl buffer (pH 7.1). The liberation of Nbs was followed spectrophotometrically at 412 nm (E_{412} = 1.28 x 10^4 M^{-1} cm^{-1}) (————). In a parallel experiment , aliquots were removed at timed intervals and both the zinc content and protein concentration were determined after Sephadex G-50 gel filtration (▲————▲).



Scheme 3.17 Reaction of 5-Aminolevulinic Acid Dehydratase with Nbs_2.

Reaction progress curves obtained when 0.5 mM Nbs_2 was reacted with: (———) reduced apoenzyme; (- - - -) reduced apoenzyme in the presence of 50 µM Pb^{++}; (— —) reduced enzyme in the presence of 100 µM Zn^{++}; (—·—) oxidised enzyme in the presence or absence of 50 µM Pb^{++} or 100 µM Zn^{++}. The reactions were carried out at 25°C in 0.1 M Tris-Hcl buffer pH 7.1.



apoenzyme was preincubated with zinc prior to the reaction with Nbs_2 or when the zinc was added to the apoenzyme together with the Nbs_2 reagent itself.

When the reduced apoenzyme was treated with Nbs_2 in the presence of 50 μM lead chloride (Scheme 3.17) the reaction did not appear to be significantly affected, although this concentration of lead is known to inhibit enzyme activity by approximately 70% (see Section 3.3.2).

Oxidation of the apoenzyme resulted in the loss of catalytic activity and was associated with the loss of the ability of groups I and II to react with Nbs_2 (Schemes 3.17 and 3.18).

3.3.8 Effect of Zinc Ions on the Oxidation of 5-Aminolevulinic Acid Dehydratase.

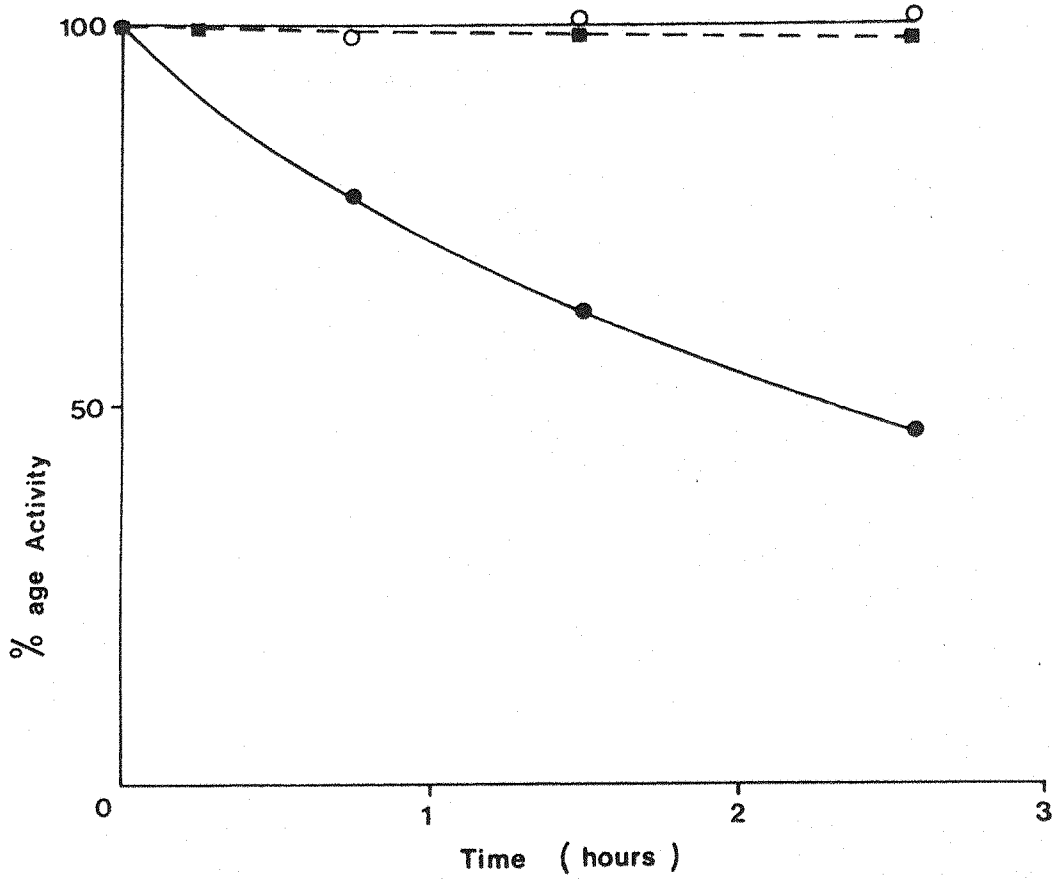
The human enzyme, in common with the bovine liver enzyme, was inactivated, albeit at a much slower rate, by oxidation of its constituent sulphydryl groups which is accompanied by the loss of two Nbs_2 reactive equivalents (groups I and II; Scheme 3.17).

Inactivation of the human apoenzyme by atmospheric oxygen ($t_{\frac{1}{2}}$ = 135 mins, Scheme 3.18) was almost completely blocked by zinc (100 μM) suggesting that in the zinc-enzyme complex the sensitive sulphydryl groups are no longer readily available for reaction.

Porphobilinogen (5 mM) did not prevent the reaction of Nbs_2 with groups I and II (as is seen with the bovine enzyme) (Seehra *et al.*, 1981). Neither the substrate ALA (5 mM) nor the substrate analogue levulinic acid (5 mM) had any effect on the reaction progress curve.

3.3.9 Stopped-Flow Spectrophotometry.

The two fastest reacting sulphydryl groups (groups I and II) of the human enzyme reacted with Nbs_2 far too rapidly to be studied by conventional spectrophotometric techniques described above and stopped-flow analysis was therefore employed. The apoenzyme or holoenzyme was mixed with a 100-fold molar excess of Nbs_2 (with or without 100 μM zinc respectively) and reaction progress curves were obtained for



Scheme 3.18 The Rate of Loss of Activity of 5-Aminolevulinic Acid Dehydratase on Oxidation by Air.

For experimental details, see methods. The enzyme was incubated in 0.1 M potassium phosphate buffer, pH 6.8, in the presence of (●——●) 0 μM Zn^{2+}, (■——■) 100 μM Zn^{2+} or (○——○) under oxygen-free nitrogen. The solutions were incubated at 37°C in open tubes of area 1.45 cm^2 and had a depth of 1 cm.

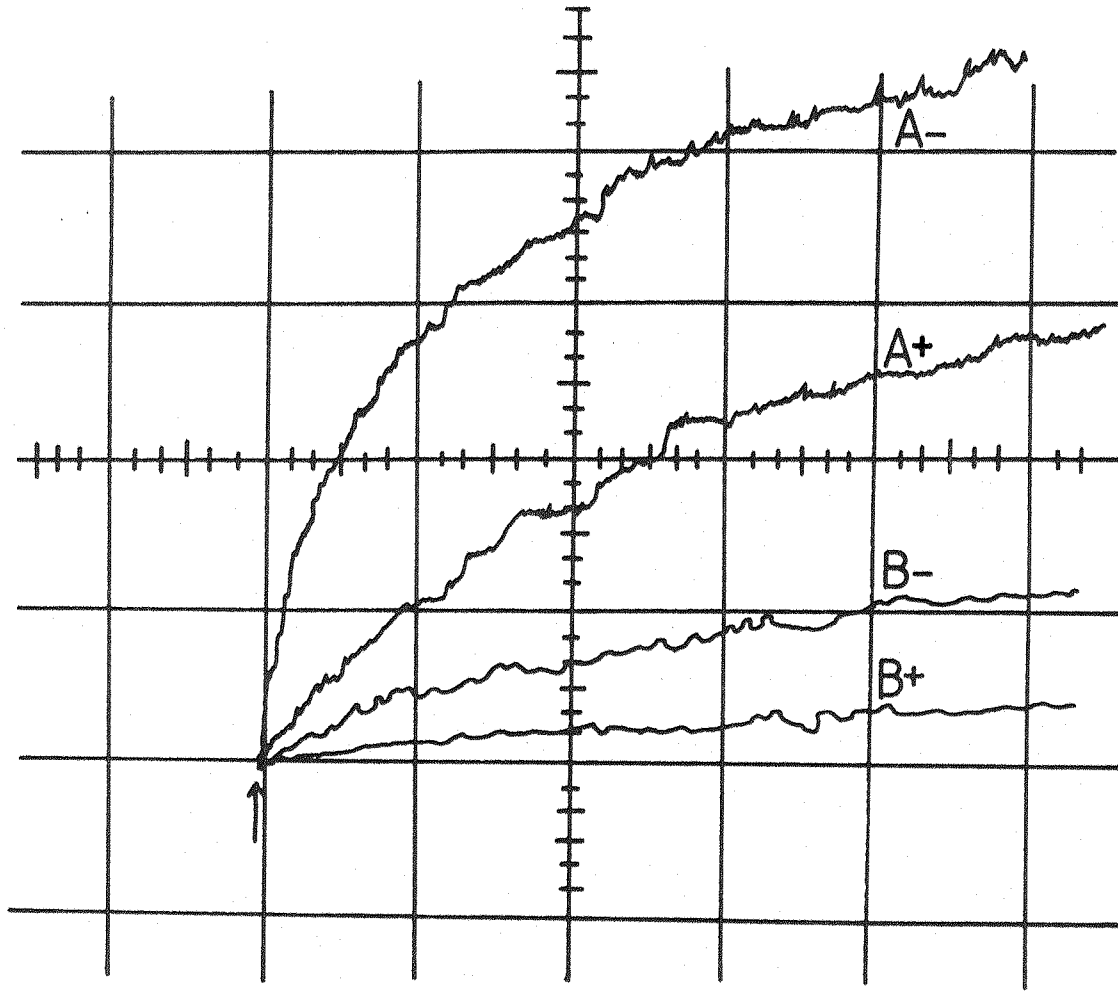
time-courses of between 0.25 and 20 seconds (Scheme 3.19). Under these conditions, the reaction can be considered as first order owing to the large excess of one reactant (Nbs_2). Close examination revealed single reaction rate constants for both group I and group II. When apoenzyme was reacted with Nbs_2 in the absence of zinc ions, first order apparent rate constants of 135 min^{-1} and 5.3 min^{-1} were obtained for the two site reactions (Curve A^-, Scheme 3.19). However, in the presence of zinc ions the reactivity of group I decreased to about 12 - 16% of that of the apoenzyme with an apparent rate constant of 18 min^{-1} (Curve A^+, Scheme 3.19). The reactivity of group II appeared to be less influenced by the presence of zinc with a rate constant of 2.1 min^{-1}.

The presence of lead ions had little effect on the reactivities of the thiol groups with Nbs_2 as judged from the progress curves obtained from conventional spectrophotometry (Scheme 3.17), although a more detailed examination by the stopped-flow technique showed a decrease in reactivity to Nbs_2 by approximately 25%. Other studies (using ^{65}zinc chloride) have shown, interestingly, that this concentration of lead ions will displace approximately 20% of enzyme-bound zinc (see Section 3.3.4).

Table 3.5 summarises the cumulative data from conventional and stopped-flow spectrophotometry of the rate constants for the four reactive sulphydryl groups in the human enzyme in the presence and absence of both zinc (100 μM) and lead (50 μM).

Table 3.5 Rate Constants for the Four Reactive Sulphydryl Groups of Human 5-Aminolevulinic Acid Dehydratase in the Absence and Presence of Zinc and Lead.

	Sulphydryl Group			
	Gp I (min^{-1})	Gp II (min^{-1})	Gp III ($\times 10^3$ $M^{-1}min^{-1}$)	Gp IV ($\times 10^2$ $M^{-1}min^{-1}$)
Apoenzyme Alone	135	5.3	1.63	2.76
Apoenzyme + 100 μM Zinc	18.0	2.1	1.43	2.5
Apoenzyme + 50 μM Lead	115	4.6	nd	nd



Scheme 3.19 Stopped-Flow Spectrophotometric Observation of the Reaction Between Nbs_2 and 5-Aminolevulinic Acid Dehydratase.

The enzyme (10 μM initial concentration) was mixed 1:1 with Nbs_2 (1 mM initial concentration) at 25°C in 0.1 M Tris-HCl buffer pH 7.1. The curve A- and A+ are reaction progress curves of apoenzyme alone or enzyme in the presence of 100 μM Zn^{2+}; the time scale was 3200 ms/division. The curves marked B- and B+ describe similar reactions except that the time scale was 100 ms/division.

The arrow indicates the point of mixing. The vertical scales represent 0.5 equivalents of Nbs released. Molarity is calculated in terms of the monomer.

3.3.10 Titration of the Human 5-Aminolevulinic Acid Dehydratase Apoenzyme with Nbs_2.

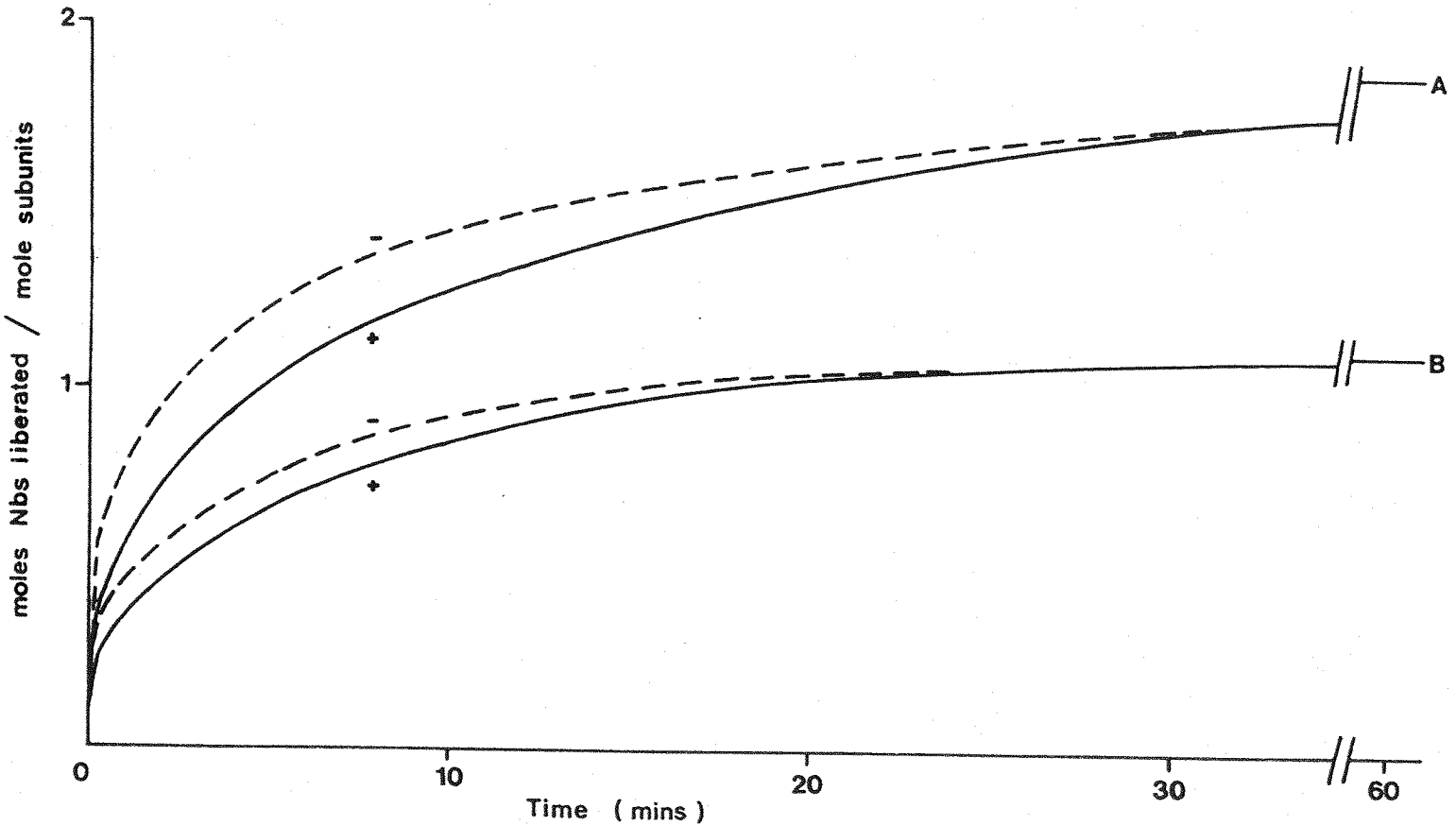
When human apoenzyme (10 nmoles in 1 ml) was titrated with an equimolar concentration of Nbs_2 (10 nmoles) at 25°C, approximately 2 mole equivalents of Nbs (19.2 nmoles) were liberated (Scheme 3.20). Similarly, when 1 mole of enzyme was titrated with 0.5 mole Nbs_2 (5 nmoles), approximately 1 mole of Nbs (10.6 moles) was released. The end-points of these two titrations were essentially unaffected by the presence of the divalent metal ions lead and zinc.

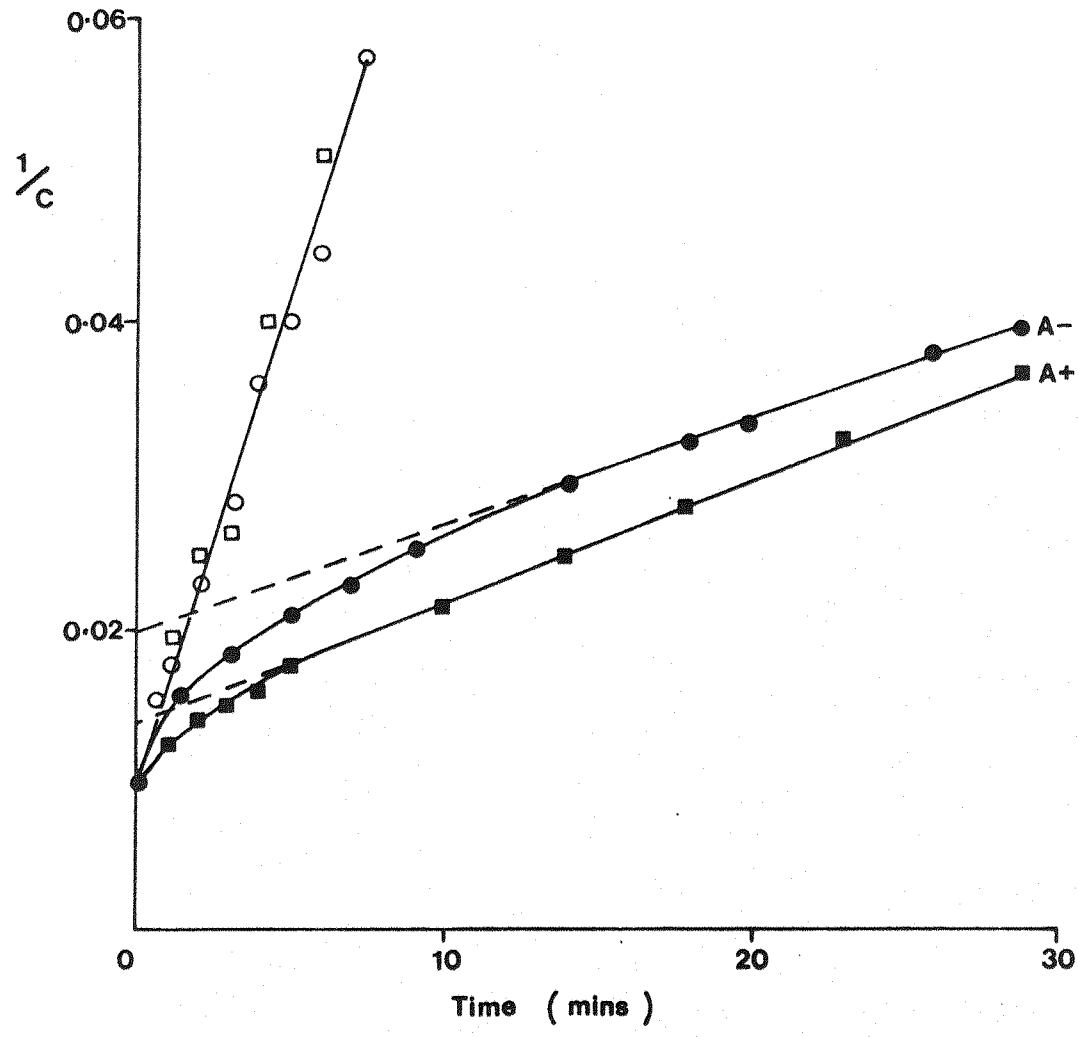
When the ^{65}zinc holoenzyme was incubated with an equimolar concentration of Nbs_2 for 30 minutes at 25°C the resultant, essentially inactive, enzyme was shown to have lost 80% of the initially bound zinc. Similarly, reaction with a half mole equivalent of Nbs_2 resulted in the release of 54% of the label from the ^{65}zinc holoenzyme and further demonstrates the close relationship between the availability of unmodified SH groups and zinc binding.

On analysis of the reaction progress curves (Scheme 3.21), again it becomes clear that the presence of zinc ions reduces the reactivity of the sulphydryl group I. The second order plot of the data from Scheme 3.20 (Curve A-) is composed of two sections: an initial curved section, the rate of which decreases to reveal a second slower reaction. Correction of the initial section of the curve by subtraction of this slower rate made it possible to estimate the rate of the fast phase in isolation. It can be noted that both reactions are of equal size, with the line of the extrapolated slow phase intercepting the axis (time = 0) at the position equivalent to 50% of the total reaction (release of Nbs). Also shown in Scheme 3.21 is the data from the same reaction performed in the additional presence of 100 μM zinc ions. It can be noted that the intercept obtained in this case by back extrapolation of the slow rate is no longer at the 50% reaction point but is now around the 80% of reaction point. The fast phase of this reaction, although similar in amplitude proceeds at approximately the same rate as that in the absence of zinc (Scheme 3.21). The slower phase appears to be similar in rate to that obtained in the absence of zinc ions, although larger in amplitude.

Scheme 3.20 Titration of 5-Aminolevulinic Acid Dehydratase with Nbs_2

Nbs_2 and enzyme (10 μM) were mixed as described in the methods. (A) Reaction of the enzyme with 1 mole Nbs_2/mol subunit in the absence (-) or presence (+) of 100 μM Zn^{2+}. (B) Reaction of the enzyme with 0.5 mol and Nbs_2/mol subunit in the absence (-) or presence (+) of 100 μM Zn^{2+}.





Scheme 3.21 Second-Order Rate Analysis of the Reaction Progress Curves from Scheme 3.20.

The curves A- and A+ from Scheme 3.20 were subjected to second order analysis. The reactant concentrations were equal at time = 0, thus in the plot above the y axis is the reciprocal of the concentration (at time t) of one of the reactants (Nbs_2). The closed circles (●) represent data from curve A- and the closed squares (■) the data from curve A+. The open circles (○) and squares (□) represent derived rates from data taken from the difference between the initial part of each rate curve and the extrapolated portions (dashed lines) of those respective curves.

3.3.11 Protein Fluorescence.

The human apoenzyme exhibited fluorescence emission at 335 nm when the endogenous tryptophan was excited by light at 280 nm. The intensity of this fluorescence was perturbed by the addition or removal of the metal ions, zinc and lead, without any shift in the wavelength of maximum emission (Table 3.6). Zinc ions increased the fluorescence intensity of the apoenzyme in a concentration dependent manner up to a maximum of between 4.5 and 5% (Expt. 1). This effect was completely reversed on addition of a 40-fold excess of EDTA (Expt 2).

A more detailed analysis of the data from Expt 1 (see Scheme 3.22) clearly demonstrated that the optimal fluorescence change occurred between 100 and 300 μM zinc. These results are in good agreement with the concentration of zinc required to maximally stimulate the human dehydratase apoenzyme (Section 3.3.1, Scheme 3.1). The results were also analysed to see if there was any correlation between the change in fluorescence intensity and enzymic activity. The data are presented in Scheme 3.23 and showed that, at activities greater than 25% of the maximum, the enzyme activity parallels the change in fluorescence. However, at low activities (< 25%) this correlation breaks down presumably due to the small amount of zinc still associated with the apoenzyme (see Section 3.2.5). Hyperbolic non-linear regression analysis of the data from Scheme 3.23 yielded a dissociation constant (K_D) for zinc of 19.9 ± 4.5 μM which correlates well with the value of ≈10 μM for the half-maximum stimulation of the apoenzyme by zinc (Scheme 3.1).

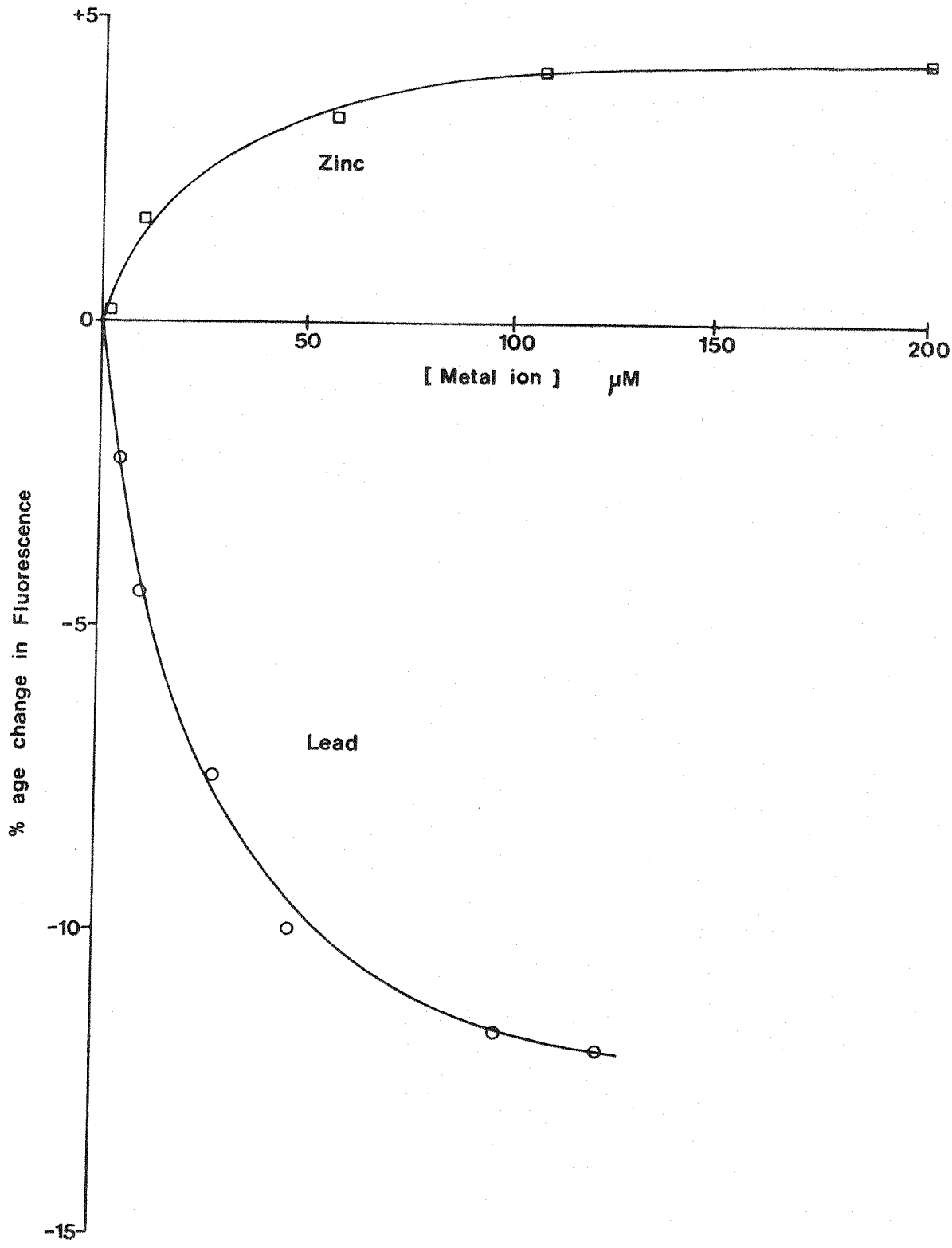
The addition of lead to the apoenzyme caused a decrease in the intensity of fluorescence (11.93% at 124 μM; Expt 3) which was not further decreased by EDTA. The data are presented in graphical form (Scheme 3.22) and revealed that the concentration of lead ions required to cause a half-maximal change in fluorescence was 26.5 ± 2.6 μM (hyperbolic non-linear regression, K_D value). This value is in good agreement with the K_i app of lead of between 10 and 30 μM (Section 3.3.2). The data in Scheme 3.24 emphasise the strong correlation between fluorescence change and loss of enzymic activity, suggesting that lead may inhibit the human dehydratase enzyme by causing a conformational change.

Addition		Metal Ion Present (μM) Zinc	Lead	% Change In Fluorescence	
Zinc (μM)	2	-	-	+ 0.19	EXPT 1
	10	-	-	+ 1.69	
	57	-	-	+ 3.31	
	108	-	-	+ 4.19	
	203	-	-	+ 4.33	
EDTA (mM)	4.9	108	-	+ 0.22	EXPT 2
LEAD (μM)	5	-	-	- 2.25	EXPT 3
	10	-	-	- 4.42	
	29	-	-	- 7.45	
	48	-	-	- 9.99	
	99	-	-	-11.62	
	124	-	-	-11.93	
ZINC (μM)	94	-	48	- 0.96	EXPT 4
	187	-	48	+ 2.50	
	93	-	65	- 2.08	
LEAD (μM)	46	203	-	+ 1.82	EXPT 5
EDTA (mM)	4.6	94	48	- 8.82	EXPT 6
	4.6	203	46	- 6.89	

Table 3.6 The Effect of Zinc and Lead Ions on the Fluorescence Intensity of the Human Dehydratase Apoenzyme.

The protein fluorescence of the human dehydratase apoenzyme (180 μg, 5.14 nmoles) at 335 nm was determined in the presence of increasing amounts of zinc in 200 μmoles potassium phosphate buffer (pH 6.8, 2 ml) containing 20 μmoles dithioerythritol (Expt 1). The additional presence of a 50 fold excess of EDTA on the fluorescence intensity was also determined (Expt 2). Similar studies were carried out to determine the effect of increasing quantities of lead on the fluorescence of the apoenzyme (Expt 3). Expts 4 and 5 show the effect of the presence of varying amounts of both zinc and lead on the protein fluorescence of the apoenzyme. In Expt 6 the additional presence of EDTA on the fluorescence intensity of the apoenzyme in the presence of both lead and zinc was determined.

In all experiments the fluorescence was excited by light at 280 nm.

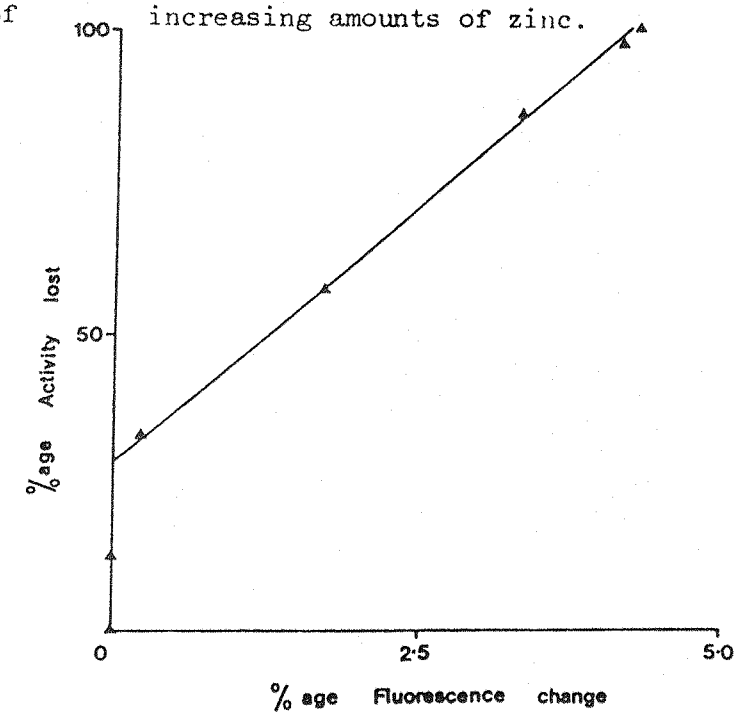


Scheme 3.22 The Effect of Zinc and Lead Concentration on the Fluorescence Intensity of the Human Dehydratase Apoenzyme.

Data from Exptrs. 1 and 2 in Table 3.5. (See Legend for Table 3.5 for experimental details).

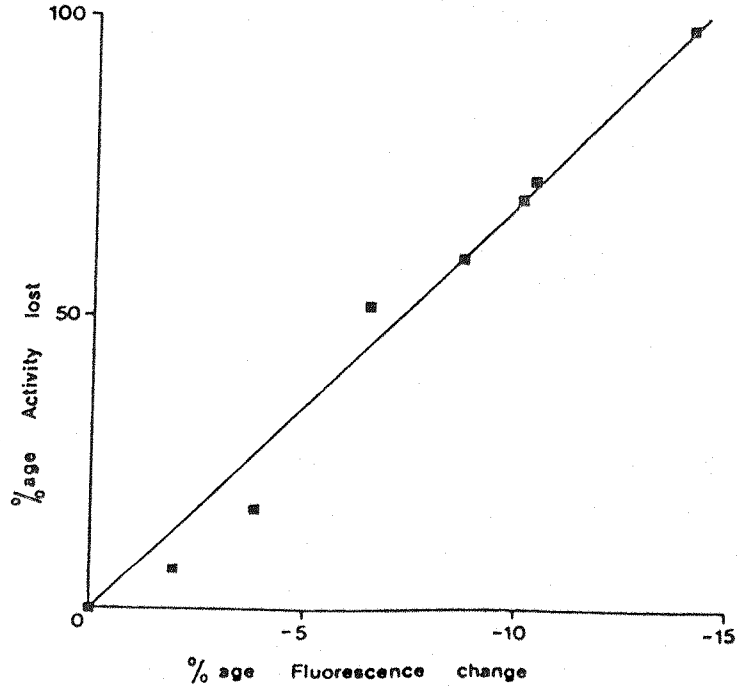
Scheme 3.23 Correlation Between Zinc Activation and the Change in Fluorescence Intensity of the Human Apoenzyme.

The fluorescence intensity (Section 3.2.10) and activity (Section 3.2.4) of the human dehydratase apoenzyme were determined in the presence of increasing amounts of zinc.



Scheme 3.24 Relationship Between Enzyme Activity and Lead-Induced Decrease in Tryptophan Fluorescence.

The activity (Section 3.2.4) and fluorescence intensity (Section (3.2.10) of the human dehydratase apoenzyme were determined in the presence of increasing quantities of lead.



Interestingly, when the lead-inhibited enzyme was treated with zinc ions the fluorescence intensity was almost completely restored to that of the holoenzyme (Expts. 4 and 5, Table 3.6). Under these conditions the enzyme is almost completely active (see Table 3.3) suggesting that the binding of zinc to the enzyme reverses any conformational change which may be induced by lead, thus stabilising the enzyme in a fully active form.

On addition of excess EDTA to the enzyme that had been treated with both lead and zinc ions there was a decrease in fluorescence intensity (Expt. 6) to levels similar to that of the lead-inhibited apoenzyme (Expt. 3), presumably due to preferential removal of zinc by the EDTA.

When the apoenzyme was oxidised with equimolar concentration of Nbs_2 the resulting protein (after isolation by gel filtration) exhibited no enzyme activity and failed to show any changes in fluorescence on addition of either lead or zinc. However, as expected, this effect was reversed by the addition of 5 mM dithioerythritol (final concentration).

The presence of porphobilinogen (5 mM) in the fluorimeter cell was shown to have no effect on the observed fluorescence changes.

3.4 Discussion.

Numerous reports in the literature have clearly demonstrated that zinc ions stimulate the activity of human 5-aminolevulinic acid dehydratase both *in vivo* and *in vitro* (see Section 3.1 for details). However, most of these studies have involved the determination of the effect of zinc in assays using red cell haemolysates; conditions which are far from ideal for investigating the interaction of zinc with a single protein (5-aminolevulinic acid dehydratase). Initial experiments were performed therefore to determine the effect of zinc ions on purified human erythrocyte 5-aminolevulinic acid dehydratase (prepared as described in Section 2.2.2). The studies revealed that maximum activation of both the native and apoenzyme occurred in the presence of between 100 and 300 μM zinc (Scheme 3.1). This value is in good agreement with data obtained by other groups in assays using erythrocyte haemolysates (Meredith *et al.*, 1977; Davis and Avram, 1980; Trevison *et al.*, 1980; Geisse *et al.*, 1983) or the purified enzyme (Anderson and Desnick, 1979). These findings are in concord with values for the concentration of zinc normally present in human blood of 135 ± 31 μM (Vallee and Gibson, 1948) and 109 ± 29 μM (Meredith and Moore, 1980) and clearly suggest that the human enzyme is fully active when present in the body. On the other hand, Despaux *et al.*, (1977) have found that the partially purified human enzyme is maximally stimulated by 1.5 μM zinc. However, this finding seems somewhat unlikely as their results also demonstrate that at blood zinc concentrations the enzyme is strongly inhibited (≈ 20% of maximum activity).

Further results in the current studies demonstrated that the purified human enzyme had an absolute requirement for zinc for catalytic activity (Scheme 3.2) and that exogenous thiol was not required as long as the oxidisable sulphydryl groups present in the enzyme were fully reduced. Previous studies on the bovine enzyme have, in most cases, demonstrated a similar dependance on zinc for activity (Cheh and Neilands, 1973, 1976; Bevan *et al.*, 1980; Jaffe *et al.*, 1984). However, Tsukamoto *et al.* (1979) found that the bovine apoenzyme was almost completely active (≈ 70% of maximum) and proposed that zinc was not essential for enzymic activity. This apparent anomaly is almost certainly attributable to the fact that in the studies of Tsukamoto *et al.*, (1979) a final enzyme concentration of 40 nM in the assay was used and also no attempts were

made to remove adventitious metal ions from the buffers and reagents. Vallee and Galdes (1984) have reported that it is virtually impossible to reduce the concentration of zinc below 10 nM using metal extraction procedures and that without these precautions the zinc concentration would be substantially higher. Consequently, when low concentrations of apoenzyme are assayed in buffers with trace metal contamination it would appear to be almost completely active (Tsukamoto et al., 1979). However, when the apoenzyme was assayed in metal "free" solutions at a concentration of between 2 and 10 μM (Cheh and Neilands, 1976; Bevan et al., 1980; Jaffe et al., 1984; this Thesis, Section 2.2.1) the activity was found to be less than 20% of that determined for the holoenzyme.

In the present work, kinetic studies revealed that the purified human enzyme showed Michaelis-Menten kinetics (Section 1.1) and no cooperativity was observed (Scheme 3.3). A K_m for the substrate, ALA, of 0.25 mM was determined and was virtually identical to the value of 0.27 mM obtained by Anderson and Desnick (1979). This value, however, was higher than that found with the partially purified enzyme (K_m=0.48 mM; Despaux et al., 1979). Further studies revealed that the K_m was unaffected by zinc, whereas V_{max} increased from 18.8 U/mg in the absence of zinc to 23.9 U/mg in the presence of zinc (Table 3.1). Thus, the activation of native human 5-aminolevulinic acid dehydratase by zinc (100 μM) is consistent with an effective increase in the number of functional catalytic sites.

The activity of the purified human enzyme was investigated in the presence of various metal ions (Schemes 3.4 and 3.8) and it was demonstrated that zinc and cadmium were the only metal ions to activite the native enzyme at low concentrations ($\lesssim$ 3 μM). These findings suggest that the human enzyme has a specific metal ion requirement which is consistent with the specificity observed for the bovine enzyme (Cheh and Neilands, 1976; Bevan et al., 1980). A similar specificity has been observed with aspartate transcarbamylase (Griffin et al., 1973). It should be noted that zinc in carboxypeptidase A can be replaced by cobalt, manganese, nickel, cadmium and mercury (Coleman and Vallee, 1961), the zinc in neutral protease by cadmium, mercury and lead (Mc Conn et al., 1967), whereas the zinc ion in aldolase can be substituted for by

manganese, iron (II), cobalt and nickel (Kobes et al., 1969). These enzymes, therefore, appear to have a much more flexible metal ion requirement than either mammalian 5-aminolevulinic acid dehydratase or aspartate transcarbamylase.

Lead has been shown to inhibit human erythrocyte 5-aminolevulinic acid dehydratase activity potently with inhibition constants (K_i's) of between 1 and 15 μM (see Section 3.1). This inhibition by lead has been used as a sensitive indicator of lead poisoning and linear relationships between $\log_{10}$ of the activity and the blood level concentrations have been demonstrated (Hernberg and Nikkanen, 1970; Lauwerys et al., 1973; Mauras and Allain, 1979). Accordingly, an investigation into the effect of lead on purified human 5-aminolevulinic acid dehydratase was performed which demonstrated a concentration dependant inhibition on the enzyme with a K_i of between 9.7 μM (Dixonplot; Scheme 3.7) and 28 μM (Scheme 3.5) depending on the method used to determine the K_i. This value agrees well with data obtained by Despaux et al., (1977) using a partially purified enzyme preparation (K_i = 15 μM). In the current study, kinetic analysis revealed that lead caused a non-competitive inhibition of the enzyme (Scheme 3.6; Table 3.1). These findings are consistent with lead binding to a site on the enzyme other than the active site which causes a loss of enzymic activity (possibly by inducing a conformational change). A more detailed analysis of the interaction of lead with the human enzyme (Dixon plot; Scheme 3.7) revealed that a higher concentration of lead was required to produce the same degree of inhibition in the presence of high substrate concentrations (Table 3.2). These data are consistent with a lower affinity of lead for the enzyme-substrate complex than for the free enzyme.

Lead inhibition of the purified human enzyme could be almost completely reversed by the addition of zinc (Scheme 3.5) in concord with findings published in the literature (Finelli et al., 1975; Haeger-Aronsen et al., 1976; Meredith and Moore, 1980). A closer examination of the interaction of lead and zinc with human 5-aminolevulinic acid dehydratase (Table 3.3) revealed that the values for the dissociation constant of lead from the native and apoenzyme were very similar (≈ 30 μM; see Section 3.3.2) and correlate well with the K_i values determined previously (see Scheme 3.5 and 3.7).

The stoichiometry of zinc binding to bovine 5-aminolevulinic acid dehydratase has been extensively studied by many groups (Cheh and Neilands, 1973, 1976; Tsukamoto et al., 1979; Bevan et al., 1980, Jaffe et al., 1984) who have shown the presence of 1 zinc binding site per subunit (8 per octamer). However, there was some conflict as to the precise stoichiometry of zinc binding which was required for maximal catalytic activity. Bevan et al., (1980) and Jaffe et al., (1984) have suggested that only 4 moles of zinc per octamer are required for optimal activity, whilst the binding of the other 4 moles of zinc per octamer are not catalytically important. On the other hand, Tsukamoto et al., (1979, 1980) have proposed that all 8 zinc molecules per octamer are required for optimal activity. Consequently, in this present study an extensive investigation into the zinc binding to the human apoenzyme using ^{65}zinc chloride was carried out. The data (Scheme 3.9) revealed a single zinc binding site per subunit (8 per octamer) with a binding constant of 90 μM (see Gibbs and Jordan, 1981). This value is somewhat at variance to the data obtained for the binding constants of zinc using different kinetic methods (see Sections 3.3.1 and 3.3.11). It is well known, however, that the determination of binding constants using gel filtration leads to an overestimate of values (Gore, 1984). The ^{65}zinc holoenzyme was found to be extremely stable provided that anaerobic conditions were maintained. An investigation into the dependance on zinc for catalytic activity clearly showed that the enzyme activity closely paralleled ^{65}zinc binding and demonstrated that the full complement of zinc (1 zinc per subunit) was required for maximal catalytic activity (Scheme 3.10). These data are concurrent with the findings of Tsukamoto et al., (1979, 1980) who investigated this relationship in the bovine enzyme.

Further studies in this current investigation demonstrated that labelled zinc was displaced from the ^{65}zinc holoenzyme by unlabelled zinc with an IC_{50} value of 100 μM (Scheme 3.12) and showed that the zinc bound to the enzyme was freely exchangeable with zinc in solution. EDTA (Scheme 3.14) and cadmium (IC_{50} = 60 μM; Scheme 3.12) were also able to displace ^{65}zinc. Interestingly, cadmium had a higher affinity for the zinc binding site than zinc itself, consistent with the stronger binding of cadmium to sulphydryl groups (Vallee and Ulmer, 1972).

Higher concentrations of cadmium, however, caused inhibition of the human enzyme (Scheme 3.4) possibly because the metal ion is interacting with an additional site on the human enzyme. Lead was also able to displace ^{65}zinc from the labelled holoenzyme but was far less effective than either cadmium or zinc (IC_{50} = 240 μM; Scheme 3.12).

Cheh and Neilands (1976) have investigated the nature of the metal binding site of bovine 5-aminolevulinic acid dehydratase by substituting zinc with cadmium, since the latter metal ion has better probe characteristics. They demonstrated that the cadmium-substituted enzyme yielded absorption and circular dichroism bands which were consistent with the formation of a cadmium-mercaptide bond. From these results, it had been suggested that, since cadmium and zinc are chemically very similar (both belong to Group IIB of the periodic table), the zinc ion is chelated to a sulphydryl residue in the dehydratase enzyme. To explore this possibility, the effect of oxidation (Scheme 3.15) and various thiophilic reagents, including Nbs_2 (Scheme 3.16), iodoacetic acid and 5-chlorolevulinic acid (Seehra and Jordan, 1981) (Table 3.4), on the zinc binding site were investigated. The data clearly highlighted that there was a close interrelationship between zinc binding and the status of reactive thiol groups in the human enzyme. Similar conclusions have been reported for the bovine enzyme (Tsukamoto et al., 1979; Bevan et al., 1980; Jaffe et al., 1984) and numerous other enzymes, including horse liver alcohol dehydrogenase (Eklund et al., 1974), superoxide dismutase (Rotilio et al., 1972), aspartate transcarbamylase (Rosenbusch et al., 1971), leucine aminopeptidase (Himmelhoch, 1969; Carpenter and Vahl, 1973) and metallothionen (Kagi and Vallee, 1961).

An extensive investigation of the involvement of sulphydryl groups in zinc binding to the human enzyme was carried out using Ellman's (1959) thiophilic reagent, Nbs_2.

Initial experiments were performed to determine the reaction of native human enzyme with Nbs_2 and demonstrated that the reaction followed an overall pattern quite similar to that of the bovine liver 5-aminolevulinic acid dehydratase (Seehra et al., 1981). There was a rapid initial release of two Nbs equivalents per subunit (groups I and II) followed by a slower release of a further two Nbs equivalents per subunit over a period of about 30 mins (groups III and IV). Thus, 32 thiol groups

titrated per octameric unit overall. Even from the spectroscopic data shown in Scheme 3.17 it was clear that zinc ions had a quite striking effect on the rate of reaction of one of the two faster reacting groups. A detailed investigation by stopped-flow analysis on this initial part of the progress curve revealed that the effect of zinc ions on the reactivity of enzyme sulphydryl groups with Nbs_2 was largely confined to the reduction in reactivity of group I; the apparent rate of reaction of group II was only marginally decreased by the presence of the metal (Scheme 3.19; Table 3.5).

The reaction of the bovine liver enzyme with Nbs_2 has been shown to result in the formation of an intramolecular disulphide bond (Tsukamoto et al., 1979; Seehra et al., 1981). That a similar mechanism was operative in the human enzyme was concluded from the reaction of enzyme with limiting amounts of Nbs_2. The data obtained in Scheme 3.20 demonstrates conclusively that the human enzyme must also contain two sulphydryl residues which are close enough to form a disulphide bridge. Two equivalents of Nbs were released from the reaction with one mole of Nbs_2 suggesting that after reaction of group I, an intramolecular rearrangement takes place involving displacement of the enzyme bound Nbs by attack from an adjacent unreacted cysteine residue (group II). In Scheme 3.21, the faster phase of the reaction (curve A-) represents the initial attack of the enzyme thiol group I on Nbs_2 with resulting liberation of one equivalent of Nbs and formation of enzyme-Nbs intermediate. The second slower phase of this curve is attributable to the intramolecular rearrangement which displaces the second equivalent of Nbs to form the S-S bond. Significantly, each phase approximated to 50% of the total release of the Nbs. The rate of the slow second phase is of course not the true rate since the two consecutive reactions are necessarily second order and first order respectively. The additional presence of zinc ions resulted in the reaction proceeding at a slow rate with only a small fast component. This suggests that the presence of zinc slows down the rate of the initial attack of Nbs_2 to a rate approximately equal to, or less than that of the intramolecular rearrangement. The small amount of fast phase is probably due to unliganded enzyme still present in solution.

These conclusions are corroborated by the experiments performed in the stopped-flow spectrophotometer (Section 3.3.9). Analysis of the data from the slow phase of each curve to determine the true rate of the intramolecular rearrangement showed that this occurred in both apo- and holoenzyme with a first order rate constant of 2.8×10^{-2} min^{-1} under these conditions. This, of course, should be independent of Nbs_2 concentration, but is slower than any of the rates measured in the conventional spectrophotometry experiments where a 50-fold excess of Nbs_2 (0.5 mM) was present. This would suggest either that modification of other thiol groups induces conformational changes resulting in an increase in the rate of the intramolecular rearrangement, or that in high concentrations of Nbs_2, both groups I and II can react directly with the reagent. This would open the possibility for intramolecular S-S bond formation by disulphide interchange forming Nbs_2 in a reaction which does not yield any additional chromophore.

Further evidence for the formation of an intramolecular S-S bridge during the reaction of groups I and II with Nbs_2 was afforded by the observation that oxidation of the native enzyme prior to reaction with Nbs_2 resulted in the loss of both of the two faster reacting components (I and II) and only two groups remained for titration, with rate constants similar to those calculated for groups III and IV in the non-oxidised enzyme (Scheme 3.17).

The reaction to form an intramolecular S-S bond on reaction with Nbs_2 has been described in several other systems apart from mammalian 5-aminolevulinic acid dehydratase (Seehra et al., 1981), notably glyceraldehyde-3-phosphate dehydrogenase (Wassarman and Majors, 1969), phosphoenol pyruvate carboxykinase (Carlson et al., 1978) and monoamine oxidase (Gornes et al., 1976). It thus appears that two SH groups are present in the structure of human 5-aminolevulinic acid dehydratase, as is the case in the bovine liver enzyme (Barnard et al., 1977; Tsukamoto et al., 1979), which are highly reactive and adjacent to one another. One of the groups binds with, or is in close proximity to, the zinc ion and the other group is close enough to permit intramolecular disulphide bond formation either with oxygen or the modified group I-Nbs intermediate.

In concord with these findings zinc was shown to protect the enzyme from oxygen inactivation (Scheme 3.18), which occurs readily in the absence of exogenous thiols, suggesting that zinc may play a crucial stabilising role in the maintenance of the reduced thiol configuration. These data agree well with the demonstration that, on exposure of the ^{65}zinc holoenzyme to oxygen in the absence of added zinc, the resultant inactivation of the enzyme was associated with the displacement of ^{65}zinc from the labelled holoenzyme (Scheme 3.15). Similarly, when the ^{65}zinc holoenzyme was treated with 1 mole Nbs_2 per mole of subunits most of the labelled zinc (≃ 80%) was displaced from the enzyme protein (see Section 3.3.10). These observations, together suggest several possibilities: (a) that zinc binds directly to sulphydryl group I; (b) that zinc is chelated in close proximity to sulphydryl group I leading to deactivation of the group by steric hindrance, and (c) that zinc elicits a conformational change which changes the reactivity of the sulphydryl group I. It is noteworthy that the effect of zinc was virtually instantaneous in inhibiting the rate of reaction of group I with Nbs_2 suggesting that if zinc was inducing any conformational change in the enzyme, the effect was extremely rapid.

The possible role of zinc in the catalytic mechanism has been discussed previously (Cheh and Neilands, 1976; Batlle and Stella, 1978; Tsukamoto et al., 1980). Although such a role for the metal ion can be envisaged, the current studies did not reveal any effect of substrate, product or substrate analogues on the reaction of Nbs_2 with group I or any of the other three reactive sulphydryl groups, suggesting that the metal ion may be participating in a structural rather than a catalytic capacity.

One of the most interesting properties of the human erythrocyte 5-aminolevulinic acid dehydratase relates to the reaction with, and inactivation by, relatively low (70 - 100 μM) concentrations of lead, and not surprisingly, it has been suggested that lead may bind to one or more of the sensitive SH groups. Accordingly, the reaction of groups I - IV with Nbs_2 was investigated in the presence of a concentration of lead (50 μM) which inhibits the human enzyme almost completely. The data shown in Scheme 3.17 demonstrates that lead had only a marginal inhibitory effect on the reactivity of the SH groups compared with zinc.

A more detailed analysis of the reaction of groups I and II under these conditions but using stopped-flow analysis revealed, however, that lead did have a measurable effect (∿ 25%) on the rate of reaction of sulphydryl group I with Nbs_2 (Table 3.5). It is interesting that at this concentration, which inhibits the enzyme about 80% (Scheme 3.5), other studies have revealed that 20 - 25% of the ^{65}zinc is displaced from the labelled holoenzyme (Schemes 3.12 and 3.13). It is therefore tempting to suggest that only at high concentrations of lead is a significant proportion of the 8 zinc binding sites occupied by this heavy metal.

Further evidence for the differences in the effects of the metals zinc and lead on the enzyme were deduced from the tryptophan fluorescence data shown in Table 3.6. Addition of increasing amounts of zinc to the apoenzyme caused a significant increase in the fluorescence intensity, accompanied by an increase in enzyme activity (Scheme 3.22 and 3.23). A more detailed examination of the data by hyperbolic non-linear regression yielded a dissociation constant for zinc of 19.9 ± 4.5 μM which is in good agreement with the value of ≈ 10 μM for half-maximal stimulation of the apoenzyme by zinc (Scheme 3.1). These results cumulatively suggest that the binding of zinc to the human dehydratase apoenzyme causes a conformational change which is associated with an increase in activity (see Scheme 3.23).

Reaction of increasing amounts of lead with the enzyme, however, caused a progressive fall in fluorescence and further analysis of the data by hyperbolic non-linear regression yielded a K_D of 26.5 ± 2.5 μM (Scheme 3.22). This value correlates well with the K_i for lead (10 - 30 μM; Section 3.3.2), which suggested that the interaction of lead with the enzyme was attributable to a non-productive conformational change resulting in a catalytically inactive enzyme. This suggestion was bourne out by the results presented in Scheme 3.24 which showed a strong correlation between the decrease in fluorescence intensity and the loss of enzymic activity.

When zinc was added to the lead-inhibited enzyme in such a concentration (200 μM) that the enzyme activity was recovered (see Table 3.3), there was a return towards the fluorescence level of the zinc

holoenzyme, although the actual lead-free level was not quite reached, possibly due to some fluorescence quenching by the lead. Interestingly, when both zinc and lead were present with the enzyme, addition of EDTA, which chelates the zinc preferentially, caused a decrease in the fluorescence to the level of the lead-inhibited enzyme together with a loss of catalytic activity.

The cumulative findings from the experiments presented in this chapter confirm that zinc and lead interact with the human 5-aminolevulinic acid dehydratase by different mechanisms. However, the studies are not conclusive as to whether the two metal ions are interacting at one site or more than one site. One possibility is that there may be two metal binding sites, one preferentially binding zinc and involving the highly reactive sulphydryl group I and a second which interacts primarily with lead. The affinity of lead for the zinc site must be low since the effect of lead on the Nbs_2 titration (Scheme 3.17) with group I is relatively small. It is possible that lead inhibits the enzyme by binding to the alternative site and eliciting a conformational change which affects the topography of the active site. Only at high lead concentrations would there be significant binding of lead to the zinc site. An alternative mechanism for the inhibition of the human enzyme by lead can be proposed if the binding of lead to one or two subunits within the dehydratase octamer promotes conformational changes in the remaining subunits rendering the other six or seven subunits inactive. The differential effects of zinc and lead on the same binding site can be explained by the different chemical properties of these metal ions. Zinc is a Group IIB transition metal and contains two electrons ($4s^2$) in the outer shell. However, zinc is a somewhat atypical transition metal in that its penultimate shell is full ($3s^2$ $3p^6$ $3d^{10}$) and consequently zinc cannot exhibit oxidation states in which the d electrons are involved. Therefore, zinc only has an oxidation status of +2 and usually forms tetrahedral complexes with organic compounds. Studies on the zinc binding site of the metalloenzymes, carbonic anhydrase, liver alcohol dehydrogenase and carboxypeptidase A, have revealed that the metal is coordinated in a distorted tetrahedral configuration (Chlebowski and Coleman, 1976; Lipscomb, 1983). Lead, on the other hand, is a Group 4B

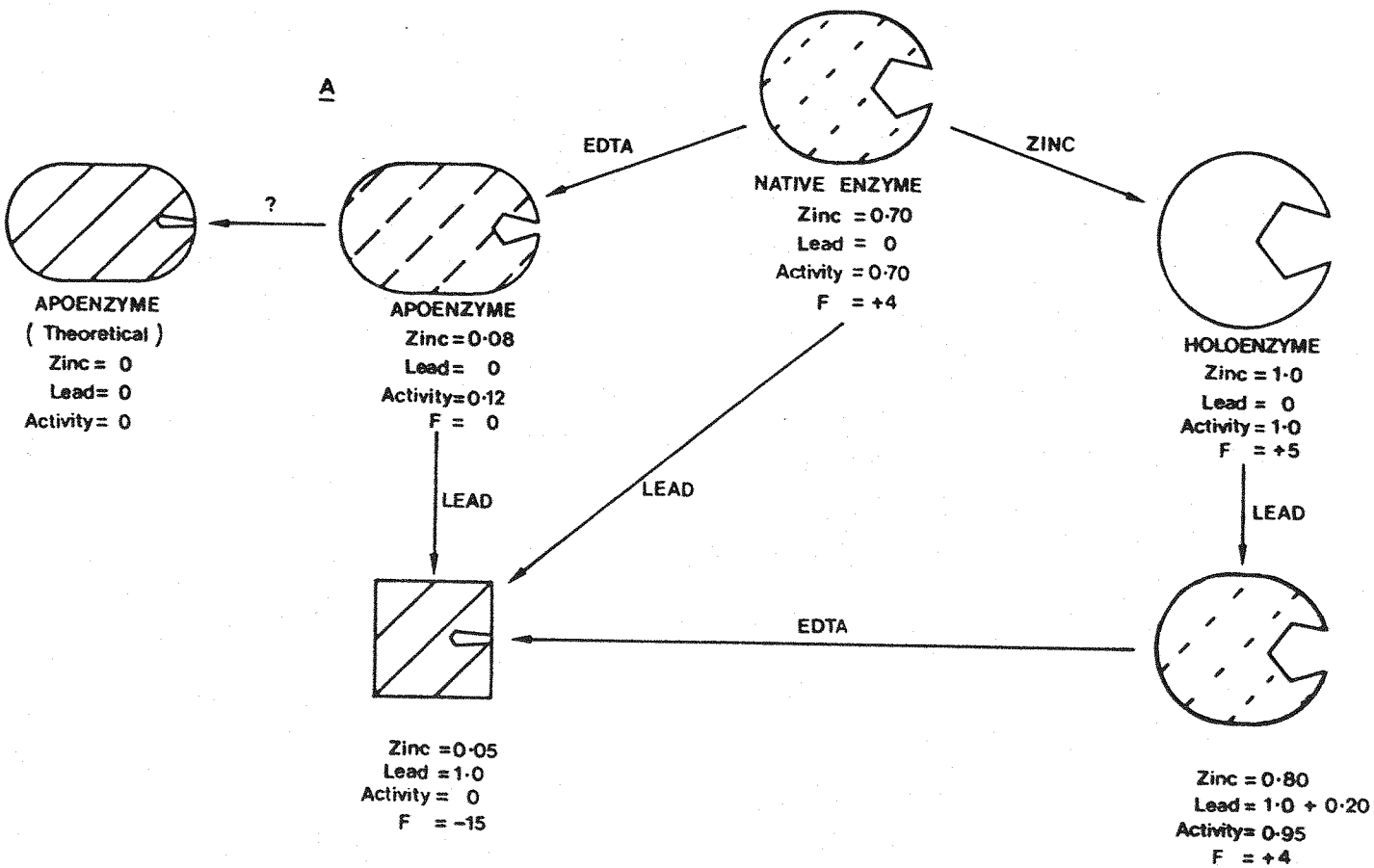
element and has four electrons in its outer shell ($6s^2$ $6p^2$). Consequently, lead can form both tetrahedral and octahedral complexes, although the latter are not so common and are less stable than the corresponding tetrahedral complexes. In addition, lead is much larger than zinc (0.120 nm compared to 0.074 nm; ionic radii) and has slightly different ligand preferences (Vallee and Ulmer, 1972) and could thus not only interact with different ligands at the zinc binding site, but could also cause structural perturbations by binding to the same ligands as zinc.

The results in this present study have shown that the dissociation constants for both lead and zinc are approximately the same (in the range of 10 - 30 μM). Therefore, if equal quantities of zinc and lead are present with the enzyme the resultant activity should be approximately 50% of maximum, assuming that there is a single metal binding site and that the octameric enzyme behaves as a symmetrical molecule. However, when the lead and zinc are present in a 1:1 molar ratio there is over 95% of maximal activity (see Table 3.3). From these findings it can be deduced that either the oligomeric enzyme behaves assymmetrically or that the metal ions must bind to different sites on the same subunit. This proposal is bourne out by the demonstration that the K_d app of lead for both the apoenzyme and the native enzyme are approximately the same (32.6 ± 8.6 μM and 29.2 ± 10.1 μM respectively; Table 3.3) and showed that lead inhibition was essentially unaffected by the presence of zinc. The finding that higher concentrations of lead are required to cause the same degree of inhibition in the presence of the substrate, ALA (Table 3.2), is consistent with the binding of lead to an allosteric site and that the conformation change induced by the binding of lead to yield a catalytically inactive enzyme is partially prevented by the presence of substrate at the active site. Thus, lead has a lower affinity for the ES complex than for the free enzyme. A two site model is further corroborated by other data; activity of the enzyme increases with the concentration of Zn^{++} up to ≈ 200 μM but then decreases with higher concentrations of the metal suggesting that the zinc first occupies its own binding site but then proceeds to occupy a second site, possibly the one which binds lead.

In summary, the results presented in this chapter suggest that lead and zinc interact primarily at different sites on the human enzyme. This two site mechanism is diagrammatically represented in Scheme 3.25. However, it was not possible to perform two key experiments which would have been of use in providing conclusive evidence for the above proposal. Firstly, the non-availability of a suitable lead isotope precluded any direct study on the binding of this metal to the enzyme in the presence and absence of zinc. Such a study would have revealed whether the amount of lead bound to the enzyme was consistent with a one site or a two site mechanism. Secondly, it was not possible to determine whether higher concentrations of lead (300 μM) could mimic the effect of zinc in inhibiting the Nbs_2 reaction with sulphydryl group I since the Nbs_2 reacted with high concentrations of lead ($\geq$ 100 μM) leading to precipitation and turbidity changes which prevented a meaningful evaluation by conventional and stopped-flow spectrophotometry.

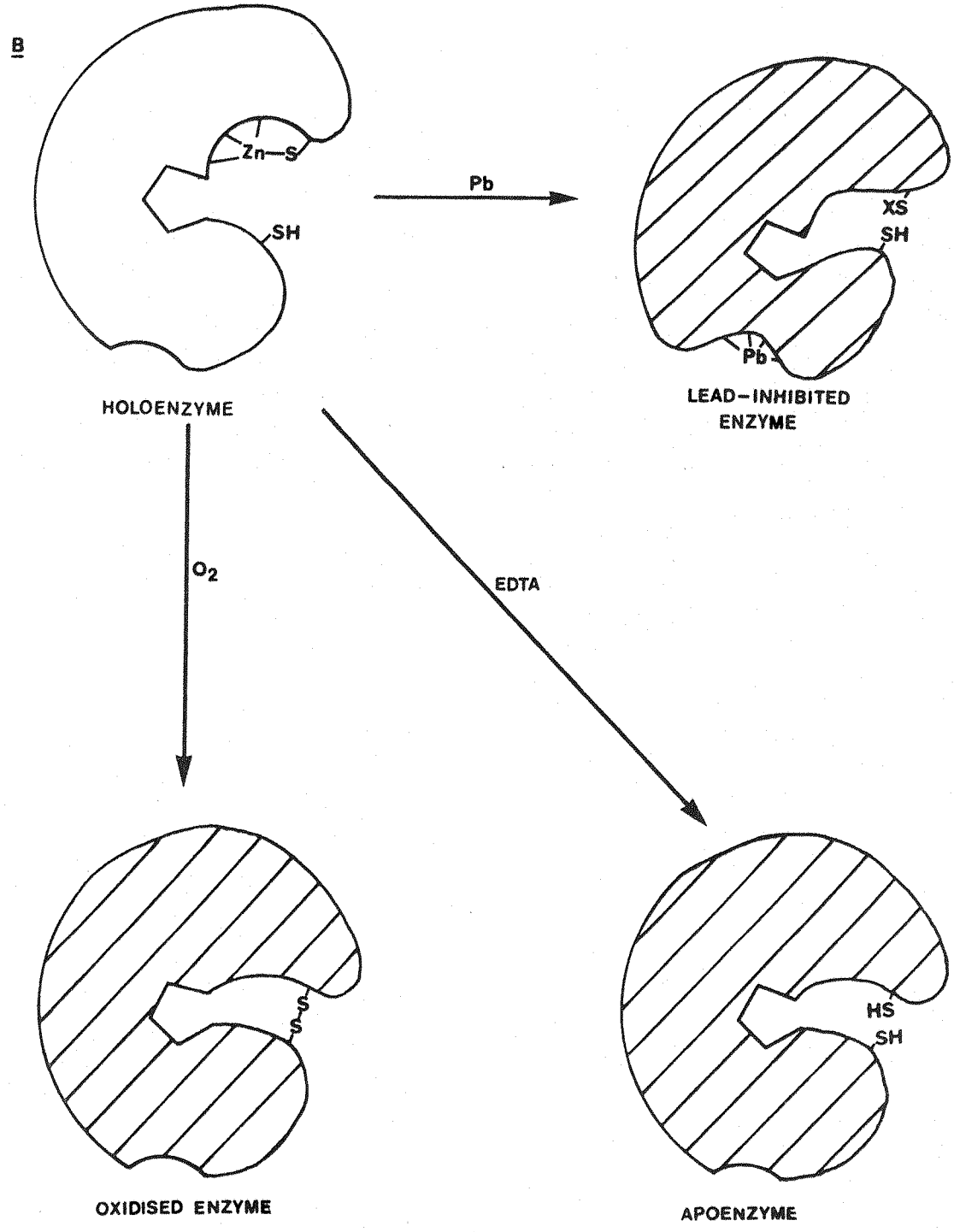
Scheme 3.25 Diagrammatic Representation of the Interaction of Lead and Zinc with Human 5-Aminolevulinic Acid Dehydratase at Two Different Binding Sites.

(a) This scheme summarises the data presented in this chapter for the various enzyme forms. The zinc and lead associated with each form is shown, with values up to a maximum of 1mole metal ion bound per subunit. Also shown is the activity (up to a maximum of 1) and fluorescence intensity (F) of the various enzyme forms.



Scheme 3.25 (cont.)

(b) Proposed two site model accounting for the presented data. (The inhibited enzyme is represented by diagonal lines ▨).



X = H or Pb

3.5 Summary.

Human 5-aminolevulinic acid dehydratase catalyses the condensation of two molecules of ALA to yield the product, porphobilinogen. Initial studies (Scheme 3.1) revealed that the presence of zinc increased the enzyme activity with maximal stimulation occurring between 100 and 300 μM zinc. Removal of zinc from the native enzyme (see Section 3.2.5) yielded an apoenzyme of low activity which could be reactivated upon addition of zinc. The concentration of zinc required to half-maximally stimulate the enzyme was 10 μM (Scheme 3.1). These results cumulatively demonstrated that zinc was essential for catalytic activity (Scheme 3.2) provided that the oxygen sensitive thiol groups in the enzyme were maintained in the reduced form. Kinetic studies revealed a K_m of 0.25 mM for the substrate, ALA (Scheme 3.2) and demonstrated that the activation by zinc was due to an increase in the number of functional active sites. The zinc binding site was investigated using ^{65}zinc chloride and showed that there was a single zinc site per subunit (8 per octamer) with a binding constant of 90 μM (Scheme 3.9), all eight zinc atoms per octamer being required for maximal catalytic activity (Scheme 3.10). The ^{65}zinc bound to the labelled human holoenzyme was shown to be freely exchangeable with zinc in solution (Scheme 3.11) and an IC_{50} value of 100 μM was determined (Scheme 3.12). This value agrees closely with the dissociation constant for the binding of ^{65}zinc to the apoenzyme. Similarly, EDTA (Scheme 3.14) was able to chelate the enzyme bound ^{65}zinc and was associated with a loss of enzymic activity.

The effect of various metal ions on the activity of the native human dehydratase enzyme were determined (Schemes 3.4, 3.5 and 3.8), but only cadmium and lead modulated catalytic activity at low concentrations ($\lesssim$ 3 μM). Cadmium was shown to stimulate the activity of the human enzyme (Scheme 3.4) and this effect is due to the close chemical relationship between cadmium and zinc which allows cadmium to interact with the zinc binding site. Cadmium was also shown to displace ^{65}zinc from the labelled holoenzyme (Scheme 3.11) with and IC_{50} of 60 μM (Scheme 3.12). The greater potency of cadmium for the zinc binding site than zinc itself is attributable to the higher affinity of cadmium for sulphydryl groups. (A reactive sulphydryl group (I) in the bovine

enzyme is thought to provide a ligand for the zinc ion) (Tsukamoto et al., 1979; Bevan et al., 1980). The inhibition of enzyme activity at higher cadmium concentrations (K_i app ≈ 100 μM; Scheme 3.4) is presumably due to metal binding at an additional low affinity metal site.

On the other hand, lead was shown to inhibit native human 5-aminolevulinic acid dehydratase potently (K_i = 10 - 30 μM; Schemes 3.5, 3.6 and 3.7) in a non-competitive fashion (Scheme 3.6; Table 3.1) and these findings are consistent with the binding of lead to an allosteric site which causes a loss of catalytic activity possibly by inducing a conformational change. The potency of the interaction of lead with the native enzyme was found to be dependant on the presence of the substrate, ALA, which revealed that lead had a higher affinity for the free enzyme than for the enzyme-substrate complex (Scheme 3.7; Table 3.2). Zinc was shown to completely reverse lead inhibition suggesting that as long as sufficient zinc is present, the enzyme remains in a viable configuration. The K_d of lead from both the apoenzyme and holoenzyme was shown to be ≈ 30 μM and demonstrated that the presence of zinc did not substantially affect the dissociation constant of lead (Table 3.3). Lead was also found to displace labelled zinc from the ^{65}zinc holoenzyme (IC_{50} = 240 μM; Scheme 3.12) but was far less effective than either cadmium or zinc. A closer examination of the interrelationship between lead inhibition and displacement of ^{65}zinc from the labelled holoenzyme (Scheme 3.13) revealed that, at concentrations of lead (100 μM) which almost completely inhibit the enzyme, only about 25% of the ^{65}zinc was displaced. These data suggest one of two possibilities: (a) that lead and zinc are interacting at different sites and that the displacement of ^{65}zinc from the holoenzyme by lead is due to the low affinity of lead for the zinc binding site, or (b) that lead and zinc are interacting at the same site, but with different ligands and coordination geometry.

Further studies were performed to investigate the nature of the zinc binding site and involved the determination of the effect of oxidation (Scheme 3.15) and various thiophilic reagents, including iodoacetic acid, and 5-chlorolevulinic acid (Table 3.4). The findings demonstrated that a close relationship existed between ^{65}zinc binding and the oxidation status of the reactive SH groups in the human enzyme. This was further corroborated by demonstrating that zinc greatly reduced the reactivity of group I towards Nbs_2 (Schemes 3.17 and 3.19; Table 3.5)

and was associated with the displacement of labelled zinc from the ^{65}zinc holoenzyme (Scheme 3.16). In the presence of an equivalent concentration of Nbs_2, two equivalents of Nbs were released from the enzyme, suggesting that an intramolecular disulphide bridge is formed between sulphydryl groups I and II (Scheme 3.20). A similar effect is observed on oxidation of the enzyme (Scheme 3.17), although the presence of zinc (100 μM) has been shown to protect the enzyme from oxidation presumably by stabilizing the reactive thiol groups in the reduced form (Scheme 3.18).

Lead (50 μM) was shown to reduce the reactivity of group I with Nbs_2 by ≈ 25% (Scheme 3.17; Table 3.5) and is in good agreement with the finding that 20 - 25% of the ^{65}zinc is displaced from the holoenzyme under these conditions (Scheme 3.13). However, it was not possible to determine whether this effect was due to a low affinity of lead for the zinc site with the lead exerting its primary effect at another site or if this was the primary site for lead interaction.

Fluorescence studies (Table 3.6) revealed that zinc binding was associated with a productive conformational change which resulted in a catalytically competent holoenzyme (Schemes 3.22 and 3.23), whereas lead causes a non-productive conformational change resulting in a catalytically inactive enzyme (Schemes 3.22 and 3.24).

In summary, the results presented in this chapter have revealed that the dissociation constants for both lead and zinc are approximately the same (10 - 30 μM). Similarly, when lead and zinc are present in equimolar concentrations the enzyme is almost completely active. These data are consistent with the interaction of the two metal ions at two different binding sites assuming that the enzyme behaves in a symmetrical manner (see Scheme 3.25). The effects of lead on the ^{65}zinc binding are attributable to a low affinity of lead for the zinc binding site.

CHAPTER 4

AN INVESTIGATION INTO THE MECHANISM OF ACTION OF HUMAN ERYTHROCYTE 5-AMINOLEVULINIC ACID DEHYDRATASE USING A SINGLE-TURNOVER REACTION TECHNIQUE

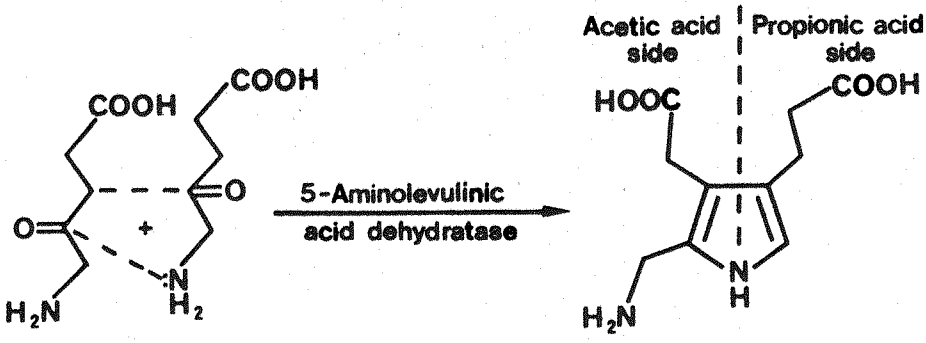
CHAPTER 4

An Investigation into the Mechanism of Action of Human Erythrocyte 5-Aminolevulinic Acid Dehydratase using a Single-turnover Reaction Technique

4.1 Introduction

5-Aminolevulinic acid dehydratase (porphobilinogen synthase; EC 4.2.1.24) was first isolated by Gibson et al.(1955) and catalyses a typical Knorr condensation reaction between two molecules of ALA (4.1) to form porphobilinogen (4.2) (Scheme 4.1), the monopyrrolic precursor of all porphyrins, chlorophylls and corrins (Akhtar and Jordan, 1978).

Scheme 4.1: The 5-aminolevulinic acid dehydratase reaction



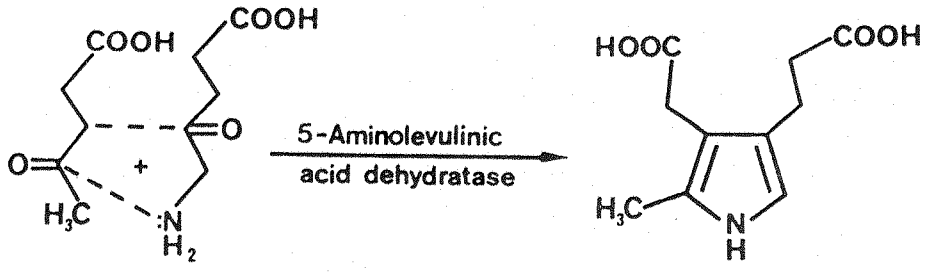
4.1 4.1 4.2

The transformation comprises of three distinct reactions which are (not necessarily in this order) an aldol condensation, the elimination of water and the formation of an intermolecular Schiff base intermediate. Comprehensive studies on the enzymatic reaction mechanism have been carried out using the enzyme isolated from Rhodopseudomonas sphaeroides (Nandi et al, 1968; Nandi & Shemin, 1968 a,b). They demonstrated that the enzyme was inactivated by $NaBH_4$ in the presence of either the substrate, ALA, or substrate analogues such as the competitive inhibitor, levulinic acid. When ^{14}C labelled ALA was used, the inactivation of the enzyme was accompanied by the incorporation of label into the enzyme protein. From these data, they suggested that a Schiff base intermediate

(4.4) was formed between a reactive amino group at the active site of the enzyme (4.3) and the substrate ALA (4.1) (Scheme 4.2) (see Section 2.3.2). Nandi (1978) subsequently isolated the modified amino acid (4.6) from acid hydrolysates of the ^{14}C labelled protein (4.5) and demonstrated that it was chromatographically and electrophoretically identical to chemically synthesised N-ε-(4-(5-aminovalerate))lysine (ALA-lysine). He concluded that the Schiff base intermediate was formed between the substrate, ALA, and an ε-amino group of a lysine residue (4.3) at the substrate binding site of 5-aminolevulinic acid dehydratase (Scheme 4.2).

Nandi and Shemin (1968b) investigated the enzyme specificity for this Schiff base formation and clearly demonstrated that only γ-oxoacids, such as ALA and levulinic acid (4.7), were able to form a Schiff base intermediate with the enzyme. They further demonstrated that heterologous pyrroles of the type (4.8) were produced on incubation of 5-aminolevulinic acid dehydratase with a mixture of ALA and levulinic acid (Scheme 4.3).

Scheme 4.3: Formation of a mixed pyrrole by 5-aminolevulinic acid dehydratase isolated from R.Sphaeroides

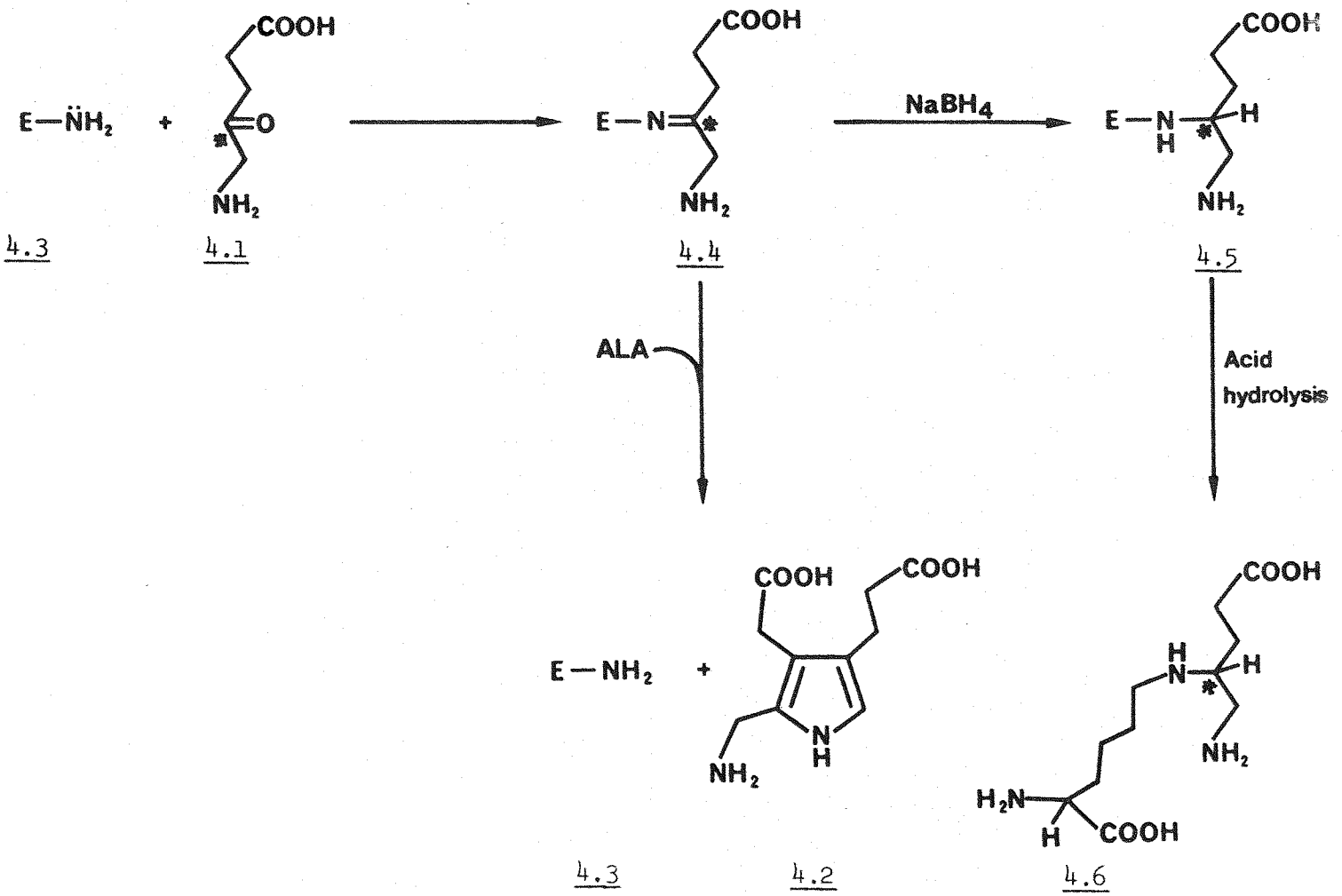


4.7 4.1 4.8

The levulinic acid cannot occupy the P side of the heterologous pyrrole since the substrate forming the P side must have an amino group in order to provide the nitrogen (atom 1) for the pyrrole ring and consequently the levulinic acid must have been incorporated into the A side (as shown in (4.8)). Nandi and Shemin argued naively that, because levulinic acid can also form a Schiff base with the enzyme as well as

Scheme 4.2 Formation of a Schiff Base Between 5-Aminolevulinic Acid Dehydratase and the Substrate, ALA, During the Biosynthesis of Porphobilinogen.

The asterisk (*) indicates the position of the label from [4-^{14}C] ALA.



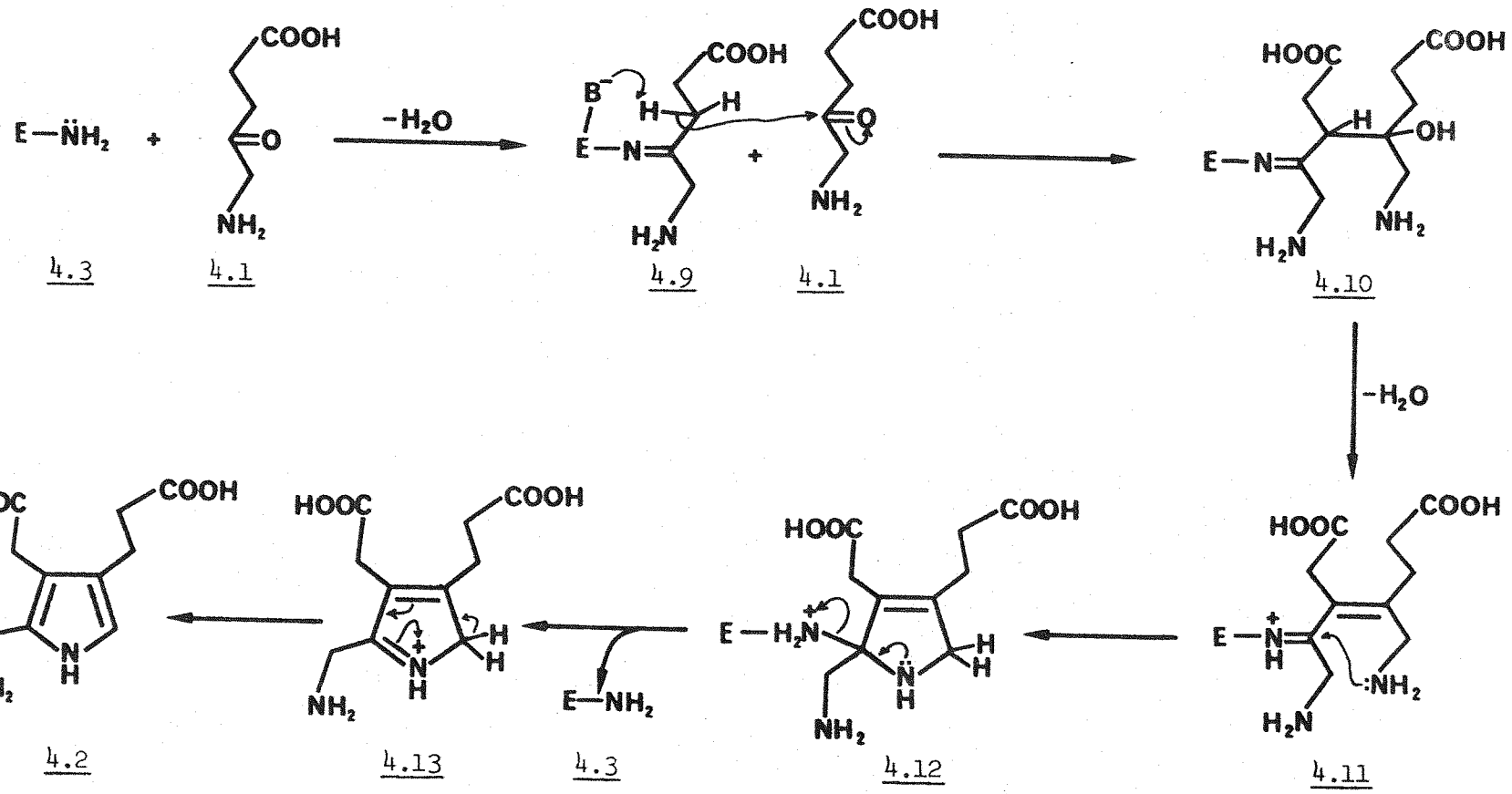
4.3 4.1 4.4 4.5

4.3 4.2 4.6

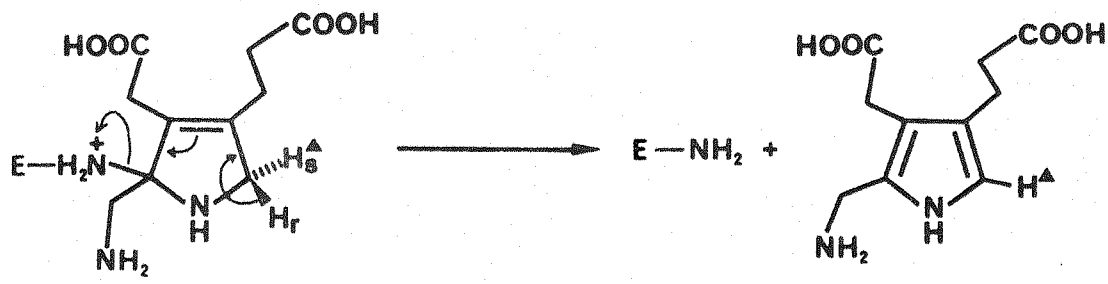
acting as a substrate in the formation of the A side, the levulinic acid must bind first to the enzyme at the A site in the formation of the heterologous pyrrole. Subsequent reaction with a molecule of ALA would yield the product (4.8). This argument was extended to the biosynthesis of porphobilinogen in which the first molecule of ALA also binds to the A side of the enzyme to form a Schiff base and consequently becomes atoms 4, 5, 6, 7, 11 and 12 (Scheme 4.1). What they had failed to appreciate was that levulinic acid need not act as a substrate at the same binding site with which it formed a Schiff base. Based on these arguments, Nandi and Shemin (1968b) went on to postulate a mechanism for the enzymic reaction (Scheme 4.4) in which the first molecule of substrate reacts with the enzyme (on the A side) to form a Schiff base and loses a proton to form a stabilised carbanion (4.9). Nucleophilic attack of this carbanion onto the carbonyl group of the second molecule of ALA in an aldol condensation reaction gives (4.10). After dehydration (4.10)→(4.11), cyclization (4.11)→(4.12), and elimination of the enzyme (4.12)→(4.13), a tautomer of the product (4.13) is released. The loss of a proton from the C-2 position of this tautomer finally yields the product, porphobilinogen (4.2). However, subsequent studies have revealed that the removal of this proton is catalysed by the enzyme. Chaudhry and Jordan (1976) demonstrated that porphobilinogen, derived from [5RS-$^3H_2$]ALA, was shown to retain 50% of the tritium present initially in the ALA. This was also demonstrated by Abboud and Akhtar (1976) who showed that [5S-^3H]ALA was converted to porphobilinogen without loss of the tritium label. These data suggest that the pro-R hydrogen atom present at C-5 of ALA is lost during the enzymic conversion to porphobilinogen. A simple modification (Scheme 4.5) of the mechanism proposed by Nandi and Shemin could account for the obligatory participation of the enzyme in this reaction whereby (4.12) is converted directly to porphobilinogen instead of via a tautomer.

Although the following mechanism has been widely accepted in the literature for many years (Cheh and Neilands, 1975; Batlle and Stella, 1978), there was no direct experimental evidence in its support. Recently, this mechanism has been shown to be unlikely by Jordan and Seehra (1980 a,b) who used the natural substrate for the enzymic reaction, ALA, to determine directly the order of addition of the two substrate molecules to the enzyme in the biosynthesis of porphobilinogen. They used a

Scheme 4.4 Mechanism of Action of 5-Aminolevulinic Acid Dehydratase Proposed by Nandi and Shemin (1968b)



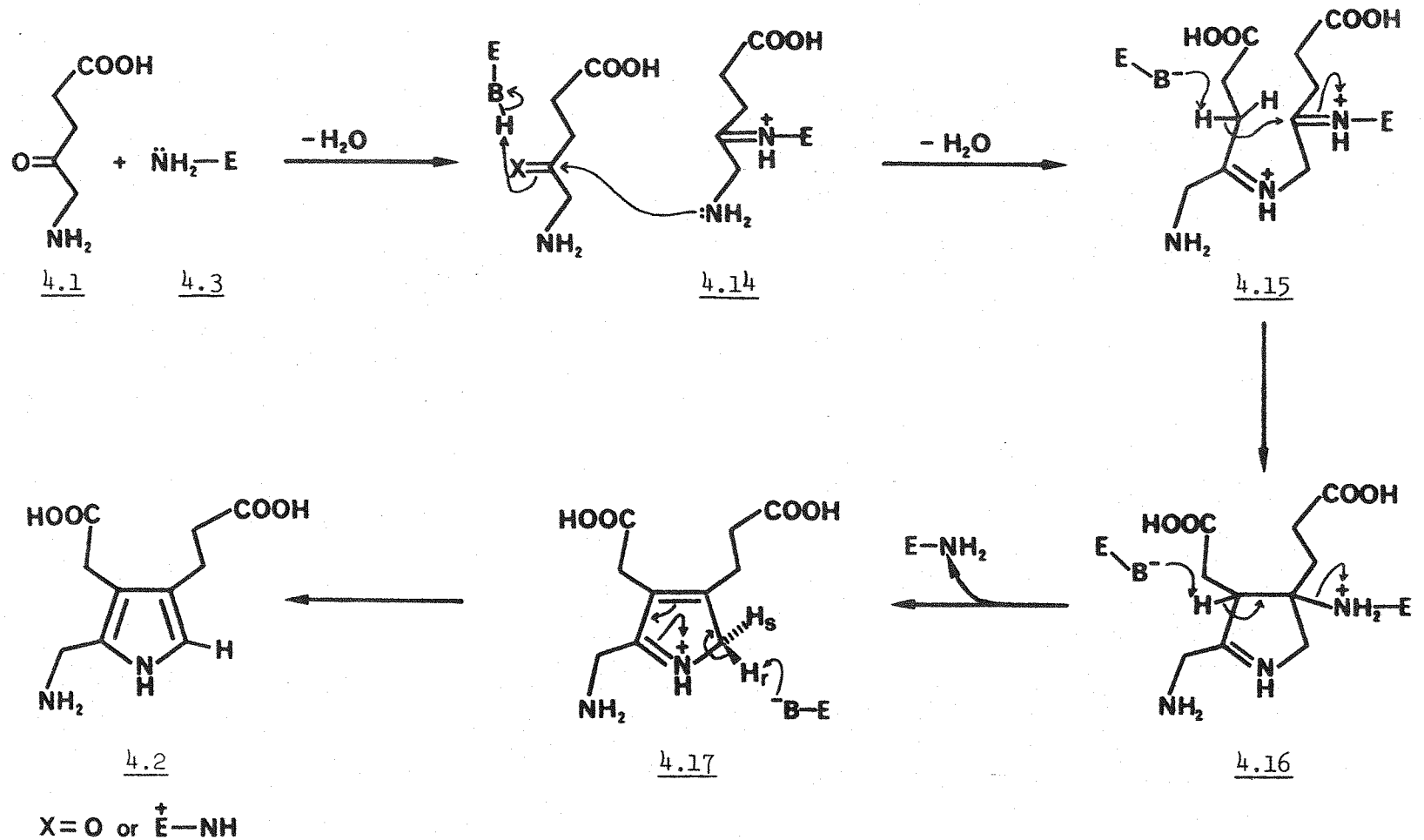
Scheme 4.5: The loss of the pro-R hydrogen atom at C-5 of ALA during the biosynthesis of porphobilinogen



4.12 4.3 4.2

single-enzyme-turnover approach whereby stoichiometric amounts of [5-^{14}C]ALA and 5-aminolevulinic acid dehydratase (isolated from both bovine liver and R.sphaeroides) were mixed initially in a rapid mixing device. The resulting enzyme-substrate complex was then quenched into a large excess of unlabelled ALA. Degradation of the resultant porphobilinogen revealed that only a small proportion of the radioactivity was present at C-11 of porphobilinogen ("acetic acid" (A) side) and hence it followed that the label must be present at C-2. They went on to prove that this assumption was correct by demonstrating that [2-^{13}C] porphobilinogen was enzymically synthesised from 5-aminolevulinic acid dehydratase which had been mixed with a stoichiometrically equivalent amount of [5-^{13}C]ALA in a single-turnover reaction using ^{13}C n.m.r. spectroscopy to reveal the location of the label (Jordan and Seehra, 1980b). These results cumulatively demonstrate that the first molecule of ALA to bind to the enzyme gives rise to the "proprionic acid" (P) side of porphobilinogen (atoms 1, 2, 3, 8, 9 and 10 in Scheme 4.1) and clearly highlights the importance of using the natural substrate in the elucidation of enzyme mechanisms. Based on these results, a mechanism for the action of 5-aminolevulinic acid dehydratase has been proposed (Jordan and Seehra, 1980a) (Scheme 4.6) in which the first molecule of substrate binds to the enzyme and forms a Schiff base with a reactive amino group at the "proprionic acid" (P) side of the enzyme (4.14). Binding and subsequent reaction of the second substrate molecule produces an intermolecular Schiff base between the two molecules of ALA (4.14)→(4.15). Deprotonation

Scheme 4.6 Mechanism of Action of 5-Aminolevulinic Acid Dehydratase Proposed by Jordan and Seehra (1980a, b).



4.1 4.3 4.14 4.15 4.16 4.17 4.2

at C-3 of the second substrate molecule (which eventually gives rise to C-4 of the product, porphobilinogen) to yield a carbanion allows an aldol condensation type reaction and the formation of a carbon-carbon bond (4.15)→(4.16). Displacement from the enzyme (4.16)→(4.17), followed by enzymic aromatization (4.17)→(4.2) yields the product, porphobilinogen. In this mechanism, the order of binding of the substrate molecules to the enzyme is clearly opposite in sequence to that proposed in the Shemin mechanism.

This Chapter describes the use of a single-enzyme-turnover reaction technique in the determination of the order of binding of the two identical substrate molecules in the biosynthesis of porphobilinogen by human erythrocyte 5-aminolevulinic acid dehydratase (prepared as described in Section 2.2.2).

4.2 Experimental

4.2.1 Materials

[5-^{14}C] Aminolevulinic acid hydrochloride was purchased from Amersham International, Bucks., U.K. 1-Fluoro-2, 4-dinitrobenzene (FDNB), dinitrophenyl-glycine (DNP-glycine) and Sigmacell 20 were purchased from Sigma Chemical Co. Ltd., Kingston-upon-Thames, London, U.K. Silica gel 60 premade thin layer chromatography (t.l.c.) plates were obtained from Merck Chemicals, Germany. Human erythrocyte 5-aminolevulinic acid dehydratase, porphobilinogen and all other reagents and materials were obtained from the same sources as described in Chapter 2 (Sections 2.2.1 and 2.2.2)

4.2.2 Single-turnover experiments

Human erythrocyte 5-aminolevulinic acid dehydratase (37.5-78.1 units; specific activity 21.7 units/mg in the presence of 0.1mM zinc ions), prepared as described in Section 2.2.2, was dissolved in 500µl of 0.1M potassium phosphate buffer (pH 6.8) containing 10mM dithioerythritol and 0.1mM zinc ions and rapidly mixed with the required amount of [5-^{14}C]ALA (20.6-804 nmol) in 500µl of the same buffer. The mixing of enzyme and substrate was achieved using a rapid mixing device with two syringe inputs which allowed the enzyme and labelled substrate to interact for a short time (≈ 100 m sec) before chasing with a large excess of unlabelled ALA (60µmol) in the same buffer (1ml). The resultant reaction mixture was incubated for 5 minutes at 37°C before isolating the labelled porphobilinogen (Scheme 4.8). Additions are shown in Table 4.1).

4.2.3 Purification of porphobilinogen

(a) Ion-exchange chromatography

The impure samples of porphobilinogen from the single-turnover reactions were diluted with unlabelled porphobilinogen (4mg) and the pH of the solution was adjusted to 7.5 by the dropwise addition of 1M acetic acid. The samples were applied to columns of Dowex 1 x 8 acetate (1.5 x 12 cm) which had been equilibrated previously with distilled water and the unreacted ALA was removed by washing the column with 80 ml of ice-cold water. The porphobilinogen was eluted from the column with 30 ml of ice-cold acetic acid (1M) and the eluate was freeze-dried.

(b) Thin layer chromatography (t.l.c.)

The lyophilised porphobilinogen was dissolved in 0.5 ml water and further purified by t.l.c. on Sigmacell 20 plates (0.75 mm thick x 10 cm x 20 cm) which were developed in n-butanol:acetic acid:water (63:27:10, by volume). The porphobilinogen, located by spraying a small area of the plate with modified Ehrlich's reagent (Mauzerall and Granick, 1956), was eluted with a small volume of 0.1M ammonium hydroxide (2-3 ml) and lyophilised immediately.

The concentration of porphobilinogen in the purified samples was determined by reacting a known volume of the sample with p-dimethylamino-benzaldehyde in 2M perchloric acid (λmax = 555 nm; $\varepsilon = 6.02 \times 10^4\ M^{-1}\ cm^{-1}$). The radioactivity associated with the porphobilinogen was determined as described in Section 4.2.6. The specific activities of the porphobilinogen samples were then calculated.

4.2.4 Degradation of porphobilinogen to glycine

The regiospecifically labelled porphobilinogen (3.04-4.44 mg) was dissolved in 500 µl of ammonium bicarbonate (10% w/v) and stirred for 10 minutes at 0°C with acetic anhydride (10 mg; 127 µmol) to yield N-acetyl porphobilinogen. After the addition of 3ml of ozone saturated formic acid (98% v/v) and 1 ml of chloroform, ozone (generated using a British Oxygen Laboratory Ozoniser by the high voltage discharge on oxygen) was bubbled through the mixture at a flow rate of approximately 0.6 mg ozone/minute for 2 hours at 0°C. The "ozonide" produced was cleaved by the addition of 1 ml of hydrogen peroxide (30% v/v) followed by incubation at room temperature for 16 hours. The mixture was freeze-dried and the residue was then neutralized (to pH 7) by the addition of dilute ammonium hydroxide (1M). Catalase (3-4 µl) was added to destroy any residual hydrogen peroxide. The pH of the solution was adjusted to 1 using 1M hydrochloric acid and it was freeze-dried. This residue was subsequently extracted with freshly distilled tetrahydrofuran (THF) (4 x 2 ml) and the extract, which contained N-acetyl glycine, was rotary evaporated to dryness (in vacuo). The resultant residue was dissolved in 0.5 ml of 6M hydrochloric acid and incubated for 16 hours at 110°C in a drawn test-tube (1 x 5.5 cm) which had been sealed under vacuum in an atmosphere of nitrogen. The Hcl was removed using a rotary vacuum pump to yield a residue of impure glycine. This glycine was purified by t.l.c. on

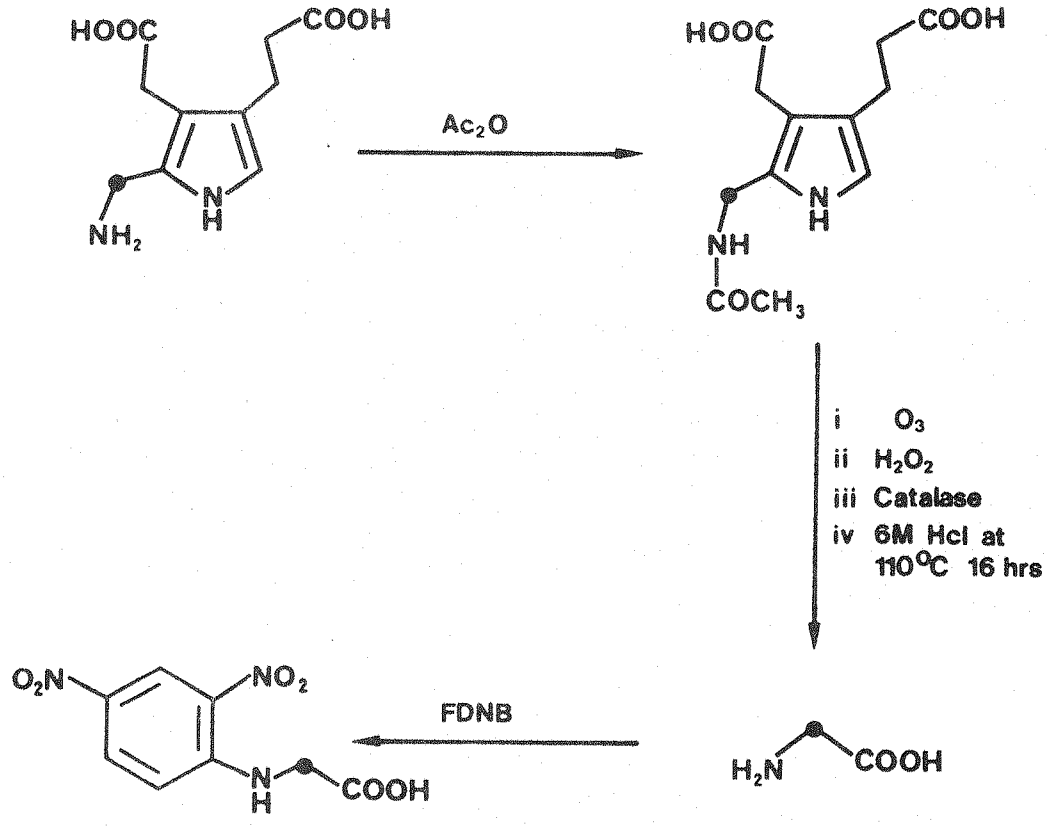
Sigmacell 20 plates (0.4 mm thick x 10 cm x 20 cm) which were developed in n-butanol:acetic acid:water (4:1:1, by volume). The glycine band was eluted with water (3-5 ml) and freeze-dried (see Scheme 4.7).

4.2.5 Preparation of DNP-glycine

The freeze-dried glycine residue was dissolved in 200µl of sodium bicarbonate (5% w/v) and mixed with 500µl of FDNB (5% v/v) in ethanol. The pH of the solution was maintained between 8 and 10 by further additions of sodium bicarbonate (5% w/v) and the solution was incubated at room temperature for 45 minutes with occasional shaking. The reaction was terminated by the addition of 2 ml of sodium bicarbonate (1% w/v) and the unreacted FDNB was extracted into diethylether (5 x 10 ml). The solution was acidified to pH 1 by the addition of 1M Hcl and the DNP-glycine was extracted into diethylether (3 x 5 ml). The etherial extract was dried over anhydrous sodium sulphite, filtered and rotary evaporated to dryness. The DNP-glycine was purified by t.l.c. on Silica gel 60 plates (1 mm x 10 cm x 20 cm) which were developed in methylene chloride:acetone:acetic acid (19:2:1, by volume) together with standard DNP-glycine. The band containing DNP-glycine (Rf ≈ 0.45) was eluted with 3-5 ml of chloroform:methanol (1:1, v/v), rotary evaporated to dryness and redissolved in methanol. The concentration of the DNP-glycine was determined spectrophotometrically (λ max = 348 mm; $\varepsilon = 1.36 \times 10^4$ M^{-1} cm^{-1} in methanol). The radioactivity associated with the DNP-glycine was determined as described in the following section. The specific activity of the DNP-glycine could then be calculated (see Scheme 4.7).

4.2.6 Determination of radioactivity

All determinations were made using a Phillips 4700 scintillation spectrometer programmed to give a quench correction to d.p.m. Samples were counted in 10 ml of toluene:methanol (4:1 v/v) containing 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6 g/l). Quench curves were constructed to determine the quenching of increasing quantities of both DNP-glycine and porphobilinogen.

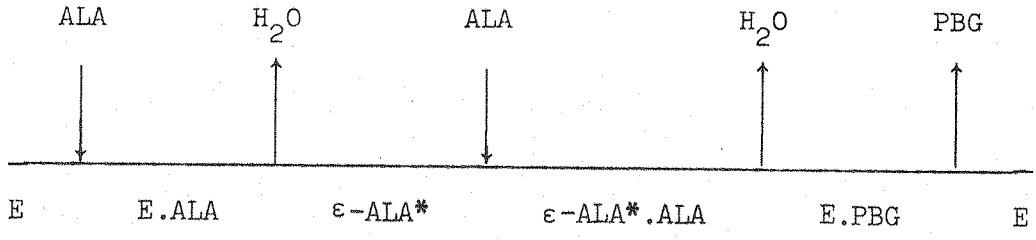


Scheme 4.7 Degradation of Regiospecifically Labelled Porphobilinogen to DNP-Glycine.

The black dot (●) shows the fate of C-11 of porphobilinogen.

4.3 Results and Discussion

The biosynthesis of porphobilinogen from two molecules of ALA by 5-aminolevulinic acid dehydratase is considered to follow a ping-pong type reaction mechanism (Nandi and Shemin, 1968b; Cleland, 1970) whereby one molecule of ALA initially binds to the enzyme to form a Schiff base intermediate with the concommitant liberation of water. Subsequent reaction with the second substrate molecule yields a second water molecule and the dimeric product, porphobilinogen.



For a bisubstrate reaction in which the two substrate molecules are different (i.e. a heterosubstrate reaction), the sequence of events which occur at the active site of the enzyme can be studied using steady-state kinetics. Under different conditions (such as varying concentrations of substrate, product and inhibitor) it is possible, in many cases, to determine the order of addition of substrates and subsequent release of products from the enzyme and also determine whether this order is obligatory or random (Chapter 1, Section 1.1). This information is essential for formulating the mechanism of action of the enzyme. However, in bisubstrate reactions where the two substrate molecules are identical (i.e. homosubstrate or homopolymerization reactions), such as the enzyme-catalysed reactions of β-oxoacyl-CoA thiolase, 5-aminolevulinic acid dehydratase and porphobilinogen deaminase, this conventional kinetic approach is of little value.

An approach to the problem of finding the order of substrate binding in the dehydratase reaction was developed by Nandi and Shemin (1968b) who utilised the substrate, ALA, together with the pseudosubstrate, levulinic acid, to demonstrate that a mixed pyrrole was produced on their co-incubation with 5-aminolevulinic acid dehydratase (as shown in Scheme 4.3). Since the levulinic acid could also bind to the enzyme through a Schiff base they suggested that it interacted solely with the A site and argued that it was at this site that the first substrate

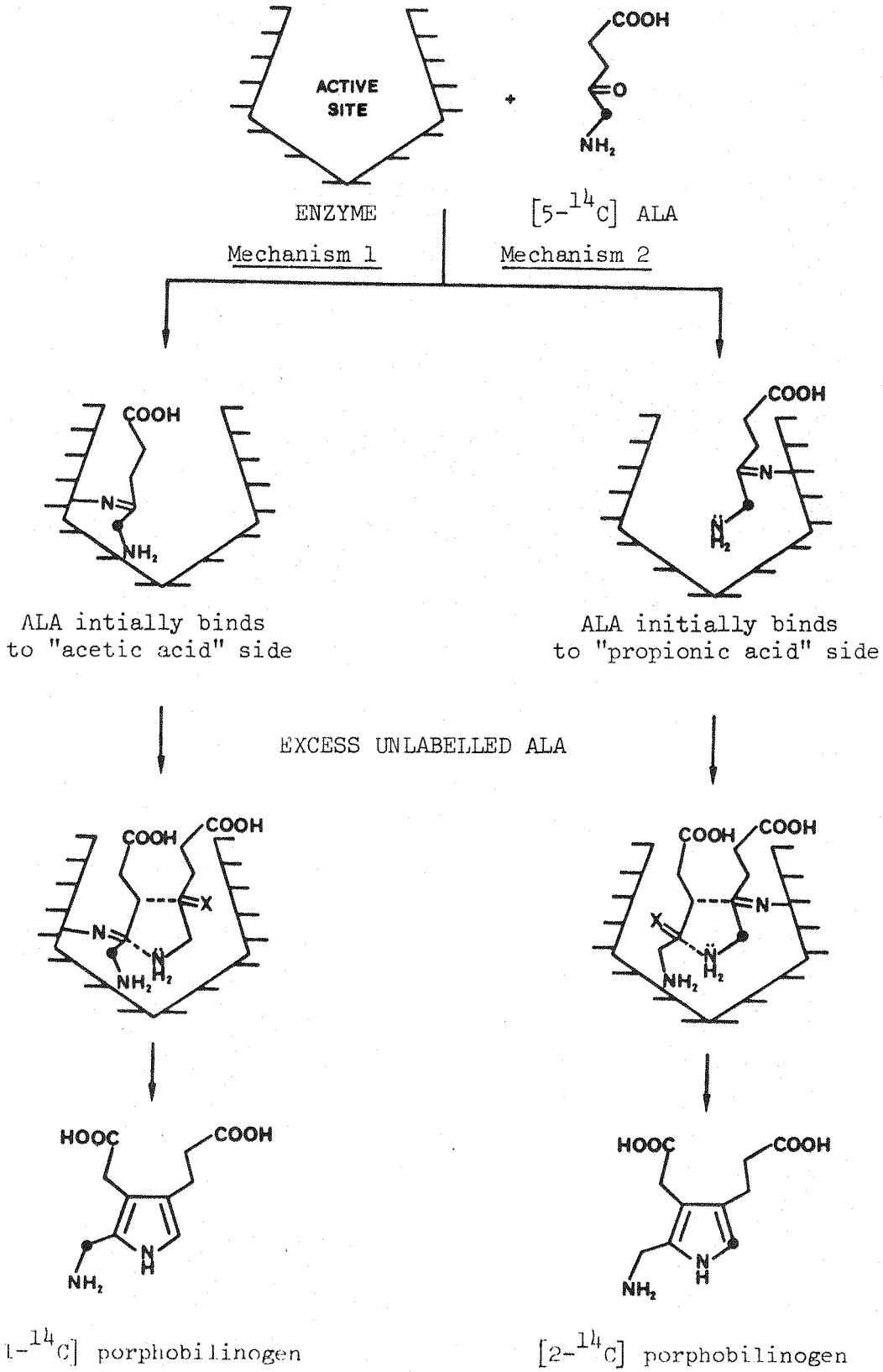
molecule binds (see Section 4.1 for further details). The use of this approach has, however, proved confusing since it cannot be unequivocally proven that the levulinic acid interacts with the same binding site both when it acts as a substrate and when it binds to the enzyme in the form of a Schiff base.

The method that we have adopted to the study of the human erythrocyte 5-aminolevulinic acid dehydratase reaction utilises only the substrate ALA, but the two identical substrate molecules which participate in the formation of porphobilinogen, can be distinguished by isotopic labelling using a single-turnover enzyme reaction technique. In conjunction with chemical analysis of the product to reveal the location of the label, this approach enables one to determine the order of addition of the two substrate molecules. This technique has been used successfully to elucidate mechanistic features of other enzymes catalysing homopolymerization reactions, including β-oxoacyl-CoA thiolase (Jordan and Gibbs, 1983) (see Chapter 5) and porphobilinogen deaminase (Jordan and Seehra, 1979; Seehra and Jordan, 1980).

Using this single-turnover enzyme reaction technique it should be possible to resolve the two broad mechanisms which are feasible for the 5-aminolevulinic acid dehydratase reaction. In the first mechanism, the labelled ALA ([5 - ^{14}C]ALA) binds to the "acetic acid" side of the active site of the enzyme to form a Schiff base. Subsequent reaction with a second molecule of unlabelled substrate will yield porphobilinogen which will be preferentially labelled at the C-11 position (Scheme 4.8, Mechanism (1)). Alternatively, in mechanism 2 the [5 - ^{14}C] ALA initially binds to the "proprionic acid" side of the active site of the enzyme and subsequent reaction with unlabelled ALA yields porphobilinogen preferentially labelled at the C-2 position (Scheme 4.8, Mechanism (2)). These two possibilities can be distinguished clearly by determining the percentage of the radioactivity present at C-2 and C-11 in the regiospecifically labelled porphobilinogen (derived from [5 - ^{14}C] ALA). This is achieved by degrading the porphobilinogen to DNP-glycine (which contains the carbon atom originating from C-11 of porphobilinogen) (Scheme 4.7) and comparing its specific activity with that of the original porphobilinogen. The percentage of radioactivity originally present at C-2 of porphobilinogen can therefore be calculated allowing one to

Scheme 4.8 Possible Fates of [5-^{14}C] ALA in the Enzymic Synthesis of Porphobilinogen Using a Single-Turnover Enzyme Reaction Technique.

The symbol (●) represents the location of the ^{14}C label.



distinguish whether the [5 - ^{14}C]ALA which was initially bound to the enzyme gave rise to the "acetic acid" (C-11) or "proprionic acid" (C-2) side of the product.

The success of a single-turnover enzyme reaction technique depends on a number of factors which are as follows. First, there must be a substantial difference between the affinity of the enzyme for the two substrate molecules so that one of the two substrate binding sites is preferentially labelled. The apparent K_m for the human dehydratase enzyme (251 ± 8μM; Section 3.3.1) almost certainly reflects the affinity for the binding of the second substrate molecule to the low affinity binding site and therefore the affinity of the enzyme for the first substrate molecule must be substantially higher if this technique is to prove successful. Second, there must be no positive cooperativity whereby the binding of the first substrate molecule to the enzyme causes an increase in affinity of the enzyme for the second molecule and results in the labelled substrate binding to both the substrate binding sites. Third, the enzyme and labelled substrate must be mixed together for a short time (50 - 100 msec) before chasing with unlabelled substrate, to allow only sufficient time for the binding of the first substrate to the enzyme. Too long a time would allow the enzyme to bind a second molecule of substrate and turnover to yield porphobilinogen which would, consequently, be labelled at both the C-2 and C-11 positions. Thus, the mixing time for the enzyme and labelled substrate must be short compared with the rate of turnover of the enzyme. Finally, the enzyme and labelled substrate should be mixed in equimolar concentrations so that there is a limited amount of substrate present and there is only sufficient substrate to occupy the higher affinity binding site. However, in order to maximise the chance of obtaining a meaningful result different ratios of labelled substrate and enzyme were used in the single-turnover experiments (i.e. 1.6, 8 and 16 moles of labelled ALA/mole of octameric enzyme or 0.2, 1 and 2 moles ALA/mole of enzyme active sites).

Accordingly, stoichiometric equivalents of [5 - ^{14}C]ALA and human erythrocyte 5-aminolevulinic acid dehydratase were mixed in a rapid mixing device (see Chapter 5, Scheme 5.6) so that one of the two ALA binding sites would be preferentially occupied. The subsequent addition of a large excess of unlabelled ALA would carry the initially bound label

into the product, porphobilinogen, thus completing a single-enzyme-turnover with respect to the labelled substrate. After purification by ion exchange chromatography and t.l.c. (Section 4.2.3), the regio-specifically labelled porphobilinogen was degraded to yield DNP-glycine to give an unambiguous evaluation of the amount of label originally present at C-11 of the porphobilinogen and therefore also at C-2 (Scheme 4.7).

The results from the degradation of porphobilinogen enzymically synthesised in single-turnover experiments from 1.6, 8 and 16 moles of [5 - ^{14}C]ALA per mole of octameric enzyme (Expts. 1, 2 and 3 respectively in Table 4.1) clearly demonstrated that the DNP-glycine contained only a small proportion of the label (originally present at C-11 of porphobilinogen) and, therefore, the label must be present at C-2. When excess [5 - ^{14}C]ALA was mixed with the enzyme (Expt. 4, Table 4.1) the resultant porphobilinogen yielded, as expected, essentially equal distribution (48:52) of radioactivity between C-11 and C-2.

From these results, it can be concluded that the first molecule of ALA to bind to human erythrocyte 5-aminolevulinic acid dehydratase is the one which gives rise to the "proprionic acid" side of porphobilinogen (atoms 1, 2, 3, 8, 9 and 10) (see Scheme 4.8, Mechanism (2)). These data are in close agreement with the findings of Jordan and Seehra (1980 a,b) and Seehra (1980) who studied the dehydratase enzyme isolated from bovine liver and the photosynthetic bacteria, R. sphaeroides using this single-enzyme-turnover reaction approach. A comparison of the results obtained using 5-aminolevulinic acid dehydratase isolated from the aforementioned sources is shown in Table 4.2.

Earlier work, presented in Chapter 2 of this thesis (Section 2.3.2), clearly demonstrated that human erythrocyte 5-aminolevulinic acid dehydratase was inactivated by $NaBH_4$ in the presence of [4 - ^{14}C]ALA and that this inactivation was accompanied by the incorporation of label into the enzyme protein. Porphobilinogen was shown to drastically reduce this incorporation into the enzyme (Section 2.3. ; Scheme 2.) and these results cumulatively suggest that a Schiff base intermediate is formed during the enzyme reaction between the substrate, ALA, and a reactive amino group at the active site of the enzyme. Further studies demonstrated that when equimolar concentrations of enzyme and [4 - ^{14}C]ALA (8 moles ALA/ mole of octameric enzyme) were mixed in a rapid mixing device and

Legend to Table 4.1

Legend to Table 4.1

Stoichiometric quantities of human erythrocyte 5-aminolevulinic acid dehydratase (8.04 - 12.85 nmol) and [5 - ^{14}C]ALA (20.5 - 804 nmol) were mixed in a rapid mixing device, so that the enzyme and ^{14}C substrate interacted for approximately 100 msec, before the mixture was "chased" with a large excess of unlabelled ALA (60 μmol). After purification by ion exchange chromatography and t.l.c. (Section 4.2.3), the regio-specifically labelled porphobilinogen was degraded to DNP-glycine (Sections 4.2.4 and 4.2.5) (Scheme 4.7) in order to determine the distribution of the label between C-11 and C-2.

(a) Specific activity of [5 - ^{14}C]ALA = 381 x 10^2 dpm/nmol (Expts. 1, and 3) and 63.5 x 10^2 dpm/nmol (Expt. 4).

(b) Expressed per mole of the octameric enzyme (MR = 285,000). The amount of enzyme used = 12.85 nmol (Expt. 1) and 8.04 nmol (Expts. 2, 3 and 4).

(c) Specific activity of human erythrocyte 5-aminolevulinic acid dehydratase = 21.7 U/mg when assayed in the presence of 0.1 mM zinc.

(d) Determined by reaction of porphobilinogen with p-dimethylaminobenzaldehyde in 2M perchloric acid (λmax = 555 nm; ε = 6.02 x 10^4 M^{-1} cm^{-1}).

(e) After purification, the yield of porphobilinogen from [5 - ^{14}C]ALA was approximately 30% (Expts. 1, 2 and 3).

(f) Determined in methanol (λmax = 348 nm; ε= 1.36 x 10^4 M^{-1} cm^{-1}).

(g) Determined by comparing the specific activities of the DNP-glycine and the original porphobilinogen.

Table 4.1 ^{14}C Radioactivity in C-11 and C-2 of Porphobilinogen Enzymically Synthesised from [5 - ^{14}C] ALA in a Single-Turnover Reaction Using Human Erythrocyte 5-Aminolevulinic Acid Dehydratase

		PORPHOBILINOGEN			DNP-GLYCINE				
Expt. No.	No. of moles of [5 - ^{14}C]ALA[a] used/mole of enzyme[bc]	Yield[d,e] (μmole)	Total Radio-activity (dpm x 10^{-3})	Specific activity (dpm x 10^{-3}/μmol)	Yield[f] (μmol)	Total Radio-activity (dpm x 10^{-3})	Specific activity (dpm x 10^{-3}/μmol)	% Radio-activity at C-11 of porphobi-linogen	% Radio-activity at C-2 of porphobi-linogen
1	1.6	19.28	118.7	6.16	3.25	1.35	0.42	6.8	93.2
2	8.0	13.32	444.4	33.37	2.13	12.08	5.67	17.0	83.0
3	16.0	15.60	1,651.3	105.85	2.57	77.53	30.17	28.5	71.5
4	EXCESS (100.0)	14.75	3,765.2	255.27	2.65	324.79	123.56	48.0	52.0

TABLE 4.2: Comparison of ^{14}C radioactivity incorporated into C-11 and C-2 of porphobilinogen enzymically synthesised from [5 - ^{14}C]ALA in a single-turnover reaction using 5-aminolevulinic acid dehydratase isolated from different sources

Source of Enzyme	No. of moles [5 - ^{14}C]ALA used/mole of enzyme[a]	Specific activity of porphobilinogen (dpm x 10^{-3}/μmol)	Specific activity of DNP-Glycine (dpm x 10^{-3}/μmol)	% Radioactivity at C-11 of porphobilinogen	% Radioactivity at C-2 of porphobilinogen
Human[b]	8	33.4	5.7	17	83
Bovine[c]	8	129	17.6	14	86
R.sphaeroides[c]	6[d]	896	360	40	60
Control	Excess	255.3	123.6	48.0	52.0

(a) Quantities refer to the number of moles of labelled ALA added in the single-turnover experiments based on a molecular weight of 280,000 - 285,000.

(b) 5-Aminolevulinic acid dehydratase purified from human erythrocytes (Chapter 2, Section 2.2.2).

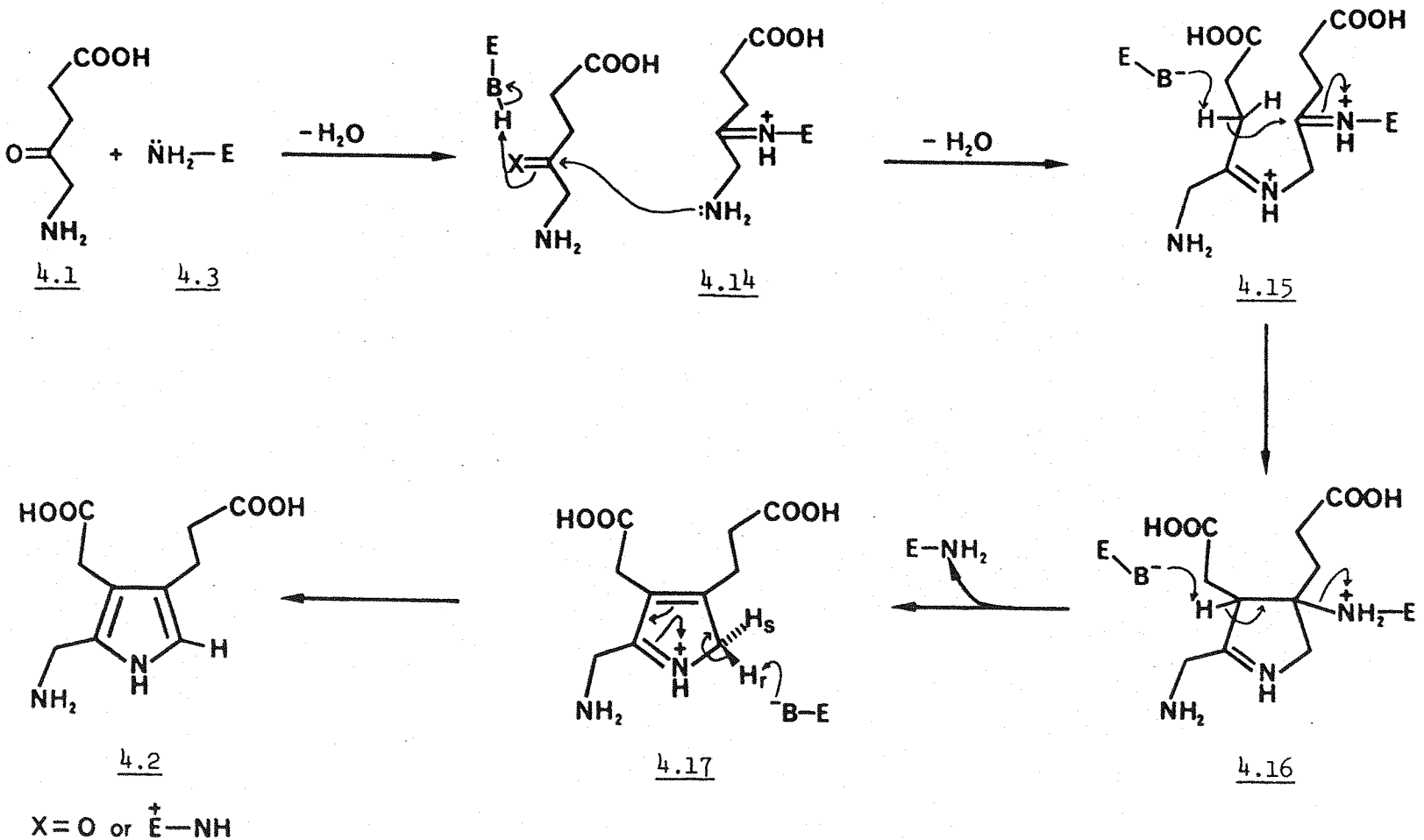
(c) Data from Jordan and Seehra (1980a) and Seehra (1980).

(d) 5-Aminolevulinic acid dehydratase from R.sphaeroides exists as a hexamic protein of subunit molecular weight 47,500

subsequently "quenched" into 1M $NaBH_4$ (at pH 6.8) the incorporation of label into the enzyme protein was between 2.8 and 3.2 moles of ALA per mole of octameric enzyme (0.35 - 0.40 moles ALA/mole of enzyme subunit, i.e. 35-40% saturation of available active sites) (data not presented). This value correlates well with the observation that, in the single-turnover experiments, approximately 30% of the labelled substrate ([5 - ^{14}C] ALA) was carried through into porphibilinogen. (The yield was determined <u>after</u> purification of the porphobilinogen by ion exchange chromotography and t.l.c.) (Table 4.1). These results suggest that the enzyme-substrate complex (a Schiff base) is a catalytically viable intermediate which can either be converted to product with a second substrate molecule or be trapped by $NaBH_4$ to yield a stabilised enzyme-substrate complex which is catalytically inactive (Scheme 4.2). This stabilised complex has been used to determine the nature of the active sites of both the human and bovine dehydratase enzymes (Chapter 2). Recent work by Nandi (1978) has demonstrated that the modified amino acid obtained from acid hydrolysates of this stabilised enzyme-substrate complex (^{14}C labelled) was chromatographically and electrophoretically identical to chemically synthesised N-ε-(4-(5-aminovalerate))lysine (ALA-lysine). From these data he concluded that a Schiff base was formed between ALA and an ε-amino group of a lysine residue at the substrate binding site of 5-aminolevulinic acid dehydratase isolated from <u>R.sphaeroides</u> (Scheme 4.2) during the enzymic conversion of ALA to porphobilinogen.

Based on these results a mechanism of action of human erythrocyte 5-aminolevulinic acid dehydratase can be proposed (Scheme 4.6) in which the first molecule of ALA binds to the "proprionic acid" side of the active site of the enzyme (Scheme 4.8, Mechanism (2)) and forms a Schiff base with a reactive amino group (4.14). Subsequent reaction with a second substrate molecule yields a Schiff base between the two molecules of ALA at the active site (4.14)→(4.15). Abstraction of a proton at C-3 of the second substrate molecule yields a stabilised carbanion which participates in the formation of a carbon-carbon bond (4.15)→(4.16). Abstraction of a second proton from the same carbon atom (originally from C-3 of the second substrate molecule) results in the cleavage of the initially formed bond between the enzyme and the first substrate molecule (4.16)→(4.17) and subsequent enzymic aromatization (4.17)→(4.18) yields the product, porphobilinogen.

Scheme 4.6 Mechanism of Action of 5-Aminolevulinic Acid Dehydratase Proposed by Jordan and Seehra (1980a, b).



This mechanism, first proposed by Jordan and Seehra (1980a), involves the formation of a Schiff base between the two substrate molecules at the active site (4.15), followed by carbon-carbon bond formation (4.15)→(4.16) and differs substantially from the mechanism proposed by Shemin (Scheme 4.4) in which the sequence for these steps is reversed. A common feature of the two mechanisms is the formation of a carbanion at C-3 of the ALA molecule giving rise to the "acetic acid" side of porphobilinogen, which is stabilised by mesomerism in the form of a protonated Schiff base. However, the two mechanisms involve a different Schiff base in this stabilisation in each case.

The results presented in Table 4.2 clearly demonstrate that the order of addition of the substrate molecules in the biosynthesis of porphobilinogen is the same for 5-aminolevulinic acid dehydratase isolated from different sources. Therefore, since the mechanism of action of all dehydratases may be considered to be identical, any new mechanism proposed must account for the observation that on incubation of ALA and levulinic acid with the R.sphaeroides dehydratase a mixed pyrrole was formed (Scheme 4.3), whereas incubations with 5-aminolevulinic acid dehydratase isolated from both bovine liver (Seehra, 1980) and human erythrocytes (unpublished observations) showed a complete absence of any mixed pyrroles. This apparent anomaly can be accounted for by a less specific requirement of the binding of the second substrate molecule in the R.sphaeroides enzyme and, therefore, levulinic acid can compete with the ALA for the "acetic acid" binding site of the enzyme and form an intermolecular Schiff base with the ALA already bound to the "proprionic acid" binding site. Subsequent condensation and aromatization of this Schiff base intermediate would yield the mixed pyrrole (4.8) (Scheme 4.3). Therefore, levulinic acid is able to bind to both the "acetic acid" and "proprionic acid" binding sites of 5-aminolevulinic acid dehydratase isolated from R.sphaeroides but it can only inactivate the enzyme at the P site. Consequently, levulinic acid acts as a competitive inhibitor of all 5-aminolevulinic acid dehydratases by forming a Schiff base at the P site of the enzyme (enzyme-inhibitor complex) which is unable to react further to yield any mixed pyrrole due to the requirement of an amino group in the formation of the pyrrole ring nitrogen (which must originate from a molecule of ALA bound to the "proprionic acid" side). Therefore, this

enzyme-inhibitor complex can either dissociate to regenerate the free enzyme or be trapped by $NaBH_4$ to yield a stable enzyme-inhibitor complex which is no longer catalytically active.

4.4 Summary

Single-turnover experiments were employed to elucidate the order of addition of the two identical substrate molecules (a homopolymerization or dimerization reaction) in the biosynthesis of porphobilinogen by human erythrocyte 5-aminolevulinic acid dehydratase. In these experiments, varying quantities of [5 - ^{14}C]ALA and enzyme (1.6, 8 and 16 moles ALA per mole of octameric enzyme) were mixed in a rapid mixing device in order that one of the ALA binding sites would be preferentially occupied. Subsequent "chasing" with a large excess of unlabelled ALA should carry the label through into the product, porphobilinogen. Analysis of the distribution of label between C-11 and C-2 of the resultant porphobilinogen (by degradation to DNP-glycine) unambiguously demonstrated that the radioactivity originated from C-2 of porphobilinogen. Therefore, the first molecule of ALA to bind to the enzyme gave rise to the "proprionic acid" side of porphobilinogen (atoms 1, 2, 3, 8, 9 and 10) (Scheme 4,8, Mechanism (2)).

Other studies on the human dehydratase enzyme demonstrated that it was inactivated by $NaBH_4$ when mixed in a rapid mixing device with an equimolar concentration of [4 - ^{14}C]ALA (1 mole ALA per mole of enzyme active sites) and that this inactivation was associated with an incorporation of label into the enzyme protein (0.35 - 0.40 moles ALA/ mole subunits at pH 6.8). This inactivation was prevented by the presence of porphobilinogen (10mM) and suggested that the initially bound ALA formed a Schiff base with a reactive amino group (probably lysine) at the active site of the enzyme. The covalently-bound enzyme-substrate complex can either react with substrate to produce porphobilinogen or with $NaBH_4$ to yield a stabilised enzyme-substrate complex which is no longer catalytically viable. From these results, a mechanism of action of human erythrocyte 5-aminolevulinic acid dehydratase was proposed (Scheme 4.6) which is similar to the mechanism of Jordan and Seehra (1980a). This mechanism is significantly different from earlier studies on the enzyme (Nandi and Shemin, 1968b) and emphasises the importance of using the natural substrate for the enzyme reaction in the elucidation of the mechanism.

CHAPTER 5

AN INVESTIGATION INTO THE MECHANISM OF ACTION OF β-OXOACYL-CoA THIOLASE FROM RAT LIVER CYTOSOL

CHAPTER 5

An Investigation into the Mechanism of Action of β-Oxoacyl-CoA Thiolase from Rat Liver Cytosol

5.1 Introduction

β-Oxoacyl-CoA thiolase (acyl-CoA:acetyl-CoA C-acetyl transferase) catalyses the thiolytic cleavage of β-oxoacyl-CoA esters (n carbon atoms) by CoA to produce acetyl-CoA and a saturated acyl-CoA ester (n-2 carbon atoms) (Hartmann and Lynen, 1961; Gehring and Lynen, 1972) according to the following reaction (equation (5.1)).

$$R\text{-}CH_2CO.CH_2CO.SCoA + CoASH \rightleftharpoons R\text{-}CH_2CO.SCoA + CH_3CO.SCoA \quad ....(5.1)$$

where R = 4 - 16 carbon atoms.

The reaction was first postulated in 1951 by Lynen et al., and shortly thereafter direct experimental evidence was obtained independently in three laboratories (Lynen et al., 1952; Green et al., 1953; Stern et al., 1953). The enzyme is ubiquitous in nature and has been studied in a wide variety of organisms including bacteria (Overath et al., 1967), yeast (Kornblatt and Rudney, 1970, 1971 a, b), plants (Cooper and Beevers, 1969) and most mammalian tissues, particularly in metabolically active organs such as the liver, heart and kidney (McGarry and Foster, 1969; Middleton, 1973).

β-Oxoacyl-CoA thiolases are involved in many important pathways including the breakdown of fatty acids, the biosynthesis of sterols, the formation of ketone bodies and play an important role in the regulation of lipid metabolism (Scheme 5.1). Their physiological functions are highlighted below.

5.1.1 Long Chain specific β-oxoacyl-CoA thiolases

Long chain specific β-oxoacyl-CoA thiolases (EC 2.3.1.16) have a wide substrate specificity and are active on β-oxoacyl-CoA substrates with chain lengths of 4-16 carbon atoms (Seubert et al., 1968). They

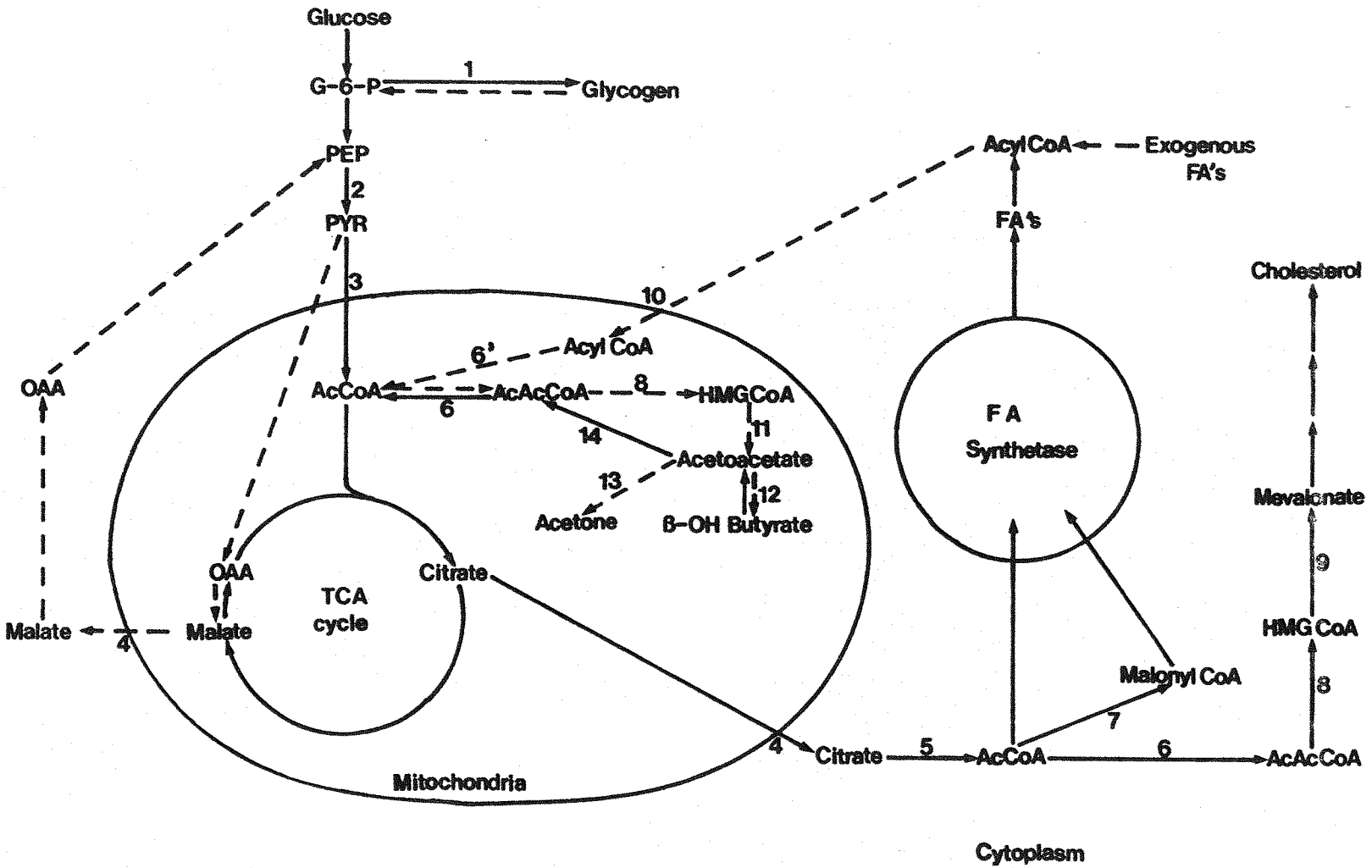
Legend to Scheme 5.1

Scheme 5.1 Pathways Involving β-Oxoacyl-CoA Thiolase

Key to Enzymes

(1) Glucogen synthetase
(2) Pyruvate kinase
(3) Pyruvate dehydrogenase
(4) Citrate-malate antiport
(5) ATP citrate lyase
(6) β-Oxoacyl-CoA thiolase (short chain specific, EC.2.3.1.9)
(6') β-Oxoacyl-CoA thiolase (long chain specific, EC.2.3.1.16).
(7) Acetyl-CoA carboxylase
(8) HMG-CoA synthase
(9) HMG-CoA reductase
(10) Carnitine acyl transferase
(11) HMG-CoA lyase
(12) β-Hydroxybutyrate dehydrogenase
(13) Acetoacetate decarboxylase
(14) Succinyl-CoA:β-oxoacyl-CoA transferase

Scheme 5.1 Pathways Involving β-Oxoacyl-CoA Thiolase



——→ Glycogenic, Glycolytic, Lipogenic, Cholesterogenic Liver

– – → Glycogenolytic, Gluconeogenic, Ketogenic Liver

are located in the mitochondrion (constituting 40% of the total thiolase activity in ox liver; Middleton, 1972) and catalyse the general reaction shown in equation (5.1) above.

Role in β-oxidation

In the β-oxidation cycle, saturated acyl-CoA esters are broken down by a series of four reactions to yield acetyl-CoA and a saturated acyl-CoA moeity, shorter by two carbon atoms. The first three reactions involve the oxidation of a methylene group (placed β to the ester group) by the enzymes, acyl-CoA dehydrogenase, enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase, to yield a β-oxoacyl-CoA ester. This ester is subsequently cleaved by long chain specific β-oxoacyl-CoA thiolases (EC 2.3.1.16) to yield acetyl-CoA and a saturated acyl-CoA ester (n-2 carbon atoms). This shortened acyl-CoA can undergo another cycle of oxidation starting with the reaction catalysed by acyl-CoA dehydrogenase.

For the complete oxidation of fatty acids to CO_2 and H_2O, the TCA cycle and the electron transport chain are also required (the former being essential for the regeneration of CoA, by the citrate synthase reaction, to allow β-oxidation to continue) with each molecule of acetyl-CoA giving rise to 12 molecules of ATP.

Work by Overath *et al.* (1969) with *Escherichia coli* showed that the enzymes of the β-oxidation cycle were co-induced when the cells were grown on media containing oleate as the sole carbon source. Genetic and biochemical analysis of mutants which were unable to grow on oleate revealed that the structural genes for the enzymes, β-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and β-oxoacyl-CoA thiolase were closely linked and indicated that these genes formed an operon.

Seubert *et al.* (1968) have also demonstrated that this mitochondrial enzyme plays an important part in the malonyl-CoA independent elongation of fatty acids.

5.1.2 Short chain specific β-oxoacyl-CoA thiolases

Short chain specific β-oxoacyl-CoA thiolases (EC 2.3.1.9) are highly specific for the four carbon (C_4) substrate, acetoacetyl-CoA, and catalyse the following reaction (equation (5.2)).

$$CH_3CO.SCoA + CH_3CO.SCoA \rightleftharpoons CH_3CO.CH_2CO.SCoA + CoASH \quad \ldots \quad (5.2)$$

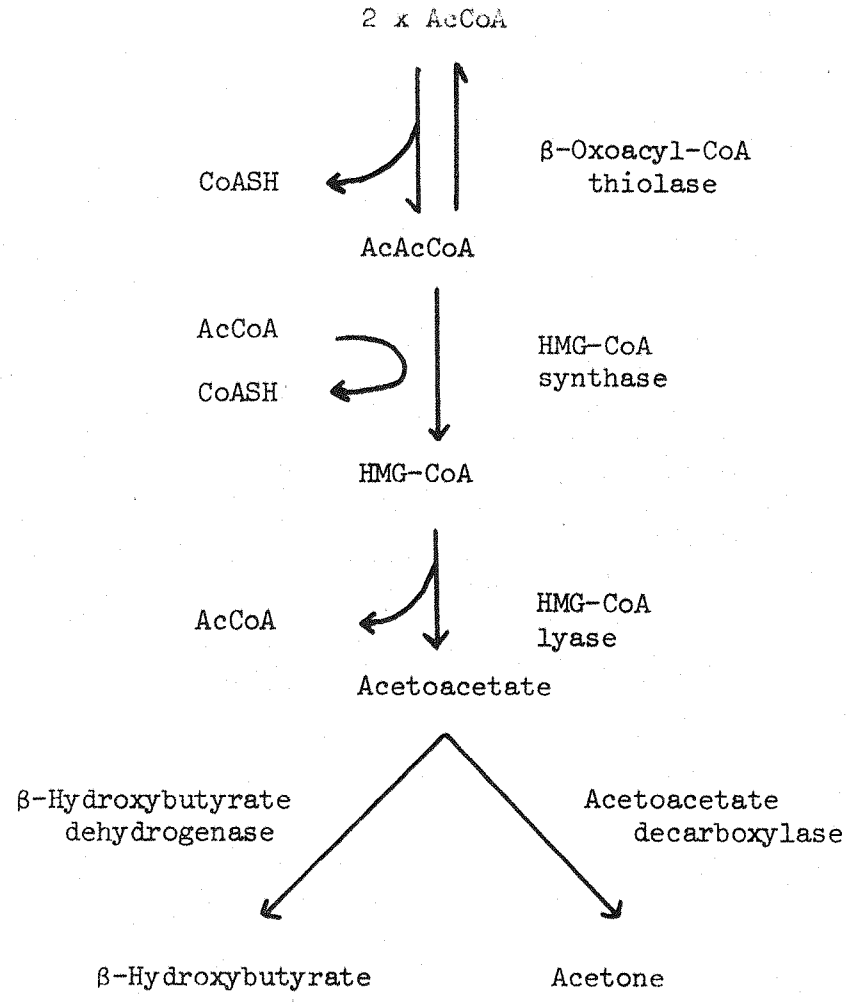
Two different forms of this short chain specific β-oxoacyl-CoA thiolase have been characterised by Middleton (1971, 1973; see Section 5.1.3) and he has further demonstrated that one form was located in the mitochondrion, whilst the other form was located predominantly in the cytoplasm. Their physiological significance is discussed below.

(a) Role in ketogenesis

During periods of excess formation of acetyl-CoA, the liver has the enzymic capacity to divert some of the acetyl-CoA derived from fatty acid or pyruvate oxidation into free acetoacetate, β-hydroxybutyrate and acetone, collectively known as the "ketone bodies" (Huth et al., 1975). The acetyl-CoA is initially converted to acetoacetyl-CoA by the mitochondrial form of β-oxoacyl-CoA thiolase (EC 2.3.1.9), which constitutes 46% of the total thiolase activity in ox liver (Middleton, 1972). The product, acetoacetyl-CoA, is converted to β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase and subsequently converted to acetoacetate by HMG-CoA lyase, an enzyme which is located solely in the matrix and inner mitochondrial membrane (Clinkenbeard et al., 1975). This free acetoacetate can be enzymically decarboxylated to yield acetone or, more importantly, can be reduced by β-hydroxybutyrate dehydrogenase to produce β-hydroxybutyrate (Scheme 5.2).

The ketone bodies, acetoacetate and β-hydroxybutyrate, can diffuse out of the liver cells into the bloodstream to the peripheral tissues, such as the heart myocardium, where the β-hydroxybutyrate is reconverted to acetoacetate and subsequently activated to its CoA ester by succinyl-CoA:β-oxoacyl-CoA transferase. The resultant acetoacetyl-CoA can be thiolytically cleaved by β-oxoacyl-CoA thiolase to yield acetyl-CoA which can then enter the TCA cycle and be utilised as an energy source. Normally the levels of ketone bodies in the blood are low, but in fasting or disease (e.g. diabetes mellitus) the levels can be extremely high. This is due to the fact that under these conditions oxaloacetate (OAA) is utilised for the maintenance of blood glucose through gluconeogenesis (see Scheme 5.1) and therefore acetyl-CoA cannot be converted into citric acid for further oxidation by the TCA cycle. The acetyl-CoA, in consequence, is diverted towards ketone body formation. Huth et al.

Scheme 5.2 Formation of ketone bodies in liver mitochondria



(1975) postulated that mitochondrial β-oxoacyl-CoA thiolase may in fact be the control point for ketogenesis.

(b) Role in cholesterogenesis

Cytoplasmic β-oxoacyl-CoA thiolase (EC 2.3.1.9) constitutes 14% of the total thiolase activity of ox liver (Middleton, 1972) and catalyses the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA (Middleton, 1971, 1973, 1974) as shown in equation (5.2) above. The reaction is the first committed stage in the biosynthesis of mevalonic acid, the direct precursor of cholesterol and other sterols. Although the reaction equilibrium strongly favours acetoacetyl-CoA cleavage (K_{eq} = 2 x 10^{-5} at pH 8.2; Stern, 1956), the net flux of acetyl-CoA to acetoacetyl-CoA and thence to mevalonic acid is made possible by the efficient conversion of acetoacetyl-CoA into HMG-CoA by the enzyme HMG-CoA synthase. This was clearly demonstrated by Clinkenbeard et al.

(1973) who used the enzyme from avian liver to show that, at equilibrium, 68% of the acetyl-CoA was converted to HMG-CoA. Further conversion of the HMG-CoA to mevalonic acid is catalysed by the microsomal enzyme, HMG-CoA reductase (Siperstein, 1970) and the resultant mevalonic acid is subsequently transformed into cholesterol and other sterols (Kornblatt and Rudney, 1971b; Clinkenbeard et al., 1975) (Scheme 5.1). Further evidence for the role of cytoplasmic β-oxoacyl-CoA thiolase in cholesterogenesis was furnished by McGarry and Foster (1969) who studied a rat liver hepatoma (3924A) and demonstrated that its inability to synthesise cholesterol was consistent with a lack of enzymes, β-oxoacyl-CoA thiolase and HMG-CoA synthase.

Interestingly, the first two stages of both the ketogenic and cholesterogenic pathways are chemically identical and involve the enzymes, β-oxoacyl-CoA thiolase and HMG-CoA synthase, which convert acetyl-CoA to HMG-CoA. The subsequent conversion of HMG-CoA to acetoacetate in the mitochondrion is achieved by HMG-CoA lyase (Bucher et al., 1960) which is localised solely in the matrix and inner mitochondrial membrane (Clinkenbeard et al., 1975). Conversely, in the cytoplasm the microsomal enzyme, HMG-CoA reductase (Bucher et al., 1960; Siperstein, 1970) converts the HMG-CoA to mevalonic acid and thence to cholesterol and other sterols. The distinction of the cytoplasmic and mitochondrial HMG-CoA pools is guaranteed by the impermeability of the inner mitochondrial membrane to CoA derivatives (McGarry and Foster, 1969).

5.1.3 Evidence for various isoenzymes of β-oxoacyl-CoA thiolase and their physiological significance

Middleton (1971, 1973) demonstrated the presence of at least three different forms of β-oxoacyl-CoA thiolase in mammalian liver which differed significantly in their catalytic and kinetic properties. Subcellular fractionation studies showed that one form of β-oxoacyl-CoA thiolase originated from the cytosol (Clinkenbeard et al., 1975) and at least two other forms of the enzyme originated from mitochondria. The cytosolic isoenzyme, isolated from ox liver, had an absolute specificity for the four carbon substrate, acetoacetyl-CoA, and gave a single band on isoelectric focussing (pI = 5.2). This form of the enzyme is believed to be involved predominantly in the biosynthesis of cholesterol and other sterols (Overath et al., 1969; Kornblatt and Rudney, 1971 a, b; Clinkenbeard, et al., 1973).

Mammalian mitochondria also contain an acetoacetyl-CoA-specific thiolase (pI = 7.8) but it differs substantially from its cytosolic counterpart. For example, the mitochondrial form of the enzyme has a seven-fold lower K_m for acetoacetyl-CoA (7μM as compared with 50μM for the cytosolic form; rat liver) and, unlike the cytosolic form, exhibits substrate inhibition when assayed in the presence of high concentrations of substrate (Middleton, 1973). The mitochondrial acetoacetyl-CoA-specific thiolase also differs from its cytosolic counterpart by being greatly stimulated by potassium ions and this form of the enzyme is involved predominantly in ketone body formation (Huth et al., 1975). The other mitochondrial isoenzyme has a much broader substrate specificity ranging from C_4 to C_{16} units (Seubert et al., 1968) and has a K_m of 10μM for acetoacetyl-CoA (rat liver). The wide specificity of this general β-oxoacyl-CoA thiolase and its intracellular localisation (matrix and inner mitochondrial membrane; Clinkenbeard et al., 1975) suggest that this form of the enzyme is involved in β-oxidation (Overath et al., 1969; Middleton, 1973; Huth et al., 1975). Middleton (1971, 1973) further demonstrated that this general β-oxoacyl-CoA thiolase gave two major peaks on isoelectric focussing (pI's of 5.7 and 6.7; ox liver) and suggested that there may be a family of closely related general β-oxoacyl-CoA thiolase isoenzymes.

The relative proportions of the different forms of β-oxoacyl-CoA thiolase vary from tissue to tissue and are discussed in greater detail by Middleton (1973) and Clinkenbeard et al. (1975).

5.1.4 Molecular and catalytic properties

Native β-oxoacyl-CoA thiolase isolated from pig heart has a molecular weight of 170,000 which, under denaturing conditions (5M guanidine-Hcl), dissociates to give single subunits of molecular weight 39,000-44,000 (Gehring and Riepertinger, 1968; Gehring and Harris, 1970a). Similar values were found for the cytoplasmic enzyme from rat liver (Middleton, 1974), whereas yeast (Kornblatt and Rudney, 1971a) and E.coli β-oxoacyl-CoA thiolases (Mazzei et al., 1970) only have a molecular weight of 140,000 (tetramer). Quantitative analysis of the N-terminal amino acid residues by reaction with dinitrofluorobenzene or phenylisothiocyanate (PITC) yielded 4 moles of valine per mole of the native enzyme (Gehring and Riepertinger, 1968) and, in conjunction with peptide

mapping and gel electrophoresis, it was concluded that β-oxoacyl-CoA thiolase consisted of four very similar and probably identical peptide chains (Gehring and Harris, 1970a).

The enzyme is inactivated by thiol modifying reagents such as iodoacetate (Lynen, 1953), N-ethylmaleimide (Gehring et al., 1968), p-chloromercuribenzoate (Mazzei et al., 1970) and various affinity labels (Holland et al., 1973) which suggested that β-oxoacyl-CoA thiolase contained an essential thiol group at or near the active site. Gehring et al. (1968) demonstrated that the enzyme was protected against inactivation by preincubation with substrate and went on to show that the incorporation of ^{14}C -labelled iodoacetamide directly paralleled the loss of enzymic activity. From the acid hydrolysate of this enzyme-inhibitor complex ^{14}C-carboxymethylcysteine was isolated thus proving that the inhibitor had reacted with a functionally important sulphydryl residue. Gehring and Harris (1968, 1970b) demonstrated that incubation of β-oxoacyl-CoA thiolase with [1 - ^{14}C] acetyl-CoA resulted in the incorporation of label into the protein (3.1 moles/mole tetramer) which suggested that the enzymic reaction proceeded via a covalent enzyme-substrate complex. They went on to isolate and sequence tryptic peptides derived from both the enzyme-substrate and enzyme-inhibitor complexes and demonstrated a common heptapeptide which had the following structure:

Val-Cys*-Ala-Ser-Gly-Met-Lys

(The asterisk (*) corresponds to the ^{14}C-labelled cysteine residue).

These data conclusively proved that both the substrate and inhibitor react with the same sulphydryl residue at the active site of β-oxoacyl-CoA thiolase.

5.1.5 Mechanism of action of β-oxoacyl-CoA thiolase

The observation that β-oxoacyl-CoA thiolase is inactivated by various sulphydryl blocking reagents (see Section 5.1.4) led Lynen (1953) to propose a mechanism based on the assumption that an active site sulphydryl residue participated directly in the catalytic reaction and involved the formation of an acyl-enzyme complex. A similar mechanism was proposed by Goldman (1954) based on purely kinetic data (equations

(5.3) and (5.4).

$$R\text{-}CH_2CO.CH_2CO.SCoA + {}^{-}S\text{-}Enz \rightleftharpoons CH_3CO.SCoA + R\text{-}CH_2CO.S\text{-}Enz \quad \ldots(5.3)$$

$$R\text{-}CH_2CO.S\text{-}Enz + CoAS^{-} \rightleftharpoons R\text{-}CH_2CO.SCoA + {}^{-}S\text{-}Enz \quad \ldots(5.4)$$

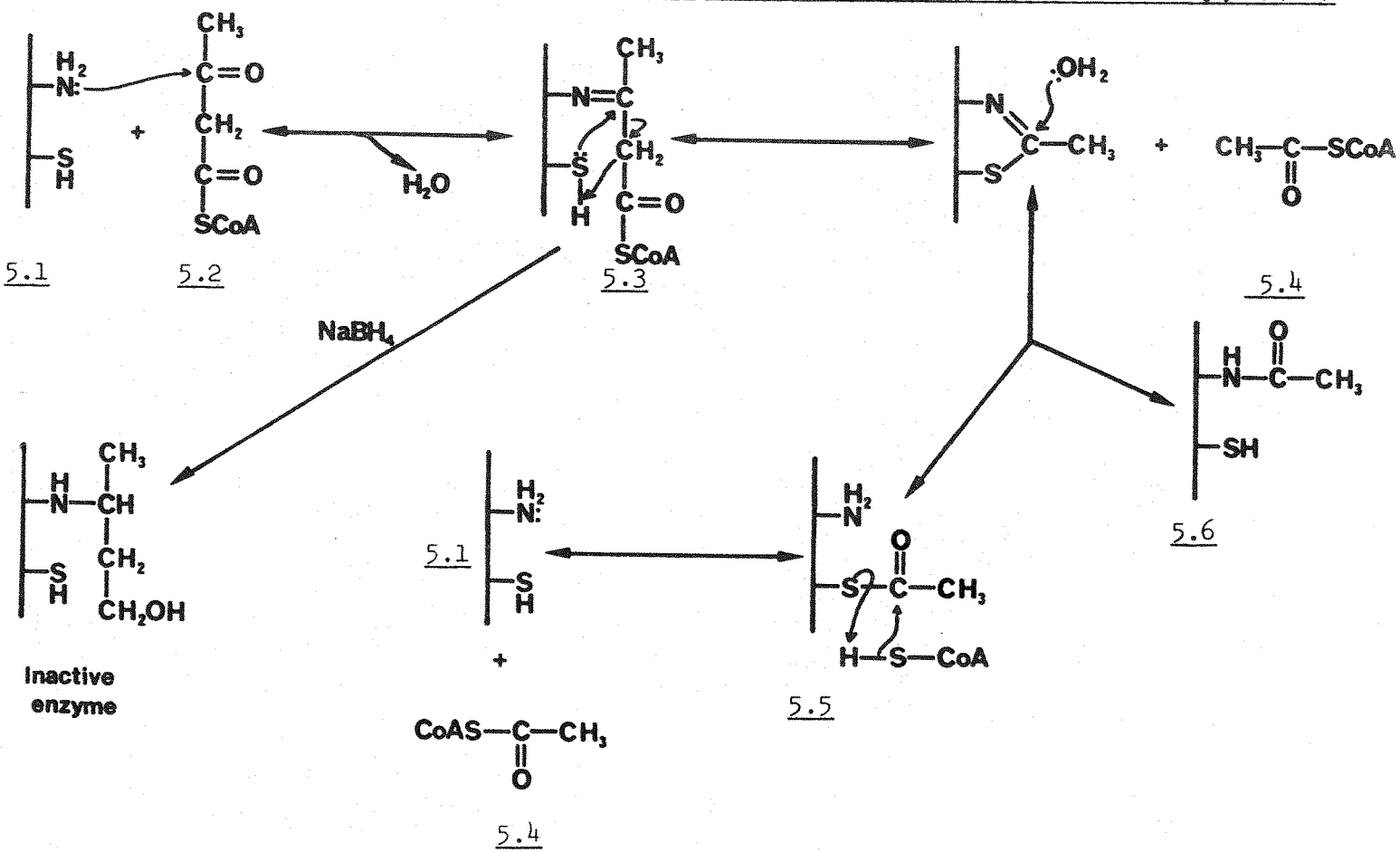
An acetyl-enzyme complex was subsequently shown to be formed between acetyl-CoA and a reactive sulphydryl residue at the active site of the enzyme (Gehring and Harris, 1968, 1970b; Section 5.1.4).

Extensive kinetic studies with the cytoplasmic (Middleton, 1974) and mitochondrial (Huth et al., 1975) forms of the enzyme have provided data consistent with a ping-pong type reaction mechanism (Cleland, 1967) involving the participation of an acetyl-enzyme intermediate (equation (5.5)).

$$\underset{}{E} \xrightarrow{AcAcCoA} E.AcAcCoA \xrightarrow{AcCoA} E\text{-}Ac \xrightarrow{CoA} E.AcCoA \xrightarrow{AcCoA} E \quad \ldots(5.5)$$

β-Oxoacyl-CoA thiolase (5.1) was shown to be inactivated by $NaBH_4$ in the presence of either acetoacetyl-CoA (5.2) or acetyl-CoA (5.4) (Kornblatt and Rudney, 1971a; Holland et al., 1973). This observation was consistent with the formation of a Schiff base intermediate (a ketimine) (5.3) during the enzymic reaction and led to the postulation that there was a catalytically important amino group at the active site of the enzyme. Salam (1982) went on to demonstrate that β-oxoacyl-CoA thiolase, which had been inactivated by the sulphydryl modifying reagent methylmethanethiosulphonate (MMTS), could be further modified by the amino group reagent carbonic anhydride. After removal of the thiomethyl blocking group from the active site sulphydryl group the enzyme was still completely inactive. From these data a role for an amino group in the catalytic mechanism of β-oxoacyl-CoA thiolase was postulated involving the formation of a ketimine intermediate (5.3) (Kornblatt and Rudney, 1971a) (Scheme 5.3).

Scheme 5.3 Postulated Mechanism of Action of β-Oxoacyl-CoA Thiolase Involving an Amino Group at the Active Site (Kornblatt and Rudney, 1971a)



However, the direct involvement of an amino group in the enzymic reaction has not been conclusively proven and it has been suggested that the amino group is involved in the formation of an abortive enzyme-N-CO.CH_3 linkage (5.6) which results from the rearrangement of the acetyl-enzyme (enzyme-S-CO.CH_3) (5.5) moiety (Jordan and Gibbs, 1983) and is discussed in greater detail in this chapter (see Results and Discussion section).

5.1.6 Stereochemistry of the β-oxoacyl-CoA thiolase reaction

Willadsen and Eggerer (1975b) studied the stereochemistry of the β-oxoacyl-CoA thiolase reaction using enzymically synthesised isomers of 3-hydroxybutyryl-CoA. In one experiment, they oxidised symmetrically tritiated (3S)-[2-$^3H_2$,3-^{14}C]-3-hydroxybutyryl-CoA (5.7) with NAD^+ in the presence of 3S-specific-3-hydroxybutyryl-CoA dehydrogenase. The acetoacetyl-CoA (5.8) produced was thiolytically cleaved by β-oxoacyl-CoA thiolase (see equation (5.2)) and subsequently hydrolysed by phosphotransacetylase to yield acetate (5.9). After purification, the acetate was found to contain 93% of the original tritium, demonstrating that both hydrogen atoms at C-2 of acetoacetyl-CoA were retained during the thiolase reaction (Scheme 5.4).

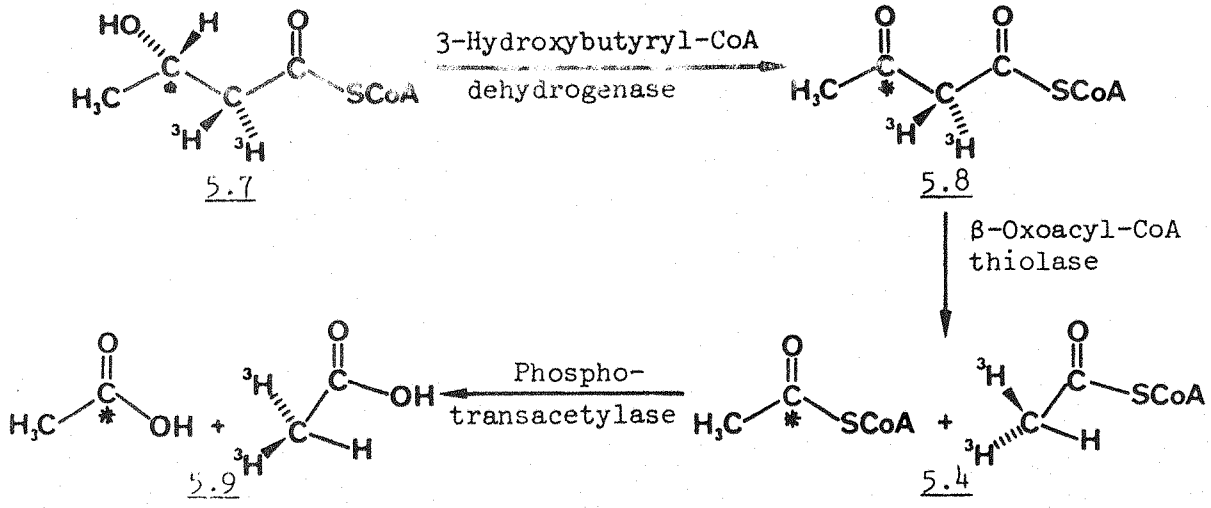
In a second experiment, stereospecifically labelled (2R,3S)-[2-^3H, 2-^2H]-3-hydroxybutyryl-CoA, generated by an enoyl-CoA hydratase exchange reaction (Willadsen and Eggerer, 1975a), was converted to chiral acetate by the same series of reactions as shown in Scheme 5.4. Subsequent conversion of the chiral acetate to malate using the enzyme malate synthase and equilibration of the malate with fumarase enabled them to determine the stereochemistry of the acetate since the pro-R hydrogen of the methylene group of malate is always removed by fumarase (Cornforth et al., 1970). The results clearly demonstrated that, in the β-oxoacyl-CoA thiolase reaction, the formation of the methyl group of acetyl-CoA derived from the C-2 chiral methylene group of acetoacetyl-CoA occurred with inversion of configuration (Scheme 5.5).

5.1.7 The effect of pH and magnesium on the equilibrium of the β-oxoacyl-CoA thiolase reaction

The equilibrium constant for the β-oxoacyl-CoA thiolase reaction in the direction of acetoacetyl-CoA synthesis was determined by Stern et

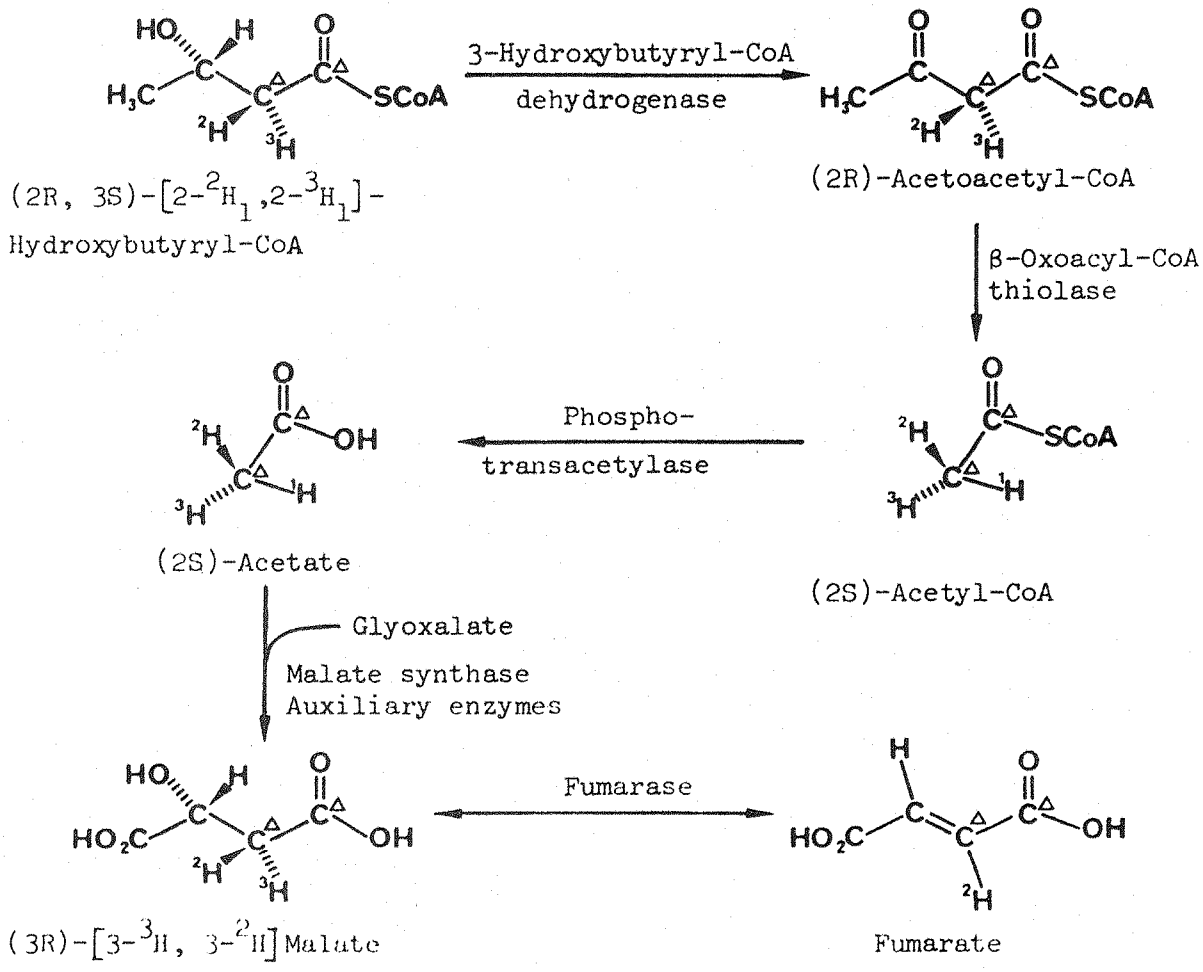
Scheme 5.4 Retention of Tritium Label in Acetate Derived from (3S)-[2-$^{3}H_2$, 3-^{14}C]-3-Hydroxybutyryl-CoA.

The asterisk (*) indicates the position of the ^{14}C label.



Scheme 5.5 The Conversion of (2R,3S)-[2-$^{2}H_1$,2-$^{3}H_1$]-3-Hydroxybutyryl-CoA to Fumarate with Inversion of Configuration at C-2 of Acetoacetyl-CoA by β-Oxoacyl-CoA Thiolase.

The triangles (△) indicate the carbon atoms in (2S)-acetate.



al., (1953) and Goldman (1954) based on the following reaction:

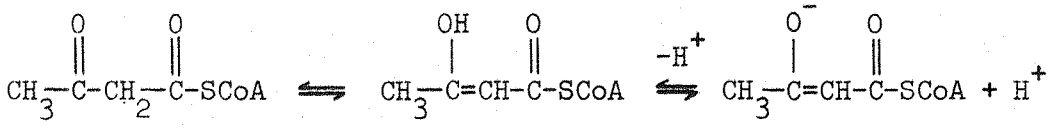
$$2AcCoA \rightleftharpoons AcAcCoA + CoA$$

The equilibrium constant (K_{eq}) is therefore:

$$K_{eq} = \frac{(AcAcCoA)(CoA)}{(AcCoA)^2}$$

The Gibbs free energy change (ΔG^{o}) associated with this reaction was found to be +22,572 kJ/mole at 16°C (pH 8.8; Goldman, 1954) and demonstrates that the equilibrium strongly favours the thiolytic cleavage of acetoacetyl-CoA by CoA.

The K_{eq} for the enzymic synthesis of acetoacetyl-CoA was shown to be pH-dependent and had a value of 2×10^{-5} at pH 8.1 (Stern et al., 1953) increasing to 6×10^{-5} at pH 8.5 and 8.7×10^{-5} at pH 8.8 (Goldman, 1954). These data demonstrate that the synthesis of acetoacetyl-CoA becomes more favourable (or less unfavourable) with increasing pH. This effect is due to the fact that acetoacetyl-CoA tautomerises between its keto (5.10) and enol (5.11) forms and this equilibrium is dependent on the concentration of H^{+} ions.



5.10 5.11 5.12

Magnesium also affects the equilibrium constant by stabilising the enolate ion (5.12) of acetoacetyl-CoA as its magnesium chelate (1 magnesium ion per acetoacetyl-CoA ion; Stern, 1956) and this effectively reduces the concentration of free acetoacetyl-CoA. Middleton (1974) further demonstrated that the magnesium had no effect on the rate of synthesis of acetoacetyl-CoA, but profoundly inhibited its rate of thiolysis. This effect is most simply explained if the keto form of acetoacetyl-CoA is considered to be the true substrate for the β-oxoacyl-CoA thiolase reaction.

In summary, the mechanism of action of β-oxoacyl-CoA thiolase generally considered to be operative involves the initial binding of acetyl-CoA to the enzyme followed by release of CoA and the establishment of an acyl-enzyme complex. Reaction of the second molecule of acetyl-CoA follows, with the concomitant breakage of the acetyl-enzyme thioester bond and formation of acetoacetyl-CoA.

The reaction of the short chain specific β-oxoacyl-CoA thiolase (EC 2.3.1.9) is unique amongst thiolases in that two molecules of the same substrate, namely acetyl-CoA, are involved. Investigation of the order of substrate binding during the synthesis of acetoacetyl-CoA, and release of acetyl-CoA during thiolysis using steady-state methods is not therefore possible. This Chapter describes an alternative approach to the study of the mechanism of action of cytoplasmic β-oxoacyl-CoA thiolase from rat liver using a single-turnover enzyme reaction procedure (see Chapter 4). This technique enables the direct determination of the order of addition of the two molecules of acetyl-CoA during the biosynthesis of acetoacetyl-CoA.

5.2 Experimental

5.2.1 Materials

[1 - ^{14}C] Acetyl-CoA and NaH$^{14}CO_3$ were purchased from the Radiochemical Centre, Amersham, Bucks. CoA and lithium acetoacetate were purchased from Sigma Chemical Co., Ltd., U.K. Cellulose phosphate and DEAE-cellulose were obtained from Whatman Ltd., Maidstone, Kent. Acetic anhydride, diketene (vinyl aceto-β-lactone), semicarbazide HCl and all other chemicals and reagents were purchased from British Drug Houses, Poole, Dorset, U.K.

5.2.2 Purification of β-oxoacyl-CoA thiolase from rat liver cytosol

Cytoplasmic β-oxoacyl-CoA thiolase (EC 2.3.1.9) was prepared from rat liver according to the method of Middleton (1974). All purification procedures were carried out at 4°C and all buffers contained 1mM 2-mercaptoethanol unless otherwise stated.

Preparation of crude homogenate

Freshly excised livers (223g) were obtained from 15 male Sprague-Dawley rats (250-300g) and were washed in 500 ml of ice cold sucrose (0.25M). The livers were homogenised in 1.5 litres of 0.25M sucrose (3 x 500 ml batches) in an MSE Atomiser for 3 minutes at low speed, followed by 2 minutes at high speed. The homogenate was then centrifuged at 8,200 rpm (12,000xg) for 10 minutes in an MSE 21 centrifuge (6 x 500ml rotor) in order to remove the membrane fractions and other cell debris.

Acid Treatment

The murky supernatant from the previous stage (1,580 ml) was stirred at about 40 rpm using a Gallenkamp overhead stirrer with a Z-shaped glass rod and the pH of the supernatant was adjusted to 5.5 by the dropwise addition of 1M acetic acid. Stirring was continued for 5 minutes at the new pH and the precipitated proteins were subsequently removed by centrifugation at 3,500 rpm (2,000xg) for 10 minutes in an MSE 21 centrifuge (6 x 500 ml rotor). The clear supernatant was carefully decanted and the pH was rapidly adjusted to 8.2 by the addition of 70ml of 1M Tris Hcl buffer (pH 8.2). 2-Mercaptoethanol was also added to the supernatant to give a final concentration of 1mM.

DEAE-cellulose chromatography

The clear supernatant (1,425ml) was applied to a DEAE-cellulose column (6.5 x 11.5cm) which had been equilibrated previously with 50 mM Tris Hcl buffer (pH 8.2). The column was washed at a flow rate of about 5ml/minute with 750ml of the same buffer to remove all unbound protein. The weakly bound protein was subsequently eluted from the column by washing the column with 1 litre of 100mM Tris Hcl buffer (pH 8.2). Cytoplasmic β-oxoacyl-CoA thiolase was then eluted with 200mM Tris Hcl buffer (1 litre) and the fractions containing enzyme activity (tubes 45-65) were pooled. This procedure gives complete separation of the cytoplasmic enzyme from any contaminating mitochondial β-oxoacyl-CoA thiolase activities (Middleton, 1973).

Chromatography on calcium phosphate

Calcium phosphate was prepared by mixing equal volumes (200ml) of 0.5M calcium chloride and 0.5M di-sodium hydrogen phosphate in a dropwise manner (over 2 hours) whilst slowly stirring the mixture at 20 rpm using a Gallenkamp overhead stirrer. The calcium phosphate slurry was carefully washed with distilled water (5 x 3 litre), poured into a column (4.5 x 6cm high) and equilibrated overnight with 200mM Tris Hcl buffer (pH 8.2).

The eluate from the DEAE-cellulose column (208ml) was applied to the column of calcium phosphate at a flow rate of 3ml/minute and washed with 0.5mM dithiothreitol in distilled water until no more protein was eluted (≈160ml). The column was subsequently washed with 10mM sodium phosphate buffer (pH 6.6) and 10ml fractions were collected. The fractions (10-33) containing enzymic activity were pooled.

Chromatography on cellulose phosphate

The ionic strength of the eluate from the previous column (241ml) was adjusted to 50mM by the addition of 10ml of 1M sodium phosphate buffer (pH 6.6). This enzyme solution was applied to a column of cellulose phosphate (4.5 x 7cm high) which had been previously equilibrated with 2 litres of 50mM sodium phosphate buffer (pH 6.6). Unbound protein was removed by washing the column with 600 ml of the same buffer. The enzyme was subsequently eluted by applying a linear gradient of sodium phosphate buffer (50-250mM; 450ml total volume; pH 6.6) to the column. The fractions containing enzymic activity

(tubes 33-39) were pooled, precipitated with $(NH_4)_2 SO_4$ (85% saturation) and the precipitate was collected by centrifugation at 20,000 rpm (50,000xg) for 10 minutes in an MSE 21 centrifuge (8 x 50 ml rotor). The pellet was resuspended in 10ml of 30mM Tris Hcl buffer (pH 8.2) containing 10% v/v glycerol and dialysed against 5 litres of the same buffer overnight. The dialysed enzyme was concentrated by ultrafiltration using an Amicon ultrafiltration cell fitted with a PM30 membrane. The concentrated enzyme (2.1ml) was made up to 50% v/v with glycerol and stored in the presence of 0.5mM dithiothreitol at -25°C. Under these conditions the enzyme maintained about 80% of its activity over a period of 2 weeks. A typical purification is shown in Table 5.1.

5.2.3 Assay of β-oxoacyl-CoA thiolase

The enzyme was assayed according to the method of Middleton (1973) as follows. The enzyme solution (5-20µl; 0.05 units max) was mixed (in a silica cuvette at 30°C) with 1ml of 0.1M Tris Hcl buffer (pH 8.2) containing 25mM magnesium chloride, 50mM potassium chloride and 28µM acetoacetyl-CoA (10µl of the solution prepared as described in Section 5.2.4). The reaction was initiated by the addition of 25µl of 5mM CoA and the change in absorption due to the breakdown of acetoacetyl-CoA (Stern, 1956) was followed using a SP8400 spectrophotometer (λmax = 303nm; εmax = 16,900 $M^{-1}cm^{-1}$). 1 unit of enzyme is defined as that required to consume 1 µmol of acetoacetyl-CoA per minute at 30°C.

5.2.4 Preparation of acyl-CoA derivatives

Acetyl-CoA was prepared as follows. CoA (30mg) was dissolved in 6ml of distilled water at 0°C in a 50ml round bottomed flask fitted with a pH electrode and stirrer. The pH was adjusted to 8.5 with aq. 0.1M NH_4OH and the thiol was determined (10 µl portions) by the method of Ellman (1959). Acetic anhydride (4.5µl) was added with stirring and the pH was maintained at 8.5 by the dropwise addition of aq. 0.1M NH_4OH. If any free thiol remained, a further amount of acetic anhydride (1.0µl) was added. After stirring for 2 minutes at pH 8.5, the pH was adjusted to 5 with 1M acetic acid and the solution was freeze-dried. The acetyl-CoA was stored at -20°C in a desicator. The purity was greater than 90% as judged by the thiol ester determination of Lipmann and Tuttle (1945).

Acetoacetyl-CoA (5.2) was prepared according to the method of Weiland and Rueff (1953) as follows. CoA (5mg) was dissolved in 1ml of 0.1M $KHCO_3$ (nitrogen saturated) and 10µl of diketene (vinyl aceto-β-lactone)(5.13) was added (see equation (5.6) for reaction).

$$\underset{\underline{5.13}}{CH_2=C(-O-)-CH_2-C=O} + CoASH \longrightarrow \underset{\underline{5.2}}{CH_3CO.CH_2CO.SCoA} \qquad ....(5.6)$$

The solution was left for 2 minutes at 0°C and the formation of the thioester was determined using a nitroprusside reagent spot test (Tonnies and Kolb, 1951; Lynen, 1951). (The reagent is prepared from 1.5g sodium nitroprusside dissolved in 5ml of 1N H_2SO_4, 90 ml of methanol and 10ml of ammonia solution (28% w/v) and filtered before use). If the reaction was incomplete a further aliquot (10µl) of diketene was added. The impure solution of acetoacetyl-CoA was adjusted to pH 1 by the addition of 1N Hcl (150µl) and excess diketene was extracted with diethylether (4 x 2ml). Nitrogen was then bubbled through the solution to remove contaminating diethylether, the pH was adjusted to 5-6 with 1M $KHCO_3$ (50µl) and the solution was stored at 0°C until required. This method gave approximately 2.8µmoles of acetoacetyl-CoA in 60% yield.

5.2.5 Synthesis of acetoacetyl-CoA with β-oxoacyl-CoA thiolase

The incubation conditions employed for the optimal yield of acetoacetyl-CoA were as follows. A final volume of 1ml contained Tris Hcl buffer pH 8.8, (100µmol), $MgSO_4 7H_2O$ (300µmol) and β-oxoacyl-CoA thiolase (9.0 units; 4.45 nmol; 195µg). The reaction was carried out in a spectrophotometer cell at 30°C and the formation of acetoacetyl-CoA was followed at 303nm. Experiments involving radioactive synthesis followed this method with only minor modifications to enzyme and substrate concentrations. Additions are shown in Table 5.2.

5.2.6 Preparation of acetyl-enzyme complex

The method of Gehring and Harris (1970b) was modified as described below. Unlabelled acetyl-enzyme (see Expt. 2, Table 5.2) was prepared from 5.1 units (2.51 nmol) of enzyme and 0.56µmol of acetyl-CoA at pH 7. The acetyl-enzyme complex was separated from small molecules by gel

filtration with Sephadex G-50 fine at 0°C (see Chapter 3, Section 3.2.3). The disappearance of the reactive enzyme thiol (Ellman, 1959) indicated that the acetylation of the enzyme was complete.

[1-^{14}C] Acetyl-enzyme was prepared by reacting enzyme (4.45 nmol) and [1-^{14}C] acetyl-CoA (22.1 nmol). The yield of complex was 50-60% (see Expts. 3, 4 and 5, Table 5.2).

5.2.7 Single-turnover experiments

Single-turnover experiments were carried out using a rapid mixing device with three syringe inputs, two of which allow enzyme and substrate (either ^{14}C or unlabelled) to interact for approximately 50msec. The third input allows a further addition of substrate (^{14}C or unlabelled) before final quenching of the reaction ∿100 msec later. In some experiments the enzyme-substrate complex was used in the first syringe and additional substrate in the second leaving the third input for a rapid quench with 0.2M NaOH (Schemes 5.6 and 5.7). Details of quantities, ^{14}C label used and composition of experiments are given in Table 5.2.

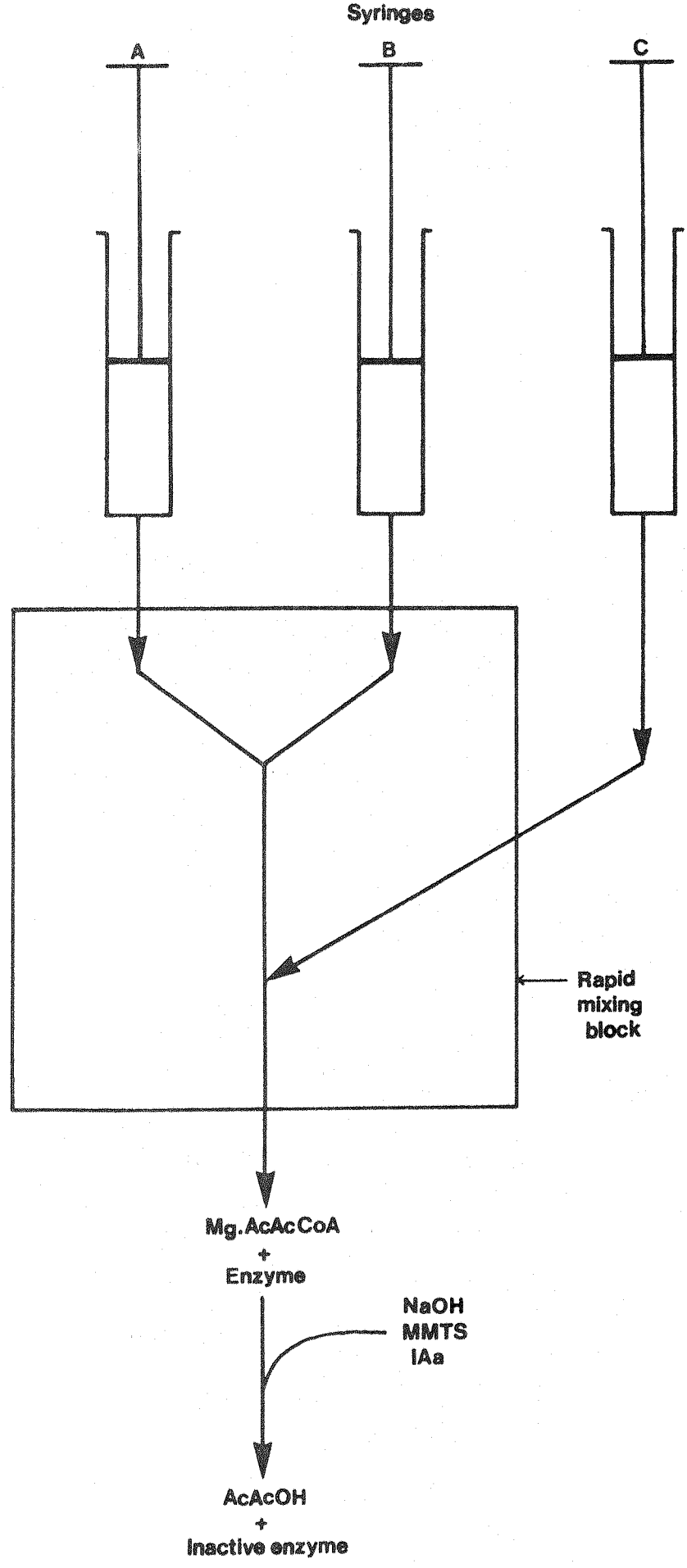
In all experiments, the formation of acetoacetyl-CoA was promoted by the inclusion of $MgSO_4$ (0.3M final concentration), which chelates the enol form of acetoacetyl-CoA (Stern, 1956; see Section 5.1.7).

The randomisation of label in acetoacetyl-CoA was minimised by utilisation of methylmethanethiosulphonate (MMTS), which reacted with the liberated CoA and prevented further reaction of the enzyme (Bloxham et al., 1978) (Schemes 5.6 and 5.7).

5.2.8 Degradation of acetoacetyl-CoA to acetone semicarbazone and barium carbonate

The radioactivity in regiospecifically labelled acetoacetyl-CoA synthesised from [1-^{14}C] acetyl-CoA was determined at the end of the enzyme incubation as follows. The pH was adjusted to 13 with 5M NaOH to terminate the enzyme reaction and to hydrolyse the thioester. After 10 minutes at 37°C, carrier lithium acetoacetate (100mg) was added and the solution was centrifuged (1000 x g) for 5 minutes to collect denatured enzyme. The supernatant was transferred into the left side of a two arm "soldier" and the pH adjusted to 4 with 5M Hcl.

Scheme 5.6 Rapid Mixing Device Used for the Single-Turnover Experiments

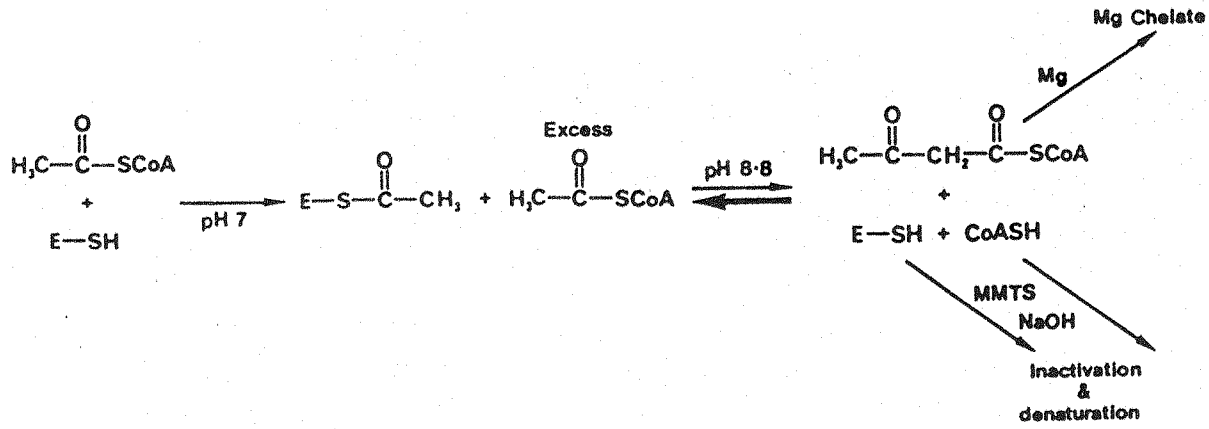


Semicarbazide (200mg) and sodium acetate (200mg) were added to the solution. Into the right arm of the "soldier" 7ml of saturated aqueous $Ba(OH)_2$ was added and the "soldier" was stoppered. Decarboxylation was allowed to proceed for 16 hours at 37°C after which time the contents of the soldier were cooled to 0°C to complete the crystallization of acetone semicarbazone. Acetone semicarbazone and barium carbonate were each isolated by filtration. Acetone semicarbazone was recrystallized from water to constant specific activity (mp. 189°C) (Scheme 5.8).

5.2.9 Counting of radioactive samples

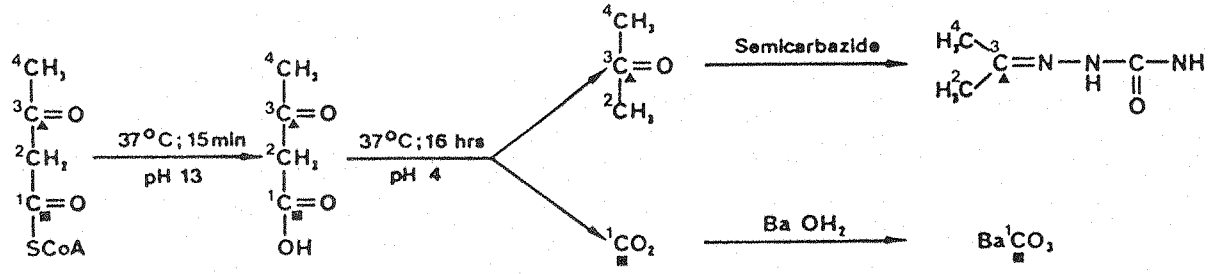
All determinations were made using a Phillips P4700 scintillation spectrometer programmed to give a quench correction to d.p.m. Samples were counted in 10ml of toluene/methanol (4:1, v/v) containing 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oza-3,4-diazole (6 g/1). Barium carbonate ($Ba^{14}CO_3$) precipitates were sonicated for 10 minutes in the scintillation vial and the suspension was allowed to settle for 1 hour before counting radioactivity. The efficiency of the counting of $Ba^{14}CO_3$ was 73.9% as determined by conversion of a known quantity of $NaH^{14}CO_3$ into $Ba^{14}CO_3$.

Scheme 5.7 Enzymic Synthesis of Regiospecifically Labelled Acetoacetyl-CoA by β-Oxoacyl-CoA Thiolase and Subsequent Degradation to Acetone Semicarbazone and Barium Carbonate.



Scheme 5.8 Degradation of Acetoacetyl-CoA to Acetone Semicarbazone and Barium Carbonate in Order to Determine the Location of the Radioactivity.

The solid triangle (▲) enables the fate of C-3 of acetoacetyl-CoA to be followed. Similarly, the solid square (■) enables the fate of C-1 of acetoacetyl-CoA to be followed.



5.3 Results and Discussion

Cytoplasmic β-oxoacyl-CoA thiolase (EC 2.3.1.9) was prepared in 10% yield from the livers of 15 Sprague-Dawley rats according to the method described in Section 5.2.2. The purified enzyme (5.0 mg) had a specific activity of 46.2-49.8 units/mg protein, although the peak fraction from the final chromatographic column had a specific activity of 81.6 units/mg, and compared very favourably with the purification of Middleton (1974), being somewhat improved with respect to the number of units obtained and the specific activity. A typical purification is shown in Table 5.1.

Polyacrylamide gel electrophoresis of the purified enzyme in the presence (Shapiro *et al.*, 1967) or absence of SDS (Weber and Osborn, 1969) yielded a single protein band which contained all the enzymic activity and demonstrated that the purified cytoplasmic β-oxoacyl-CoA thiolase was essentially homogeneous (Scheme 5.9).

The mechanism by which β-oxoacyl-CoA thiolase from mammalian liver cytoplasm catalyses the formation of acetoacetyl-CoA from two molecules of acetyl-CoA is considered to follow a ping-pong type reaction mechanism (Middleton, 1974; Huth *et al.*, 1975) in which one of the acetyl-CoA molecules initially binds to the enzyme, establishing an acyl-enzyme complex, with concomitant liberation of CoA. The acetyl-enzyme complex reacts with a second molecule of acetyl-CoA to yield the product acetoacetyl-CoA (equation (5.2)).

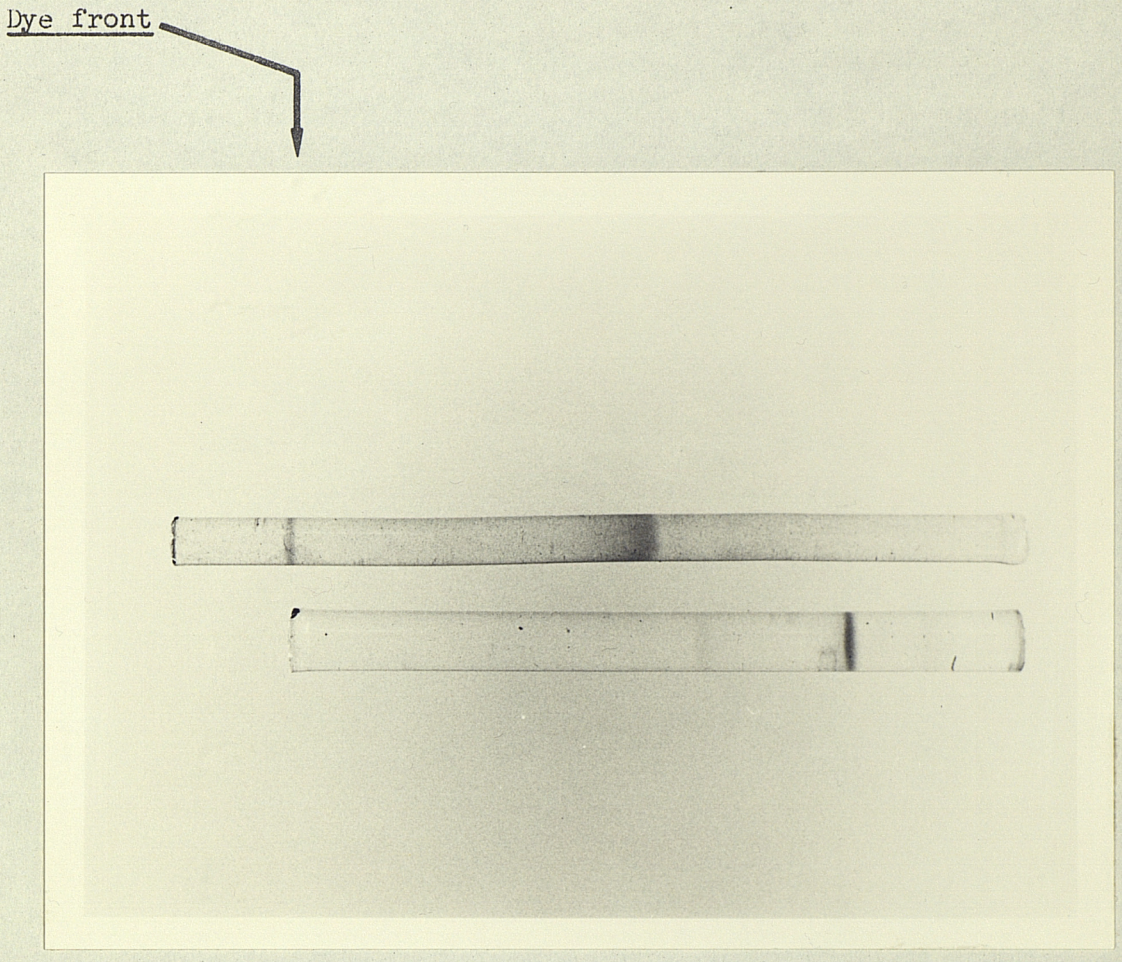
Acetyl-CoA has the unique property of acting either as a nucleophilic or as an electrophilic reagent. Two broad mechanisms are thus possible for the β-oxoacyl-CoA thiolase reaction. In mechanism (1) acetyl-CoA interacts with the enzyme with release of CoA and the establishment of an acyl-enzyme complex. Nucleophilic attack by the second acetyl-CoA molecule on the electrophilic thioester carbonyl group of the acetyl-enzyme complex liberates the product acetoacetyl-CoA. In this mechanism the initially bound acyl unit becomes incorporated into C-3 and C-4 of the product (Scheme 5.10).

Equally feasible is the alternative mechanism (2) in which the initially bound acyl unit acts as a nucleophile, reacting with the

Table 5.1 Purification of β-oxoacyl-CoA thiolase from rat liver cytosol (Method of Middleton, 1974)

	(mls) Volume	(u) Total Units	(mgs) Total Protein	(u/mg) Specific Activity	(%) Yield	Purification Factor
Homogenate	1,580	2,302	35,968	0.064	100	
pH 5.5 supernatant	1,425	1,685	8,930	0.189	73.2	3.0
DEAE cellulose	208	958	1,549	0.618	41.6	9.7
Calcium phosphate	241	743	336	2.21	32.3	34.6
Cellulose phosphate	80	333	5.25	63.4	14.5	991.1
Concentrated enzyme	2.1	249	5.00	49.8	10.8	778.1

1 unit = 1 μmol of acetoacetyl-CoA consumed per minute at 30°C.



Scheme 5.9 Polyacrylamide Gel Electrophoresis of β-Oxoacyl-CoA Thiolase.
Polyacrylamide gel electrophoresis of rat liver β-oxoacyl-CoA thiolase was performed as described in Section 2.2.5. The upper gel is a 5% non SDS polyacrylamide gel of the purified enzyme whilst the lower gel is a 15% SDS polyacrylamide gel.

carbonyl group of acetyl-CoA, the displaced CoA being used to cleave the acyl-enzyme bond to furnish acetoacetyl-CoA. In this latter mechanism, the initially bound acyl unit becomes incorporated into C-1 and C-2 of the product (Scheme 5.10). By analogy with long chain specific β-oxoacyl-CoA thiolases and from the study of exchange reactions (Gehring and Lynen, 1972) the mechanism (1) has been considered the most likely and has been favoured for several years.

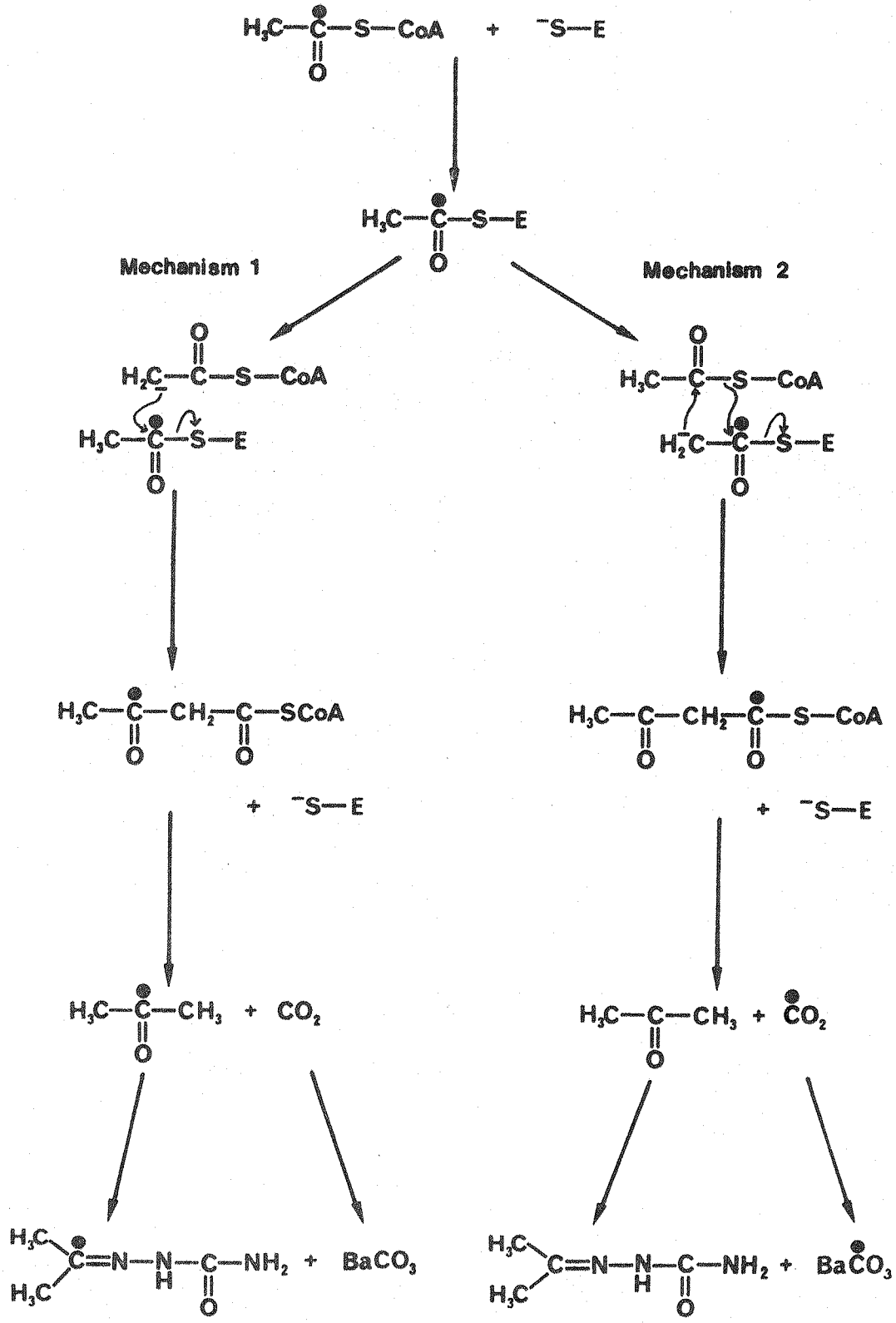
A knowledge of the order of binding of substrates to an enzyme is of paramount importance for the formulation of the reaction mechanism. In heterosubstrate reaction studies much information about the order of substrate binding can be obtained from steady-state kinetics (Cleland, 1967). However, in homopolymerisation reactions of the β-oxoacyl-CoA thiolase type, where more than one molecule of the same substrate is involved, this approach is of little use. In this case the order of binding of the two identical substrate molecules can only be delineated by using a single-enzyme-turnover reaction in which one substrate molecule is distinguished from the other by isotopic labelling (see Chapter 4).

Accordingly, β-oxoacyl-CoA thiolase is mixed with a stoichiometric equivalent of [1-^{14}C]acetyl-CoA on anticipation that one of the two substrate binding sites will be preferentially occupied by the label. Subsequent addition of a large quantity of unlabelled acetyl-CoA, to complete the turnover of the ^{14}C intermediate complex, should thus result in the formation of acetoacetyl-CoA in which the majority of the label is located regiospecifically either at C-1 or at C-3 depending on which of the two acetyl-CoA binding sites was occupied initially by the labelled substrate.

Degradation of the acetoacetyl-CoA to determine the location of the label would thus provide direct evidence for the order of binding of the substrates to the enzyme and thus allow important mechanistic conclusions. This type of single-enzyme-turnover approach has been used successfully to elucidate mechanistic features of the enzymes, 5-amino-levulinic acid dehydratase (Jordan and Seehra, 1980a,b; this thesis, see Chapter 4) and porphobilinogen deaminase (hydroxymethylbilane synthase) (Jordan and Seehra, 1979) both of which catalyse homopolymerization reactions in tetrapyrrole biosynthesis.

Scheme 5.10 Possible Mechanism for the β-Oxoacyl-CoA Thiolase Reaction.

The black dot (●) enables the fate of the carbonyl carbon atom to be followed in both mechanisms.



Several difficulties associated with the study of the β-oxoacyl-CoA thiolase reactions were overcome as follows. Firstly, the enzyme reaction normally favouring thiolysis ($K_{eq} = 6 \times 10^{-5}$ at pH 8.5; Goldman, 1954) was encouraged in the direction of acetoacetyl-CoA synthesis by carrying out incubations in the presence of magnesium ions (0.3M final concentration). This stabilises the enol form of acetoacetyl-CoA as its magnesium chelate and effectively reduces the concentration of free acetoacetyl-CoA (Stern, 1956; Middleton, 1974). Secondly, the reaction was carried out in a rapid-mixing device at 0°C such that the enzyme was in contact with the initial labelled substrate for 50 msecs, reducing the chance of both of the acetyl-CoA binding sites being sequentially occupied by label. Thirdly, after reaction with excess unlabelled acetyl-CoA, the reaction mixture was quenched into thiophilic reagents, which had the dual effect of removing CoA and of inactivating the enzyme, thus reducing the reverse reaction and any attendant exchange (see Schemes 5.6 and 5.7).

The rapid-mixing device employed was fitted with three syringe inputs. This apparatus allows the enzyme and labelled substrate (in approximately stoichiometric quantities) to interact for ∿ 50 msec before the unlabelled substrate is added through the third input. The solution was then passed into methylmethanethiosulphonate and iodoacetamide at alkaline pH to terminate the reaction (Scheme 5.6).

Thus, enzyme and [1-^{14}C]acetyl-CoA were initially mixed, excess unlabelled acetyl-CoA was added and after termination of the reaction the acetoacetyl-CoA was degraded to acetone and CO_2 (Scheme 5.8). The proportion of radioactivity in C-3, as acetone semicarbazone, and C-1, as $BaCO_3$, could then be determined accurately. The ratio of label in C-3 and C-1 in this experiment (Expt. 1 in Table 5.2) was 68:32 showing that the molecule of acetyl-CoA initially bound to the enzyme became C-3 and C-4 in acetoacetyl-CoA. The converse experiment in which unlabelled acetyl-CoA was initially added to the enzyme, followed by [1-^{14}C] acetyl-CoA gave, as expected, the converse distribution of label in acetoacetyl-CoA with a C-3:C-1 ratio of 43:57 (Expt. 2 in Table 5.2). These results favour the mechanism (1) discussed above.

In experiments, in which the ^{14}C acetyl-enzyme complex was used (Expts. 3, 4 and 5) approximately 50-60% of the radioactivity remained associated with the enzyme pellet after termination of the enzyme reaction, even when a 2,500 fold excess of acetyl-CoA with respect to ^{14}C acetyl-enzyme complex was used. This is attributable to an abortive enzyme-N-CO.CH_3 linkage formed by rearrangement of the enzyme-S-CO.CH_3 (acetyl-enzyme) moiety under the experimental conditions employed. In order to determine that the acyl moiety of the acetyl-enzyme complex (Gehring and Harris, 1970) was incorporated into positions C-3 and C-4 of acetoacetyl-CoA, [1-^{14}C]acetyl-enzyme was prepared (see Section 5.2.6), and subsequently allowed to react with excess unlabelled acetyl-CoA (Expts. 3 and 4). Degradation of the acetoacetyl-CoA showed that the C-3:C-1 ratio was 86:14 and 83:17 respectively, indicating that the reaction course using the [1-^{14}C]acetyl-enzyme complex was the same as in the single-turnover experiments starting from [1-^{14}C]acetyl-CoA. If the reaction using [1-^{14}C]acetyl-enzyme complex was repeated (Expt. 5) but termination was delayed for 2 minutes the distribution of label was less marked and showed a C-3:C-1 ratio of 57:43 presumably due to equilibration with residual CoA.

Experiment 6, in which excess [1-^{14}C]acetyl-CoA was added to β-oxoacyl-CoA thiolase, yielded, as expected, an equal (50:50) distribution of radioactivity between C-3 and C-1 in the acetoacetyl-CoA. In control experiments, negligible counts were incorporated into acetoacetyl-CoA (Expt. 7).

Legend to Table 5.2

Legend to Table 5.2

Enzyme-single-turnover experiments with β-oxoacyl-CoA thiolase

All experiments were terminated with 0.2M NaOH (300 µl). In experiments 1 and 2 the NaOH was in the termination vessel; in experiments 3, 4 and 7 the NaOH was in syringe input C. In experiments 5 and 6 2 minutes was allowed to elapse before termination. $MgSO_4.7H_2O$ (150 µmol) and methylmethanethiosulphonate (MMTS; 5 µmol) were present in all experiments. Quantities of enzyme and acetyl-CoA are as shown. All samples, after termination and the addition of carrier lithium acetoacetate (100 mg), were processed as described in the Experimental Section to give acetone semicarbazone and $BaCO_3$ (Scheme 5.8). The specific activity of the [1-^{14}C]acetyl-CoA was 56.6 Ci/mol. Enzymic conversions of acetyl-CoA into acetoacetyl-CoA were typically about 24% (Expt. 6).

Table 5.2 Enzyme-Single-Turnover Experiments with 3-Oxoacyl-CoA Thiolase.

Experiment	syringe input A	syringe input B	syringe input C	specific activity dpm/mmole		ratio of specific activities C3:C1 acetoacetic acid
				acetone semicarbazone	$BaCO_3$	
1	enzyme 4.45 nmol	^{14}C acetyl-CoA 22.1 nmol	acetyl-CoA 1.78 µmol	5,203	2,450	68 : 32
2	acetyl-enzyme 2.51 nmol	^{14}C acetyl-CoA 22.1 nmol	acetyl-CoA 1.78 µmol	1,636	2,133	43 : 57
3	^{14}C acetyl-enzyme 4.45 nmol	acetyl-CoA 1.78 µmol	-	631	104	86 : 14
4	^{14}C acetyl-enzyme 4.45 nmol	acetyl-CoA 11.8 µmol	-	1,100	229	83 : 17
5	^{14}C acetyl-enzyme 4.45 nmol	acetyl-CoA 1.78 µmol	-	1,927	1,430	57 : 43
6	enzyme 4.45 nmol	^{14}C acetyl-CoA 70 nmol	-	78,053	79,387	50 : 50
7	buffer	^{14}C acetyl-CoA 22.1 nmol	-	36	0	-

5.4 Summary

Single-turnover enzyme reactions were employed with β-oxoacyl-CoA thiolase purified from rat liver cytosol (Middleton, 1974) to determine the order of binding of the two molecules of acetyl-CoA to the enzyme during the formation of acetoacetyl-CoA. This type of single-enzyme turnover approach has been used successfully to elucidate the mechanistic features of the enzymes, 5-aminolevulinic acid dehydratase (Jordan and Seehra, 1980a,b; Chapter 4 of this thesis) and porphobilinogen deaminase (Jordan and Seehra, 1979) both of which catalyse homopolymerization reactions in the biosynthesis of tetrapyrroles.

The labelled acetoacetyl-CoA was enzymically synthesised by mixing equimolar quantities of [1-^{14}C]acetyl-CoA and enzyme in a rapid mixing device so that one of the acetyl-CoA binding sites would be preferentially occupied. Subsequent quenching of the reaction by the addition of a large excess of unlabelled acetyl-CoA would then incorporate the label into acetoacetyl-CoA. Alternatively, unlabelled acetyl-CoA was used initially followed by a chase of [1-^{14}C] acetyl-CoA. Degradation of the resulting acetoacetyl-CoA was employed for the determination of the distribution of the ^{14}C label.

A major difficulty associated with the study of the β-oxoacyl-CoA thiolase reaction for the formation of acetoacetyl-CoA is that the enzyme reaction favours the thiolytic cleavage of this dimeric molecule to yield two molecules of acetyl-CoA (K_{eq} = 2 x 10^{-5} at it's pH optimum of 8.1; Stern et al., 1953). Consequently, in order to study the order of addition of the two acetyl-CoA molecules, it is necessary to shift the equilibrium of the β-oxoacyl-CoA thiolase reaction more towards the synthesis of acetoacetyl-CoA, so that sufficient quantities could be synthesised for the determination of the distribution of the ^{14}C label. This was achieved in the following manner. First, the enzyme reaction was carried out at pH 8.8, conditions which are known to make the synthesis of acetoacetyl-CoA more favourable (K_{eq} shifts to 8.7 x 10^{-5}; Goldman, 1954). Second, the reaction was carried out in the presence of a high concentration of magnesium ions (0.3 M) which chelate the enol form of the enzymically synthesised acetoacetyl-CoA (Stern, 1956) (Section 5.1.7) and prevent the back reaction to acetyl-CoA which would result in the randomisation of the label in the regiospecifically labelled

acetoacetyl-CoA. This randomisation was further minimised by the inclusion of methylmethanethiosulphonate in the termination vessel which reacts with both the liberated CoA and the active site sulphydryl group of the free enzyme hence preventing further reaction (Bloxham, et al., 1978).

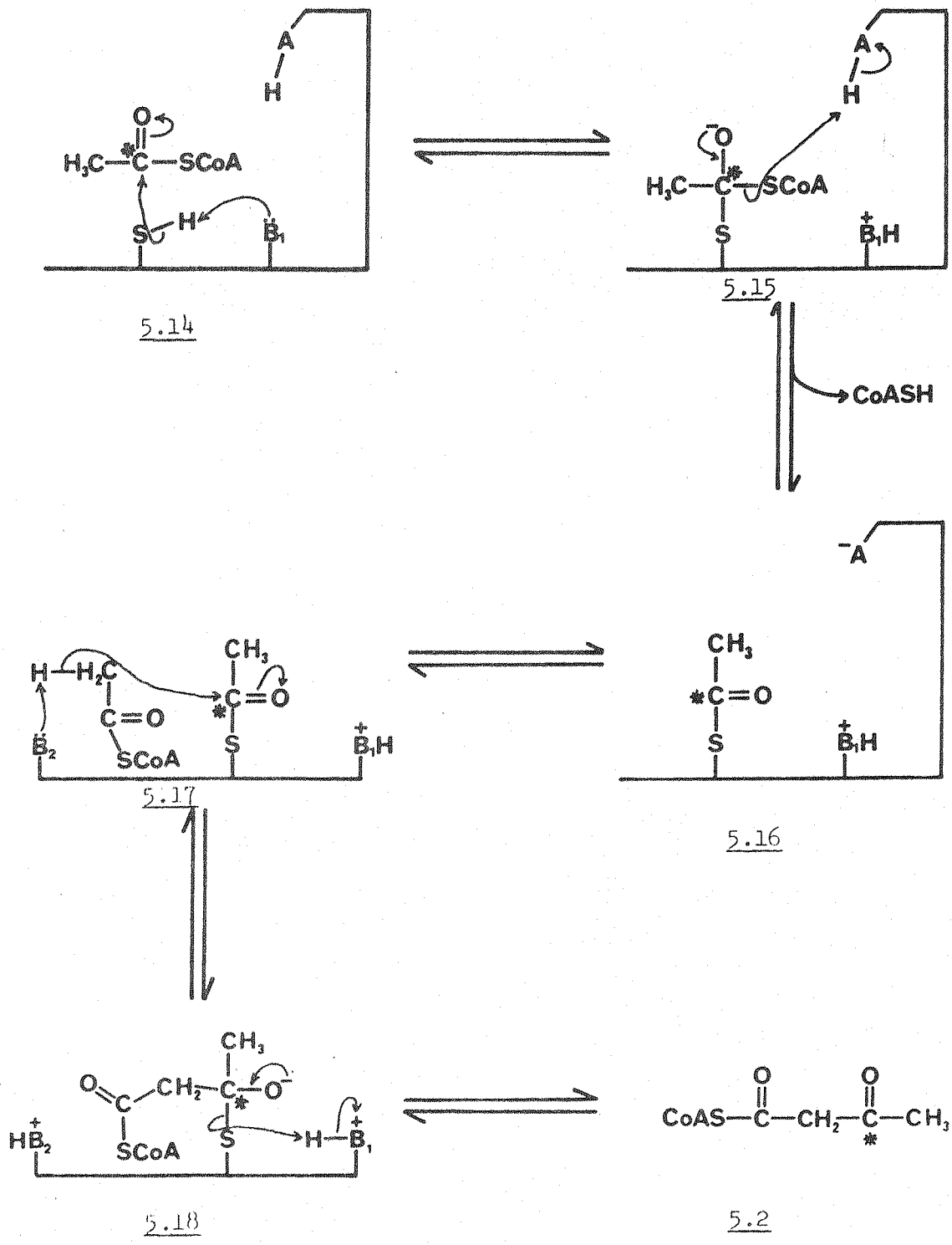
Thus, enzyme and [1-^{14}C] acetyl-CoA were mixed and subsequently chased with excess unlabelled acetyl-CoA. Degradation of the resultant acetoacetyl-CoA to acetone semicarbazone and barium carbonate, originating from C-3 and C-1 of acetoacetyl-CoA respectively (Section 5.2.8; Scheme 5.8), clearly demonstrated that a larger proportion of the radioactivity was in C-3 (Expt. 1 in Table 5.2). This result showed that the molecule of acetyl-CoA initially bound to the enzyme gave rise to C-3 and C-4 of acetoacetyl-CoA (Mechanism (1) in Scheme 5.10). In the converse experiment, in which unlabelled acetyl-CoA was initially mixed with the enzyme and subsequently quenched into an excess of [1-^{14}C] acetyl-CoA, the radioactivity was incorporated preferentially into C-1 (Expt. 2 in Table 5.2). Similar results were obtained when the [1-^{14}C] acetyl-enzyme complex was isolated by gel filtration (Section 5.2.6) before reaction with an excess of unlabelled acetyl-CoA in a rapid-mixing device (Expts. 3, 4 and 5), the radioactivity in these experiments appearing largely in C-3 of acetoacetyl-CoA.

These results provide the first direct evidence for the order of addition of the two molecules of acetyl-CoA during the enzymic synthesis of acetoacetyl-CoA and conclusively demonstrate that, of the two molecules of acetyl-CoA that are bound to the enzyme and subsequently converted to acetoacetyl-CoA, it is the one giving rise to C-3 and C-4 of acetoacetyl-CoA that is bound initially to the enzyme in the form of an acetyl-enzyme intermediate complex. These results are consistent with the mechanism of action of β-oxoacyl-CoA thiolase which has been favoured in the literature since the original proposal by Lynen (1953) and is shown in Scheme 5.11. In this mechanism, deprotonation of the active site sulphydryl group by a basic group (B_1) allows nucleophilic attack of the carbonyl group of the first acetyl-CoA molecule (5-14)→(5.15). Subsequent protonation by an acidic group (HA) helps the removal of the CoA leaving group to yield the acetyl-enzyme complex (5.15)→(5.16). Proton abstraction from C-2 of the second molecule of acetyl-CoA by the

basic group, B_2, allows nucleophilic attack onto the carbonyl group of the first acetyl-CoA molecule (5.17)→(5.18). Finally, reprotonation of the enzyme sulphydryl group releases the product, acetoacetyl-CoA (5.18)→(5.2).

Scheme 5.11 Mechanism of Action of β-Oxoacyl-CoA Thiolase from Rat Liver Cytosol.

The asterisk (*) indicates the location of the label derived from [1-^{14}C] acetyl-CoA.



REFERENCES

REFERENCES

Abboud, M.M. and Akhtar, M. (1976). J. Chem. Soc. Chem. Comm. 1976, 1007-1008.

Abdulla, M. and Haeger-Aronsen, B. (1971). Enzyme 12, 708-710.

Abdulla, M. and Svensson, S. (1979). Scand. J. Clin. Invest. 39, 31-36.

Abdulla, M., Haeger-Aronsen, B. and Svensson, S. (1976). Enzyme 21, 248-252.

Akhtar, M. and Jordan, P.M. (1978). In Comprehensive Organic Chemistry., (Ollis, W.D., ed.), Vol 5, 1121-1163, Pergamon Press, London.

Akhtar, M., Emery, V.C. and Robinson, J.A. (1984). In The Chemistry of Enzyme Action, (Page, M.I., ed.), Chapter 9, 303-372, Elsevier, Amsterdam.

Albery, W.J. and Knowles, J.R. (1976). Biochemistry 15, 5627-5630 and 5631-5640.

Ainsworth, S. (1977). Steady-State Enzyme Kinetics, The MacMillan Press Ltd., London and Basingstoke.

Al-Shammary, F.J. (1984). Ph.D. Thesis, University of Southampton.

Anderson, P.M. and Desnick, R.J. (1979). J. Biol. Chem. 254, 6924-6930.

Anderson, P.M. and Desnick, R.J. (1980). J. Biol. Chem. 255, 1993-1999.

Baloh, R.W. (1974). Arch. Environ. Health. 28, 198-208.

Barnard, G.F., Itoh, R., Hohberger, L.H. and Shemin, D. (1977). J. Biol. Chem. 252, 8965-8974.

Batlle, A.M. del C. and Stella, A.M. (1978). Int. J. Biochem. 9, 861-864.

Batlle, A.M. del C., Ferramola, A. and Grinstein, M. (1967). Biochem. J. 104, 244-249.

Batlle, A.M. del C., Stella, A.M., Ferramola, A.M., Sopena, Y., Wider de Xifra, E.A. and Sancovich, H.A. (1978). Int. J. Biochem. 9, 401-406.

Battersby, A.R. and McDonald, E. (1975). In Porphyrins and Metalloporphyrins, (Smith, K.M., ed.), 61-116, Elsevier, Amsterdam.

Bell, R.M. and Koshland, D.E., Jr. (1971). Science 172, 1253-1256.

Benkovic, S.J. and de Maine, M.M. (1982). Adv. Enzymol. 53, 45-82.

Berry, A. (1983). Ph.D. Thesis, University of Southampton.

Berry, A. and Jordan, P.M. (1981). Biochem. J. 195, 177-181.

Berry, A., Jordan, P.M. and Seehra, J.S. (1981). FEBS Letts. 129, 220-224.

Bevan, D.R., Bodlaender, P. and Shemin, D. (1980). J. Biol. Chem. 255, 2030-2035.

Blake, C.C.F. (1979). In Companian to Biochemistry. Selected Topics for Further Study, Vol. 2, Chapter 10, 281-326, Longman, London.

Blake, C.C.F., Koenig, D.F. Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1965). Nature 206, 757-761.

Blow, D.M. (1969). Biochem. J. 112, 261-268.

Blow, D.M. (1976). Accts. Chem. Res. 9, 145-152.

Blow, D.M., Birktoft, J.J. and Hartley, B.S. (1969). Nature 221, 337-340.

Bloxham, D.P., Chalkley, R.A., Coghlin, S.J. and Salam, W.H. (1978). Biochem. J. 175, 999-1011.

Border, E.A., Cantrell, A.C. and Kilroe-Smith, T.A. (1976a). Brit. J. Ind. Med. 33, 85-87.

Border, E.A., Cantrell, A.C. and Kilroe-Smith, T.A. (1976b). Environ. Res. 11, 319-325.

Bradford, M.M. (1976). Anal. Biochem. 72, 248-254.

Breslow, R. and Wernick, D. (1977). Proc. Natl. Acad. Sci. USA 74, 1303-1307.

Brevet, A., Roustan, C., Desvages, G., Pradel, L.-A. and van Thoai, N. (1973). Eur. J. Biochem. 39, 141-147.

Briggs, G.E. and Haldane, J.B.S. (1925). Biochem. J. 19, 338-339.

Brown, A.J. (1902). J. Chem. Soc. 81, 373-388.

Boyer, P.D. (1959). Arch. Biochem. Biophys. 82, 387-410.

Bucher, N.L.R., Overath, P. and Lynen, F. (1960). Biochim. Biophys. Acta 40, 491-501.

Bustos, N., Stella, A.M., Wider de Xifra, E.A. and Batlle, A.M., del C.(1980). Int. J. Biochem. 12, 745-749.

Cantor, C.R. and Timasheff, S.N. (1982). In The Proteins, (Neurath, H., and Hill, R.L., eds.), 3rd edn., Vol. 5, Chapter 2, 145-306, Academic Press, New York.

Carpenter, F.H. and Vahl, J.M. (1973). J. Biol. Chem. 248, 294-304.

Carraway, K.L. and Koshland, D.E., Jr. (1972). Methods in Enzymology 25, 616-623.

Carty, R.P. and Hirs, C.H.W., (1968). J. Biol. Chem. 243, 5244-5253.

Chance, B. (1943). J. Biol. Chem. 151, 553-577.

Chandrika, S.R. and Padmanaban, G. (1980). Biochem. J. 191, 29-36.

Chang, C.S., Sassa, S. and Doyle, D. (1984). Biochim. Biophys. Acta 797, 297-301.

Chang, J.Y., Brauer, D. and Wittman-Liebold, B. (1978). FEBS Letts. 93, 205-214.

Chaudhry, A.G. and Jordan, P.M. (1976). Biochem. Soc. Trans. 4, 760-761.

Chaudhry, A.G., Gore, M.G. and Jordan, P.M. (1976). Biochem. Soc. Trans. 4, 301-303.

Cheh, A.M. and Neilands, J.B. (1973). Biochem. Biophys. Res. Comm. 55, 1060-1063.

Cheh, A.M. and Neilands, J.B. (1976). Structure and Bonding 29, 123-170.

Chlebowski, J. and Coleman, J.E. (1976). In Metal Ions in Biological Systems, (Sigel, H., ed), Vol. 6, Chapter 1, 1-140, Dekker, New York.

Cleland, W.W. (1963a). Biochim. Biophys. Acta 67, 104-137.

Cleland, W.W. (1963b). Biochim. Biophys. Acta 67, 173-196.

Cleland, W.W. (1967). Adv. Enzymol. 29, 1-32.

Cleland, W.W. (1967). Ann. Rev. Biochem. 36, 77-112.

Cleland, W.W. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol.2, Chapter 1, 1-65, Academic Press, New York.

Cleland, W.W. (1977). Adv. Enzymol. 45, 273-387.

Cleland, W.W., O'Leary, M.H. and Northrop, D.B. (eds.), (1977). Isotope Effects on Enzyme-Catalysed Reactions, University Park Press, Baltimore.

Clinkenbeard, K.D., Reed, W.D., Mooney, R.D. and Lane, M.D. (1975). J. Biol. Chem. 250, 3108-3116.

Clinkenbeard, K.D., Sugiyama, T., Moss, J., Reed, W.D. and Lane, M.D. (1973). J. Biol. Chem. 248, 2275-2284.

Cohen, L.A. (1968). Ann. Rev. Biochem. 37, 683-726.

Cohen, L.A. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 1, Chapter 3, 147-211, Academic Press, New York.

Cohn, M. (1949). J. Biol. Chem. 180, 771-781.

Cohn, M. (1970). Quart. Rev. Biophys. 3, 61-89.

Cohn, M. and Reed, G.H. (1982). Ann. Rev. Biochem. 51, 365-394.

Coleman, D. (1966). J. Biol. Chem. 241, 5511-5517.

Coleman, J.E. (1967). J. Biol. Chem. 242, 5212-5219.

Coleman, J.E. and Gettins, P. (1983). Adv. Enzymol. 55, 381-452.

Coleman, J.E. and Vallee, B.L. (1961). J. Biol. Chem. 236, 2244-2249.

Coletti-Previero, M-A., Previero, A. and Zuckerkandl., E. (1969). J. Mol. Biol. 39, 493-501.

Cookson, G.H., Rimington, C. and Kennard, O. (1953). Nature 171, 875-876.

Cooper, T.G. and Beevers, H. (1969). J. Biol. Chem. 244, 3514-3520.

Cornforth, J.W., Redmond, J.W., Eggerer, H., Buckel, W. and Gutschow, C. (1970). Eur, J. Biochem. 14, 1-13.

Cornish-Bowden, A. (1979). Fundamentals of Enzyme Kinetics, Butterworths, London-Boston.

Crestfield, A.M., Stein, W.H. and Moore, S. (1963). J. Biol. Chem. 238, 2413-2420.

Criddle, R.S., McMurray, C.H. and Gutfreund, H. (1968). Nature 220, 1091-1095.

Cuatrecasas, P., Fuchs, S. and Anfinsen, C.B. (1968). J. Biol. Chem. 243, 4787-4798.

Dahlquist, F.W., Rand-Meir, T. and Raftery, M.A. (1968). Proc. Natl. Acad. Sci. USA 61, 1194-1198.

Davis, J.R. and Avram, M.J. (1978). Toxicol.and Appl. Pharmacol. 44, 181-190.

Davis, J.R. and Avram, M.J. (1980). Toxicol. and Appl. Pharmacol. 55, 281-290.

De Bruin, A. (1968). Med. Lavoro 59, 411-418.

De Toma, F. and Abeles, R.H. (1970). Fed. Proc. 29, 461.

Despaux, N., Bohuon, C., Comoy, E. and Boudene, C. (1977). Biomedicine 27, 358-361.

Despaux, N., Comoy, E., Bohuon, C. and Boudene, C. (1979). Biochemie 61, 1021-1029.

Dixon, M. (1953). Biochem. J. 55, 170-171.

Doudoroff, M., Barker, H.A. and Hassid, W.Z. (1947). J. Biol. Chem. 168, 725-732.

Dowd, J.E. and Riggs, D.S. (1965). J. Biol. Chem. 240, 863-869.

Doyle, D. (1971). J. Biol. Chem. 246, 4965-4972.

Doyle, D. and Schimke, R.T. (1969). J. Biol. Chem. 244, 5449-5459.

Dresel, E.I.B. and Falk, J.E. (1953). Nature 172, 1185.

Drum, D.E., Harrison, J.H. (IV), Li, T.-K., Bethune, J.L. and Vallee, B.L. (1967). Proc. Natl. Acad. Sci. USA 57, 1434-1440.

Eadie, G.S. (1942). J. Biol. Chem. 146, 85-93.

Eady, R.R., Lowe, D.J. and Thorneley, R.N.F., (1978). FEBS Letts. 95, 211-213.

Eisenberg, D. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 1, Chapter 1, 1-89, Academic Press, New York.

Eisenthal, R. and Cornish-Bowden, A. (1974). Biochem. J. 139, 715-720.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T. and Bränden, C.-I. (1974). FEBS Letts. 44, 200-204.

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

Engel, P.C. (1977). Enzyme Kinetics. The Steady-State Approach., Chapman and Hall, London and New York.

Erlanger, B.F., Cooper, A.G. and Cohen, W. (1966a). Biochemistry 5, 190-196.

Erlanger, B.F., Vratsanos, S.M., Wasserman, N. and Cooper, A.G. (1966b). Biochem. Biophys. Res. Comm. 23, 243-245.

Falk, J.E., Dresel, E.I.B. and Rimington, C. (1953). Nature 172, 292-294.

Feinstein, G., Bodlaender, P. and Shaw, E. (1969). Biochemistry 8, 4949-4955.

Feldman, F. and Butler, L.G. (1972). Biochim. Biophys. Acta 268, 698-710.

Fersht, A.R. and Jakes, R. (1975). Biochemistry 14, 3350-3356.

Findlay, D., Herries, D.G., Mathias, A.P., Rabin, B.R. and Ross, C.A. (1962). Biochem. J. 85, 152-153.

Finelli, V.N., Klauder, D.S., Karaffa, M.A. and Petering, H.G. (1975). Biochem. Biophys. Res. Comm. 65, 303-312.

Finelli, V.N., Murthy, L., Peirano, W.B. and Petering, H.G. (1974). Biochem. Biophys. Res. Comm. 60, 1418-1424.

Foster, R.L. (1980). The Nature of Enzymology, (Baron, P.J., ed.), Croom Helm, London.

Fridovich, I. and Westheimer, F.H. (1962). J. Amer. Chem. Soc. 84, 3208-3209.

Frieden, E.M., Roberts, P.B., Bray, R.C., Lowe, D.J., Nautner, G.N., Rotilio, G. and Calabrese, L. (1974). Biochem. J. 139, 49-55.

Fromm, H.J. (1963). J. Biol. Chem. 238, 2938-2944.

Fujita, H., Orii, Y. and Sano, S. (1981). Biochim. Biophys. Acta 678, 39-50.

Fujita, H., Sato, K. and Sano, S. (1982). Int. Arch. Occup. Environ. Health. 50, 287-297.

Gehring, U. and Harris, J.I. (1968). FEBS Letts. 1, 150-152.

Gehring, U. and Harris, J.I. (1970a). Eur. J. Biochem. 16, 487-491.

Gehring, U. and Harris, J.I. (1970b). Eur. J. Biochem. 16, 492-498.

Gehring, U. and Lynen, F. (1972). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 7, 391-405, Academic Press, New York.

Gehring, U. and Riepertinger, C. (1968). Eur. J. Biochem. 6, 281-292.

Gehring, U., Riepertinger, C. and Lynen, F. (1968). Eur. J. Biochem. 6, 264-280.

Geisse, S., Brüller, H.-J. and Doss, M. (1983). Clin. Chim. Acta 135, 239-245.

George, D.J. and Phillips, A.T. (1970). J. Biol. Chem. 245, 528-537.

Gibbs, P.N.B. and Jordan, P.M. (1981). Biochem. Soc. Trans. 9, 232-233.

Gibbs, P.N.B., Chaudhry, A.G. and Jordan, P.M. (1984). Eur. J. Biochem. In Press.

Gibson, K., Neuberger, A. and Scott, J. (1955). Biochem. J. 61, 618-629.

Glazer, A.N. (1976). In The Proteins, (Neurath, H., and Hill, R.L., eds.), 3rd edn., Vol. 2, Chapter 1, 1-103, Academic Press, New York.

Gold, A.M. and Osber, M.P. (1972). Arch. Biochem. Biophys. 153, 784-787.

Goldman, D.S. (1954). J. Biol. Chem. 208, 345-357.

Gore, M.G. (1984). Personal Communication.

Gore, M.G., Jordan, P.M. and Chaudhry, A.G. (1976). Biochem. Soc. Trans. 4, 762.

Granick, S. and Mauzerall, D. (1958). J. Biol. Chem. 232, 1119-1140.

Grazi, E., Cheng, T. and Horecker, B.L. (1962). Biochem. Biophys. Res. Comm. 7, 250-257.

Green, D.E., Goldman, D.S., Mii, S. and Beinert, H. (1953). J. Biol. Chem. 202, 137-150.

Griffin, J.H., Rosenbusch, J.P., Blout, E.R. and Weber, K.K. (1973). J. Biol. Chem. 248, 5057-5062.

Gurba, P., Sennett, R. and Kobes, R. (1972). Arch. Biochem. Biophys. 150, 130-136.

Gurne, D., Chen, J. and Shemin, D. (1977). Proc. Natl. Acad. Sci. USA 74, 1383-1387.

Gutfreund, H. (1959). In The Enzymes, (Boyer, P.D., Lardy, H., and Myrback, K., eds.), 2nd edn., Vol. 1, Chapter 5, 233-258, Academic Press, New York.

Gutfreund, H. (1971). Ann. Rev. Biochem. 40, 315-344.

Gutfreund, H. (1972). Enzymes: Physical Principles, John Wiley, London.

Haeger-Aronsen, B., Abdulla, M. and Fristedt, B.I. (1971). Arch. Environ. Health. 23, 440-445.

Haeger-Aronsen, B., Abdulla, M. and Fristedt, B.I. (1974). Arch. Environ. Health. 29, 150-153.

Haeger-Aronsen, B., Abdulla, M. and Shutz, A. (1976). Arch. Environ. Health. 31, 215-219.

Halford, S.E. (1974). In Companion to Biochemistry. Selected Topics for Further Study., Vol. 1, Chapter 5, 197-226, Longman, London.

Hanson, K.R. and Havir, E.A. (1972). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 7, Chapter 3, 75-166, Academic Press, New York.

Hartley, B.S. and Kilby, B.A. (1954). Biochem. J. 56, 288-297.

Hartmann, G. and Lynen, F. (1961). In The Enzymes, (Boyer, P.D., Lardy, H. and Myrback, K., eds.), 2nd edn., Vol. 5, 381-386, Academic Press, New York.

Hartridge, H. and Roughton, F.J.W. (1923). Proc. Roy. Soc. Ser. A. 104, 376-430.

Hass, L.F. and Byrne, W.L. (1960). J. Amer. Chem. Soc. 82, 947-954.

Hellmut, M. and Beyersmann, D. (1983). Enzyme 29, 260-266.

Henkart, P. and Dorner, F. (1971). J. Biol. Chem. 246, 2714-2720.

Hernberg, S. and Nikkanen, J. (1970). Lancet 1970, 63-64.

Hernberg, S. and Nikkanen, J. (1972). Pracov. Lek. 24, 77-83.

Hess, G.P. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 3, Chapter 7, 213-248, Academic Press, New York.

Hexter, C.S. and Westheimer, F.H. (1971). J. Biol. Chem. 246, 3928-3933.

Hill, R.D. and Laing, R.R. (1967). Biochim. Biophys. Acta 132, 188-190.

Himmelhoch, S.R. (1969). Arch. Biochem. Biophys. 134, 597-602.

Hiromi, K. (1979). Kinetics of Fast Enzyme Reaxtions. Theory and Practice., Halsted Press, New York.

Hodgins, D.S. and Abeles, R.H. (1969). Arch. Biochem. Biophys. 130, 274-285.

Hofstee, B.H.J. (1952). J. Biol. Chem. 199, 357-364.

Holbrook, J.J. and Jeckel, R. (1969). Biochem. J. 111, 689-694.

Holland, P.C., Clark, M.G., and Bloxham, D.P. (1973). Biochemistry 12, 3309-3315.

Horecker, B.L., Pontremoli , S., Ricci, C. and Cheng, T. (1961). Proc. Natl. Acad. Sci. USA 47, 1949-1955.

Howard, J.K. (1978). J. Toxicol. Environ. Health. 4, 51-57.

Hughes, M.N., (1972). The Inorganic Chemistry of Biological Processes., Wiley-Interscience, London.

Hupe, D.J. (1984). In The Chemistry of Enzyme Action., (Page, M.I., ed.), Chapter 8, 271-301, Elsevier, Amsterdam.

Husain, S.S. and Lowe, G. (1968). Biochem. J. 108, 855-859 and 861-866.

Huth, W., Jonas, R., Wunderlich, I. and Seubert, W. (1975). Eur. J. Biochem. 59, 475-489.

Inagami, T., Patchormik, A. and York, S.S. (1969). J. Biochem. 65, 809-819.

Jaffe, E.K., Salowe, S.P., Chen, N.T. and DeHaven, P.A., (1984). J. Biol. Chem. 259, 5032-5036.

Jakoby, W.B. and Wilchek, M. (eds.), (1977). Methods in Enzymology 46, Academic Press, New York.

Jeffcoat, R., Hassall, H. and Dagley, S. (1969). Biochem. J. 115, 977-983.

Jencks, W.P. (1969). In Catalysis in Chemistry and Enzymology., McGraw-Hill, New York.

Johnson, L.N. and Phillips, D.C. (1965). Nature 206, 761-763.

Jolley, C.J. and Yankeelov, J.A., Jr. (1972). Biochemistry 11, 164-169.

Jordan, P.M. and Gibbs, P.N.B., (1983). Biochem. J. 213, 153-158.

Jordan, P.M. and Seehra, J.S. (1979). FEBS Letts. 104, 364-366.

Jordan, P.M. and Seehra, J.S. (1980a). J. Chem. Soc. Chem. Comm. 1980, 240-242.

Jordan, P.M. and Seehra, J.S. (1980b). FEBS Letts. 114, 283-286.

Jordan, P.M., Gore, M.G. and Chaudhry, A.G. (1976). Biochem. Soc. Trans. 4, 762-763.

Kägi, J.H.R. and Vallee, B.L. (1960). J. Biol. Chem. 235, 3188-3192.

Kagi, J.H.R. and Vallee, B.L. (1961). J. Biol. Chem. 236, 2435-2442.

Kajimoto, M., Kondo , M., Niwa, M., Suzuki, T., Kimura, H., Sasaki, A. and Urata, G. (1983). Arch. Toxicol. 52, 1-11.

Kapke, G. and Davis, L. (1975). Biochemistry 14, 4273-4276.

Kennedy, E.P. and Koshland, D.E., Jr. (1957). J. Biol. Chem. 228, 419-431.

Kenyon, G.L. and Reed, G.H. (1983). Adv. Enzymol. 54, 367-426.

Klinman, J.P. (1978). Adv. Enzymol. 46, 415-494.

Kobes, R.D., Simpson , R.T., Vallee, B.L. and Rutter, W.J. (1969). Biochemistry 8, 585-588.

Kollman, P. (1984). In The Chemistry of Enzyme Action, (Page, M.I., ed.), Chapter 2, 55-71, Elsevier, Amsterdam.

Komai, H. and Neiland, J.B. (1968). Arch. Biochem. Biophys. 124, 456-461.

Komai, H. and Neilands, J.B. (1969). Biochim. Biophys. Acta 171, 311-320.

Kondo , M., Kajimoto, M., Kimura, H., Suzuki, T., Sasaki, A., Niwa., M. and Urata, G. (1981). Arch. Biochem. Biophys. 208, 189-194.

Kornblatt, J.A. and Rudney, H. (1970). Fed. Proc. 29, 674.

Kornblatt, J.A. and Rudney, H. (1971a). J. Biol. Chem. 246, 4417-4423.

Kornblatt, J.A. and Rudney, H. (1971b). J. Biol. Chem. 246, 4424-4430.

Koshland, D.E. Jr., (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 1, 341-396, Academic Press, New York.

Kreutzer, M., Schmidt, M., Stadler, E. and Zeitler, H.-J. (1977). Hoppe-Seyler's Z. Phsiol. Chem. 358, 1081-1091.

Kuo, L.C. and Makinen, M.W. (1982). J. Biol. Chem. 257, 24-27.

Laghai, A. (1977). Ph.D. Thesis, University of Southampton.

Lauwerys, R.R., Buchet, J.-P. and Roels, H.A. (1973). Brit. J. Ind. Med. 30, 359-364.

Layne, P.P. and Najjar, V.A. (1979). Proc. Natl. Acad. Sci. USA 76, 5010-5013.

Liedgens, W., Grützmann, R. and Schneider, H.A.W. (1980). Z. Naturforsch. 35, 958-962.

Liedgens, W., Lütz, C. and Schneider, H.A.W. (1983). Eur. J. Biochem. 135, 75-79.

Liljas, A., Kannan, K.K., Bergsten, P.-C., Waara, I., Fridborg, K., Strandberg, B., Carlbom, U., Järap, L., Lovgren, S. and Petef, M. (1972). Nature 235, 131-137.

Lindskog, S. (1963). J. Biol. Chem. 238, 945-951.

Lindskog, S. (1966). Biochemistry 5, 2641-2646.

Lineweaver, H. and Burk, D. (1934). J. Amer. Chem. Soc. 56, 658-666.

Lingner, B. and Kleinschmidt, T. (1983). Z. Naturforsch. 38, 1059-1061.

Lipmann, F. and Tuttle, L.C. (1945). J. Biol. Chem. 159, 21-28.

Lipscomb, W.N. (1983). Ann. Rev. Biochem. 52, 17-34.

Lowry, O.H., Rosebrough, N.J., Farr, L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.

Lynen, F. (1951). Ann. Chem. 574, 33-37.

Lynen, F. (1953). Fed. Proc. 12, 683-691.

Lynen, F., Reichert, E. and Rueff, L. (1951). Ann. Chem. 574, 1-32.

Lynen, F., Wesseley, L., Wieland, O. and Rueff, L. (1952). Angew. Chem. 64, 687.

Macfarlane, N. and Ainsworth, S. (1972). Biochem. J. 129, 1035-1047.

Mahler, H.R., Baker, R.H., Jr. and Shiner, V.J., Jr. (1962). Biochemistry 1, 47-52.

Makinen , M.W., Kuo, L.C., Dymowski, J.J. and Jaffer, S. (1979). J. Biol. Chem. 254, 356-366.

Makinen, M.W., Yamamura, K. and Kaiser, E.T. (1976). Proc. Natl. Acad. Sci. USA 73, 3882-3886.

Markus, M., Hess, B., Ottaway, J.H. and Cornish-Bowden, A. (1976). FEBS Letts. 63, 225-230.

Matthews, B.W. (1977). In The Proteins, (Neurath, H., and Hill, R.L., eds.), 3rd edn., Vol. 3, Chapter 4, 403-590, Academic Press, New York.

Maurus, Y. and Allain, P. (1979). Enzyme 24, 181-187.

Mauzerall, D. and Granick, S. (1956). J. Biol. Chem. 219, 435-446.

Mazzei, Y., Negrel, R. and Ailhaud, G. (1970). Biochim. Biophys. Acta 220, 129-131.

McColl, K.E.L. and Goldberg, A. (1980). Clinics in Haematol. 9, 427-444.

McConn, J.D., Tsuri, D. and Yasunobu, K.T. (1967). Arch. Biochem. Biophys. 120, 479-486.

McGarry, J.D. and Foster, D.W. (1969). J. Biol. Chem. 244, 4251-4256.

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

Meloche, H.P., Ingram, J.I. and Wood, W.A. (1966). Methods Enzymol. 9, 520-536.

Meredith, P.A. and Moore, M.R. (1978). Biochem. Soc. Trans. 6, 760-762.

Meredith, P.A. and Moore, M.R. (1980). Int. Arch. Occup. Environ. Health. 45, 163-168.

Meredith, P.A., Moore, M.R. and Goldberg, A. (1974). Biochem. Soc. Trans. 2, 1243-1245.

Meredith, P.A., Moore, M.R., and Goldberg, A. (1977). Enzyme 22, 22-27.

Metcalfe, J.C., Birdsall, N.J.M. and Lee, A.G. (1974). In Companion to Biochemistry. Selected Topics for Further Study, Vol. 1, Chapter 3, 139-162, Longman, London.

Michaelis, L. and Menten, M. (1913). Biochem. Z. 49, 333-369.

Middleton, B. (1971). Biochem. J. 125, 69P.

Middleton, B. (1972), Biochem. Biophys. Res. Comm. 46, 508-515.

Middleton, B. (1973). Biochem. J. 132, 717-730.

Middleton, B. (1974). Biochem. J. 139, 109-121.

Mildvan, A.S. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 2, Chapter 9, 445-536, Academic Press, New York.

Mildvan, A.S., Leigh, J.S., Jr. and Cohn, M. (1967). Biochemistry 6, 1805-1818.

Miller, W.M. and Richards, J.H. (1969). J. Amer. Chem. Soc. 91, 1498-1507.

Miziorki, H.M. and Lane, M.D. (1977). J. Biol. Chem. 252, 1414-1420.

Miziorki, H.M., Clinkenbeard, K.D., Reed, W.D. and Lane, M.D. (1975). J. Biol. Chem. 250, 5768-5773.

Moore, M.R. (1972). Biochem. J. 129, 43P-44P.

Moore, M.R., Beattie, A.D., Thompson, G.G. and Goldberg, A. (1971). Clin. Sci. 40, 81-88.

Morihara, K. and Nagami, K. (1969). J. Biochem. 65, 321-323.

Morris, D. and Grewaal, D.S. (1971). Eur. J. Biochem. 22, 563-572.

Morris, D., Maneckjee, A. and Hebb, C. (1971). Biochem. J. 125, 857-863.

Morris, H.R., Williams, D.H. and Ambler, R.P. (1971). Biochem. J. 125, 189-201.

Mullen, E., Johnson, A.H. and Akhtar, M. (1981). FEBS Letts. 130, 187-193.

Nakayama, H., Tanizawa, K. and Kanaoka, Y. (1970). Biochem. Biophys. Res. Comm. 40, 537-541.

Nandi, D.L. (1978). Z. Naturforsch. 33, 799-800.

Nandi, D.L. and Shemin, D. (1968a). J. Biol. Chem. 243, 1231-1235.

Nandi, D.L. and Shemin, D. (1968b). J. Biol. Chem. 243, 1236-1242.

Nandi, D.L. and Shemin, D. (1973). Arch. Biochem. Biophys. 158, 305-311.

Nandi, D.L. and Waygood, E.R. (1967). Can. J. Biochem. 45, 327-331.

Nandi, D.L., Baker-Cohen, K.F. and Shemin, D. (1968). J. Biol. Chem. 243, 1224-1230.

Neuberger, A. and Scott, J.J. (1953). Nature 172, 1093-1094.

Nishimura, J.S., Kenyon, G.L. and Smith, D.J., (1975). Arch. Biochem. Biophys. 170, 461-467.

O'Leary, M.H. and Baughn, R.L. (1972). J. Amer. Chem. Soc. 94, 626-630.

O'Leary, M.H. and Westheimer, F.H. (1968). Biochemistry 7, 913-919.

O'Leary, M.H., Urberg, M. and Young, A.P. (1974). Biochemistry 13, 2077-2081.

Overath, P., Pauli, G. and Schairer, H.U. (1969). Eur. J. Biochem. 7, 559-574.

Overath, P., Raufuss, E.-M., Stoffel, W. and Ecker, W. (1967). Biochem. Biophys. Res. Comm. 29, 28-33.

Page, M.I. (1984a). In The Chemistry of Enzyme Action., (Page, M.I., ed.), Chapter 1, 1-54, Elsevier, Amsterdam.

Page, M.I. (1984b). In The Chemistry of Enzyme Action., (Page, M.I., ed.), Chapter 7, 229-269, Elsevier, Amsterdam.

Palm, D. (1966). Z. Naturforsch. 21, 540-546.

Patthy, L. and Smith, E.L. (1975). J. Biol. Chem. 250, 557-564 and 565-569.

Phillips, D.C. (1966). Scientific American 215, 78-90.

Phillips, D.C. (1967). Proc. Natl. Acad. Sci. USA 57, 484-495.

Pocker, Y. and Sarkanen, S. (1978). Adv. Enzymol. 47, 149-274.

Popjak, G. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 2, Chapter 3, 115-215, Academic Press, New York.

Potter, L.T., Glover, V.A.S. and Saelens, J.K. (1968). J. Biol. Chem. 243, 3864-3872.

Rando, R. (1974). Biochemistry 13, 3859-3863.

Rashed, N. and Rabin, B.R. (1968). Eur. J. Biochem. 5, 147-150.

Richards, F.M. and Wyckoff, H.W. (1971). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 4, Chapter 24, 647-806, Academic Press, New York.

Richards, J.H. (1970). In The Enzymes, (Boyer, P.D. ed.), 3rd edn., Vol. 2, Chapter 6, 321-333, Academic Press, New York.

Riley, W.D. and Snell, E.E. (1968). Biochemistry 7, 3520-3528.

Riordan, J.F., McElvany, K.D. and Borders, C.L. (1977). Science 195, 884-886.

Roberts, G.C.K., Dennis, E.A., Meadows, D.H., Cohen, J.S. and Jardetzky, O. (1969). Proc. Natl. Acad. Sci. USA 62, 1151-1158.

Roels, H.A., Buchet, J.-P. and Lauwerys, R.R. (1974). Int. Arch. Arbeitsmed. 33, 277-284.

Rose, I.A. (1979). Adv. Enzymol. 50, 361-395.

Rose, I.H. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 2, Chapter 5, 281-320, Academic Press, New York.

Rose, Z.B. (1980). Adv. Enzymol. 51, 211-253.

Rosen, O.M., Hoffee, P. and Horecker, B.L. (1965). J. Biol. Chem. 240, 1517-1524.

Rosso, R.G. and Adams, E. (1967). J. Biol. Chem. 242, 5524-5534.

Rotilio, G., Calabrese, L., Bossa, F., Barra, D., Agró, A.F. and Mondovi, B. (1972). Biochemistry 11, 2182-2187.

Rutter, W.J. (1964). Fed. Proc. 23, 1248-1257.

Salam, W.H. (1982). Ph.D. Thesis, University of Southampton.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Sassa, S. and Kappas, A. (1983). J. Clin. Invest. 71, 625-634.

Satre, M. and Kennedy, E.P. (1978). J. Biol. Chem. 253, 479-483.

Sawyer, C.B. and Kirsch, J.F. (1975). J. Amer. Chem. Soc. 97, 1963-1964.

Schirch, L. (1982). Adv. Enzymol. 53, 83-112.

Schmid, R. and Shemin, D. (1955). J. Amer. Chem. Soc. 77, 506-507.

Schmidt, D.E., Jr., Nigh, W.G., Tanzer, C. and Richards, J.H. (1969). J. Amer. Chem. Soc. 91, 5849-5854.

Schneider, H.A.W. (1970). Z. Planzenphysiol. Bd. 62, 328-342.

Schneider, H.A.W. and Liedgens, W. (1981). Z. Naturforsch. 36, 44-50.

Schoellman, G. and Shaw, E. (1963). Biochemistry 2, 252-255.

Schroeder, D.D. and Shaw, E. (1971). Arch. Biochem. Biophys. 142, 340-350.

Schuberth, J. (1966). Biochim. Biophys. Acta 122, 470-479.

Schwartz, J. (1963). Proc. Natl. Acad. Sci. USA 49, 871-878.

Seehra, J.S. (1980). Ph.D. Thesis, University of Southampton.

Seehra, J.S. and Jordan, P.M. (1980). J. Amer. Chem. Soc. 102, 6841-6846.

Seehra, J.S. and Jordan, P.M. (1981). Eur. J. Biochem. 113, 435-446.

Seehra, J.S., Gore, M.G., Chaudhry, A.G. and Jordan, P.M. (1981). Eur. J. Biochem. 114, 263-269.

Segel, I.H. (1975). Enzyme Kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems., Wiley-Interscience, New York.

Seltzer, S., Hamilton, G.A. and Westheimer, F.H. (1959). J. Amer. Chem. Soc. 81, 4018-4024.

Seubert, W., Lamberts, L., Kramer, B. and Ohly, B. (1968). Biochim. Biophys. Acta 164, 498-517.

Shafer, J., Baronowsky, P., Laursen, R., Finn, F. and Wesheimer, F.H. (1966). J. Biol. Chem. 241, 421-427.

Shaltiel, S. and Soria, M. (1969). Biochemistry 8, 4411-4415.

Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967). Biochem. Biophys. Res. Comm. 28, 815-820.

Shaw, D.C., Stein, W.H. and Moore, S. (1964). J. Biol. Chem. 239, PC 671-PC 673.

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

Shaw, E. (1970b). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 1, Chapter 2, 91-146, Academic Press, New York.

Shaw, E. and Glover, G. (1970). Arch. Biochem. Biophys. 139, 298-305.

Shedlarski, J.L. and Gilvarg, C. (1970). J. Biol. Chem. 245, 1362-1373.

Shemin, D. (1972). In The Enzymes, (Boyer, P.D. ed.), 3rd edn., Vol. 7, 323-337, Academic Press, New York.

Shemin, D. (1976). Phil. Trans. R. Soc. Lond. B. 273, 109-115.

Shemin, D. and Russell, C.S. (1953). J. Amer. Chem. Soc. 75, 4873-4874.

Shemyakin, M.M., Ovchinnikov, Y.A., Vinogradova, E.I., Kiryushkin, A.A., Feigina, M.Y., Aldanova, N.A., Alakhov, Y.B., Lipkin, V.M., Miroshnikov, A.I., Rosinov, B.V. and Kazaryan, S.A. (1970). FEBS Letts. 7, 8-12.

Shetty, A. and Miller, G. (1969). Biochem. J. 114, 331-337.

Shulman, R.G., Navon, G., Nyluda, B.J., Douglass, D.C. and Yamane, T. (1966). Proc. Natl. Acad. Sci. USA 56, 39-44.

Sigler, P.B., Blow, D.M., Matthews, B.W. and Henderson, R. (1968). J. Mol. Biol. 35, 143-164.

Silverstein, E. and Sulebele, G. (1969). Biochemistry 8, 2543-2550.

Silverstein, R., Voet, J., Reed, D. and Abeles, R.H. (1967). J. Biol. Chem. 242, 1338-1346.

Simpson, R.T., Riordan, J.F. and Vallee, B.L. (1963). Biochemistry 2, 616-622.

Singer, S.J. (1967). Adv. Prot. Chem. 22, 1-54.

Siperstein, M.D. (1970). Curr. Topics Cell. Regn. 2, 65-100.

Smith, I. (1976). In Chromatographic and Electrophoretic Techniques, Vol. 2, Chapter 4, 66-89, on "Thin Layer Electrophoresis" by Ritschard, W.J., Heinemann.

Snell, E.E. and Di Mari, S.J. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 2, Chapter 7, 335-370, Academic Press, New York.

Spande, T.F., Witkop, B., Degani, Y. and Patchornik, A. (1970). Adv. Prot. Chem. 24, 97-260.

Spector, L.B. (1972). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 7, Chapter 12, 357-389, Academic Press, New York.

Spector, L.B. (1982). Covalent Catalysis by Enzymes., Spring-Verlag, New York.

Stafforini, D.M., Polo, C.F., Stella, A.M., Wider de Xifra, E.A. and Batlle, A.M. del C. (1980). Int. J. Biochem. 12, 757-760.

Stark, G.R. (1970). Adv. Prot. Chem. 24, 261-308.

Stella, A.M. and Batlle, A.M. del C. (1977). Int. J. Biochem. 8, 353-358.

Stern, J.R. (1956). J. Biol. Chem. 221, 33-44.

Stern, J.R., Coon, M.J. and del Campillo, A. (1953). Nature 171, 28-30.

Stewart, P.R. and Rudney, H. (1966). J. Biol. Chem. 241, 1222-1225.

Sykes, B.D. and Scott, M.D. (1972). Ann. Rev. Biophys. Bioeng. 1, 27-50.

Takahashi, K. (1968). J. Biol. Chem. 243, 6171-6179.

Taylor, J.S. and Coleman, J.E. (1973). J. Biol. Chem. 248, 749-755.

Theorell, H. and Chance, B. (1951). Acta Chemica Scand. 5, 1127-1144.

Thiers, R.E. (1957). Methods Biochem. Anal. 5, 273-335.

Thomas, J.O. (1974). In Companion to Biochemistry. Selected Topics for Further Study., Vol. 1, Chapter 2, 87-138, Longman, London.

Thomasino, J.A., Zuroweste, E., Brooks, S.M., Lerner, S.I. and Finelli, V.N. (1977). Arch. Environ. Health. 32, 244-247.

Thompson, J., Jones, D.D. and Beasley, W.H. (1977). Brit. J. Ind. Med. 34, 32-36.

Timasheff, S.N. (1970). In The Enzymes, (Boyer, P.D., ed.), 3rd edn., Vol. 2, Chapter 8, 371-444, Academic Press, New York.

Tipton, K.F. (1974). In Companion to Biochemistry. Selected Topics for Further Study., (Bull, A.T., Lagnado, J.R., Thomas, J.O., and Tipton, K.F., eds.), Vol. 1, Chapter 6, 227-252, Longman, London.

Tipton, K.F. (1979). In Companion to Biochemistry, Selected Topics for Further Study., (Bull, A.T., Lagnado, J.R., Thomas, J.O., and Tipton, K.F., eds.), Vol. 2, Chapter 11, 327-382, Longman, London.

Toennies, G. and Kolb, J.J. (1951). Anal. Chem. 23, 823-826.

Tomokuni, K. (1979). J. Toxicol. Sci. 4, 11-17.

Trevisan, A., Gori, G.P., Zangirolami, A., Benevento, C., Rosa, A. and Chiesura, P. (1980). Enzyme 25, 33-36.

Tsukamoto, I., Yoshinaga, T. and Sano, S. (1975). Biochem. Biophys. Res. Comm. 67, 294-300.

Tsukamoto, I., Yoshinaga, T. and Sano, S. (1979). Biochim. Biophys. Acta 570, 167-178.

Tsukamoto, I., Yoshinaga, T. and Sano, S. (1980). Int. J. Biochem. 12, 751-756.

Vallee, B.L. and Galdes, A. (1984). Adv. Enzymol. 56, 283-430.

Vallee, B.L. and Gibson, J.G. (1948). J. Biol. Chem. 176, 445-457.

Vallee, B.L. and Riordan, J.F. (1978). In Molecular Interactions and Activity in Proteins., 197-223, Excerpta Medica, Amsterdam.

Vallee, B.L. and Ulmer, D.D. (1972). Ann. Rev. Biochem. 41, 91-128.

Vilkas, M. (1978). In Enzyme-Activated Irreversible Inhibitors., (Seiler, N., Jung, M.J. and Koch-Weser, J., eds.), 323-335, Elsevier, Amsterdam.

Wada, O., Yano, Y., Ono, T. and Toyokawa, K. (1972). Ind. Health. 10, 59-61.

Walsh, C. (1979). Enzymatic Reaction Mechanisms., Freeman, San Francisco.

Warren, S., Zerner, B. and Westheimer, F.H. (1966). Biochemistry 5, 817-823.

Wassarman, P. and Majors, J. (1969). Biochemistry 8, 1076-1082.

Weber, H., Seibl, J. and Arigoni, D. (1966). Helv. Chim. Acta 49, 741-748.

Weber, K. and Osborn, M. (1969). J. Biol. Chem. 244, 4406-4412.

Weiland, T. and Rueff, L. (1953). Angew. Chem. 65, 186-187.

Weissberg, J. and Voytek, P. (1974). Biochim. Biophys. Acta 364, 304-319.

Westall, R.G. (1952). Nature 170, 614-616.

Wigfield, D.C., Farant, J.-P. and MacKeen, J.E. (1982). J. Anal. Toxicol. 6, 123-126.

Wilkinson, G.N. (1961). Biochem. J. 80, 324-332.

Willadsen, P. and Eggerer, H. (1975a). Eur. J. Biochem. 54, 247-252.

Willadsen, P. and Eggerer, H. (1975b). Eur. J. Biochem. 54, 253-258.

Wilson, E., Burger, H.P. and Dowdle, E.B. (1972). Eur. J. Biochem. 29, 563-571.

Wolochow, H., Putnam, E.W., Doudoroff, M., Hassid, W.Z. and Barker, H.A. (1949). J. Biol. Chem. 180, 1237-1242.

Wu, W.H., Shemin, D., Richards, K.E. and Williams, R.C. (1974a). Fed. Proc. 33, 1478.

Wu, W.H., Shemin, D., Richards, K.E. and Williams, R.C. (1974b). Proc. Natl. Acad. Sci. USA 71, 1767-1770.

Yamada, M. (1972). Biochim. Biophys. Acta 279, 535-543.

Zeitler, H.-J., Kreutzer, M. and Andondonskata-Renz, B. (1982). Int. J. Biochem. 14, 787-795.