PURIFICATION AND PROPERTIES OF MITOCHONDRIAL δ-AMINOLEVULINIC ACID SYNTHETASE

A thesis submitted for the degree

of

Doctor of Philosophy

by

Malcolm J. Whiting, B.Sc.(Hons.)

from

The Department of Biochemistry,
University of Adelaide.

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Truth of any kind breeds ever new and
better truth.

Carlyle.

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SUMMARY

1. δ-Aminolevulinic acid (ALA) and aminoacetone (AA) synthetases in liver mitochondria from guinea pigs dosed with the drug DDC have been localised as either within the mitochondrial matrix or possibly loosely bound to the inner mitochondrial membrane.

2. A cytoplasmic form of ALA synthetase, accounting for approximately 30% of the total enzyme activity in liver after allowing for leakage from mitochondria, has been demonstrated in guinea pig liver 24 hours after DDC administration. This finding is in agreement with previous reports of extra-mitochondrial ALA synthetase activity in rat liver.

3. Electron microscopy studies of liver mitochondria from DDC-treated guinea pigs showed no specific structural alterations that could be correlated with an increase in ALA synthetase activity or porphyrin production.

4. ALA synthetase, extracted from porphyric guinea pig mitochondria by sonication or freeze-drying was found to be excluded from Sephadex G-200, and other studies revealed that activity was associated with a large aggregate. In contrast, AA synthetase behaved as a soluble enzyme with a molecular weight of 64,000. By treating the aggregate with both NaCl and dithioerythritol, ALA synthetase activity

was released to yield a soluble enzyme of molecular weight 77,000, as determined by Sephadex gel chromatography.

5. The purification of solubilised mitochondrial ALA synthetase was initially attempted from porphyric guinea pig liver. In later studies, a relatively fast, reproducible method, involving Sephadex chromatography, ammonium sulphate fractionation and affinity chromatography and giving a 40-fold purification, was developed for solubilised enzyme from rat liver mitochondria.

6. Some preliminary kinetic studies of the purified enzyme from rat liver mitochondria were carried out, using an assay system containing chemically synthesised succinyl-CoA. True Michaelis constants for glycine and succinyl-CoA were determined as 19 mM and 200 μM respectively. Hemin inhibited the enzyme by 50% at a concentration of 10 μM. The addition of glycine and succinyl-CoA to the enzyme surface was shown to occur by a non-sequential reaction mechanism.

7. The molecular weight values of the solubilised mitochondrial and cytoplasmic forms of ALA synthetase from rat liver, determined under identical conditions by Sephadex chromatography, were 77,000 and 178,000 respectively. Antibodies prepared in rabbits against the purified mitochondrial enzyme cross-reacted with the cytoplasmic enzyme,

although not to the same extent.

The above findings are discussed in terms of the hypothesis that cytoplasmic ALA synthetase is a precursor of the mitochondrial enzyme.

8. Other immunological studies failed to detect the presence of an enzymically inactive, but immunologically reactive precursor of ALA synthetase in liver of normal rats. This supports the proposal that the drug-induced increase in ALA synthetase activity is due to *de novo* synthesis of enzyme.

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STATEMENT

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

MALCOLM J. WHITING

PUBLICATIONS

Whiting, M.J. and Elliott, W.H. (1970)
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ABBREVIATIONS

The abbreviations used in this thesis are acceptable to the Journal of Biological Chemistry, or are defined in the text, with the following exceptions.

AA	aminoacetone
AA pyrrole	2,4-dimethyl-3-acetyl-pyrrole
AIA	allylisopropylacetamide
ALA	δ-aminolevulinic acid
ALA pyrrole	2-methyl-3-acetyl-4-propionic acid-pyrrole
copro	coproporphyrin
cyclic AMP	adenosine-3',5'-monophosphate
DDC	3,5-dicarbethoxy-1,4-dihydrocollidine
hemin	ferric protoporphyrin IX
MW	molecular weight
PBG	porphobilinogen
proto	protoporphyrin
uro	uroporphyrin
M. phlei	*Mycobacterium phlei*
N. crassa	*Neurospora crassa*
N. tabacum	*Nicotiana tabacum*
P. shermanii	*Propionibacterium shermanii*
R. spheroides	*Rhodopseudomonas spheroides*
S. itersonii	*Spirillum itersonii*

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Porphyrins probably originated abiogenically in the 'primitive soup' when oxygen appeared in the Earth's atmosphere at a relatively late stage in evolution [1].

In the beginning period of the existence of life, organisms which were able to use porphyrins as photocatalysts were placed at a selective advantage, since they were then able to utilise visible light as an additional source of energy [2].

As life has evolved, porphyrin derivatives have become an essential part of metabolic processes; the ability to synthesise porphyrins is possessed by almost all species. Cytochromes occur in virtually all aerobic organisms; hemoproteins are vital to the maintenance of normal metabolism in animal cells; chlorophylls are essential for the process of photosynthesis in plants.

This thesis is concerned with studies in mammalian liver of the properties of the enzyme δ-aminolevulinic acid (ALA) synthetase, which catalyses the first step in the biosynthesis of porphyrins and heme. As is discussed later, this step is rate-limiting in the heme biosynthetic pathway in almost all systems studied, and is therefore a logical control point to regulate production of porphyrins and heme.

The following literature review provides background information as an introduction to the field of ALA synthetase and heme biosynthesis. Firstly, some details of this enzyme in various systems are described and our present knowledge of

the other enzymic steps involved in the formation of heme are summarised.

Secondly, factors which operate to regulate the level of ALA synthetase activity in bacterial and animal cells, and control heme synthesis, are discussed. Some suggested molecular mechanisms by which regulation of activity may occur are also presented.

Finally, the 'porphyrias' a group of metabolic disorders characterised by greatly increased formation, accumulation and excretion of porphyrins and/or their precursors, are mentioned, with a discussion on possible sites of genetic defects in the hepatic porphyrias.

1.2. THE ENZYMOLOGY OF THE HEME BIOSYNTHETIC PATHWAY

The study of the biosynthesis of heme began in 1945 when it was demonstrated by Shemin that labelled glycine was incorporated into the heme molecule [3]. Since this time, all of the reactions involved in the heme biosynthetic pathway have been elucidated and demonstrated in a variety of cell-free systems, and studies of the enzymes catalysing the individual steps carried out. A summary of the steps involved in the pathway is shown in Fig. 1-1.

The enzymes are compartmentalised, at least in mammalian liver [4], such that the initial enzyme δ-aminolevulinic acid (ALA) synthetase is in normal conditions detected in mitochondria; the next three enzymes occur in the cytoplasm; and the last two enzymes are again found in the mitochondrion. In fact, recent work has localised ALA synthetase in the matrix of the mitochondrion, and ferrochelatase the last enzyme of the pathway, on the inner mitochondrial membrane [5,6]. Another recent finding is that a cytoplasmic form of ALA synthetase exists in rat liver [7,8]. This form of the enzyme has been proposed to be in transit from its cytoplasmic site of synthesis to the mitochondrion, and this hypothesis and other data relevant to the relationship between the two forms of enzyme will be discussed in detail later in this thesis.

Some of the reactions in the pathway have been characterised in more detail than others, but studies are continuing on all steps in a variety of systems. The purpose

Fig. 1-1.

The porphyrin-heme biosynthetic pathway,

showing the formation of both type I and III series of isomers.
A = acetyl; P = propionyl; V = vinyl; M = methyl.

The enzymes catalysing the individual steps are:

1. ALA synthetase
2. ALA dehydratase
3. Uroporphyrinogen III synthetase
4. Uroporphyrinogen III decarboxylase
5. Coproporphyrinogen III oxidase
6. Ferrochelatase.

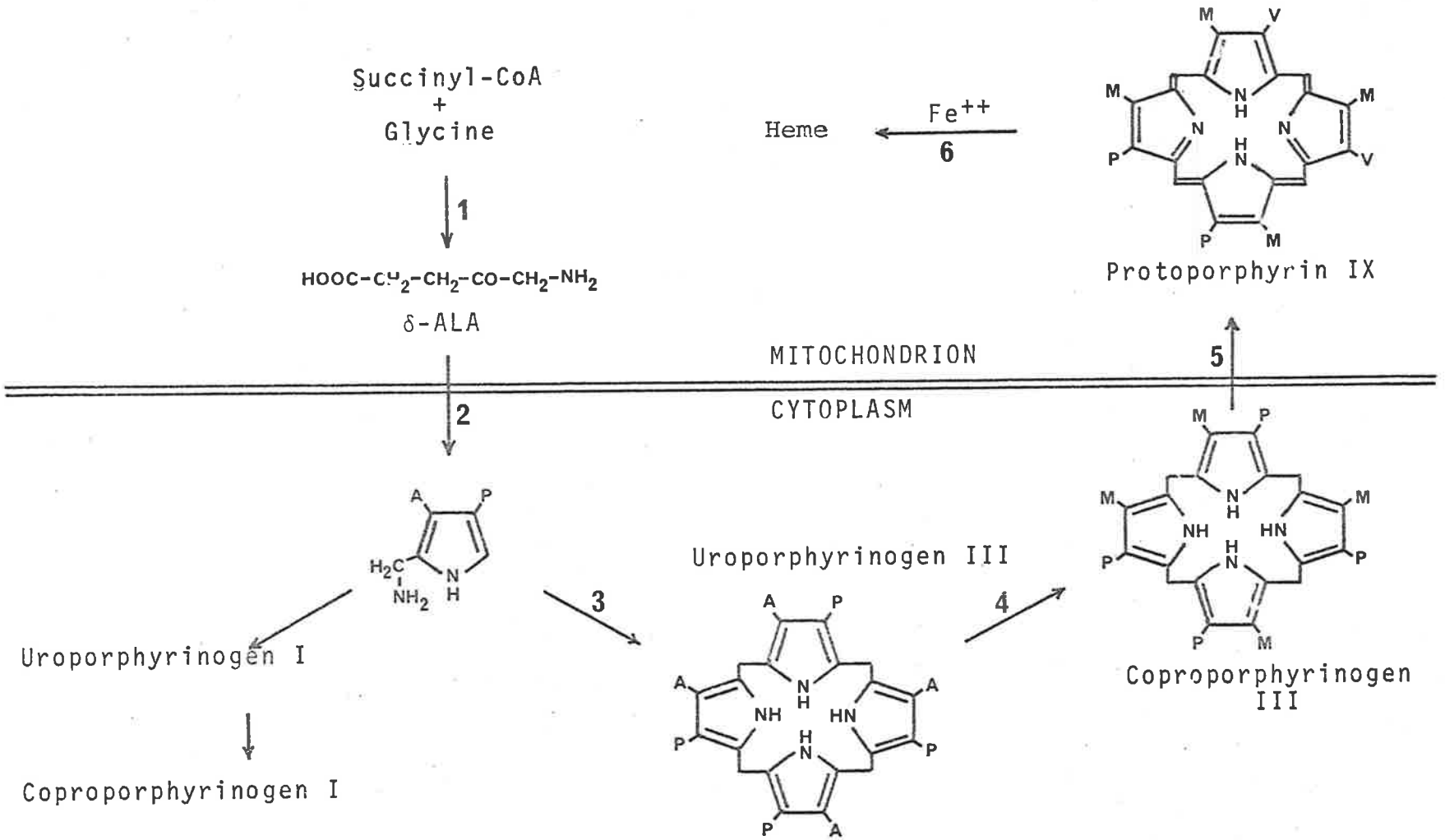

Succinyl-CoA
+
Glycine
1
HOOC-CH2-CH2-CO-CH2-NH2
δ-ALA
Heme
Fe++
6
Protoporphyrin IX
MITOCHONDRION
CYTOPLASM
2
5
3
4
Uroporphyrinogen III
Uroporphyrinogen I
Coproporphyrinogen I
Coproporphyrinogen III

FIG. 1-1

of the following section is to describe what is known about the enzymes catalysing the individual reactions, with emphasis on more recent work that has been published in the last five years. Several excellent reviews are available which cover work until 1967 [9-14], and these are recommended to the reader for a more detailed coverage of earlier work. No attempt therefore has been made, in this section, to refer to all papers published before 1967.

Step 1. δ-Aminolevulinic Acid (ALA) Synthetase (Succinyl-CoA - glycine succinyltransferase)

ALA synthetase, the first enzyme of heme biosynthesis, requires pyridoxal 5-phosphate as a co-factor, and catalyses the condensation of glycine and succinyl-CoA to form ALA, carbon dioxide and CoA.

$$\text{Succinyl-CoA} + \text{Glycine} \rightarrow \text{ALA} + CO_2 + \text{CoA}$$

The enzyme has been detected in cells from a number of sources, including *R. spheroides* [15], *S. itersonii* [16], *P. shermanii* [17], yeast [18], spinach [19], soybean callus [20], *N. crassa* [21], mouse spleen [22,23], bone marrow [24,25], rat Harderian gland [26], mammalian and avian reticulocytes [9], mammalian and avian liver [27,28], and rat kidney [29].

Unless large amounts of porphyrin derivatives, such as bacteriochlorophyll in photosynthetic bacteria, or heme proteins, such as cytochromes in liver and hemoglobin in reticulocytes, are being made by the cell, the level of detectable ALA synthetase is very low. The enzyme has proved to be very difficult to measure in plant systems, and no

significant activity could be detected in chloroplasts and proplasmids competent of making heme and chlorophylls, even using a sensitive radiochemical assay [30]. In many systems which have high levels of ALA dehydratase and other enzymes of the pathway, ALA synthetase cannot be detected.

In all of the above systems, ALA synthetase has been accepted as the rate-limiting enzyme of the heme biosynthetic pathway, with a few exceptions [16,17]. Thus, only small amounts of enzyme may be necessary to meet the porphyrin requirements of the cell. Alternatively, inhibitors may be present in the cell to control ALA synthetase activity, as has been reported for *R. spheroides*, or ALA could be generated by an alternative metabolic pathway in some systems, as discussed by Tait [14].

Although the second enzyme of the heme pathway, ALA dehydratase, may be of regulatory importance in a few cases [16-18,21,31,32], the formation of ALA represents the control step in heme biosynthesis for most systems. This is best illustrated in mammalian and avian liver, since levels of ALA synthetase can be drammatically elevated by a variety of drugs and steroids [27,28,33,34], and this results in an over-production of porphyrins and/or their precursors, such that liver cells fluoresce under ultraviolet light.

Many factors are now known to increase levels of ALA synthetase in liver. These increases are prevented by inhibitors of RNA and protein synthesis [28,35], and are therefore thought to result from new enzyme synthesis. The next section (1.3B) discusses these factors more fully in

terms of the control of heme production.

Although initial attempts at purifying ALA synthetase were frustrated by its instability, this difficulty is now being overcome, and reports have recently appeared on the purification of the enzyme from *R. spheroides* [36,37], rat liver [38] and rabbit reticulocytes [39].

The *R. spheroides* enzyme has been purified 1200 to 1300-fold [36,37] and has a molecular weight of approximately 60,000. Kikuchi's group [36] has reported the existence of two forms of the enzyme, which can be separated on DEAE-Sephadex due to their different charges at pH 7.4. The two forms appear to be subject to different control mechanisms, and increases in enzyme activity can be induced by different stimuli [40]. Also, one form of the enzyme can exist in an inactive state, which has a slightly greater molecular weight than the active enzyme [41]. The presence of a low molecular weight activator and an inhibitor of ALA synthetase in extracts of *R. spheroides* has also been reported [42,44]. These findings are discussed in Section 1.3A.

The purified enzyme from *R. spheroides* is sensitive to end product inhibition by hemin. No evidence of allosteric behaviour was observed in kinetic studies with substrates glycine or succinyl-CoA. The activity of the enzyme in crude extracts was greatly affected by oxidising and reducing reagents, such that potassium ferricyanide markedly stimulated activity, while dithiothreitol was inhibitory. In contrast, no effect of these compounds was observed with purified enzyme [37].

ALA synthetase in the mammalian liver cell exists after drug treatment in the cytoplasm and mitochondria [7,8]. The cytoplasmic enzyme has been purified 200-fold by Scholnick *et al.* [45]. The presence of divalent or monovalent cations was necessary to stabilise and activate the enzyme, and to prevent enzyme aggregation. Sucrose density gradient centrifugation gave a molecular weight for the enzyme of approximately 150,000. While the crude enzyme was inhibited by heme only by high, non-physiological levels, purified cytosol ALA synthetase was inhibited 50% by a concentration of 2.10^{-5} M.

ALA synthetase from rabbit reticulocytes has been purified 4,400-fold to apparent homogeneity by disc gel electrophoresis [39]. The molecular weight of the enzyme from Sephadex G-200 chromatography was around 200,000, and the enzyme was inhibited 40% by hemin at a concentration of 10^{-5} M.

A mechanism for the ALA synthetase catalysed reaction has been suggested from studies with the *R. spheroides* enzyme [46], and is shown in Fig. 1-2. Pyridoxal 5-phosphate is postulated to be bound to the enzyme through the phosphate group by ionic forces, and by a covalent linkage produced by the formation of a Schiff base between an amino group on the enzyme and the aldehyde group of pyridoxal 5-phosphate.

Pyridoxal 5-phosphate then transiminates with the amino group of glycine while still linked to the enzyme. A stabilised carbanion of glycine makes a nucleophilic attack

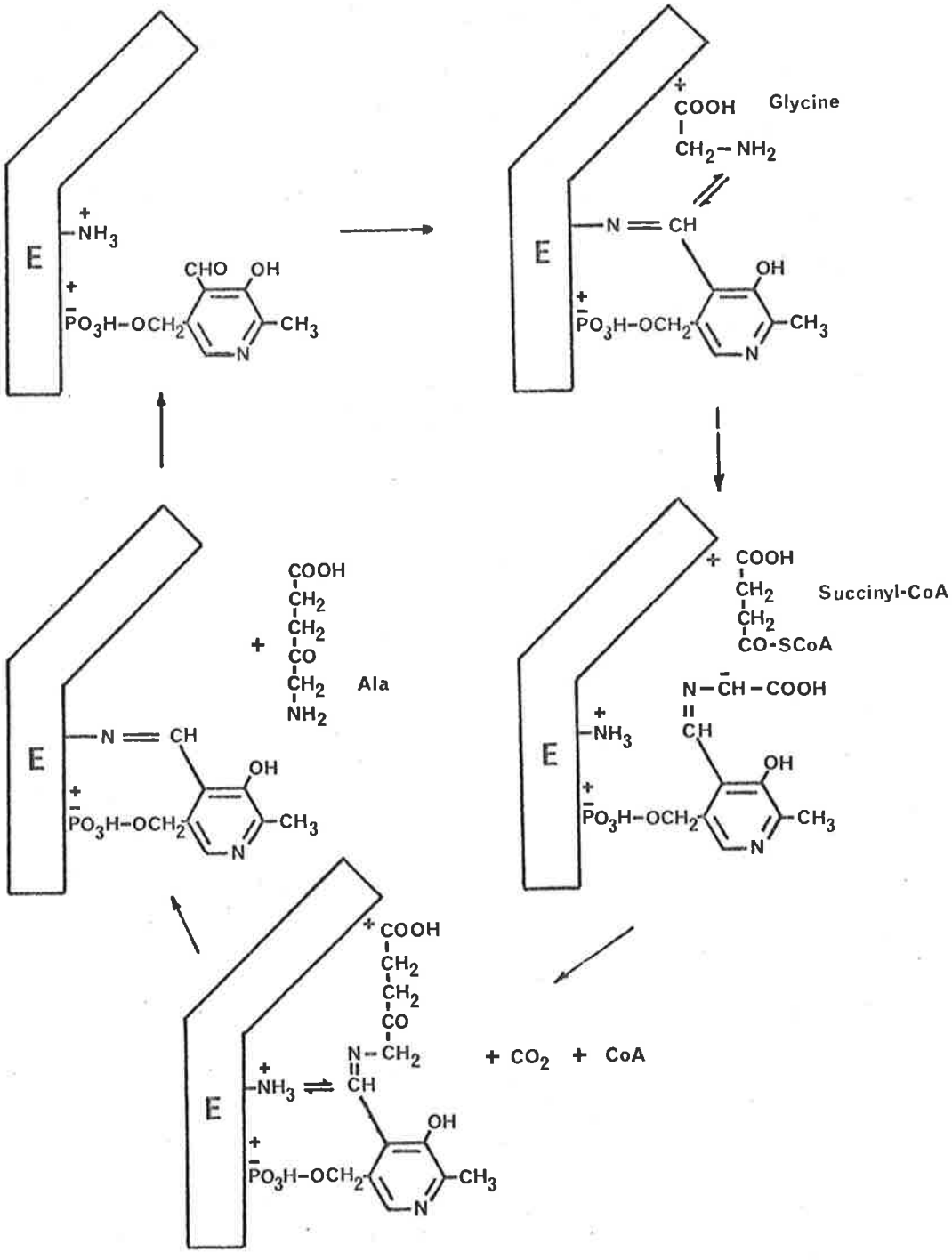


Fig. 1-2.

Proposed mechanism of enzymic synthesis of ALA from glycine and succinyl-CoA by ALA synthetase (based on that of Shemin [46]). See the text for details.

on succinyl-CoA, displacing CoA. The α-amino-β-ketoadipate formed spontaneously decarboxylates and ALA is released from the enzyme by the reverse of the transimination process.

The formation of a carbanion from glycine by stereospecific loss of a proton has recently been demonstrated by Akhtar and Jordan [47,48]. They prepared two species of glycine in which the α-hydrogen atoms were stereospecifically labelled with tritium. On incubating these molecules with enzyme from *R. spheroides* and succinyl-CoA, they found one of the hydrogen atoms was stereo-specifically removed, whereas the hydrogen atom in the other configuration was retained.

The condensation of an acyl-CoA derivative with the α-carbon atom of an amino acid has also been observed in the reaction of acetyl-CoA and glycine to form aminoacetone. The enzyme catalysing this reaction has been demonstrated in chicken reticulocytes [49] and is readily detectable in normal mammalian liver [50]. Some studies have been carried out in this laboratory on the partially purified enzyme from sheep liver mitochondria [51].

More recently, a similar reaction in which palmityl-CoA condenses with the α-carbon atom of serine has been demonstrated in the synthesis of sphingosine [52].

Step 2. δ-Aminolevulinic Acid Dehydratase (5-Aminolevulinate hydro-lyase [adding δ-aminolevulinate and cyclising], E.C. 4.2.1.24)

ALA dehydratase catalyses the condensation of two

molecules of ALA to form the pyrrole, porphobilinogen (PBG). The specific activity of this enzyme in animal and bacterial cells is much higher than that of ALA synthetase, with few exceptions [16].

The enzyme has been purified from a wide variety of sources including cow liver, human erythrocytes, soy bean callus tissue, wheat leaves, yeast and *R. spheroides* (see Tait [14]). More recently the properties of the enzyme have been studied in *S. itersonii* [16], *M. phlei* [53], *N. tabacum* [54] and mouse liver [55], and compared with properties previously reported for the enzyme from other sources.

The enzymes from mouse liver [55] and *R. spheroides* [56] have a molecular weight of 250,000, and consist of six subunits of equal molecular weight (40,000). Thiol groups are known to be an essential requirement for enzyme activity in all systems, but other properties differ with enzyme sources. Inhibition by heme is significant for enzyme from *R. spheroides* [57] and mammals [58], but not from *N. tabacum* [54] or *S. itersonii* [16]. ALA dehydratase of *R. spheroides* has many of the characteristics of an allosteric enzyme [59], but no evidence has been reported to suggest allosteric control of the mammalian or plant enzyme. EDTA is a potent inhibitor of enzyme from mammalian sources [60-62], except that from the mouse [63], while enzyme from *R. spheroides* is insensitive to this compound [15]. Magnesium ions activate enzyme preparations from *S. itersonii* [16], *N. tabacum* [54], and *R. spheroides* [59]. Shemin [46] has

suggested that all dehydratases contain a metal ion, but in some cases, such as for *R. spheroides*, strong binding of the metal prevents its removal by EDTA.

The mechanism of PBG synthesis has been studied using purified enzyme from *R. spheroides* by Shemin and co-workers, and is discussed in detail in a recent review [46].

Step 3. Uroporphyrinogen III Synthetase or Porphobilinogenase

The condensation of four molecules of PBG to form uroporphyrinogen III (or uro'gen III) constitutes one of the most intricate steps in porphyrin and heme biosynthesis. From early work on plant systems [64,65], the enzyme complex involved in this step, which has been termed uro'gen III synthetase, or porphobilinogenase was known to consist of two different enzymes. These are PBG deaminase, which catalyses the conversion of PBG to uro'gen I, and uro'gen isomerase or uro'gen III cosynthetase, which is responsible for the formation of uro'gen III. The two enzymes can be distinguished on the basis of their heat stability, the isomerase being unstable at 60-70°C for short times.

The mechanism of the overall reaction sequence is largely unknown, although hypothetical reaction mechanisms have been proposed (see Tait [14]). Recent work has resulted in the separation and purification of the two enzymes from cow liver [66], avian erythrocytes [67], and cultured soybean callus cells [68], andkinetic studies have been performed on these preparations [66,69] in an effort to elucidate some

aspects of the reaction mechanism.

The mode of action of the isomerase is of particular interest, since alone it has no action on either PBG or uro'gen I. Evidence has now been obtained for a polypyrrolic intermediate, which in combination with PBG, acts as substrate in the formation of uro'gen III by purified isomerase preparations from various sources [70]. A hypothesis which is based on that of Cornford [71], has been proposed for the formation of uro'gens I and III from PBG [68]. This scheme suggests that the isomerase functions at a step after the condensation of two molecules of PBG to form a dipyrrol, but before the insertion of the fourth molecule of PBG. The result is the insertion of a PBG molecule "back the front" in the tetrapyrrole ring to form the type III isomer.

Step 4. Uroporphyrinogen III Decarboxylase

Uroporphyrinogen III (or uro'gen III) decarboxylase catalyses the conversion of the octacarboxylic compound, uro'gen III, to the tetracarboxylic coproporphyrinogen (copro'gen) III by decarboxylation of four acetic acid sidechains to four methyl groups. The enzyme has been studied in plant, animal and bacterial cells, and recently reports have appeared of a 220-fold purification of the enzyme from chicken erythrocytes to apparent homogeneity on polyacrylamide gels [72], and a 21-fold purification from mouse spleen [73].

Since intermediate porphyrins with 7, 6 and 5

carboxyl groups can be detected in incubation mixtures, the decarboxylation reaction has been proposed to occur in a step-wise manner, with the first decarboxylation proceeding more rapidly than the last three. However, only one component with decarboxylase activity has been found in chicken erythrocytes, and it is therefore thought that this enzyme must have more than one active site [72].

The enzyme from mouse spleen decarboxylates uro'gens I and III at the same rate [73], but the type III isomer has been found to be decarboxylated twice as rapidly as the type I in chicken [72] and rabbit [74] reticulocytes, giving some degree of specificity to this enzyme.

Step 5. Coproporphyrinogen III Oxidase

The enzyme catalysing the step Coproporphyrinogen III to Protoporphyrin IX is coproporphyrinogen (copro'gen) oxidase. Two propionic acid chains of the tetrapyrrole, copro'gen III are converted to vinyl groups. The protoporphyrinogen IX so formed is then oxidised to protoporphyrin. The enzyme from liver mitochondria has been purified to electrophoretic homogeneity and has a molecular weight of 80,000 [75]. Studies with this enzyme have shown an absolute requirement for oxygen for the reaction to occur; no other electron acceptor could substitute. This has led to the suggestion that in an oxygenase type reaction, propionic acid side chains are converted into β-hydroxypropionic acid; dehydration then yields acrylic acid and subsequent decarboxylation yields a vinyl group [76]. Indeed, chemically

synthesised 2,4-bis-β-hydroxypropionic acid deuteroporphyrinogen, an expected intermediate for this reaction mechanism, is converted to protoporphyrinogen by the enzyme [76].

Since certain bacteria can synthesise cytochromes or bacteriochlorophylls during growth under anaerobic conditions, a mechanism using another electron acceptor must exist for this oxidative reaction. Tait [77] has shown that extracts of *R. spheroides* grown semi-anaerobically in light can convert copro'gen III to protoporphyrin in the absence of oxygen if extracts are supplemented with ATP, methionine or 5-adenosylmethionine and $MgSO_4$.

Additional studies suggested that under anaerobic conditions a flavoprotein and iron are involved in the removal of hydrogen from copro'gen, and that $NAD(P)^+$ is the final hydrogen acceptor [78].

The aerobic reaction in *E. coli* is thought to be similar to that demonstrated in liver mitochondria, although rather surprisingly the reaction could not be demonstrated in some other heme-containing bacteria [79].

Step 6. Ferrochelatase or Heme Synthetase (Protohaem Ferro-lyase, E.C. 4.99.1.1)

Ferrochelatase catalyses the last step of heme biosynthesis, inserting Fe^{++} ions into protoporphyrin to form heme. The enzyme has been detected in cell-free systems from liver, bone marrow, reticulocytes, yeast and bacteria, and more recently in isolated spinach chloroplasts [80] and plant mitochondria [81]. The enzyme also has a wide

distribution in rat tissues and activity has been assayed in liver, kidney, heart and brain [29].

Ferrochelatase is a particulate enzyme; it is located in mitochondria of animal and yeast cells, and is probably attached to the cell membrane in bacteria. Solubilisation of the enzyme from chicken erythrocyte stroma has been accomplished by detergent treatment, and evidence obtained that phospholipids are important for high enzyme activity [82].

In general, the enzyme is not specific for Fe^{++}, and will incorporate Zn^{++} or Co^{++}, and other metal ions at lower efficiency. Extracts of *R. spheroides* have been shown to contain two ferrochelatases, one being a soluble form and the other particulate [83]. The significance of the soluble form is uncertain.

Other studies have shown that particulate *R. spheroides* ferrochelatase is sensitive to inhibition by magnesium protoporphyrin and heme [83]. Since heme is known also to inhibit ALA synthetase and ALA dehydratase in this organism, the regulation of heme and chlorophyll synthesis by feedback effects is now complex. Heme has also been reported to inhibit rat liver mitochondrial ferrochelatase [84].

1.3. THE CONTROL OF THE HEME BIOSYNTHETIC PATHWAY

Under normal metabolic conditions, porphyrin and heme biosynthesis is efficiently regulated and very little accumulation of porphyrins or the precursors, ALA and PBG, occurs. However, the controls which operate on the pathway can be interfered with by various treatments, leading to disordered metabolism and over-production of porphyrins and their precursors. The study of how these disorders are brought about can provide information on the mechanisms of normal control processes that occur *in vivo*.

As described in Section 1.2, the focal point of control of flux in the pathway is ALA synthetase, because it is the rate-limiting step in almost all systems studied, and therefore regulates porphyrin production.

The following section is mainly concerned with factors that increase or decrease the amount of ALA synthetase in the cell. An increase is defined as 'induction', and a decrease as 'repression'; these terms are used without any mechanistic implications of how the effects are brought about.

To illustrate the types of control known to operate, three cell types in which levels of ALA synthetase activity can fluctuate in response to various stimuli are described. These are the bacterial cell, *R. sphaeroides*, the mammalian and avian liver parenchymal cell, and the erythroid cell.

1.3 A. Control of ALA Synthetase Activity in *R. spheroides*

R. spheroides is a photosynthetic bacterium which has been used in studies on the regulation of tetrapyrrole biosynthesis because under appropriate conditions of growth, bacteriochlorophyll is synthesised in large amounts, as well as heme. The first step leading specifically to bacteriochlorophyll production involves the incorporation of Mg^{++} into protoporphyrin; an alternative to heme formation which is not present in animal cells thus exists for the metabolic fate of protoporphyrin. Bacteriochlorophyll synthesis by far predominates under anaerobic-illuminated conditions of bacterial growth, but its synthesis is inhibited by high aeration or by high light intensity. ALA synthetase has been suggested as a major locus of these effects since the synthesis of this enzyme is repressed by oxygen or light [85,86].

Recent studies on the role of ALA synthetase in the regulation of tetrapyrrole synthesis in *R. spheroides* reveal a complex situation. The purification of the enzyme [36] has resulted in the isolation of two forms of enzyme, and these appear to be subject to control by different environmental conditions [40]. Levels of form 1 were greatly increased by anaerobic growth of cells, but levels of form 2 were increased only when cultures were illuminated as well as incubated anaerobically.

While these increases are due to new protein synthesis [40], the activity of existing enzyme also appears subject to several control mechanisms. Form 1 has been

shown to be present in active and inactive states [36,41]. The enzyme in the inactive state was found to be slightly larger (MW 100,000) than the active enzyme (MW 80,000). Conversion to the active state was accomplished by a protein component from *R. spheroides*, or by a protein fraction from rat liver mitochondria [41], and is therefore thought to involve some enzymic modification of the ALA synthetase molecule.

Another type of control is revealed by the studies of Marriott *et al.* [43,44]. They reported an unusual spontaneous activation of ALA synthetase in extracts of cells grown semi-anaerobically, when stored at 4°C for 1 hr. This effect was dependent on the cell concentration of the suspensions used, and further studies indicated a labile, heat-stable activator of low molecular weight was involved. Oxygenated cells were shown to contain a low molecular weight inhibitor which prevented the activation, and a specific reversible inhibitor of ALA synthetase with somewhat similar properties was also reported by Tuboi *et al.* [42]. The structures of the activator and inhibitor, and the nature of their interaction with ALA synthetase is still unknown. A similar activation phenomenon has also recently been reported for ALA synthetase in a plant system [20].

As well as these findings having possible regulatory significance, heme is known to affect ALA synthetase activity in *R. spheroides* in two ways. Firstly it is a potent feedback inhibitor [15,37]; secondly, as well as decreasing the activity of ALA synthetase present, heme is known to

repress the synthesis of this enzyme [86,87]. Thus many factors are now known to control the amount and activity of ALA synthetase in *R. spheroides*.

1.3 B. The Control of ALA Synthetase Activity in Liver

Studies on the role of ALA synthetase in the production of porphyrins and heme in liver began in 1963 when a dramatic increase in hepatic ALA synthetase activity was detected in guinea pigs dosed with the drug, DDC [25]. Solomon and Figge [88] had previously reported that this chemical disturbed normal porphyrin metabolism, producing a marked increase in hepatic concentrations of protoporphyrin and coproporphyrin.

Since this time, much work has been carried out on the study of the control of ALA synthetase production in liver. A lot of the progress made in this field is due to the development of an *in vitro* system by Granick [28]. Using chick embryo liver cells in culture, ALA synthetase could be measured in a semi-quantitative fashion indirectly by porphyrin fluorescence, since ALA synthetase is rate-limiting. Many compounds could thus be quickly tested for an effect on ALA synthetase activity by their inclusion in the growth medium. Other advantages of this system are the knowledge that compounds under test are having a direct action on the liver cell, and that useful experimental manipulations with the cells, which are not possible using *in vivo* systems, can be carried out. The validity of this technique has recently been confirmed by

direct assay of ALA synthetase in large-scale cultures [89-91].

Attempts to obtain an *in vitro* system in mammalian liver whereby ALA synthetase levels can be altered under well-defined conditions have been initiated in this laboratory. The results of studies using isolated perfused rat liver were reported recently [92,93]. Many factors and compounds are now known to affect, directly or indirectly, the amount of active ALA synthetase present in the liver cell. These may be classified as various drugs, steroids, heme, glucose, vitamin E, lead, and ferric citrate.

Studies describing the effects of each of these compounds in turn on ALA synthetase activity in liver, and their possible mechanisms of action, will now be discussed.

Factors Affecting the Level of ALA Synthetase Activity in the Liver Cell

(i) Drugs

From studies *in vitro* [28,91,94] and *in vivo* [27,33], a wide variety of foreign chemicals are known to increase the level of ALA synthetase in mammalian and avian liver cells. The 'experimental porphyria' produced by these compounds has attracted great interest because of its resemblance biochemically to human porphyrias, genetically determined diseases in man [see Section 1.4]. Also, a large number of the inducing drugs have been implicated as causing attacks of porphyria in man.

The increase is prevented by inhibitors of RNA

and protein synthesis [28,35], and is thought to be due to increased synthesis of enzyme, rather than decreased degradation, since inducing chemicals do not affect the half-life of ALA synthetase in the chick embryo liver cell system *in vitro* [89]. Also, an attempt to distinguish between these two possibilities has been made using mathematical models [95,96], and using a half-life for rat liver ALA synthetase of 65-70 min [35,95]. However, conclusive evidence regarding these points awaits the preparation of a specific ALA synthetase antibody for direct measurement of enzyme levels using established radioisotopic techniques.

The chemical structures and steric configurations of the inducing chemicals seem unlimited in their diversity, and it is very difficult to envisage a common site of action for such a wide range of compounds. Marks *et al.* [97-100] have searched for a relationship between chemical structure and 'porphyrin-inducing' activity by synthesising various analogues of well-known inducers, and suggest a critical feature of inducing compounds is an ester or amide group which is sterically hindered from hydrolysis. De Matteis [94] has suggested that a correlation exists between lipid solubility and porphyrinogenic activity of inducing compounds. There is now evidence that all drugs do not have similar effects. This had been indicated earlier by the finding that different patterns of porphyrins and porphyrin precursors were produced in experimental animals by different drugs. For example, DDC caused a very much

greater accumulation of protoporphyrin in livers of rabbits [101] and mice [102] than did AIA.

Recently, Sassa and Granick [89] have proposed that these two compounds stimulate ALA synthetase synthesis at different points in the protein biosynthetic machinery. From studies with inhibitors of RNA and protein synthesis, DDC appeared to act at a transcriptional level, while AIA acted at the translational level.

The association of other metabolic changes with experimental porphyria in animals, including the stimulation of synthesis of several other liver enzymes, indicates that a drug-induced increase of ALA synthetase may be only one aspect of a more basic alteration of liver metabolism involving protein biosynthesis. Although studies with radioactive orotic acid and leucine in tissue culture showed that AIA did not bring about a detectable change in the overall rates of RNA and protein synthesis [28,29], AIA is known to cause many metabolic changes within the liver when administered *in vivo*. A list of these changes includes an increase in liver size with hypertrophy of the smooth endoplasmic reticulum [103,104]; increased liver protein synthesis [105], increased fatty acid synthesis [106], increased cholesterol synthesis [107], increases in the enzymes tyrosine aminotransferase [104,108,109], tryptophan pyrrolase [109,110], NADPH-cytochrome C reductase [111], glucose-6-phosphate dehydrogenase [111] and ALA dehydratase [35,111], and at later times, increases in the drug metabolising enzymes [104,105,112].

Other effects that have been observed are increased microsomal heme breakdown [113-115], increased levels of the heme-binding serum glycoprotein hemopexin [116], and behavioural changes [117].

Elder in this laboratory has also shown that AIA given to rats can depress RNase activity in microsomal and cytoplasmic liver fractions [118]. Similar findings have been reported in phenobarbital-treated rats [119-121]. Further studies have shown, however, that the induction of ALA synthetase precedes any significant change in RNase activity, so the two effects appear unrelated. Some of the other broad effects of AIA on protein synthesis could possibly result from increased protein synthesis if this depression results in decreased m-RNA breakdown. Nevertheless, it is clear that levels of ALA synthetase rise very quickly, and at early times there is a striking increase in this enzyme as compared with other proteins.

Increased heme production would be expected to result after drug treatment, and this is indeed the case as shown by Marver [122] for the compounds phenobarbital and AIA. However, the question arises as to what is the function, if any, of the increased heme produced. It now seems likely that the majority (around 70%) is involved in the synthesis of the microsomal cytochromes, notably cytochrome P-450 [122], with some heme being devoted to mitochondrial cytochrome formation [123]. This leads to an interesting control mechanism since cytochrome P-450 is the hemoprotein considered to be the terminal oxidase involved

in drug metabolism. Increased levels of ALA synthetase may then be required after drug administration to produce increased heme, for incorporation into cytochrome P-450. The stimulation of certain hepatic microsomal drug oxidations, and subsequent detoxification and excretion of the drug then occurs.

This relationship between increased heme synthesis and increased drug metabolism has been studied by Baron and Tephly using the drugs phenobarbital and benzpyrene [124-128]. Their findings support the above concept in that when heme synthesis is partially blocked by 3-aminotriazole, induction of cytochrome P-450 by phenobarbital is impaired; that is, increased cytochrome P-450 synthesis requires increased heme synthesis. However, De Matteis and Gibbs [129] have recently published evidence suggesting that the stimulation of the drug-metabolising system and an increase in ALA synthetase are likely to result from different actions of the drugs.

If a primary effect of drugs is to increase ALA synthetase synthesis, what are the molecular mechanisms involved in this process? It is difficult to formulate a unifying hypothesis because of the structural diversity of the drugs, as already described. Granick has proposed a scheme based on our knowledge of gene expression in bacterial cells [see Section 1.3B (iii)]. An alternative theory that avoids this difficulty is that all drugs may interfere with the metabolism of a physiological compound which is the true inducer of ALA synthetase. Steroids

are known to be alternative substrates to drugs for the drug metabolising enzymes [130,131] and it seems possible that drugs might compete with steroids for the microsomal enzymes, increasing the concentration of an endogenous steroid to a level sufficient for induction at ALA synthetase. The next section discusses the role of steroids in the induction process.

One other hypothesis to briefly mention is that of Labbe *et al.* [132-134], who have proposed that the concentration of NADH or the NADH/NAD^+ ratio of the cell is involved in the control of the level of ALA synthetase. They have correlated the ability of various compounds to inhibit NADH oxidase, prepared from beef heart mitochondria, and their ability to produce porphyrin accumulation in chick embryo liver cells [133]. Also, Gadjos and Gadjos-Török [135] have suggested intracellular levels of ATP may be important in controlling the expression of hepatic ALA synthetase.

(ii) Steroids

Initial studies by Granick [28] showed that in addition to various drugs, some sex steroids, such as progesterone, testosterone and estradiol, were moderately active in inducing porphyrin synthesis in chick embryo liver parenchymal cells in culture. None of the corticosteroids tested was active.

Further work showed that some metabolites of testosterone and progesterone were much more potent inducers of porphyrin formation than were the original steroids

themselves [34,136,137]. The induction was sensitive to inhibitors of RNA and protein synthesis and therefore probably represented new enzyme synthesis. Investigations of the structural requirements for activity revealed only steroids with 19 or 21 carbon atoms and a 5β-H configuration (A:B cis) were potent inducers.

That the porphyrin-inducing action of these steroid metabolites was mediated through the induction of ALA synthetase was confirmed by assaying the activity of the enzyme in whole livers from 16-day-old chick embryos after treatment with the steroids [138]. Derivatives of progesterone used as contraceptive steroids are also active in this system [139].

On the basis of these findings, it has been suggested that steroids may be the physiological regulators of heme biosynthesis in man. This idea is attractive because the episodic, spontaneous nature of acute intermittent porphyria, and its relation to puberty, pregnancy and the menstrual cycle can then be explained in terms of an endocrine imbalance in affected individuals (see Section 1.4).

Against this hypothesis, is the finding that 5β-H steroids do not increase ALA synthetase activity *in vivo* in mammalian liver [138]. Granick has postulated that additional mechanisms may exist for controlling heme formation in mammalian cells, but recent work in this laboratory using isolated perfused rat liver has shown that progesterone and a 5β-H metabolite of this steroid, pregnanolone, are good inducers of ALA synthetase in this

system [92,93].

As well as sex steroids, the corticosteroids may play an indirect role in the induction of ALA synthetase *in vivo*, since the drug, AIA, administered to adrenalectomised rats, only produced a small rise in hepatic ALA synthetase activity [140]. When hydrocortisone was injected into these rats in addition to AIA, the levels of ALA synthetase activity reached the normal high value obtained in intact rats with AIA alone. Also, Matsuoka *et al.* [141] have reported that hydrocortisone greatly stimulated the increase in ALA synthetase activity in rats after AIA treatment, but hydrocortisone alone had no affect.

This apparent 'permissive' role of hydrocortisone in ALA synthetase induction *in vivo* is not understood.

(iii) Heme

While both drugs and steroids increase the amount of ALA synthetase present in the liver cell, heme appears to repress or decrease the synthesis of this enzyme. Studies *in vivo* [110,142-144] and *in vitro* [28,89-91,136,145] have shown that when heme (or hemin) is administered with an inducing chemical, such as AIA, the effect of the AIA is nullified and very little increase in ALA synthetase activity results. This effect does not appear to be due to heme altering the stability of the enzyme [89]. Some other metalloporphyrins also prevent induction [28,136,146].

Although end product inhibition by heme may be a controlling factor of ALA synthetase activity in liver

[8,45], this mechanism alone cannot explain the decreases of ALA synthetase activity produced by heme, since it is apparent that a reduced amount of enzyme results from its effect on cells. A molecular mechanism to explain heme repression of ALA synthetase has been proposed by Granick [28,137]. This model, as shown in Fig. 1-3, is based on the Jacob-Monod scheme for gene regulation in bacteria, and also accounts for drug- and steroid-mediated induction of ALA synthetase.

The operator of the structural gene for ALA synthetase is proposed to bind a repressor protein, which prevents transcription of the gene and the production of m-RNA. The repressor protein is made up of an apo-repressor protein, which is synthesised from a regulator gene, and a co-repressor, heme, which enables the apo-repressor to bind to the operator site. Drugs and steroids induce ALA synthetase formation by competing with heme for, or displacing heme from, its binding site on the apo-repressor protein. This allows production of ALA synthetase mRNA, and its translation to form active enzyme.

The intracellular concentration of heme thus determines whether the operator site is open or blocked, and therefore whether transcription and translation occur. If heme is utilised in the synthesis of various liver hemo-proteins, such as cytochrome P-450, or is degraded to bile pigments, the concentration will fall to a sufficiently low level to allow 'de-repression' of the ALA synthetase gene, synthesis of ALA synthetase and formation of more heme.

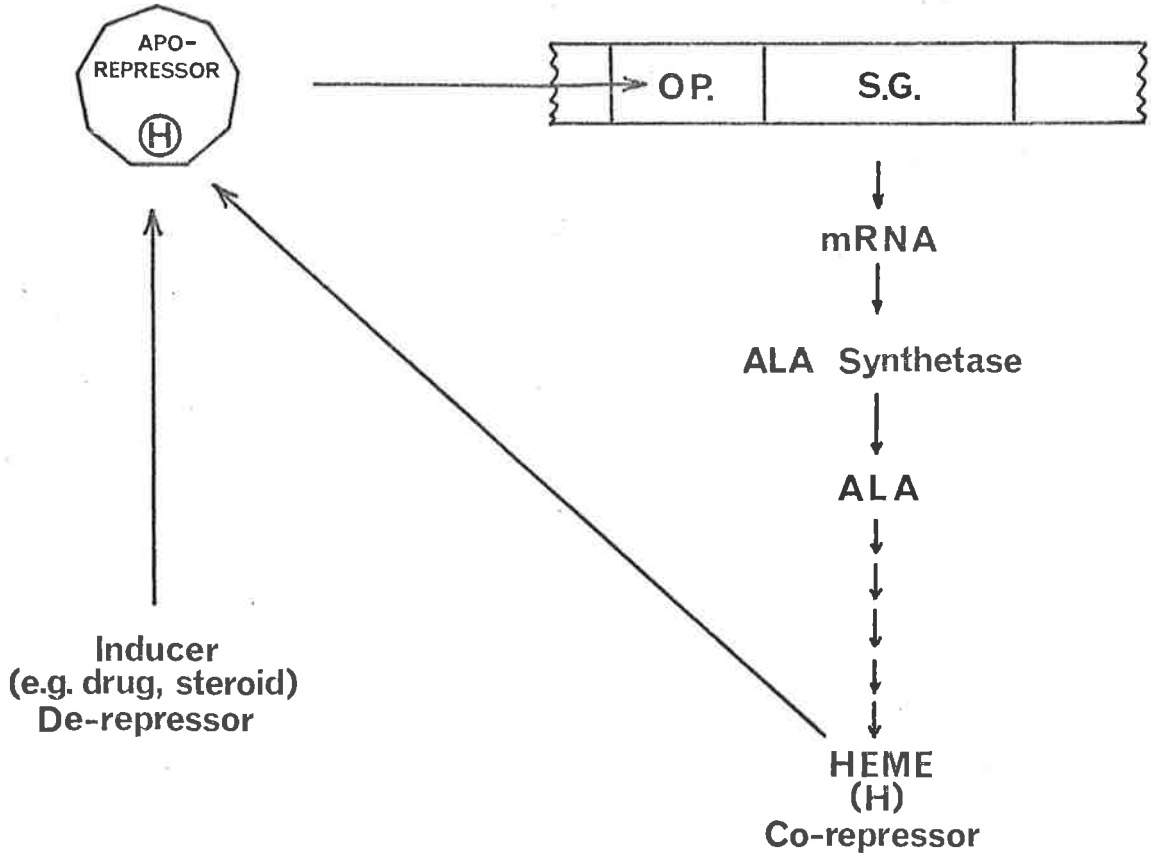


Fig. 1-3.

Control mechanism for heme synthesis,

postulated by Granick [28,137]. Details are discussed in the text.

From this type of control, it can be predicted that the level of ALA synthetase might oscillate between certain limits, and cyclic variations of ALA synthetase activity have been observed in rat liver after the administration of heme [143], and in yeast [18].

While satisfactory as a working hypothesis, this scheme is difficult to test in animal cells. From more recent work, Sassa and Granick [89] have suggested heme may act at a translational level, rather than at a transcriptional level. Also, Marver [122] has shown that the phenobarbital induction of microsomal proteins and enzymes in rats is also repressed by heme. Thus, ALA synthetase may not be the only enzyme induced by drugs and repressed by heme.

Further complications have arisen following work from Kikuchi's laboratory [147,148]. Administration of hydrocortisone with AIA and hemin to rats prevented the repression of ALA synthetase activity observed in the absence of hydrocortisone. Also, hemin appeared to affect the distribution of enzyme between mitochondrial and cytosol fractions from liver.

In spite of the complexity of the role of heme, its concentration in the liver parenchymal cell emerges as a critical factor in controlling the level of ALA synthetase activity, and it therefore seems relevant to now consider how heme is degraded.

Heme is catabolised via biliverdin to bilirubin. Since the bilirubin formed is almost entirely the IXα

isomer, it is generally accepted that the initial step of heme catabolism involves cleavage specifically at the α-methine bridge of heme to yield biliverdin IXα.

Controversy exists over the mechanism of this specific cleavage *in vivo*. A microsomal heme oxygenase, which is the rate-limiting enzyme in the oxidative breakdown of heme to bilirubin and bile pigments, has recently been described and characterised [149-152]. The precise cellular location for the reaction catalysed is disputed, but the reticuloendothelial system seems to be a primary site, since high heme oxygenase activity is found in spleen and bone marrow [153]. Other cell types may play a role in heme catabolism. For example, radioactive hemin was shown to gain access to hepatic parenchymal cells, where it was localised in the microsomal fraction [122]. Also, the effect of hemin on ALA synthetase formation in cultured cells [28,89-91,136,145] shows that it must be at least partly taken up by parenchymal cells. The significance of this degradative system then, in removing heme from liver parenchymal cells is uncertain.

The existence of a unique heme oxygenase enzyme has been disputed by O'Carra and Colleran [154]. They have presented evidence that hemoproteins themselves are able to cleave heme in the required specific manner. Apo-hemoproteins were envisaged as specific heme oxidases with the heme binding sites being equivalent to active sites. *In vivo* heme breakdown could then occur non-enzymically by coupled oxidation of hemoproteins with ascorbate in the

presence of NADPH.

Another idea is that heme and hemoprotein degradation is mediated by lipid peroxidation. Heme is known to be rapidly decomposed by lipid peroxides [155], and when NADPH-dependent lipid peroxidation was stimulated in rat liver microsomes *in vitro* [156], so was microsomal heme breakdown (including cytochrome P-450).

Although the actual relevance of these mechanisms of heme breakdown to the liver parenchymal cell is uncertain, the potent porphyrinogenic compounds, AIA and DDC or metabolites of these compounds have recently been shown to initially decrease cytochrome P-450 content in mammalian liver [112,115,157-159]. Further studies with AIA have shown increased microsomal heme turnover, with the production of 'green pigments' in liver, is the cause of the decrease [115,160]. The mechanism by which these pigments are produced from heme is obscure, but it has been suggested that random cleavage of the four methine bridges of free heme would give rise to a mixture of unphysiological biliverdin isomers, that would not be converted into bilirubin [154,160]. These could be the 'green pigments' that accumulate.

In any case, increased microsomal heme turnover in liver caused by AIA (and probably DDC) may be related to the observed increases in ALA synthetase activity. Heme might be diverted from a repressor function, allowing increased ALA synthetase production [115,160]. This may explain why other drugs, such as phenobarbital, induce only slight increases in ALA synthetase compared with AIA and

DDC. Phenobarbital does not decrease cytochrome P-450 levels in liver [112,124], and the stimulation of ALA synthetase induction by decreased hepatic heme levels thus may not occur.

Another possible way DDC may decrease hepatic heme levels, leading to ALA synthetase induction, is by inhibiting the conversion of protoporphyrin to heme [128,161].

In summary, heme plays a very important central role in the regulation of ALA synthetase production in liver, and many compounds may be interpreted as having their observed effects on ALA synthetase activity by altering hepatic heme levels, either by affecting its rate of synthesis, or its rate of breakdown.

(iv) Glucose

In experiments measuring the increase of ALA synthetase in rat liver produced by the drug, AIA, Tschudy *et al.* [33] found that simultaneous administration of glucose markedly depressed the observed increase in activity. This phenomenon became known as the 'glucose effect' because of its superficial similarity to that of catabolite repression in bacteria, and explained why it was necessary to starve animals for at least 24 hr to produce chemical porphyria.

Later studies [35] showed that glucose administration 3 hr prior to or together with AIA was as effective in blocking the induction of ALA synthetase as actinomycin D. When glucose was given to rats after ALA synthetase had been induced by AIA, the activity of the enzyme declined rapidly. However, glucagon injection of rats failed to increase the

level of ALA synthetase [35], although a small increase in enzyme activity has been detected in chick embryo liver after glucagon treatment [162].

The 'glucose effect' may not be restricted to experimental porphyria since high carbohydrate diets have proved to be a good therapy for the human disease, acute intermittent porphyria [163]. Because of complex hormonal interactions that occur *in vivo* upon glucose administration, 'glucose repression' is best studied *in vitro* to elucidate the compounds that are directly affecting the liver. Granick found that glucose, glucagon and insulin had no effect on AIA-induced fluorescence in chick embryo liver cells [28]. However, UDP-glucuronic acid did prevent steroid induction of porphyrin production *in vitro*, and Granick [136] has suggested that perhaps *in vivo*, glucose is converted to UDP-glucuronic acid, leading to increased glucuronidation and thus inactivation, of inducing substrates.

Studies in this laboratory using the perfused rat liver by Edwards and Elliott [92,93] have shown the addition of glucose to the perfusion medium does not repress increases in ALA synthetase activity. However, insulin did repress by 40% the progesterone-induced increase of ALA synthetase, and cyclic-AMP at a concentration of 10^{-4} M was a potent inducer of enzyme activity.

Thus, glucose may be having its observed *in vivo* effect on ALA synthetase in mammalian liver by alteration of insulin levels, which in turn would affect cyclic-AMP levels. Catabolite repression in bacteria is now known to be mediated

through cyclic-AMP [164] as probably is glucose repression of the inducible mammalian liver enzyme serine dehydratase [165].

(v) Vitamin E

The induction of experimental porphyria in rats by AIA has recently been shown to be prevented by prior administration of vitamin E [166]. This control is mediated by inhibiting increases in the activities of ALA synthetase and ALA dehydratase. On the other hand, vitamin E-deficient rats have decreased ability to synthesise heme in the liver [167], but show increased susceptibility to the induction of ALA synthetase by AIA.

On the basis of these results, Nair *et al.* [166] have suggested two forms of ALA synthetase exist; a normal constitutive enzyme being vitamin E-dependent, and an inducible enzyme, the synthesis of which is inhibited by vitamin E.

Thus vitamin E appears to play a role in the regulation of heme synthesis. In fact, a remission in human porphyria has been observed after vitamin E treatment [168]. An interesting possibility is that since vitamin E is an inhibitor of lipid peroxidation [169], it may have its effect by preventing heme breakdown, leading to repression of ALA synthetase synthesis.

(vi) Lead

Lead poisoning is regarded as a disorder of heme

synthesis, since porphyrins and their precursors are over-produced and excreted in this condition (see [170,171] for reviews). Heme synthesis in both hepatic and erythropoietic cells is affected, and an anemia is often associated with lead poisoning.

Lead has been proposed to affect the activities of sulfhydryl enzymes of the heme pathway, in particular ALA dehydratase and ferrochelatase, producing the observed pattern of porphyrin excess. Although it might be expected that an increase in ALA synthetase activity would occur if heme production was reduced sufficiently to relieve heme repression in liver, Gibson and Goldberg [172] did not find a significant increase in liver from lead-poisoned rabbits. Also, Stein *et al.* [108] found no increase in hepatic ALA synthetase levels in rats dosed with lead acetate for 5 days, although ALA dehydratase levels were reduced.

These results contrast with recent findings that intravenous administration of lead acetate to mice resulted in a depression of heme synthesis and a 3-fold increase of ALA synthetase activity in 24 hr [173] and that 10^{-3} M lead acetate can increase ALA synthetase activity in the perfused rat liver [92,92]. Also, it has very recently been demonstrated that lead acetate can increase ALA synthetase activity in cultured chick embryo cells [91].

(vii) Ferric Citrate

Orally administered ferric citrate was shown to result in a marked synergistic effect on the induction of hepatic ALA synthetase produced by AIA in rats [108]. Ferric citrate alone produced little or no induction. The increased enzyme levels were thought to be due to increased synthesis, but the mechanism involved is uncertain. One possibility is that since ALA dehydratase levels in livers of rats given AIA and ferric citrate were less than levels in rats given AIA alone, this enzyme may become rate-limiting for heme production, resulting in de-repression of the ALA synthetase gene, as already discussed [see section 1.3B (iii)]. Also, heme degradation may be increased by ferric citrate, since ferric chloride has been shown to stimulate lipid peroxidation and microsomal heme breakdown [150].

1.3 C. Control of ALA Synthetase Activity in Erythroid Cells

ALA synthetase is the rate-limiting step in heme formation in erythroid cells, as well as in liver [175,176]. Levere *et al.* [177], using cultured chick blastoderms as a source of erythroid cells, have shown that steroids with a 5β-H configuration stimulate hemoglobin formation by increasing ALA synthetase activity. These 5β-H steroids have recently been shown to have erythropoietic activity in mice [178,179] and had previously been shown to stimulate ALA synthetase activity in chick embryo liver cells in culture [see section 1.3 B (ii)]. However, unlike in liver

cells, foreign chemicals, such as AIA or DDC, had no effect on heme synthesis in erythroid cells. This suggests differences exist between liver and erythroid cells in the regulatory mechanisms controlling ALA synthetase activity, and studies in mice [180] have confirmed this proposal, since erythropoietic ALA synthetase was increased by hypoxia and erythropoietin, while liver ALA synthetase remained relatively constant.

Other studies with rabbit bone marrow cells in culture have shown that erythropoietin enhances ALA synthetase activity [181], and that this effect may be mediated by cyclic-AMP [182]. Although testosterone may stimulate erythropoiesis by augmenting production of erythropoietin, the effect of 5β-H steroids is not related to this factor [179].

It seems possible then that in erythroid cells, a common mechanism of action of steroid metabolites and erythropoietin in stimulating erythropoiesis is via the induction of ALA synthetase.

1.4. THE PORPHYRIAS: HUMAN DISEASES INVOLVING DISORDERED PORPHYRIN METABOLISM

The porphyrias are regarded as metabolic disorders of porphyrin metabolism in man because increased amounts of porphyrins and/or their precursors are produced and subsequently excreted. They are almost all genetically determined. On the basis of the localisation of the disorders, the porphyrias have been classified into two main groups, erythropoietic and hepatic. The erythropoietic porphyrias consist of two distinct varieties, congenital porphyria and erythropoietic protoporphyria, which differ in the type and amount of porphyrin over-produced. A comprehensive review of the biochemical and clinical features of these conditions has been undertaken by Schmid [11] and others [183-185], and they will not be discussed further here.

The hepatic porphyrias may be subdivided into four classes, on the basis of the clinical features and the patterns of porphyrin and porphyrin precursor excreted, as shown in Table 1-1 [for reviews of the hepatic porphyrias, see 11, 12, 183, 185, 186]. As is summarised in this table, all types are inherited in autosomal dominant fashion, except for symptomatic porphyria, which is thought to be an acquired condition in most cases.

The genetically determined hepatic porphyrias are all characterised by episodic attacks, which may include abdominal pain, constipation, hypertension, and a variety of neurological and mental disturbances. Attacks are

Table 1-1. A Summary of the Hepatic Porphyrias

Type of Hepatic Porphyria	ALA, PBG, Porphyrins Excreted	ALA Synthetase	Other Heme Biosynthetic Enzymes	Inher-itance	Pptd by Drugs?	Clinical Features
Acute Intermittent Porphyria (AIP)	ALA, PBG (U)+	Increased [187,188]	ALA dehydratase inc. [188]; uro'gen synthet. norm. [188], dec. [90,195]	Dominant (Latent cases)	Yes - Barbit-urates, etc.	Episodic with neurological, but no cutaneous symptoms; ass. with pregnancy or menst.cycle
Variegate (or mixed) Porphyria	Copro,proto (F)+; ALA,PBG & porphyrins (U) in attacks	Increased [189,190]		Dominant (Latent cases)	Yes - Barbit-urates, etc.	As for AIP, but cutaneous symptoms observed
Hereditary Copro-Porphyria	Copro (F); ALA, PBG, uro, copro (U)	Increased [191,192]	ALA dehydratase inc. [191]; ferrochelatase norm. [191]	Dominant (Latent cases)	Yes - Barbit-urates, etc.	As for AIP, cutaneous symptoms rarely observed
Symptomatic Porphyria (Porphyria Cutanea Tarda)	Mainly uro (U); some copro (U & F)	Increased [189,190, 193,194]; normal [191]	ALA dehydratase norm. [191] ferrochelatase norm. [191]	Acquired not inherited	Yes - Alcohol estrogen etc.	No neurological symptoms; only dermatological lesions

+ (U) - urine; (F) faeces.

often provoked by barbiturates, sulphonamides, steroids and other compounds. In addition, cutaneous lesions, essentially similar to those seen in symptomatic porphyria, are observed in variegate porphyria. Photosensitivity is probably due to photodynamically active porphyrins accumulating in the skin, but a direct causal relationship between porphyrins and/or their precursors and neurological symptoms has not been established.

As indicated in Table 1-1, an increase in ALA synthetase measured in liver biopsy and autopsy samples has now been reported for all forms of hepatic porphyria, and is most likely the reason for the increased production of porphyrins and their precursors in the porphyrias. Whether the primary genetic lesion in these conditions is a gene directly controlling ALA synthetase production, however, is uncertain.

It has been suggested that, in terms of the Jacob-Monod scheme of genetic regulation, an operator constitutive mutation could produce the observed genetic and biochemical features of the disease [28,196]. However, constitutional differences in the activities of enzymes beyond ALA synthetase would be required to produce the pattern of porphyrin and porphyrin precursor excretion in each of the various types of porphyria, and this does not seem likely.

A second possibility is that mutation has resulted, either directly or indirectly, in partial blocks at various enzymic steps in the heme biosynthetic pathway,

leading to the observed patterns of porphyrin or porphyrin precursor accumulation [186,191]. Also, if intracellular heme levels were decreased because of these mutations, ALA synthetase synthesis could be increased as a secondary effect by release of heme repression. Consistent with this idea, conversion of PBG to porphyrins in liver was found to be decreased by 50% in patients with acute intermittent porphyria in two laboratories [90,195], although earlier studies had not detected such a decrease [188]. Partial enzyme blocks at the appropriate steps for other forms of porphyria have not yet been demonstrated, but this theory is attractive since it can account for the unique biochemical features of each type of porphyria.

Both the above theories cannot explain the neurological symptoms associated with the genetically inherited porphyrias, unless it is assumed that porphyrin precursors produce neurological dysfunction. Early experimental work does not support this idea [197,198], but recent work, which is discussed fully in Chapter 8 of this thesis, has shown that ALA and PBG may yet be responsible for the neurological disorders in porphyria [199].

A third possible site for the genetic lesion is outside the heme biosynthetic pathway, perhaps in the drug metabolising enzyme system [200]. Such a theory need not require that ALA or PBG be neurotoxic since the accumulation of endogenous substances normally metabolised in liver microsomes may result in the production of toxins which affect the nervous system. The induction of ALA

synthetase could result in an unknown way as a secondary effect of this mutation, or be produced directly, also by an altered level of an endogenous compound.

Endogenous substances, or their metabolites, capable of inducing ALA synthetase have been isolated from patients with acute intermittent porphyria by two groups of workers. Firstly, Goldberg *et al.* [201] have identified excessive porphyrinogenic steroid metabolites in the urine of patients with acute intermittent porphyria and have shown the precursor of one of these compounds, dehydroepiandrosterone, can cause significant elevations in ALA synthetase when administered to rats. Also, excessive 17-ketosteroid excretion was detected in two patients in relapse with hereditary coproporphyria, while patients in remission showed normal excretion levels [192].

Secondly, Kappas *et al.* [202] have demonstrated the presence in the plasma of several acute intermittent porphyria patients of a specific substance capable of inducing porphyrin synthesis in chick embryo liver cells in culture. This substance is of low molecular weight, ethanol soluble, heat stable and trypsin insensitive, and could thus possibly be a steroid. Plasma from normal individuals or from porphyric patients in remission was ineffective, but further studies [203] have shown that normal human serum contains a protein component which inhibits porphyrin formation and masks the biological activity of the inducing substance. Thus, normal human serum contains separate factors which may be involved in the regulation

of heme formation in liver, and further studies on the relevance of these factors to acute intermittent porphyria are awaited with great interest.

As well as being studied from a medical viewpoint, porphyria appears to have been of considerable significance historically. Macalpine and Hunter have stated [204] following their investigations into the possible reasons for the reported insanity of King George III that 'a picture unfolded which revealed the purple thread of porphyria running through the royal houses from the Tudors to the Hanoverians, and from the Hanoverians to the present day'.

Thus, they have suggested that George III, and some of his blood relatives, including Mary, Queen of Scots, James VI and I, George IV and Princess Charlotte, suffered from variegate porphyria [204,205]. If correct, the great influence this disease had on English history is illustrated in the following quotation from their book [204]: 'Porphyria may justly be called a royal malady and command the historian's respect. It caused directly two national disasters; the Regency Crisis in 1788 and the catastrophe of 1817, when Princess Charlotte died in childbed with her infant. This tragedy threatened the Hanoverian succession with extinction and left the nation without an heir apparent until the birth in 1819 of Victoria'.

1.5. AIMS OF THIS PROJECT

The molecular events that regulate gene expression in higher organisms are not known, although much effort is being directed at this fundamental problem of biochemistry. Perhaps one of the most advanced systems in the study of how the expression of a specific gene is controlled is that of Tomkins *et al.*, who have conducted experiments with cultured rat hepatoma cells, in which glucocorticoids induce the synthesis of tyrosine aminotransferase without affecting overall cell growth or the synthesis of general cellular protein or RNA [206].

Initial work on the study of induction of ALA synthetase in liver cells by drugs and steroids has shown that this system has features, some unique, which may prove advantageous in any study of the molecular mechanisms involved in the control of gene expression. These are firstly, both intact animals and isolated cells in culture are available to study the induction of ALA synthetase. Secondly, the drug-stimulated increase in ALA synthetase is rapid, and large changes in enzyme activity can be detected in a few hours. This is related to the fact that the half-life of ALA synthetase is 60-70 min [35,95], and that the mRNA for ALA synthetase also turns over very rapidly compared to other enzymes, and has been calculated to have a half-life of 1 to 3 hours [35,141]. Since the time course of approach to a new steady-state level of enzyme is solely dependent on the half-life of the

enzyme [207], the level of an enzyme which turns over rapidly can be altered significantly in short times.

Thirdly, the repression of the enzyme by heme means that different mechanisms may be involved in the control of ALA synthetase gene expression than for other systems. Finally, any studies on the control of ALA synthetase production in liver may be relevant to the hepatic porphyrias, and may suggest new treatments for these diseases.

Work in this laboratory is aimed at eventually understanding, at the molecular level, the details of how the ALA synthetase gene is controlled in mammalian liver. Experiments are being carried out using perfused rat liver to investigate factors that affect levels of ALA synthetase activity in this system [92,93].

As part of this overall study, it is necessary to purify and characterise the enzyme, and gain an understanding of its properties. At the commencement of this project, only preliminary attempts at purifying ALA synthetase had been reported. Studies by Irving [208] had shown that ALA synthetase from guinea pig liver mitochondria was unstable after extraction from mitochondria, and could not be stored for any length of time without loss of activity. The aim of this project, then, was to further investigate methods of purification, with particular attention to the stabilisation of the enzyme. It was hoped that ultimately, the properties of the purified enzyme could be determined, and an antibody prepared against

the purified enzyme for use in immunological experiments, with radio-isotopes to measure directly the rates of enzyme synthesis and degradation before and after drug treatment.

During the course of this work, the existence of a cytoplasmic form of ALA synthetase was reported by other workers [7,8]. This finding was confirmed, and another aim of this project became the investigation of the relationship between cytoplasmic and mitochondrial forms of ALA synthetase in liver.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

All materials used in the work in this thesis are described below, but some methods are described in later chapters, where immediately relevant to the results presented.

2.1. MATERIALS

(a) Chemicals

ATP (disodium salt, 98% pure), CoA (85% pure), GSH and pyridoxal 5-phosphate were products of the Sigma Chemical Co. Solutions of these compounds used in ALA synthetase assays were adjusted to pH 7.4 with NaOH and stored at -15°C.

Also from the Sigma Chemical Co. were DL-α-aminoadipic acid (Grade II, 98% pure), 5'-AMP, 5,5'-dithiobis (2-dinitrobenzoic acid) (Ellman's reagent), dithioerythritol (Cleland's reagent), hemin (ferric protoporphyrin, bovine, type I), α-ketoglutarate, p-hydroxymercuribenzoic acid, pyridoxamine 5-phosphate, tris (Trizma grade), and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide.

Other chemicals used and their sources were DDC, Eastman Kodak; sucrose (A.R.), C.S.R. Co., Sydney; ammonium sulphate (enzyme grade), Mann Research Laboratories; δ-ALA hydrochloride, cyclic AMP, protamine sulphate, N-ethylmaleimide, all from Calbiochem; oxaloacetate, California Corp. for Biochem. Research; γ-aminobutyric acid,

ε-aminocaproic acid, γ-amino-N-valeric acid hydrochloride, all from Cyclochemical; avidin, Nutritional Biochem. Corp., and cyanogen bromide, Ajax Chemicals Ltd., Sydney.

Acetyl phosphate was prepared from acetic anhydride, K_2HPO_4 and LiOH, and twice recrystallised [209]. Solutions of acetyl phosphate were stored at -15°C.

(b) Chromatographic Materials

Sepharose 4B and all grades of Sephadex used were from Pharmacia; DEAE-, CM- and AE-celluloses were from Whatman; hydroxylapatite was from Clarkson Chemical Co. All these materials were handled according to the manufacturers' recommendations.

(c) Isotopes

^{14}C-1,4-succinic acid or ^{14}C-2,3-succinic acid used in radiochemical assays of ALA synthetase was obtained from the Radiochemical Centre, Amersham.

(d) Proteins and Enzymes

Proteins used as markers of known molecular weight in Sephadex chromatography or sucrose density gradient centrifugation were Albumin, bovine Cohn fraction V, Sigma; Catalase, beef liver twice crystallised, Sigma; α-Chymotrypsinogen, crystalline A grade, Calbiochem; γ-Globulin, human Cohn fraction II, Commonwealth Serum Laboratories; Hemoglobin, bovine twice crystallised, Mann Research Laboratories; Lactate Dehydrogenase, rabbit muscle type I, Sigma; and

Ovalbumin, twice crystallised, Mann Research Laboratories.

Enzymes used and their sources were Lipase, porcine pancreas B grade, California Corp. for Biochem. Research; and Snake Venom, Crotalus atrox, Sigma.

(e) Solutions

Ehrlich reagent was prepared by the method of Urata and Granick [50]. To 168 ml of glacial acetic acid were added 40 ml of 70% perchloric acid, 4.0 g of p-dimethylaminobenzaldehyde, and 0.7 g of $HgCl_2$; the solution was diluted to 220 ml with water.

Scintillation Fluid was a solution of 0.3% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-2-(4-methyl-4-phenyloxazolyl)benzene in toluene.

Freud's Complete Adjuvant was purchased from the Commonwealth Serum Laboratories, Melbourne, Australia.

(f) Buffers

PEST buffer for the preparation of mitochondria consisted of 0.1 mM pyridoxal 5-phosphate, 0.1 mM EDTA, 0.25 M sucrose and 5 mM tris-HCl, pH 7.4, and was stored at 4°C.

TP buffer was 0.02 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, pH 7.4.

TDP buffer was 0.05 M tris-HCl, 1 mM dithioerythritol, 0.01 M pyridoxal 5-phosphate, pH 7.6.

Many other buffers were used for column chromatography, and are described in the text.

2.2. PREPARATION OF BACTERIAL ENZYMES

(a) Succinyl-CoA Synthetase (succinate:CoA ligase (ADP); E.C. 6.2.1.5.)

The enzyme was partially purified from *E. coli* (Crooke's strain) by a method based on that of Hildebrand and Spector [210]. Bacteria were sonically disrupted and the extract treated with streptomycin sulphate and ammonium sulphate as described [210], before the redissolved pellet was dialysed against 0.05 M tris-HCl, 0.05 M KCl, pH 7.2 overnight. The enzyme solution was then clarified by centrifugation at 10,000 x g for 10 min, and dispensed into small vials for storage at -15°C. The enzyme, with a specific activity at approx. 14 μmoles of succino-hydroxamic acid synthesised/30 min/mg protein when assayed by the method of Kaufman [211], was stable for many months.

(b) Phosphotransacetylase

This enzyme was kindly supplied by Mr. R.L. Walsh. Crude extracts of enzyme were prepared from *E. coli* (strain B) grown overnight in nutrient broth. The harvested and washed cells, resuspended in 1.5 to 2 volumes of 0.1 M tris-HCl, pH 7.8, containing 0.14 M magnesium acetate and 0.06 M KCl, were disrupted in a French press [51]. The resultant suspension was centrifuged at 105,000 x g for 30 min and the pellet discarded. The supernatant, dialysed overnight against the same buffer, was stored at -15°C in small vials. The average protein concentration of the extracts was

4 mg/ml, and the activity was 500 μmoles acetyl phosphate degraded/15 min/ml solution, as assayed by the method of Stadtman [212].

2.3. ENZYME ASSAYS

(a) ALA Synthetase

ALA synthetase was assayed radiochemically, or colorimetrically based on the method of Irving and Elliott [213]. The following amounts of reagents (μmoles) in a final volume of 1.0 ml were used in the colorimetric assay: tris-HCl, pH 7.4, 50; glycine, 100; potassium succinate, 10; magnesium chloride, 20; CoA, 0.3; glutathione, 2; ATP, 15; pyridoxal 5-phosphate, 1; and succinyl-CoA synthetase, 2 units. EDTA (10 μmoles) was included in assays of the cycloplasmic enzyme. Incubations were carried out at 37° for 30 min. All reagents except CoA and glutathione were adjusted to pH 7.4.

The procedure for the colorimetric estimation of ALA formed was varied according to the source and purity of the enzyme preparation being assayed.

For mitochondria and mitochondrial extracts, the reaction was stopped with 1.5 ml of 0.3 M trichloroacetic acid; 0.5 ml of water was then added and the mixture centrifuged. The protein-free supernatant (2.5 ml) was mixed with 0.75 ml of 1 M sodium acetate, and 0.1 ml of acetylacetone, and heated at 100° for 15 min. After cooling, 1 ml of this solution was mixed with 1 ml of Ehrlich's reagent [50], and the optical density of the solution read

at 552 mμ after 10 min.

To the remainder of the solution was added 0.05 ml of 0.5 M disodium hydrogen phosphate and 0.15 ml of 1 N NaOH, and the mixture ether extracted [28]. The optical density of the aqueous phase was measured as above, and the amount of ALA formed in the assay, corrected for AA contamination, was calculated according to the formula of Granick [28], modified for this assay. When partially purified samples of enzyme were assayed, the ether extraction was omitted, since AA synthetase activity was not present.

To estimate low ALA synthetase activities (0 - 20 mμmole ALA formed), the sensitivity of the assay was increased in the following way. Incubations were stopped with 0.3 ml of 22.5% trichloroacetic acid, and centrifuged. 1.0 ml of supernatant was mixed with 0.25 ml of 2 M sodium acetate and 0.05 ml of acetylacetone, to give a pH of 4.6, and the mixture heated at 100°C for 15 min. When cool, 1.3 ml of Ehrlich's reagent was added and the optical density read at 552 mμ, 10 min after mixing. The extinction coefficient for ALA pyrrole was taken as 5.8×10^4 M [28]. One unit of ALA synthetase activity is defined as the amount of enzyme catalysing the formation of 1 mμmole of ALA in 30 min by this assay.

(b) AA Synthetase

AA synthetase was measured by the procedure of Walsh [51], in which acetyl-CoA was generated enzymically

from CoA and acetyl phosphate. Incubation mixtures contained in 0.5 ml the following reagents (μmoles): tris-HCl, pH 7.4, 25; glycine, 75; acetyl phosphate, 2.5; CoA, 0.21; glutathione, 1; and *E. coli* phosphotransacetylase, prepared as has been described, 0.05 ml.

Mixtures were incubated at 37°C for 30 min and the reaction stopped by the addition of 1.5 ml of 0.2 M trichloroacetic acid. The AA in the protein-free supernatant was then estimated [50]. One unit of AA synthetase activity is defined as the amount of enzyme catalysing the formation of 1 mμmole of AA in 30 min by this assay.

2.4. TREATMENT OF ANIMALS

Male guinea pigs (400 - 500 g) from the Institute of Medical and Veterinary Science, Adelaide, were starved for 40 hr and dosed orally with a suspension of 2 g of DDC in 10 ml of water.

Albino Wistar rats (250-300 g) from the Central Animal House, Waite Institute, Adelaide, were starved for 24 hr, and lightly anaesthetised with ether. 0.5 g of DDC was then administered in 2 ml as a suspension in a 15% (v/v) tragcanth mucilage via a stomach tube.

2.5. PREPARATION OF MITOCHONDRIA

After drug treatment, animals were starved for a further 24 hr, then anaesthetised with ether, killed by heart puncture, and their livers removed and immediately chilled in cold buffer. Mitochondria were isolated and

washed in PEST buffer essentially by the method of Schneider and Hogeboom [214]. After resuspending in TP buffer to a protein concentration of 60 - 100 mg/ml, mitochondria were freeze-dried, unless otherwise stated.

Levels of ALA synthetase activity obtained in mitochondria generally varied from 200-300 units/g liver for guinea pigs, and from 350-600 units/g liver for rats.

2.6. PROTEIN DETERMINATIONS

Estimations of protein were carried out using the Lowry method [215] for samples of purified enzyme, and the biuret method [216] for samples of crude enzyme or mitochondria. Bovine serum albumin was used as standard.

CHAPTER 3

THE INTRACELLULAR DISTRIBUTION OF ALA SYNTHETASE AND ELECTRON MICROSCOPY STUDIES OF PORPHYRIC GUINEA PIG LIVER

3.1. INTRODUCTION

(a) The Intracellular Distribution of ALA Synthetase in Porphyric Guinea Pig Liver

Although it was shown by Granick in 1963 [27] that a dramatic biochemical effect of the drug DDC was a greater than forty-fold elevation of ALA synthetase activity in guinea pig liver mitochondria, the actual distribution of enzyme amongst the subcellular fractions and in particular its intramitochondrial location was not determined.

Later studies on the synthesis of this enzyme in rats demonstrated that ALA synthetase turned over very rapidly, and the half-life for the enzyme has been calculated to be 70 min [35,95]. Since it has been proposed on the basis of turnover rates of 8-10 days for inner membrane components that the inner mitochondrial membrane may turn over as a unit [217,218], the determination of the intramitochondrial location of this enzyme could be relevant to mitochondrial biogenesis. A finding of ALA synthetase being attached to the inner membrane would infer that not all inner membrane components are assembled *en bloc*. Established techniques were therefore used in an effort to localise ALA synthetase to a specific mitochondrial fraction.

It was also originally reported by Granick that ALA synthetase activity could be detected in a high speed supernatant from a porphyric liver homogenate [27]. This possibility of an extramitochondrial form of ALA synthetase was not investigated thoroughly until 1969, when it was

independently reported by two laboratories that the post-mitochondrial supernatant from an AIA-induced rat liver homogenate contained 40-50% of the total enzyme activity [7,8].

In the present work, this claim was tested in guinea pig liver, using DDC as the inducing chemical, and assaying for mitochondrial matrix marker enzymes, citrate synthase and glutamate dehydrogenase, as a control for mitochondrial breakage during preparation of the post-mitochondrial fraction.

During these experiments, the intracellular distribution of the related enzyme, aminoacetone (AA) synthetase, was also determined.

(b) An Electron Microscopy Study of Liver Cells and Isolated Mitochondria Before and After Treatment of Guinea Pigs with DDC

Since the original observation by Granick and Urata [27] that porphyric guinea pig mitochondria increased in diameter by 15% with an increase in the area of the cristae, more detailed histological and electron microscopy studies have been performed on liver from AIA-treated rats [103,219,220]. The most noticeable finding was a proliferation of the smooth endoplasmic reticulum in porphyric rats as compared with controls.

The observation of mitochondrial enlargement became especially interesting when it was reported that the increase of pyruvate carboxylase in diabetic sheep liver

was accompanied by enlargement of many mitochondria to several times their normal size [221]. These mitochondria were fragile and were disrupted to membrane fragments by normal procedures for isolation of mitochondria.

With these studies in mind, guinea pig liver slices and isolated mitochondria, from starved controls and animals dosed with DDC, were compared under the electron microscope.

3.2. METHODS

(a) Glutamate Dehydrogenase Assays

Glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase (deaminating), E.C. 1.4.1.2) was assayed at 30° by a method based on that of Colman and Frieden [222], as modified by Taylor et al. [221]. The reaction mixture contained 100 mM tris-HCl, pH 7.4, 5 mM α-ketoglutamate, 5 mM ADP, 50 mM NH_4Cl, 0.3 mM NADH and enzyme. Controls contained no α-ketoglutarate. One unit of enzyme is defined as the amount of enzyme necessary to form 1 μmole product in 1 min at 30°.

(b) Citrate Synthase Assays

Citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), E.C. 4.1.3.7) was assayed at 30° by a modification of the method of Srere [223], as described by Taylor *et al.* [221], in which the reduction of 5,5'-dithiobis-2-(2-nitrobenzoate) was followed spectrophotometrically at 412 mμ. The control was the rate in the absence of oxalo-

acetate. One unit of enzyme is defined as the amount of enzyme necessary to form 1 μmole product in 1 min at 30°.

(c) Electron Microscopy

Samples were prepared by Miss P.Y. Dyer and examined in a Siemens Elmiskop I electron microscope, with an 80 kV and 50 μm objective aperture.

Membrane fractions were layered on a carbon-coated grid, and negatively stained with 2% (w/v) uranyl acetate.

Liver samples were pre-fixed for 120 min in 2% (w/v) glutaraldehyde, washed, fixed with 1% (w/v) osmium tetroxide, dehydrated in acetone and embedded in araldite. Sections were cut with glass knives, and stained with uranyl acetate and lead acetate. Isolated mitochondrial pellets were treated similarly.

3.3. RESULTS

(a) Enzyme Distribution Studies

(i) Intramitochondrial Localisation

Techniques for the separation of inner and outer mitochondrial membranes have been developed [224,225], and the method adopted was that of Parsons and Williams [224]. This involves the swelling of mitochondria in phosphate buffer to rupture outer membranes and release them from the intact inner membrane containing the matrix.

Using 40 g wet weight of porphyric guinea pig

liver, crude inner membrane matrix and outer membrane fractions were prepared by differential centrifugation and these fractions purified on sucrose gradients. Electron microscopy was used to examine purified fractions for cross-contamination. In Fig. 3-1(a), the characteristic closed vesicle appearance of the outer membrane can be seen. No detectable amounts of inner membrane were found in this fraction. Fig. 3-1(b) shows a sample from the inner membrane-matrix preparation. Clearly seen are mitochondrial images, some in the stage of bursting with protrusions of unfolding cristae. A few outer membranes were found in this preparation.

The crude and purified fractions were assayed for ALA synthetase, AA synthetase and succinyl-CoA synthetase after freezing and thawing, and the results are shown in Table 3-1. For all three enzymes, no significant activity was associated with the outer membrane fraction, and the activity recovered was in the inner membrane-matrix fraction.

Since this method does not separate the inner membrane from the mitochondrial matrix, a portion of the inner membrane-matrix fraction was sonicated for 15 sec, using a Dawe Soniprobe (Type 1130A; 20 Kc/sec) at maximum setting with a current of approximately 4 mamp. This treatment disrupts the inner mitochondrial membrane and releases matrix enzymes, but does not solubilise inner membrane enzymes, such as succinate dehydrogenase or cytochrome oxidase [226,227]. The suspension was then

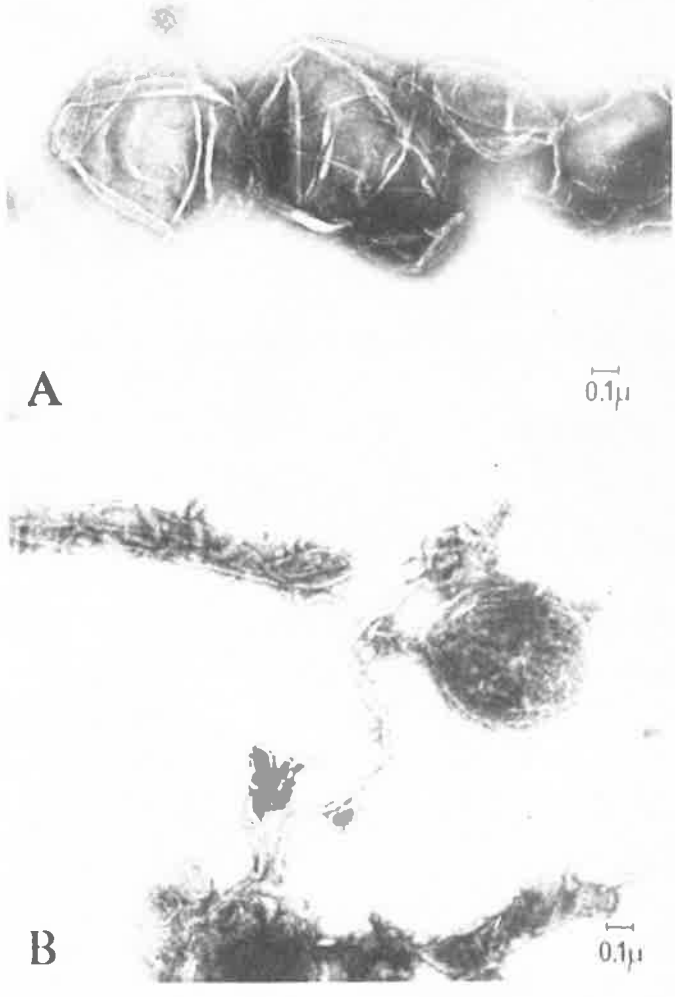


Fig. 3-1.
Electron micrographs of isolated mitochondrial membranes.

A. Outer membrane fraction; B. Inner membrane-matrix fraction.

Table 3-1

The Intramitochondrial Distribution of ALA Synthetase, AA Synthetase and Succinyl-CoA Synthetase in Porphyric Guinea Pig Liver

Fractions were prepared using the methods of Parsons and Williams [224], as described in the text, and were frozen-thawed once before assay. ALA synthetase was assayed using the radiochemical method of Irving and Elliott [213], and AA synthetase as described in Section 2.3; one unit of these enzymes is defined as the amount catalysing the formation of one mμmole of aminoketone in 30 min. Succinyl-CoA synthetase was assayed by the method of Kaufman [211]; one unit of this enzyme is the amount catalysing the formation of one μmole of succinohydroxamic acid in 30 min.

FRACTION	TOTAL PROTEIN (mg)	ALA SYNTHETASE Total Activity (units)	%	AA SYNTHETASE Total Activity (units)	%	SUCCINYL-CoA SYNTHETASE Total Activity (units)	%
Mitochondria	242	3350	100	40400	100	9600	100
Crude Inner Membrane-Matrix	173	2416	68	21440	53	7200	75
Crude Outer Membrane	15	210	12	2800	7	800	8
Purified Inner Membrane-Matrix	160	1488	42	15360	38	4640	48
Purified Outer Membrane	4	80	2	0	0	500	5

centrifuged at 105,000 x g for 60 min, and the supernatant and resuspended pellet fractions assayed for ALA synthetase activity. 75% of the activity recovered was in the supernatant.

This is a similar result to that obtained when whole mitochondria are sonicated and centrifuged. In this case, from 80-100% of the activity measured in the mitochondria may be recovered in the supernatant (see Chapter 4). AA synthetase is also found in the supernatant after sonication.

It is therefore concluded that ALA synthetase and AA synthetase are matrix enzymes or are loosely bound to the inner mitochondrial membrane and released by sonication.

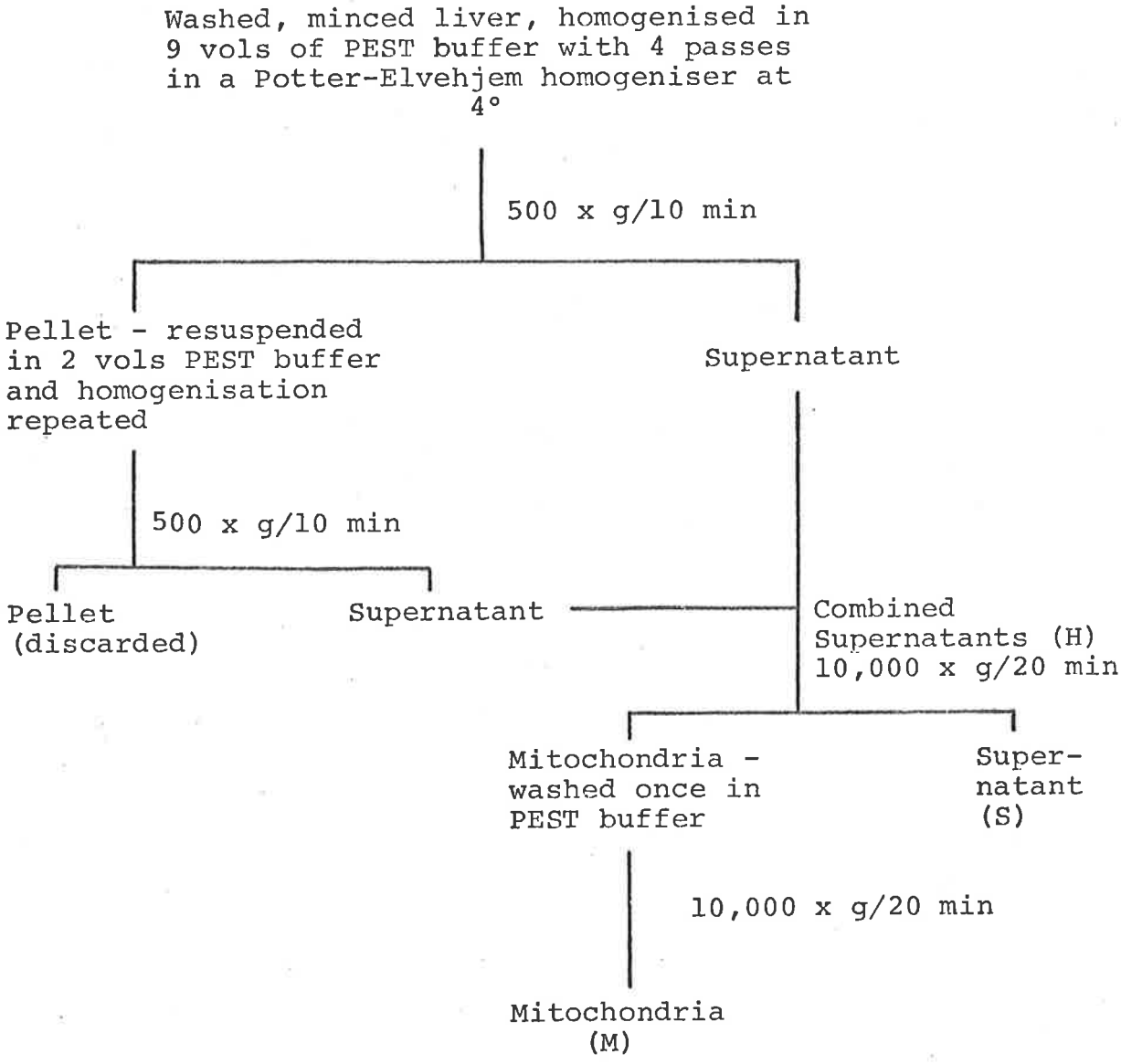
(ii) Intracellular Localisation

The fractionation scheme used to prepare the liver fractions homogenate (H), mitochondria (M) and post-mitochondrial supernatant (S) is shown in Fig. 3-2. All samples were frozen-thawed once and sonicated (as above) for 2 x 15 sec before assay for the enzymes ALA synthetase and AA synthetase, as well as glutamate dehydrogenase and citrate synthase. These latter two enzymes are regarded as exclusively mitochondrial in origin [227-230], and are localised within the matrix of the mitochondria [231-233]. They thus serve as convenient marker enzymes to test for mitochondrial breakage in cell fractionation experiments.

The results of two separate experiments are shown

Fig. 3-2. Flow Sheet for Liver Cell Fractionation

(Based on the method of Schneider and Hogeboom, [214]).



in Table 3-2. The product of the radiochemical assays was verified as ALA by thin layer chromatography of the pyrrole, as described by Irving and Elliott [213]. Whether the enzyme appearing in the supernatant fraction is expressed as a % of the original activity (S/H) or as a % of the activity recovered (S/M+S), it can be seen from the matrix marker enzymes that approximately 10% breakage of mitochondria occurred during the fractionation procedure. However, the amount of ALA synthetase, which has been shown also to be a matrix enzyme, appearing in fraction (S) was 35-40%. Thus, after allowing for mitochondrial breakage and assuming no preferential leakage of ALA synthetase occurs from the mitochondria, these studies reveal 25-30% of the total ALA synthetase activity present in guinea pig liver 24 hr after dosage with DDC is truly extramitochondrial or 'soluble'. A small amount of AA synthetase was also found in the cytosol fraction.

(b) Electron Microscopy Studies

Electron micrographs of liver sections from porphyric guinea pigs, and control animals starved for an equivalent period, are shown in Fig. 3-3. They show the appearance of the liver cells in each to be very similar, with the only readily noticeable difference being a proliferation of smooth endoplasmic reticulum in the porphyric liver, as has already been described [103,219,220].

The size distribution of mitochondria in the

Table 3-2

The Intracellular Distribution of ALA Synthetase and AA Synthetase in Porphyric Guinea Pig Liver

Fractions were prepared and sonicated as described in the text (H = 500 x g supernatant; S = 10,000 x g supernatant; M= mitochondria). ALA Synthetase was assayed colorimetrically, or radiochemically as in Table 3-1, and the total activity per fraction expressed as units of enzyme (x 10^{-2}). EDTA was added to a concentration of 10 µmole/ml for assays of fractions H and S. Control assays allowed for PBG present in fractions H and S. AA synthetase was assayed as in Table 3-1 and total activity per fraction expressed as units of enzyme (x 10^{-3}). Glutamate dehydrogenase and citrate synthase were assayed as described in Section 3.2, and total activity of these two enzymes per fraction expressed as units (x 10^{-1}).

Enzyme	Expt.	Total Units of Enzyme Per Fraction			Recovery %	Activity in Cytosol %	
		H	M	S		S/H	S/M + S
ALA Synthetase	1*	36	23	14	103	39	38
	2*	39	20	13	85	33	39
	2+	43		16		37	
AA Synthetase	1	507	316	80	78	16	20
	2	380	234	56	76	15	19
Glutamate Dehydrogenase	1	340	240	25	79	7	9
	2	380	230	25	67	7	10
Citrate Synthase	1	24	17	2.0	77	8	11
	2	24	16	1.4	73	6	8

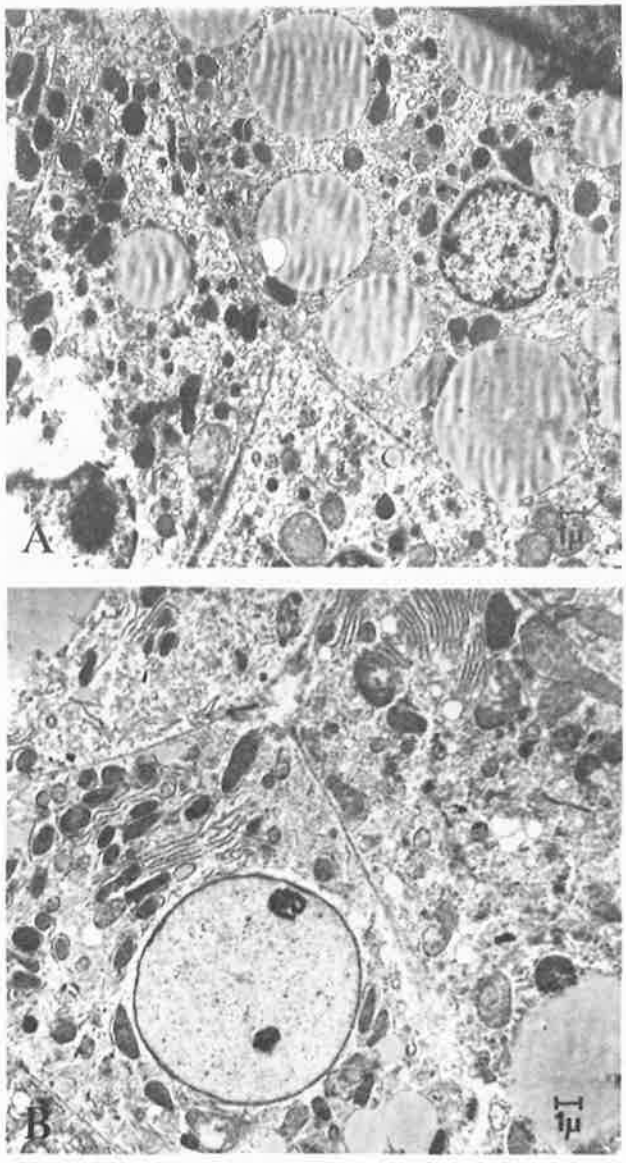


Fig. 3-3.
Electron micrographs of liver sections from porphyric and starved guinea pigs.
A. Starved; B. Porphyric.

sections was examined by estimating the area of individual mitochondria, but while the porphyric tissue appeared to contain slightly larger mitochondria than the control, the difference was of doubtful significance. Mitochondria were structurally intact *in situ* or when isolated, and no differences between porphyric and control samples were detected.

3.4. DISCUSSION

Soon after guinea pig mitochondrial ALA synthetase was found to be a matrix enzyme or to be loosely associated with the inner membrane in this laboratory, results were published independently by two groups leading to the same conclusion in rat liver.

Zuyderhoudt *et al.* [5], by fractionating mitochondria after sonication or digitonin treatment, found that ALA synthetase activity was distributed identically to the matrix marker enzyme glutamate dehydrogenase.

A more detailed study, using two different methods of disruption and known marker enzymes to label all fractions, was carried out by McKay *et al.* [6]. They localised ALA synthetase in the mitochondrial matrix also, and ferrochelatase, the final enzyme in the heme biosynthetic pathway, to the inner membrane.

Since only the first, sixth and seventh enzymes in the heme biosynthetic pathway are mitochondrial, Sano and Granick [4] have postulated this intracellular compartmentalisation of heme synthesis may serve a regulatory

function. As noted by McKay *et al.* [6], the transport of intermediates across mitochondrial membranes to and from the matrix or inner membrane could be an important factor in the control of heme synthesis.

Also, since ferrochelatase, which catalyses the formation of heme, is found inside the mitochondrion with ALA synthetase, a convenient way of controlling the pathway would be by end-product (heme) inhibition of the ALA synthetase reaction, a mechanism known to occur in *R. spheroides* [15,37]. This possibility will be further discussed in Chapter 6.

The work reported here also confirms the existence of a cytoplasmic form of ALA synthetase in the porphyric liver cell. Although enzyme activity had previously been detected in the post-mitochondrial supernatant of a liver homogenate [7,8], no assays of matrix marker enzymes were reported. The possibility therefore, that mitochondrial breakage could lead to the observed cytosol activity was not satisfactorily eliminated. An actual case of mitochondrial fragility resulting in up to 40% of the total hepatic enzyme activity being detected in the post-mitochondrial supernatant has been reported for pyruvate carboxylase in diabetic sheep liver [221]. The experiments reported here negate this type of explanation for ALA synthetase.

Thus, a possible interpretation for all these findings is that ALA synthetase is synthesised on cytoplasmic ribosomes and is subsequently transferred through the outer

membrane of the mitochondrion to the matrix. The high turnover rate of ALA synthetase [35,95] would seem to require that ALA synthetase can be inserted into, and deleted from, the intact mitochondrion.

The electron microscopy studies showed no specific structural alterations in liver mitochondria that could be directly correlated with an increase in ALA synthetase activity or porphyrin production. The proliferation of the endoplasmic reticulum is a common non-specific effect which occurs in liver after the administration of a variety of drugs. It is probably related to the increase in activity of the drug-metabolising enzymes of the smooth endoplasmic reticulum [234,235]. This change therefore only reflects cellular adaptation to metabolise foreign compounds, such as DDC.

Since this electron microscopy work was carried out, a detailed account of a more extensive study in rats, using light and electron microscopy, has been published, with similar conclusions [104].

CHAPTER 4

THE SOLUBILISATION OF ALA SYNTHETASE FROM GUINEA PIG LIVER MITOCHONDRIA

4.1. INTRODUCTION

In this laboratory, studies have been in progress for some time with the aim of obtaining pure mitochondrial ALA synthetase. Preliminary work carried out with guinea pig mitochondria by Irving [208] resulted in only a 4-6 fold purification of the enzyme. The method used involved sonicating frozen-thawed mitochondria for 15 sec to extract the enzyme into a non-sedimentable form when centrifuged at 105,000 x g for 1 hr, followed by ammonium sulphate fractionation, and Sephadex G-100 chromatography. Using this procedure, ALA synthetase activity was recovered from the Sephadex column in the void volume, but was very unstable. Storage of the enzyme solution resulted in the appearance of a precipitate and a rapid loss of activity over a few days, thus making any further attempts at purification very difficult.

With these findings in mind, initial experiments were undertaken to investigate methods of extracting the enzyme from mitochondria with attention to ways of stabilising enzyme activity.

4.2. RESULTS

(a) Disruption of Mitochondria, and Extraction of ALA Synthetase Activity

Three methods were compared for their ability to solubilise ALA synthetase activity from mitochondria. These were freeze-thawing, freeze-drying, and freeze-thawing plus sonication. After treatment, disrupted mitochondria

were centrifuged at 105,000 x g for 1 hr, and the supernatants and resuspended pellets assayed for ALA synthetase activity and protein. The range of results for the supernatant fractions is shown in Table 4-1.

Although sonication of mitochondria resulted in the best yield of ALA synthetase activity in the supernatant, much mitochondrial protein was also solubilised. This was difficult to separate from ALA synthetase activity in subsequent purification steps. Therefore, while freeze-drying and resuspending the mitochondria did not give as high a yield of solubilised enzyme as sonication, the enzyme recovered was of higher specific activity, and this method of disrupting the mitochondria was eventually adopted for all experiments. Also, it had the advantage of allowing mitochondrial preparations to be conveniently stored as a freeze-dried powder. The properties of the extracted enzyme were investigated by a number of methods, as described below.

(b) Sephadex Chromatography of the Extracted Enzyme

When enzyme extracted from mitochondria by freeze-drying, or sonication, was loaded on a Sephadex G-100 column (2.5 x 60 cm) and eluted with TP buffer (0.02 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, pH 7.4), almost all activity was excluded from the column and was recovered in the void volume. This experiment was repeated using Sephadex G-200, with the same result. Fig. 4-1 shows the type of profile obtained. The behaviour of the related enzyme,

Table 4-1

The Extraction of ALA Synthetase from Disrupted Mitochondria

Mitochondria were isolated from porphyric guinea pig liver, as described in Section 2.5. Samples were either frozen-thawed once or twice, freeze-dried and resuspended to the original volume with cold water, or sonicated for 2 x 15 sec with a 'Soniprobe' (Type 1130A, Dawe Instruments) at maximum setting with a current of 4 mamps. Treated suspensions were then centrifuged at 105,000 x g for 1 hr, and the supernatant assayed for ALA synthetase activity and protein.

Method of Disruption of Mitochondria	% Activity Recovered in Supernatant	% Protein Recovered in Supernatant
1. Freeze-thawing	20-40	20-40
2. Freeze-drying and Resuspending	50-75	40-50
3. Freeze-thawing and Sonication	80-95	70-85

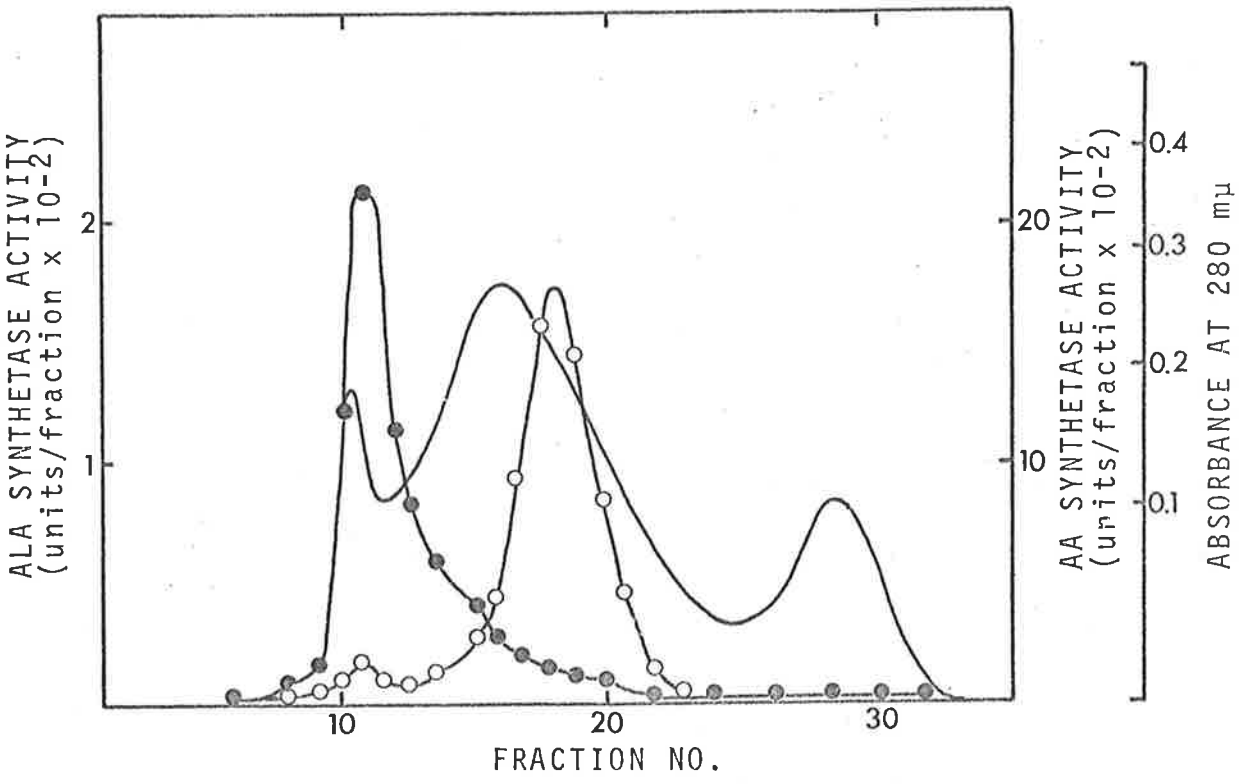


Fig. 4-1.

Chromatography of ALA synthetase, extracted from freeze-dried mitochondria, on Sephadex G-200.

The column was equilibrated in TP buffer. The powder from freeze-dried mitochondria was resuspended in the original volume of cold water, centrifuged at 105,000 x g for 1 hr, and the supernatant collected and freeze-dried. A solution prepared by dissolving 0.11 g of freeze-dried supernatant in 2 ml of cold water was loaded onto a column (2.5 x 30 cm) of Sephadex G-200 and eluted at 16 ml/hr with TP buffer. The absorbance at 280 mμ was measured continously with a LKB Uvicord II recording spectrophotometer, ———; fractions were assayed colorimetrically for ALA synthetase, ●———●; and AA synthetase, o———o .

AA synthetase, was also examined under these conditions. In contrast to ALA synthetase, AA synthetase was retarded on the column, and behaved as a soluble enzyme of molecular weight around 64,000, as determined by comparing the elution volumes of the enzyme and various proteins of known molecular weight.

From this study the alternatives existed that either ALA synthetase was an enzyme of very high molecular weight, exceeding 500,000, or it was contained in a large aggregate.

(c) Sepharose 4B Chromatography of the Extracted Enzyme

Since ALA synthetase activity was excluded by Sephadex G-200, a sample was chromatographed on Sepharose 4B, which is reported to fractionate proteins in the higher molecular weight range of 3.10^5 to 3.10^5. Conditions were identical to those used for G-200 chromatography, and the elution profiles of ALA and AA synthetases activities are shown in Fig. 4-2. ALA synthetase activity was observed to elute over a wide range of molecular weight, with a small amount of activity being excluded from the column. AA synthetase, however, eluted in a reasonably narrow, symmetrical peak as previously observed on Sephadex G-200.

(d) Sedimentation of ALA Synthetase Extract on Sucrose Density Gradients

Mitochondria were sonicated and centrifuged, as described in Table 4-1. The supernatant extract was freeze-

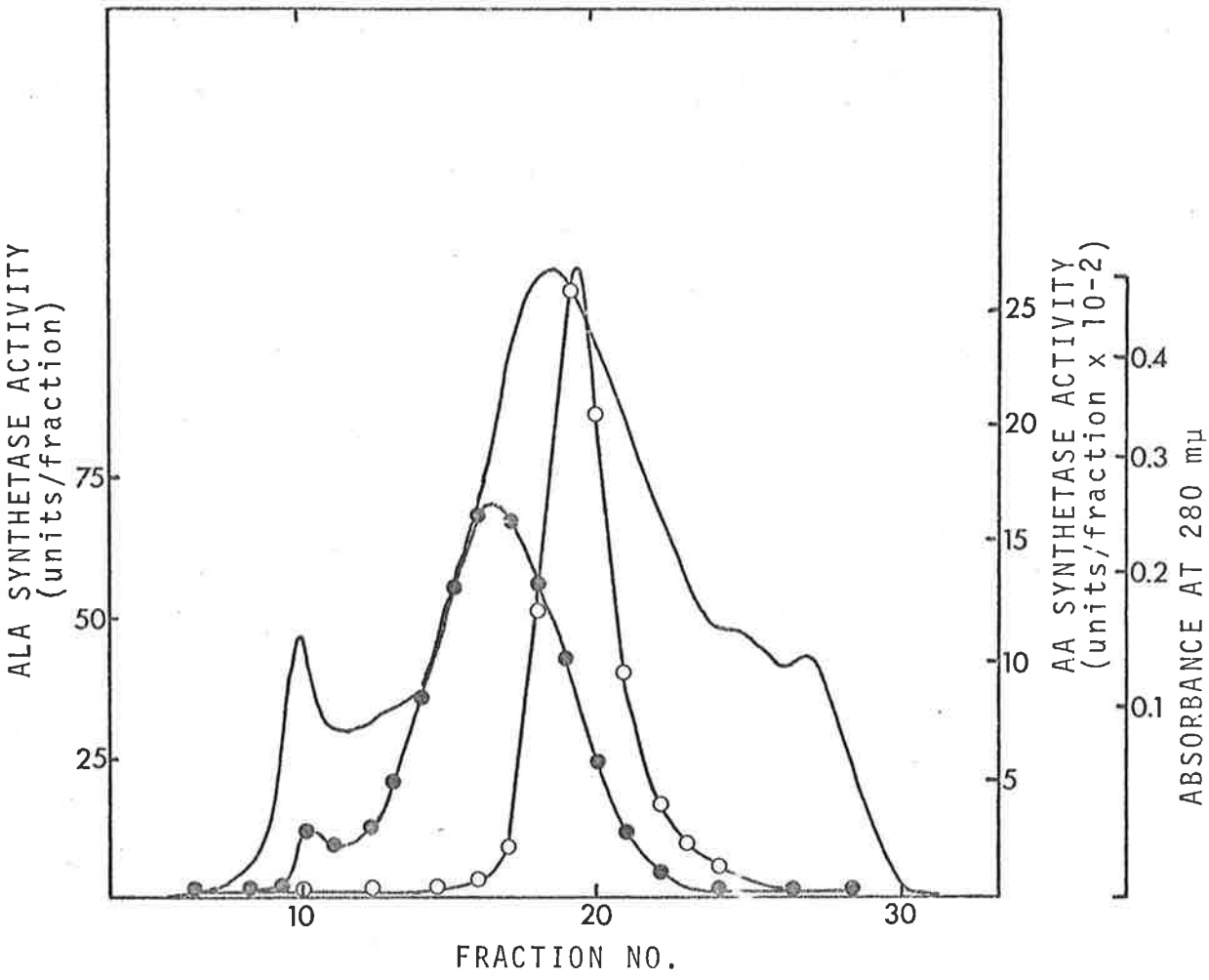


Fig. 4-2.

Chromatography of ALA synthetase, extracted from freeze-dried mitochondria, on Sepharose 4B.

The column was equilibrated in TP buffer. The experiment was carried out as in Fig. 4-1, except that 0.15 g of freeze-dried supernatant was dissolved in 2 ml of water and loaded on a column (2.5 x 35 cm) of Sepharose 4B. The absorbance at 280 mμ was measured as in Fig. 4-1, ———— ; fractions were assayed colorimetrically for ALA synthetase, ●———● , and AA synthetase, o———o .

dried, and 0.1 g of the powder plus 1-2 mg of hemoglobin, was dissolved in 1.0 ml of cold water. This solution was loaded on a 15-30% linear sucrose gradient, containing 20 mM tris-HCl, 0.1 mM pyridoxal 5-phosphate, pH 7.4, and centrifuged at 38,000 r.p.m. for 16 hr in a Beckman SW41 rotor. Separate gradients were also loaded with a mixture of the marker proteins, hemoglobin and catalase, for comparison. The distribution of ALA synthetase through the gradient, as seen in Fig. 4-3, was very broad, with much activity of molecular weight greater than that of catalase (MW 250,000). Identical results were obtained with enzyme extracted from freeze-dried mitochondria. The majority of AA synthetase activity appeared to sediment similarly to hemoglobin, although some activity trailed into regions of higher molecular weight.

In contrast, when the extracted enzyme was diluted with TP buffer to a protein concentration of 24 mg/ml and collected as a 33-50% ammonium sulphate fraction as described by Irving [208], a sedimentation profile was obtained on sucrose gradients as in Fig. 4-4. ALA synthetase activity now sedimented as a defined peak of molecular weight around 100,000. This form of the enzyme, however, was still not completely soluble, as shown by later studies. The sedimentation behaviour of AA synthetase was unaffected by the above treatments.

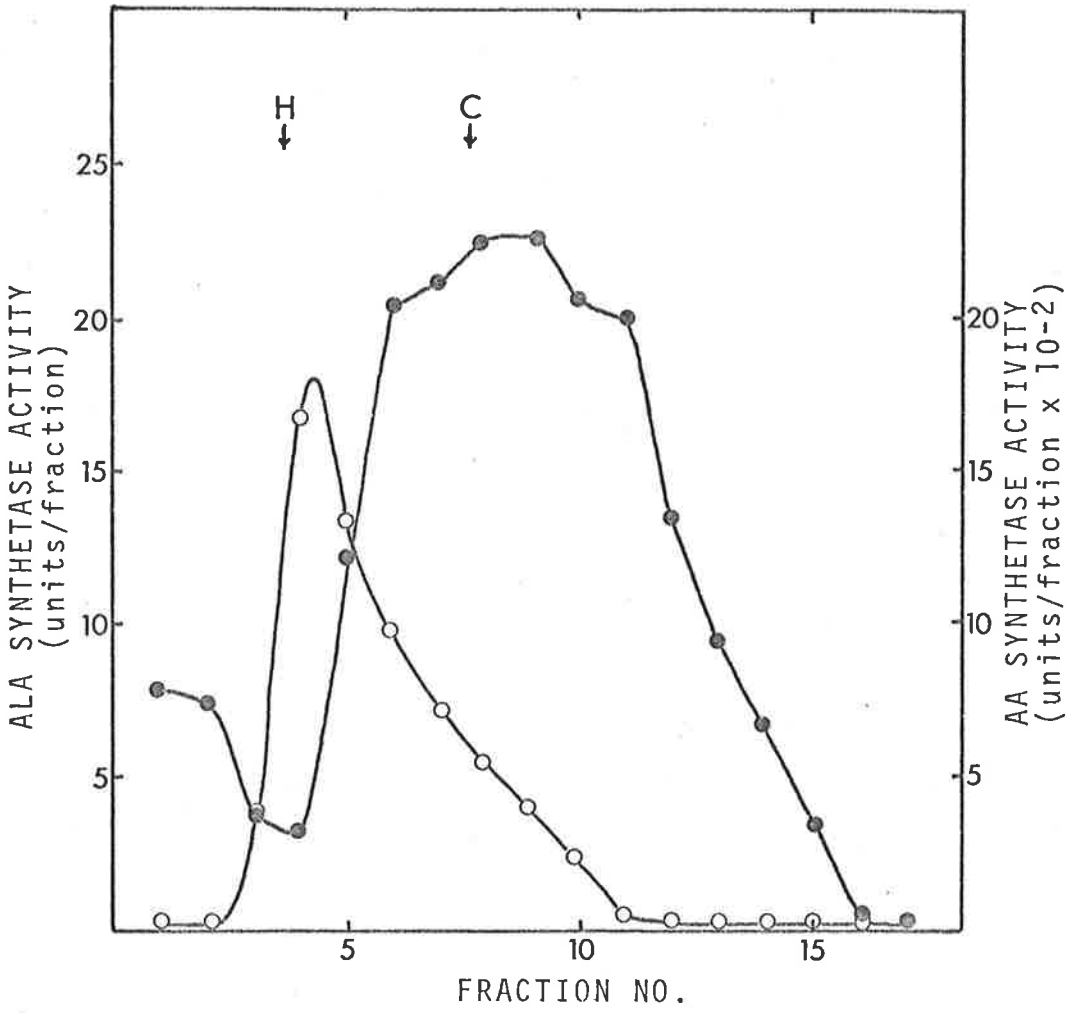


Fig. 4-3.

Sucrose density gradient centrifugation of ALA synthetase, extracted from sonicated mitochondria.

Details are described in the text. After centrifugation, tubes were pierced at the bottom and 0.7 ml fractions collected. Each fraction was assayed colorimetrically for ALA synthetase, ●——● , and AA synthetase, o——o. Hemoglobin (H) was detected by its absorbance at 413 mμ, and catalase (C) was measured by following hydrogen peroxide hydrolysis at 230 mμ.

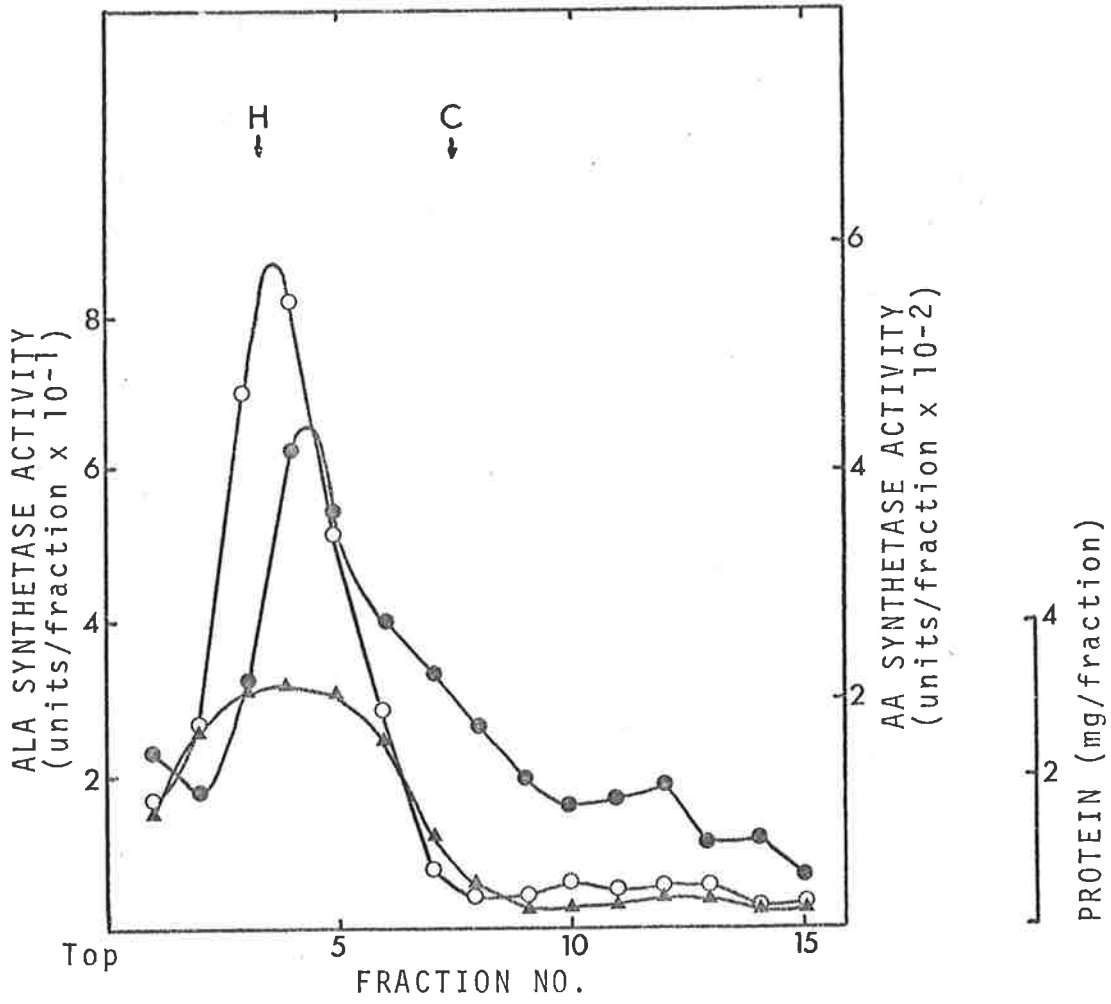


Fig. 4-4.

Sucrose density gradient centrifugation of ALA synthetase, extracted from sonicated mitochondria and fractionated with ammonium sulphate.

Enzyme was extracted from mitochondria as for Fig. 4-3 and precipitated with ammonium sulphate as described in the text. To the redissolved precipitate was added 1-2 mg of hemoglobin and 0.5 ml of solution was loaded and sedimented on a sucrose gradient as described in Fig. 4-3. Fractions were assayed radiochemically for ALA synthetase, ●——●, and colorimetrically for AA synthetase, o——o. Protein was also determined, ▲——▲.

(e) Chromatography of the Extracted Enzyme on DEAE-Cellulose at pH 7.2

Fractionation of the enzyme extract was next attempted using a DEAE-cellulose column (3 x 20 cm), equilibrated in 10 mM tris-HCl, 0.1 mM pyridoxal 5-phosphate, pH 7.4. Upon passing the crude extract through the column, all ALA synthetase activity was bound. However, elution with a linear sodium chloride gradient gave only a 2 to 3-fold purification of the enzyme, since enzymic activity was released from the column over a wide range of salt concentration along with most of the bound protein.

(f) Acetone Fractionation of the Extracted Enzyme

To the mitochondrial extract, acetone cooled to -50° was added dropwise to a final concentration of 40% (v/v) and the solution then stirred slowly for 60 min in a -15° cold room. The precipitate was collected by centrifugation at 10,000 x g for 15 min, and redissolved in TP buffer. About 50% of the initial activity was recovered by this procedure, and the solution chromatographed on a Sephadex G-150 column (2.5 x 50 cm) equilibrated in TP buffer. Most of the ALA synthetase activity was excluded from the column, and overall, the profile was not significantly different from that obtained by direct chromatography of the extract without acetone treatment.

From the results of the experiments described

to this point, it was concluded that ALA synthetase extracted from mitochondria was contained in a large aggregate. In support of this conclusion, electron microscopic examination of the fractions from Sephadex G-200 containing ALA synthetase activity showed large aggregates were present in the solution along with some fragmented membranous material.

(g) Apparent Solubilisation of ALA Synthetase from the Aggregate by Salt Treatment

Using the enzymically active fractions eluted from a Sephadex G-200 column on which mitochondrial extract had been chromatographed, ways of disrupting the aggregate, to release ALA synthetase activity, were investigated. Centrifugation of these fractions at 200,000 x g for 1 hr, or 150,000 x g for 2 hr, sedimented up to 70% of the activity as a small brown pellet. The remainder of the activity formed a gradient down the centrifuge tube, suggesting there was a range in the aggregate size. A means of testing various treatments for any effect in solubilising ALA synthetase was now available. The active pellet fraction was resuspended in the solution under test, and the suspension recentrifuged. By assaying the supernatant and pellet fractions, any solubilising effect could be observed.

Table 4-2 shows that triton X-100 and digitonin, as well as lipase and snake venom, were ineffective. However, when the pellet was resuspended in

Table 4-2

Solubilisation of ALA Synthetase Activity from the Pelleted Aggregate to a Non-Sedimentable Form

The fractions corresponding to the void volume from a Sephadex G-200 column (2.5 x 30 cm) loaded with mitochondrial extract and eluted with TP buffer were pooled (volume 20 ml). Equal volumes (2 ml) were then dispensed into centrifuge tubes, which were centrifuged at 130,000 x g for 90 min. The pellets were resuspended in 2 ml of the solution under test, allowed to stand at room temperature for 30 min, and recentrifuged at 105,000 x g for 1 hr. Supernatants and pellets resuspended in 2 ml of TP buffer were assayed for ALA synthetase activity colorimetrically.

Resuspension Solution	% Enzymic Activity Recovered in	
	Supernatant	Pellet
1. TP buffer	4	58
2. TP, 1 mM dithioerythritol	5	55
3. TP, 0.5 mM EDTA	4	72
4. TP, 0.5 M KCl	67	20
5. TP, Snake Venom, 0.1 mg/ml	13	53
6. TP, Lipase, 0.4 mg/ml	7	60
7. TP, Triton X-100, 1% (v/v)	18	52
8. TP, Digitonin, 1.2 mg/ml	7	53
9. TP, Digitonin, 3.0 mg/ml	18	57

buffer containing 0.5 M KCl (or NaCl), a major fraction of the activity was now not sedimented by a 1 hr centrifugation at 105,000 x g. In fact, enzyme activity was linearly released from the pelleted aggregate by increasing salt concentration up to 0.8 M, as shown in Fig. 4-5. Electron microscopy on the resuspended pellet material confirmed that treatment with salt dispersed much of the aggregated material present.

(h) Sephadex Chromatography of the Salt Extracted Enzyme

To test if the enzyme obtained by treating the aggregate with KCl or NaCl was truly soluble, the following experiment was performed. Mitochondrial extract, obtained by sonicating mitochondria and removing debris by centrifugation at 105,000 x g for 1 hr, was freeze-dried. 0.2 g of the powder was redissolved in 2 ml of TP buffer containing 0.6 M NaCl, and chromatographed on a Sephadex G-200 column (2.5 x 35 cm), equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.2 M NaCl, pH 7.4. The activity profile is seen in Fig. 4-6. Evidently, salt treatment had produced smaller aggregates which were still excluded from Sephadex G-200, although now not pelleted by ultracentrifugation.

(i) A Method for the Complete Solubilisation of ALA Synthetase from the Aggregate

The complete solubilisation of ALA synthetase

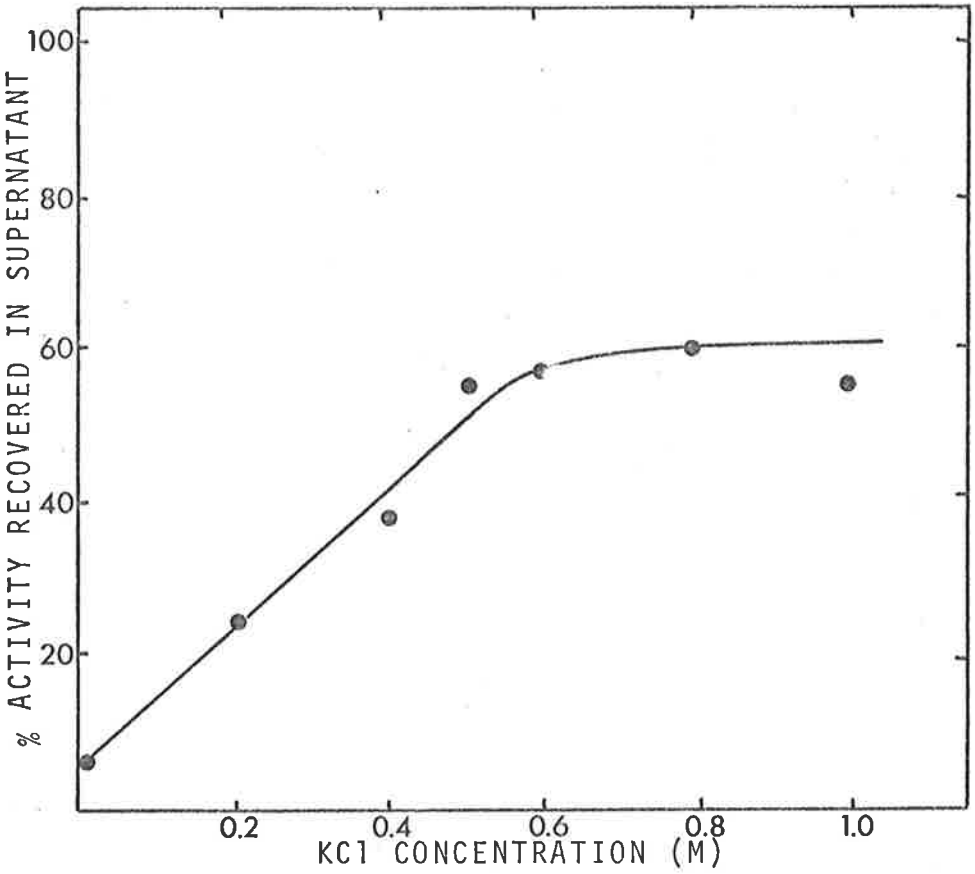


Fig. 4-5.

Release of ALA synthetase activity from the aggregate by increasing salt concentration.

The aggregate was collected from a Sephadex G-200 column (4 x 30 cm), and pelleted as described in Table 4-2. Pellets were resuspended in 2 ml of TP buffer containing KCl at various concentrations, up to 1.0 M. After standing at 4°C for 15 min, solutions were centrifuged at 105,000 x g for 45 min. Supernatants were assayed for ALA synthetase activity, ●——●.

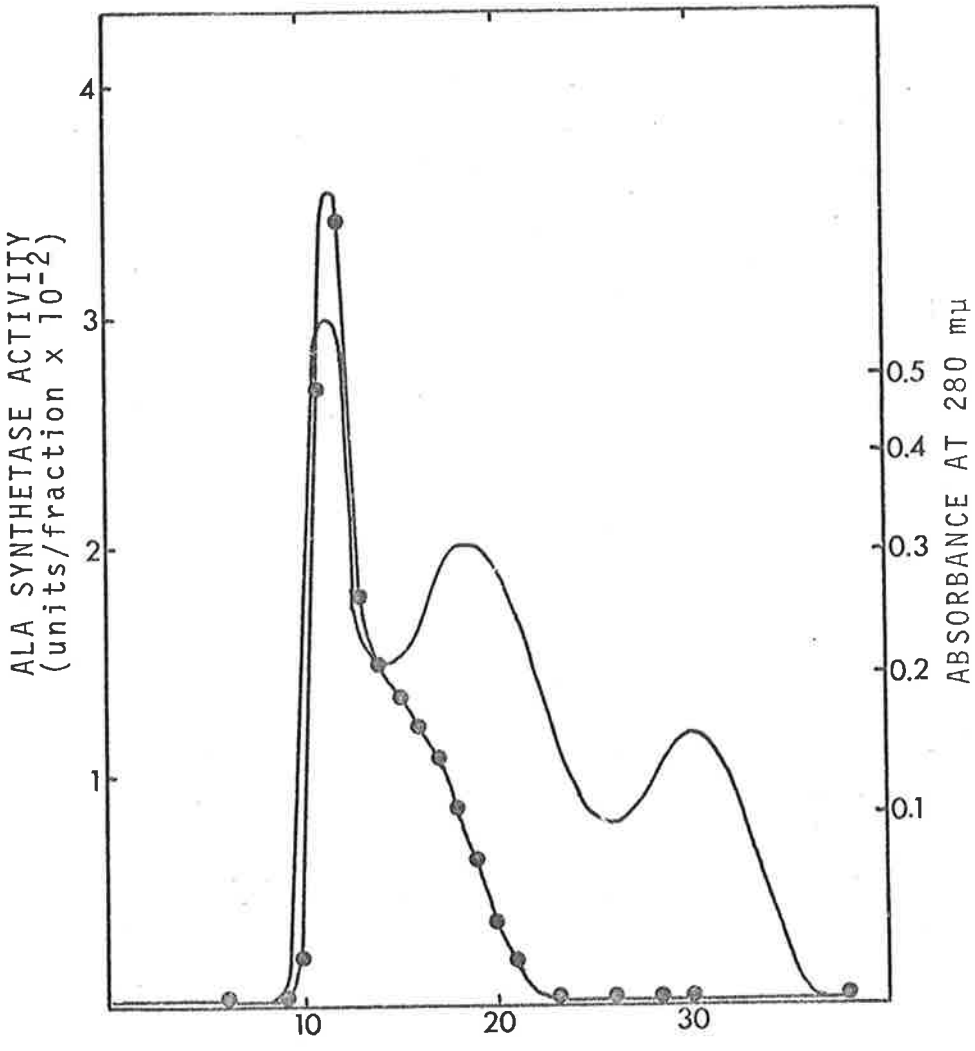


Fig. 4-6.

Chromatography of ALA synthetase extract, containing NaCl (0.6 M), on Sephadex G-200.

The column was equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.2 M NaCl, pH 7.4. Other details are described in the text. The absorbance of the eluate at 280 mμ was measured as in Fig. 4-1, ——— ; fractions were assayed colorimetrically for ALA synthetase activity, ●———●.

from the aggregate was finally accomplished by treating the aggregate with NaCl (0.8 M) and dithioerythritol (1 mM) together. Both high ionic strength and reducing conditions were required; dithioerythritol alone had no effect (see Table 4-2). The change in activity profile when the treated extract was chromatographed on Sephadex G-200 is seen in Fig. 4-7. The powder from the freeze-dried supernatant extract (0.2 g) from sonicated mitochondria was resuspended in 2 ml of TP buffer containing 0.6 M NaCl and 1 mM dithioerythritol, and chromatographed on a Sephadex G-200 column (2.5 x 35 cm) equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.2 M NaCl, 0.1 mM dithioerythritol, pH 7.4. Most activity was now retarded on the column and eluted as a symmetrical peak. Virtually no activity remained excluded from the column when the salt concentration of the suspending buffer was raised to 0.8 M, and that of the elution buffer to 0.5 M.

The fact that the continued presence of high ionic strength and reducing conditions are required to maintain ALA synthetase activity in a soluble form is shown in Table 4-3. Freeze-dried extracted enzyme was resuspended in the buffers shown, and the size of the enzyme determined by chromatography on a Sephadex G-200 column, not necessarily equilibrated in the same buffer. The only combination to produce truly soluble enzyme was that where NaCl and dithioerythriol were present in both the suspending and eluting buffers. When enzyme was solubilised by suspension in NaCl and dithioerythritol, but chromatographed

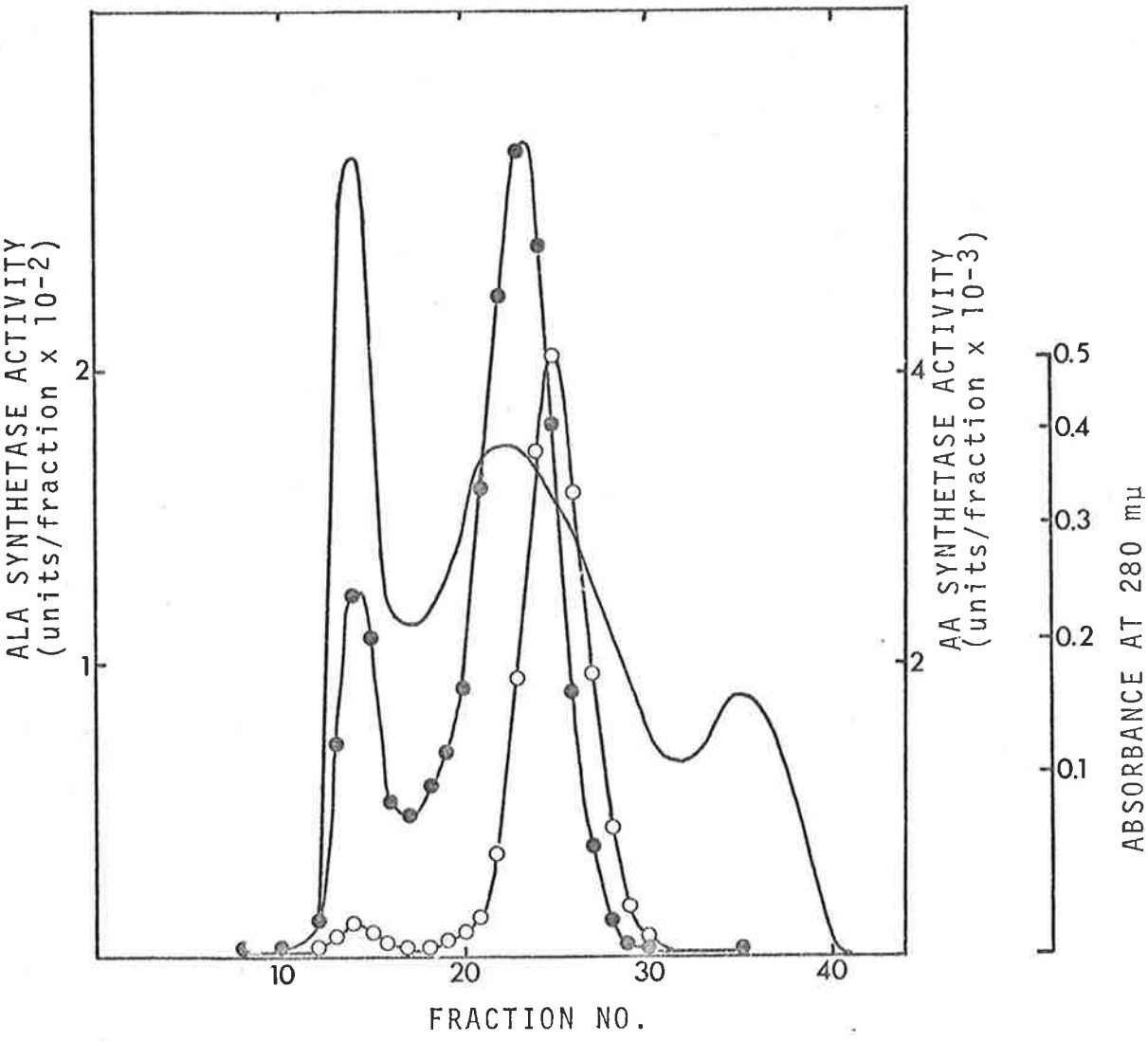


Fig. 4-7.

Chromatography of ALA synthetase extract, containing NaCl (0.6 M) and dithioerythritol (1 mM), on Sephadex G-200.

The column was equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.2 M NaCl, 0.1 mM dithioerythritol, pH 7.4. Other details are described in the text. The absorbance at 280 mμ was measured as in Fig. 4-1, ——————; fractions were assayed colorimetrically for ALA synthetase, ●————●, and AA synthetase, o————o.

Table 4-3

The Solubilisation of Crude ALA Synthetase with NaCl and Dithioerythritol, as Determined by Sephadex G-200 Chromatography.

Mitochondria (protein concentration 100 mg/ml) in TP buffer were freeze-dried and resuspended in cold water to the original volume. The suspension was centrifuged at 105,000 x g for 1 hr, and the supernatant, containing 80% of the total activity, was freeze-dried. An amount of the freeze-dried powder equivalent to 1500 units of enzyme was suspended in the buffers indicated, and loaded on a Sephadex G-200 column (2.5 x 35 cm). Elution was carried out with the buffers shown, and 0.7 ml of each fraction assayed for ALA synthetase activity to determine whether the enzyme was aggregated or soluble.

Suspension Buffers pH 7.4	Elution Buffers pH 7.4	State of Enzyme	% Activity Recovered
TP	TP	Aggregate	114
TP, 0.6 M NaCl	TP	Aggregate	63
TP, 0.6 M NaCl	TP, 0.6 M NaCl	Aggregate	144
TP, 0.6 M NaCl, 1 mM DTE*	TP, 1 mM DTE	Aggregate	90
TP, 0.6 M NaCl, 1 mM DTE	TP, 0.2 M NaCl, 1 mM DTE	80% Soluble 20% Aggregate	167

*Dithioerythritol.

in the absence of NaCl, re-aggregation occurred.

The total ALA synthetase activity recovered from columns was always greater than 100% when sodium chloride was present in the elution buffer. Sodium chloride may therefore be stimulating ALA synthetase activity, as has been reported for the purified cytosol enzyme [45].

(j) The Molecular Weights of AA and Solubilised ALA Synthetases from Guinea Pig Liver Mitochondria

A Sephadex G-150 column (1.3 cm x 120) was equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.3 M KCl, 0.1 mM dithioerythritol, pH 7.4, and the elution volumes of proteins of known molecular weight determined (Fig. 4-8). Proteins used and their molecular weight values were chymotrypsinogen A (MW 25,000), ovalbumin (MW 45,000), bovine serum albumin (MW 67,000), lactate dehydrogenase (MW 140,000) and γ-globulin (MW 160,000). The molecular weights of AA synthetase and ALA synthetase were determined on this column by a comparison of their elution volumes with those of the marker proteins, according to Andrews [236]. Values obtained were 64,000 for AA synthetase, and 73,000 for ALA synthetase.

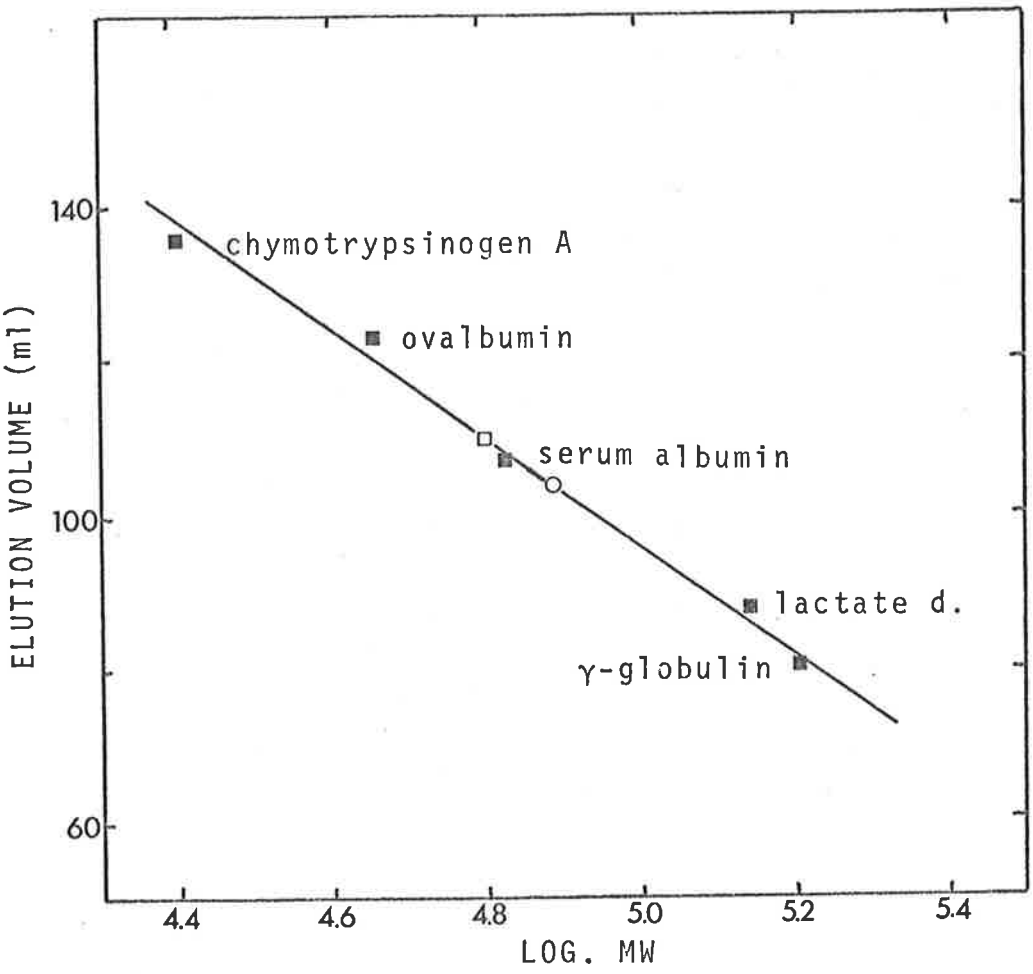


Fig. 4-8.

Determination of the molecular weights of solubilised mitochondrial ALA synthetase and AA synthetase,

from their elution volumes on a calibrated Sephadex G-150 column (1.3 x 120 cm). A sample of mitochondrial extract, containing added NaCl (0.6 M) and dithioerythritol (1 mM), was fractionated on the column using the buffer described in the text. Elution volumes of ALA synthetase (○), and of AA synthetase (□) are shown.

4.3. DISCUSSION

The findings reported here indicate that ALA synthetase extracted from guinea pig liver mitochondria by freeze-drying or freeze-thawing plus sonication is associated with a large aggregate. This accounts for the difficulty encountered in purifying the enzyme in preliminary attempts which included Sephadex and Sepharose gel chromatography, cellulose ion-exchange chromatography, sucrose density gradient centrifugation and acetone precipitation.

Using a particulate fraction from livers of AIA-treated rats, Kaplan [38] has reported similar results in attempts at purifying ALA synthetase. By extracting mitochondria with deoxycholate, he obtained an enzyme preparation, in which most of the enzymic activity remained in the supernatant after centrifugation at 140,000 x g for 2 hr, but efforts to further purify the enzyme were frustrated by low yields and inconsistent results.

It is apparent from the results presented in this chapter that different size aggregates containing enzyme activity can be isolated from mitochondria by different techniques. Most of the aggregate extracted by freeze-drying or sonication, after having been chromatographed on a Sephadex G-200 column in TP buffer, was sedimentable when centrifuged at 200,000 x g for 1 hr or 150,000 x g for 2 hr. This contrasts with the results of Kaplan [38], but can be explained on the basis that his purification

sequence included an ammonium sulphate fractionation. The present work shows that treatment of the aggregate with a high salt concentration disperses the aggregate so that activity, although still excluded when chromatographed on a Sephadex G-200 column, is now no longer pelleted by ultracentrifugation.

The physical nature of the aggregate is not known. It may consist entirely of protein or be part of a mitochondrial membrane. Electron microscopic studies of enzymically active fractions eluted from Sephadex G-200, showed the presence of both protein aggregates and membrane pieces and vesicles. Kaplan could not detect any lipid, or activity of the inner mitochondrial membrane enzymes, succinic dehydrogenase and cytochrome oxidase, in his preparation [38], and he favoured the idea of hydrophobic interactions between a group of proteins released from the mitochondria as accounting for the complex.

Whether the aggregation phenomenon has physiological significance with respect to the localisation of the enzyme within the mitochondrion, is difficult to assess. It could be an artefact produced upon disruption of the mitochondria. On the other hand, since it is thought that ALA synthetase is synthesised extra-mitochondrially on cytoplasmic ribosomes and subsequently transferred into the mitochondrion [7,8] it is feasible that a specific site exists inside the mitochondrion for localising this enzyme. As described in Chapter 3, ALA synthetase is a matrix enzyme, or may be loosely bound to the inner mitochondrial membrane.

The aggregate could then, represent protein from the inner membrane or cristae to which the enzyme becomes attached once inside the mitochondrion.

In support of this idea, the solubility of a mitochondrial enzyme may be taken broadly as an index of its intramitochondrial localisation. Thus, matrix enzymes can be isolated as water-soluble lipid-free proteins, merely by disrupting the mitochondrial membranes, and allowing their release. Other proteins and enzymes which are intrinsic components of membranes acquire the characteristics of water-soluble proteins only when treated by physical, chemical or enzymic methods. These include homogenisation or sonication; extraction with low ionic strength solutions, concentrated salt solutions, urea, alkali, metal chelators or organic solvents; or lipase, phospholipase, or proteolytic enzyme digestion. Examples of the solubilisation of mitochondrial enzymes using one, or a combination, of these methods are known and are summarised by Penefsky and Tzagoloff [237]. Finally, some macromolecular enzyme complexes are known, which are composed of proteins and associated phospholipids, with the latter frequently being necessary for enzyme activity. To extract and purify these complexes from membranes requires dispersing agents such as bile salts (e.g., deoxycholate), organic solvents (e.g., n-butyl alcohol) or synthetic detergents (e.g., triton X-100, sodium dodecyl sulphate).

The finding that ALA synthetase became a truly soluble enzyme with a molecular weight of 78,000 only

after the combined treatment with 0.8 M NaCl and 1 mM dithioerythritol is somewhat unusual, but indicates the enzyme may be specifically bound by disulphide linkage and electrostatic interactions to membrane protein inside the mitochondrion. It appears that since dithioerythritol alone had no effect in solubilising enzyme activity, a high salt concentration is necessary to disperse the aggregate and expose disulphide bonds which link ALA synthetase to other protein.

Methods which have been used successfully in solubilising membrane-associated enzymes had no effect in solubilising ALA synthetase activity, although the optimum conditions for each treatment may not have been used. These were enzymic digestion with lipase and snake venom, suspension in the detergents triton X-100 and digitonin, treatment with the chelating agent EDTA, and acetone precipitation.

High salt concentrations have been used, however, for extracting other enzymes and proteins from mitochondria or mitochondrial fragments. Most noteworthy is the observation that phosphorylating submitochondrial electron transport particles, prepared by sonicating bovine heart mitochondria, extracted with 2 M NaCl or 0.5 M NaBr in the presence of 1 mM dithiothreitol lose ATPase activity [238]. Restoration of activity was achieved by adding back salt-free extract, which was shown to contain the subunits of mitochondrial ATPase. Also, cytochrome c has been extracted from mitochondria by treatment firstly with

0.015 M KCl to modify mitochondrial structure, and then by 0.15 M KCl to release the protein [239].

CHAPTER 5

PURIFICATION OF SOLUBILISED MITOCHONDRIAL ALA SYNTHETASE

5.1. INTRODUCTION

For the purification of solubilised liver mitochondrial ALA synthetase, a good source of enzyme was required. Unfortunately, normal experimental animals such as rats and guinea pigs, and also calves, have very low levels of ALA synthetase in the liver. Even using a sensitive radiochemical assay, significant enzyme activity is not detectable in normal guinea pig liver [208,213]. This meant that the isolation of the enzyme from normal liver was not feasible, so that to obtain sufficient quantities of ALA synthetase to permit preparative scale purification, it was necessary to treat animals with drugs to increase hepatic levels of enzyme activity.

Since a method for dosing guinea pigs with the ALA synthetase-inducing drug, DDC, was already available [27], initial studies were carried out using guinea pig liver. In later work, a purification method was developed for ALA synthetase from rat liver mitochondria, using the findings established for guinea pig liver. Rat liver unexpectedly proved to be a better source of enzyme than guinea pig liver, because although the average liver size per animal was much smaller, rats were dosed with DDC by a more efficient method using a stomach tube, and much greater levels of enzyme per gram of liver were obtained.

5.2. METHODS

(a) Acrylamide Gel Electrophoresis

Analytical disc gel electrophoresis in 7.5% polyacrylamide gels was carried out at pH 9.5 by the procedure of Ornstein and Davis [240]. Staining was carried out with 1% amido black in 7% acetic acid, and gels were destained in 7% acetic acid over several days.

Before running on polyacrylamide gels in sodium dodecylsulphate (SDS), purified ALA synthetase was reduced and S-carboxymethylated by the method of Kemp [241]. 10 mg of protein was dialysed against distilled water, freeze-dried, and taken up in 5 ml of a solution containing 0.1 M β-mercaptoethanol, 8 M urea and 0.5 M ethylamine, pH 10.5. After 3 hr at 37°C, 3 ml of 3 M tris-HCl, pH 8.0, were mixed with the solution to give a final pH of 8.7. 1 ml of 1.6 M iodoacetate, pH 8.2, was then added and the solution allowed tc stand for 20 min at room temperature, before the removal of excess iodoacetate by 0.15 ml of β-mercaptoethanol. After further incubation at 37°C for 30 min, another 1 ml of iodoacetate was added, the solution incubated as before, and treated with 0.2 ml of β-mercaptoethanol. The solution was finally dialysed against distilled water and freeze-dried.

Polyacrylamide gel electrophoresis in SDS was carried out by the method of Shapiro, Vinuela and Maizel [242], as modified by Weber and Osborn [243]. The S-carboxymethylated protein was dissolved in 4 M urea,

1% SDS, 0.005% bromophenol blue, loaded on a 5% polyacrylamide gel, and run for 4 hr with a current of 7 mamps per tube. Gels were stained with coomassie blue by the method of Fairbanks *et al.* [244].

(b) Preparation of Affinity Chromatography Substrate

Aminoethyl-cellulose was first washed and succinylated in aqueous solution at pH 6.0 with succinic anhydride, by the method of Cuatrecasas [245]. 2 g of aminoethyl-cellulose was suspended in 150 ml of cold water and 15 g of succinic anhydride added in an equal volume of water at 0°C. The pH was raised to 6.0, and maintained at this value with 20% NaOH over a period of approx. 3 hr. The suspension was then allowed to stand overnight at 4°C.

The succinyl-aminoethyl-cellulose was washed with 0.05 N NaOH, and to ensure all amino groups were blocked, acetylation with 1 ml of acetic anhydride in 200 ml 50% ethanol was carried out at room temperature for 30 min. The cellulose derivative was then rewashed with 0.05 N HCl and a portion titrated with 0.01 N KOH. From the shape of the titration curve, all amino groups on the cellulose appeared to be blocked.

Pyridoxamine 5-phosphate was coupled to the succinyl-aminoethyl-cellulose at pH 4.7, using a water-soluble carbodiimide [246]. The washed succinyl-aminoethyl-cellulose was suspended in 180 ml of water and 500 mg of pyridoxamine 5-phosphate added. The pH of

the suspension was adjusted to 4.7. 5.0 g of 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide were dissolved in 15 ml of water and added to the stirred suspension. The pH was maintained at 4.7 over 2 hr with 1 N KOH, and stirred for a further 4 hr. The final cellulose adsorbent was washed with 6 litres of 0.1 M NaCl, and equilibrated in a column (1.3 x 13 cm) with 0.05 M tris-HCl, pH 7.6, containing 1 mM dithioerythritol.

(c) Preparation of Crude Cytosol ALA Synthetase

The method used was essentially that of Scholnick *et al.* [8]. Livers from rats dosed with DDC were washed with cold buffer, weighed and homogenised in an equal volume of 0.25 M sucrose, 0.01 M potassium phosphate, 1 mM EDTA, 0.89% NaCl, pH 7.0. The homogenate was centrifuged at 5,000 x g for 15 min, 40,000 x g for 15 min, and 105,000 x g for 60 min. The final supernatant was termed crude cytosol enzyme.

5.3. RESULTS

A. Purification of Solubilised ALA Synthetase from Guinea Pig Liver Mitochondria

(a) Purification Method

From the results in Chapter 4, the following method was developed to purify guinea pig liver mitochondrial ALA synthetase in a soluble form.

Step 1. Isolation and Extraction of Liver Mitochondria

Guinea pigs were dosed orally with DDC; liver mitochondria were subsequently isolated as described in Section 2.5. These were suspended in TP buffer, freeze-dried, resuspended in cold water to the original volume, and centrifuged at 105,000 x g for 1 hr. The supernatant was collected and assayed for ALA synthetase activity and protein.

Step 2. Sephadex G-200 Chromatography and Isolation of the Enzymically Active Aggregate

Mitochondrial extract (6-10 ml) was loaded onto a Sephadex G-200 column (4 x 30 cm) equilibrated in TP buffer, and eluted overnight at a flow rate of 20 ml/hr. Fractions of approximately 8 ml were collected. Most enzymic activity was excluded from the column, as already described in Chapter 4. The fractions corresponding to the void volume were pooled and centrifuged at 150,000 x g for 2 hr at 4°C. The supernatant usually contained only about 20% of the original activity after this treatment, the remainder being isolated in a small brown pellet.

Step 3. Treatment of the Aggregate with NaCl and Dithioerythritol

The pellet fraction was resuspended in 15-20 ml of 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.8 M NaCl, 1 mM dithioerythritol buffer, pH 7.6, and recentrifuged at 150,000 x g for 1 hr. Assay of the supernatant revealed that 50-60% of the original activity was now recovered in

this fraction, although the method was not entirely reproducible, and lower recoveries were sometimes observed.

The solution was freeze-dried, and redissolved in water to one-fifth of the original volume.

Step 4. Sephadex G-150 Chromatography

The concentrated, solubilised enzyme (3-6 ml) was loaded onto a Sephadex G-150 column (2.5 x 35 cm), equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.25 M NaCl, 0.1 mM dithioerythritol, pH 7.4, and eluted overnight with this buffer at a flow rate of 25 ml/hr. When fractions were assayed for enzymic activity, the profile seen in Fig. 5-1 was obtained. Not all of the ALA synthetase activity was solubilised, but much of the protein was still excluded from the Sephadex and was separated from the main peak of ALA synthetase activity. The most active fractions were pooled.

A summary of the whole procedure is shown in Table 5-1. The method would be expected to be reasonably selective for ALA synthetase since the aggregate was separated from soluble mitochondrial proteins and directly treated with NaCl and dithioerythritol. Only proteins which are solubilised by this treatment should then be retarded by Sephadex G-150 and collected in the pooled fractions from Step 4. Unfortunately, a wide variation was seen in the specific activity of the enzyme obtained from the final steps. Also, the overall yield of purified enzyme

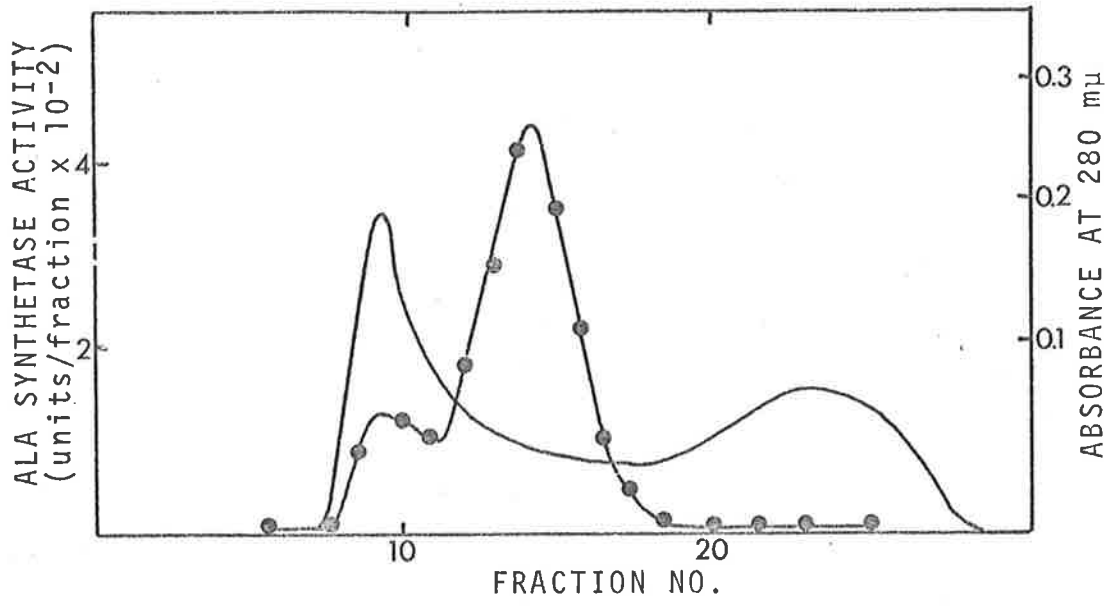


Fig. 5-1.

Chromatography of ALA synthetase, solubilised from the isolated aggregate, on Sephadex G-150.

Details are described in the text; the elution buffer was 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.25 M NaCl, 0.1 mM dithioerythritol, pH 7.4. The absorbance of the eluate at 280 mμ was determined continuously using a LKB Uvicord II recording spectrophotometer, ————; fractions were assayed colorimetrically for ALA synthetase, ●——●.

Table 5-1

The Purification of Guinea Pig Liver Mitochondrial ALA Synthetase

Details of the methods used are described in the text.

Fraction	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)
Mitochondria (from 50g liver)	10	15,300	1270	12	100
Extract from Freeze-drying	6	11,250	360	31	73
Sephadex G-200 Fractions	60	7,200	102	71	47
Treatment of Aggregate with NaCl-DTE	15	4,000	33	120	26
Sephadex G-150 Fractions	35	1,700	10	170	11

was low (around 10%).

(b) Some Properties of the Purified Guinea Pig Liver Mitochondrial Enzyme

For subsequent studies of the purified guinea pig enzyme, it was preferable to use a concentrated enzyme solution. Freeze-drying the enzyme fractions from Step 4 above, and redissolving the powder was partially successful, but limited by the amount of NaCl present. (This could not be removed by dialysis, because of the instability of the enzyme during this procedure.) Pervaporation, vacuum filtration through a collodion membrane at 4°C for several hours, or ultrafiltration of the solution using an Amicon UM-20E membrane under a nitrogen pressure of 50 lb/sq.in. for 1 hr were also unsatisfactory, and resulted in 50% or less recovery of activity.

The concentrated enzyme that was obtained using these methods lost most of its activity over a period of a few days at 4°C. Of the compounds added in an effort to stabilise the enzyme, the substrate glycine had no effect, while sucrose did slightly preserve activity. Dithioerythritol at a concentration of 1 mM or greater however, was most effective, and resulted in 60-80% recovery of ALA synthetase activity after several days storage at 4°C.

Eventually, a method of concentrating the enzyme was found which gave up to 85% recovery of ALA synthetase activity. A series of steps were carried out,

in which dry Sephadex G-25 with a water regain of 2.5 ml/g, was added to the enzyme solution containing 1 mM dithioerythritol. Anough Sephadex was added to take up half the volume of solution, and after standing for 10 min at 4°C, the suspension was centrifuged at 1,000 x g for 10 min in a filter constructed to retain the Sephadex beads above the filtrate. In this way, 30 ml of enzyme solution was concentrated to 3-4 ml in three steps with no change in the salt concentration of the enzyme buffer.

Electrophoresis of samples of this solution on acrylamide gels showed the presence of several protein bands with much protein remaining at the top of the gel (presumably an aggregate). As it seemed unlikely that the enzyme was homogeneous at this stage, various methods were investigated for possible further purification.

(c) Attempts at Further Purification of Guinea Pig Liver Mitochondrial ALA Synthetase

(i) Cellulose Ion-Exchange Chromatography

This technique was tried since methods used in the purification procedure to this stage had not fractionated proteins on the basis of their electrical charge. The stability of the enzyme in different buffers over a range of pH values was first determined. Samples of purified enzyme were incubated at 4°C for 22 hr at the test pH, and then assayed for remaining enzyme activity at pH 7.4. As seen in Fig. 5-2, the enzyme is stable under these conditions from pH 7 to 9.

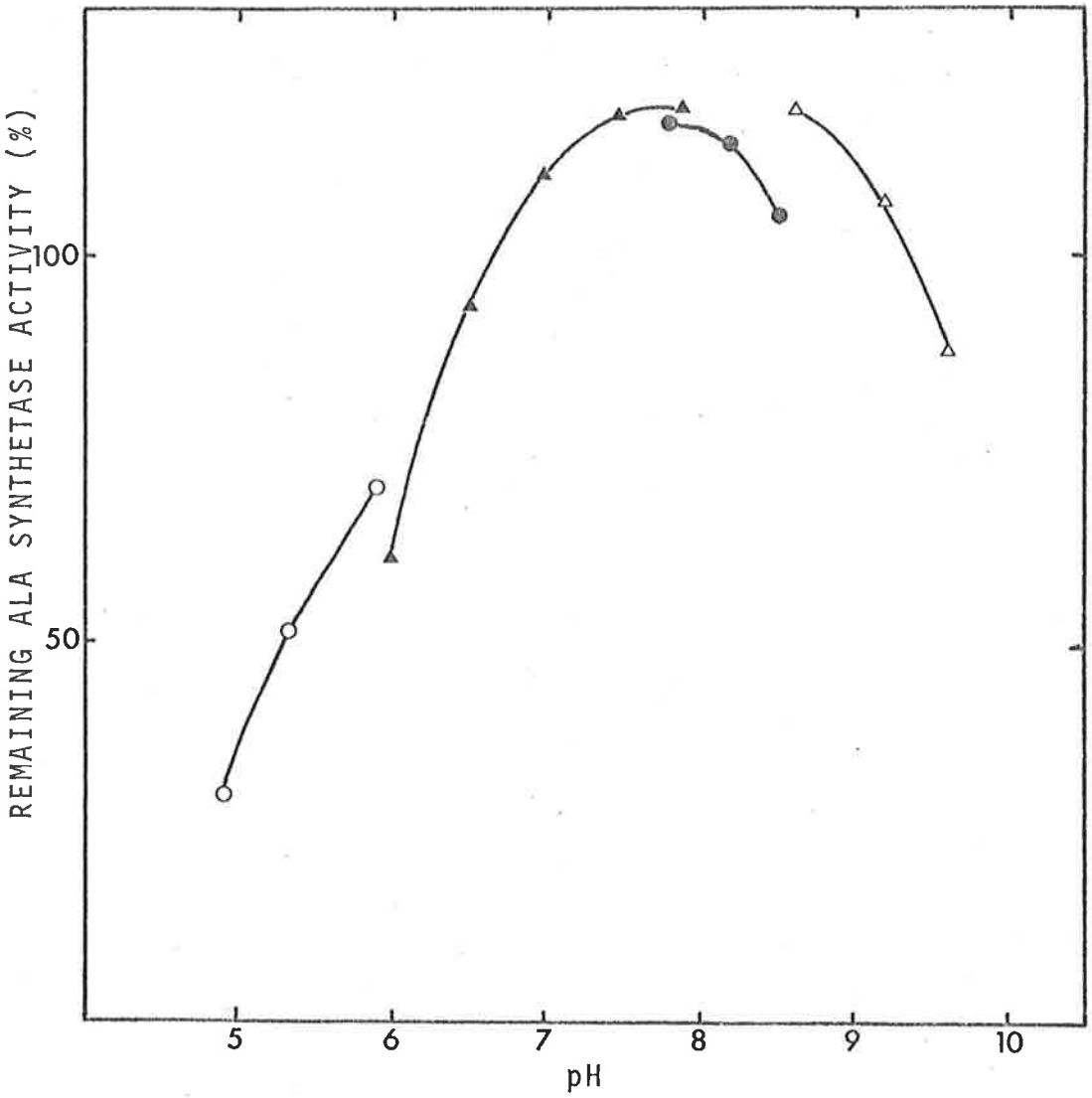


Fig. 5-2.

Stability of purified guinea pig mitochondrial ALA synthetase at various pH values.

Purified enzyme was incubated at 4°C for 22 hr in buffers at different pH values. The ALA synthetase activity remaining after this time was assayed at pH 7.4 and expressed as a % of the activity present before incubation. Buffers used were succinic acid - NaOH, ○ ; sodium phosphate, ▲ ; tris-HCl, ● ; glycine-NaOH, △ .

Batch experiments showed that the enzyme would bind to DEAE-cellulose at pH 8.9, but only when the ionic strength of the enzyme solution was less than 0.1. It did not bind to CM-cellulose at pH 7.4. For chromatography on DEAE-cellulose, it was therefore necessary to dilute the concentrated enzyme with 0.01 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.2 mM dithioerythritol, pH 8.9, to reduce the NaCl concentration in the enzyme solution.

Experiments with a small DEAE-cellulose column (1.3 x 3 cm) equilibrated at pH 8.9 in this buffer gave a 2-fold purification of enzyme with 80% recovery when the enzyme was eluted from the column with a NaCl gradient.

(ii) Hydroxyl-Apatite Chromatography

A small column (1.3 x 3 cm) of hydroxylapatite was equilibrated in 0.05 M sodium phosphate, 0.1 mM pyridoxal 5-phosphate, 0.2 mM dithioerythritol, pH 8.0. Concentrated enzyme from Step 4 of the purification procedure was diluted 3-fold with this buffer to reduce the NaCl concentration so that ALA synthetase would be adsorbed to the hydroxylapatite. After washing unadsorbed protein through the column, ALA synthetase activity was released with buffer containing 0.2 M sodium phosphate, 0.1 mM pyridoxal 5-phosphate, 0.2 mM dithioerythritol, pH 8.0. Approximately 80% of the enzyme activity was recovered in this step, with a 4-fold purification.

(iii) Chromatography on a Sepharose-Antibody Column

The concentrated enzyme from Step 4 was injected into a rabbit as follows. About 5 mg of protein (specific activity 240 units/mg protein) was emulsified in Freud's adjuvant and injected subcutaneously at several periscapular sites. Then 14 days later, a second series of injections with 4 mg of protein (specific activity 100 units/mg protein) was given without adjuvant. When blood was collected one week later from an ear vein, the serum was found to contain several cross-reacting species, as detected by the Ouchterlony double diffusion test (see Section 7.2), but did not precipitate ALA synthetase activity in immunological titrations.

An attempt was made to utilise this finding in an additional purification step. The γ-globulin fraction was isolated from the serum as described in Section 7.2, and reacted with cyanogen bromide-activated Sepharose 4B to covalently link antibodies to the agarose matrix [247]. The Sepharose derivative was then extensively washed, packed into a column (1 x 7 cm), and equilibrated with 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.3 M NaCl, 0.2 mM dithioerythritol, pH 7.4.

Concentrated enzyme solution was slowly passed through this column. It was hoped that some contaminating antigenic proteins would be immobilised on the matrix in the column and that ALA synthetase would pass through unretarded. However, no activity was detected in the

eluate. Whether the enzyme was bound to the matrix, or was denatured on passing through the matrix, is unknown.

B. Purification of Solubilised ALA Synthetase from Rat Liver Mitochondria

(a) Purification Method

Since attempts at purifying mitochondrial ALA synthetase from guinea pig liver met only with limited success, the purification of the enzyme from rat liver was undertaken to allow a comparison with the guinea pig enzyme. The properties of the rat enzyme were thought to be similar, since Kaplan [38] had reported it was extracted from liver mitochondria as an aggregate, which he was unable to disrupt by a number of methods. Other findings were also reported which agreed with those established in this laboratory for the guinea pig enzyme.

The purification method was based on studies with the guinea pig enzyme, but contained some modifications. ALA synthetase was solubilised in the mitochondrial extract, and then fractionated from other soluble proteins in three steps, including a new step involving affinity chromatography.

Step 1. Isolation and Extraction of Liver Mitochondria

Mitochondria were isolated from rat liver, freeze-dried and extracted as already described for guinea pig liver. The enzyme in the extract was then solubilised by

the addition of solid NaCl and dithioerythritol to concentrations of 0.8 M and 1 mM respectively.

Step 2. Sephadex G-150 Chromatography of the Mitochondrial Extract

A Sephadex column (4 x 30 cm), equilibrated in 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.5 M NaCl, 0.1 mM dithioerythritol, 0.1 mM EDTA, pH 8.0, was loaded with 6-10 ml of mitochondrial extract and eluted overnight at a flow rate of 20 ml/hr. The resulting ALA synthetase profile is seen in Fig. 5-3. As predicted from studies with the guinea pig enzyme, virtually all activity was solubilised and retarded on the column. The most active fractions were pooled, and EDTA added from a 0.1 M stock solution, pH 8.0, to give a final concentration of 1 mM.

Step 3. Ammonium Sulphate Fractionation

Solid ammonium sulphate was slowly added to the gently stirred solution to a final concentration of 50%. Stirring was continued for an additional 30 min, and the precipitate collected by centrifugation at 10,000 x g for 20 min. After being drained, the precipitate was redissolved in the minimum volume of 0.05 M tris-HCl, 1 mM dithioerythritol, pH 7.6.

Step 4. Affinity Chromatography

The redissolved enzyme was desalted on a Sephadex G-25 column, equilibrated in 0.05 M tris-HCl, 1 mM dithio-

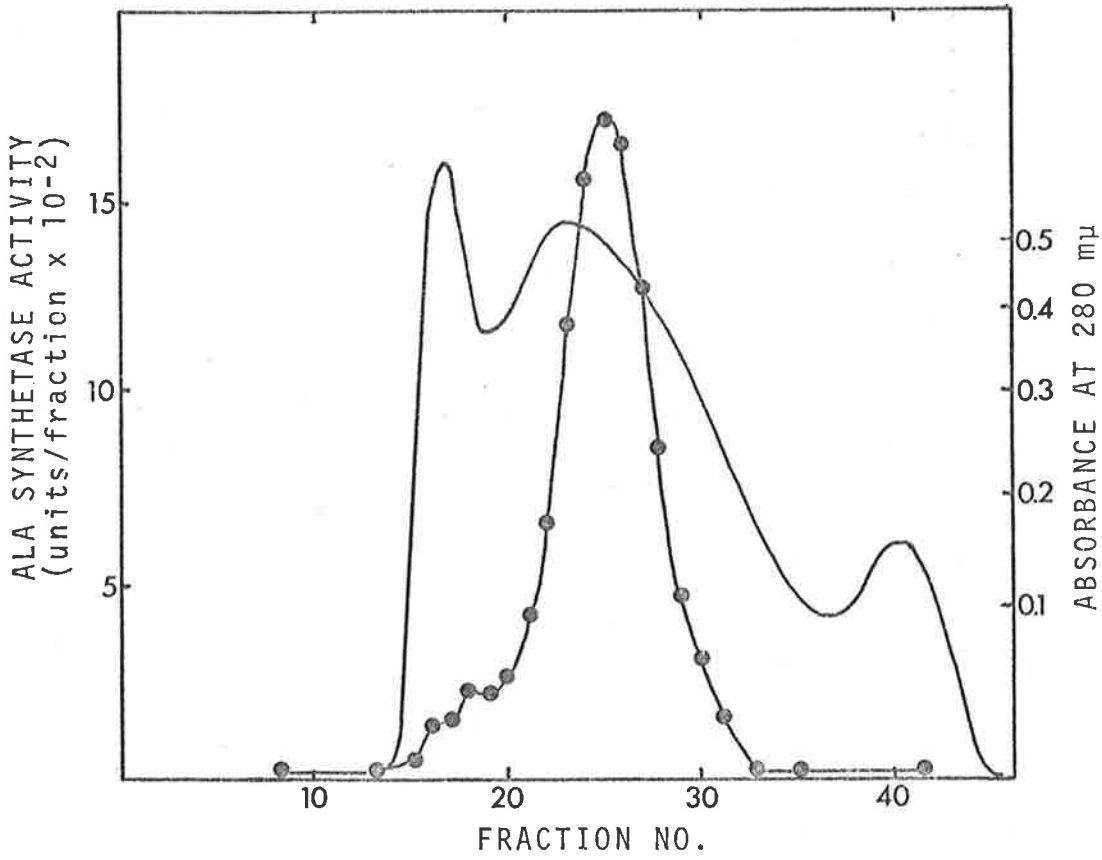


Fig. 5-3.

Sephadex G-200 chromatography of rat mitochondrial extract, containing NaCl (0.8 M) and dithioerythritol (1 mM).

Details are described in the text. The absorbance at 280 mμ was measured as in Fig. 5-1, ———; fractions were assayed colorimetrically for ALA synthetase activity, ●———●.

erythritol, pH 7.6, to remove last traces of pyridoxal 5-phosphate and ammonium sulphate. The protein was then passed through a column (1.3 x 13 cm) packed with the affinity chromatography substrate, prepared as described in Section 5.2. About 70% of ALA synthetase activity was bound to the column, which was washed with 0.05 M tris-HCl, 1 mM dithioerythritol, pH 7.6. ALA synthetase was then eluted with 20 ml of 0.05 M tris-HCl, 1 mM dithioerythritol, 0.01 M pyridoxal 5-phosphate, pH 7.6. The protein so obtained was termed purified enzyme. Before next use, the column was washed with the eluting buffer containing 0.5 M NaCl, and re-equilibrated in 0.05 M tris-HCl, 1 mM dithioerythritol, pH 7.6.

A typical fractionation is shown in Table 5-2. The overall yield for the method is approximately 20% with the affinity chromatography step giving a 10 to 12-fold purification.

(b) Some Properties of the Purified Rat Liver Mitochondrial Enzyme

The purified enzyme was reasonably stable when stored in 0.05 M tris-HCl, 1 mM dithioerythritol, 0.01 M pyridoxal 5-phosphate, pH 7.6. After 10 days at -15°C, 75% of the activity was preserved, while storage at 4°C for the same time resulted in 60% recovery of activity.

The purity of the enzyme was investigated using 5% polyacrylamide gel electrophoresis of the S-carboxymethylated protein in sodium dodecylsulphate, as described in

Table 5-2

The Purification of Rat Liver Mitochondrial ALA Synthetase

Details of the methods used are described in the text.

Fraction	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)
Mitochondria (from 20g liver)	14	12,900	990	13	100
Extract from Freeze-drying	10	9,200	460	20	71
Sephadex G-150 Fractions	105	9,050	210	43	70
Ammonium Sulphate Fractionation (0-50%)	4.4	5,500	119	46	43
Affinity Chromatography	35	2,980	6.2	530	23

Section 5.2. As seen in Fig. 5-4, one major band, which contained 70% of the total protein as determined by quantitative densitometry, along with some minor bands and aggregated material at the origin, was obtained. However, it is not known which band corresponds to ALA synthetase.

Previous studies with crude guinea pig liver enzyme indicated that in the absence of NaCl, reaggregation of the enzyme occurred. A sample of purified rat liver enzyme, stored in 0.05 M tris-HCl, 1 mM dithioerythritol, 0.01 M pyridoxal 5-phosphate, pH 7.6, and freeze-dried, was chromatographed on a Sephadex G-150 column with this buffer to check for reaggregation of enzymic activity. All activity was found to still elute as expected for the solubilised enzyme, indicating the material with which the enzyme was aggregating had evidently been removed during the purification procedure.

(c) The Molecular Weight of Solubilised Mitochondrial Rat Liver ALA Synthetase, and a Comparison with the Cytosol Enzyme

The molecular sizes of both mitochondrial and cytosol forms of ALA synthetase were determined after chromatography on a Sephadex G-150 column by comparing their elution volumes with those of proteins of known molecular weight, according to the method of Andrews [236]. Proteins used as markers were α-chymotrypsinogen (MW 25,000), ovalbumin (MW 45,000), bovine serum albumin (MW 67,000),

Fig. 5-4.

Electrophoresis of S-carboxymethylated ALA synthetase preparation on a 5% polyacrylamide gel in sodium dodecylsulphate [243].

and lactate dehydrogenase (MW 140,000). Samples of crude solubilised mitochondrial enzyme, and crude cytosol enzyme prepared as in Section 5.2, were eluted separately through the column with buffer, containing 0.05 M sucrose, 0.01 M sodium phosphate, 0.3 M NaCl, 0.1 mM EDTA, 0.01 M pyridoxal 5-phosphate, 0.1 mM dithioerythritol, pH 7.2, and the elution volumes of ALA synthetase noted (see Fig. 5-5). Values obtained for the molecular weights by this method were 77,000 for the mitochondrial enzyme, and 178,000 for the cytosol enzyme.

To establish the two enzymes as distinct species, a mixture of mitochondrial and cytosol enzymes, containing added NaCl (0.8 M) and dithioerythritol (1 mM), was chromatographed on the same column. As shown in Fig. 5-6, two peaks of ALA synthetase activity were obtained, one corresponding to mitochondrial enzyme, and the other to cytosol enzyme. The activities eluted exactly as expected from chromatography of individual enzyme samples.

5.4. DISCUSSION

The experiments described in this chapter have led to the development of a procedure reliably giving up to a 40-fold purification of mitochondrial ALA synthetase from rat liver, based on the increase in specific activity of the enzyme. The final purity of the enzyme is uncertain because the inability to identify ALA synthetase on a polyacrylamide gel makes experiments using this technique difficult to interpret. However, it should be possible

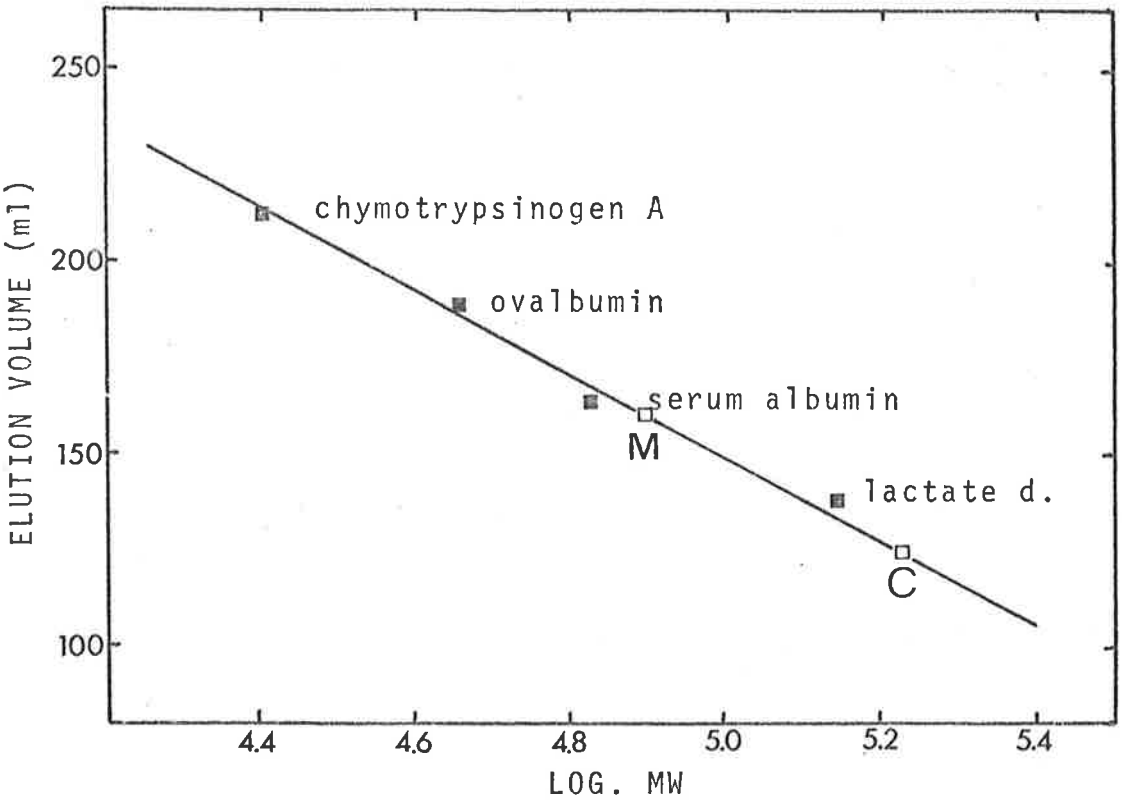


Fig. 5-5.
Determination of the molecular weights of mitochondrial (M) and cytosol (C) forms of ALA synthetase, by gel filtration on a calibrated Sephadex G-150 column (2.2 x 75 cm). Details are described in the text.

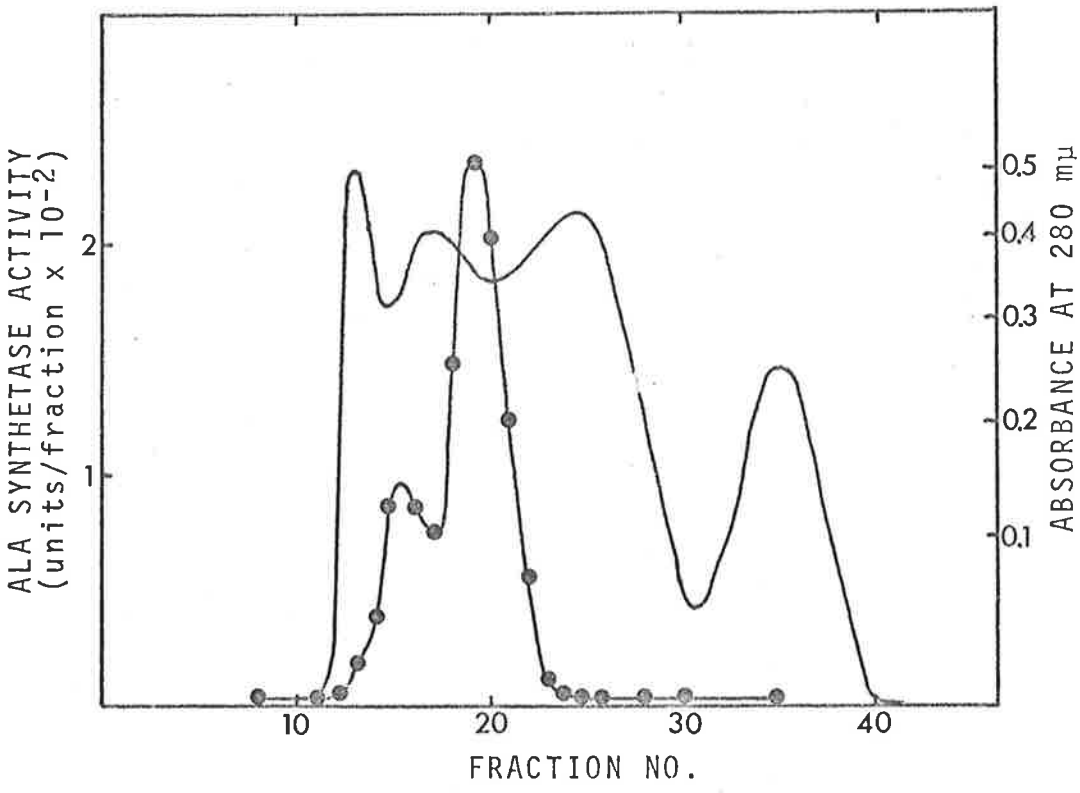


Fig. 5-6.

Chromatography of a mixture of mitochondrial and cytosol forms of ALA synthetase on Sephadex G-150.

Crude mitochondrial enzyme (1,000 units, 45 mg protein) was mixed with crude cytosol enzyme (120 units, 130 mg protein), and solid NaCl and dithioerythritol added to give final concentrations of 0.8 M and 1 mM respectively. This solution was loaded onto a Sephadex G-150 column (2.2 x 75 cm), and eluted with 0.05 M sucrose, 0.01 M sodium phosphate, 0.3 M NaCl, 0.1 mM EDTA, 0.01 mM pyridoxal 5-phosphate, 0.1 mM dithioerythritol, pH 7.2. The absorbance at 280 mμ was measured as in Fig. 5-1, ————; ALA synthetase activity was determined colorimetrically, ●— ——●.

to include another purification step, perhaps iso-electric focussing or hydroxyl-apatite chromatography, in the overall method, and work is continuing in this laboratory on this aspect.

The finding that the purified rat enzyme does not reaggregate in the absence of NaCl is of considerable interest, and implies that there may be a specific protein fraction, which is removed during the purification, capable of binding to ALA synthetase. Such protein(s) may have physiological significance with regard to any postulated mechanisms of transport of ALA synthetase from the cytosol into the mitochondrion, and localisation of ALA synthetase within the mitochondrion [7,248].

The mitochondrial form of ALA synthetase has not been previously purified from liver in a soluble form. As discussed in Chapter 4, Kaplan [38] was unable to solubilise ALA synthetase from rat liver mitochondria and succeeded only in a partial purification of an enzymically-active aggregate. The cytosol form of the enzyme, however, has been purified 200-fold from rat liver [45], although only the details of preliminary study reporting a 20-fold purification have been published [8]. Its molecular weight as determined by sedimentation on a sucrose gradient was 150,000 in the presence of NaCl, and this value compares reasonably well with 178,000, determined in this laboratory by gel filtration.

The only other data concerning the molecular sizes of the two forms of ALA synthetase from rat liver

has been published by Hayashi *et al*. [249]. A value of 115,000 was reported for the molecular weight of the mitochondrial enzyme, solubilised by sonication treatment and fractionated by ammonium sulphate. However, this enzyme preparation was not well characterised and is almost certainly an aggregate, partly dissociated by the high salt concentration encountered in the ammonium sulphate fractionation. Studies already reported (see Section 4.2(d)) have shown that the sedimentation behaviour of ALA synthetase extracted from sonicated mitochondria is changed from a broad disperse profile to a defined peak of activity of molecular weight around 100,000 by ammonium sulphate treatment.

Other studies on the molecular weight of the cytosol enzyme gave values of around 600,000, as determined by Sepharose 4B chromatography, but 178,000 from sedimentation behaviour on a sucrose gradient [249]. The reason for the difference between these values is unexplained, but aggregation of the cytosol enzyme has been reported [45] and may be causing these apparent discrepancies.

The studies reported in this chapter show a definite difference in molecular size between mitochondrial and cytosol ALA synthetases. The molecular weight value of 77,000 determined for solubilised rat liver mitochondrial ALA synthetase was in agreement with the value previously determined for the guinea pig enzyme. Mixing experiments indicated that the cytosol enzyme (MW 178,000) was not dissociated by NaCl and dithioerythritol, and that the two

enzymes were therefore distinct forms.

Reports of the purification of ALA synthetase isolated from other sources, including rabbit reticulocytes and *R. spheroides* have recently appeared, and are discussed in Section 1.2. Thus, although ALA synthetase from many sources has long been regarded as an unstable enzyme, and has been difficult to purify (e.g., ref.13), methods are now being developed which overcome these problems.

Some kinetic properties of the purified rat liver mitochondrial enzyme are described in Chapter 6, and some immunological properties in Chapter 7.

CHAPTER 6

PRELIMINARY KINETIC STUDIES OF PURIFIED RAT LIVER ALA SYNTHETASE

6.1. INTRODUCTION

Since the complete solubilisation of mitochondrial ALA synthetase has not been previously reported, no kinetic properties of this form of the enzyme are known. However, the aggregated form of the enzyme has been studied, and some kinetic properties reported for the guinea pig enzyme by Irving [208], and for the rat enzyme by Kaplan [38].

On the other hand, ALA synthetase from the cytosol of rat liver has been purified, and studied kinetically [8,45], but this enzyme has a different molecular weight from the mitochondrial enzyme (see Chapter 5), and might, therefore, have different properties. In an effort to obtain information on the relationship between the cytosol and mitochondrial enzymes, some properties of purified, solubilised mitochondrial ALA synthetase were determined for comparison with those of the cytosol and aggregated mitochondrial forms of the enzyme.

In addition, Scholnick *et al.* [8,45] have shown that hemin inhibits purified cytosol ALA synthetase. If heme inhibition of ALA synthetase is an important control mechanism used by the cell to regulate the heme biosynthetic pathway, it would seem essential for the mitochondrial enzyme to be involved, rather than the cytosol enzyme. This is because ALA synthetase is probably only functional inside the mitochondrion, where succinyl-CoA is generated, and could conveniently be controlled by heme synthesised inside the mitochondrion by ferrochelatase,

an inner membrane enzyme [6]. Since aggregated preparations of mitochondrial ALA synthetase have been shown to be inhibited by high concentrations of hemin [38,208], experiments were carried out to determine whether hemin inhibited the purified solubilised enzyme.

6.2. METHODS

(a) Synthesis of Succinyl-CoA

Succinyl-CoA was prepared by a method based on that of Simon and Shemin [250]. Firstly, CoA was reduced with sodium borohydride as follows. 12 mg of CoA were dissolved in 0.5 ml of cold water and a drop of bromothymol blue added; 1 M tris was added dropwise until the indicator turned blue. Then 1-2 mg of sodium borohydride were added and the solution allowed to stand in ice for 5 min with occasional shaking. The reaction was terminated with a drop of 6 N HCl, the frothing allowed to subside, and the total volume made up to 5 ml with cold water.

The reduced CoA was then converted to succinyl-CoA as follows. Succinic anhydride (2 mg) was added to the solution and 1 M tris added to give a pH between 7.0 and 7.5. The solution was maintained in this pH range while standing in ice for 30 min, and shaken frequently. Two drops of 6 N HCl were then added, and the mixture extracted 3 times with an equal volume of cold ether. Residual ether was removed by evaporation *in vacuo* and the succinyl-CoA formed estimated by reaction with hydroxylamine [251].

Spectrophotometric determinations, titration of

sulfhydryl groups with 5,5'-dithiobis-2(2-nitrobenzoate) [25] and assays by the hydroxamic acid method [251] indicated greater than 90% conversion of CoA to succinyl-CoA by this method.

(b) Preparation of Hemin Solutions

Hemin was prepared as a 1 mM stock solution by the method of Burnham and Lascelles [15].

6.3. RESULTS

A. Development of a New Assay System Using Chemically Synthesised Succinyl-CoA

Before the kinetic properties of the purified mitochondrial enzyme could be studied, it was necessary to develop a new assay system which utilised added succinyl-CoA. Any observations could then be directly attributable to an effect on ALA synthetase, and not on a succinyl-CoA generating system. The properties of such a system were examined to provide the basis of a reliable assay method.

(a) Determination of the pH Optimum of the Purified Enzyme

The pH optimum of the enzyme was established by determinating the V_{max} of the enzyme at pH values of 7.2, 7.6, 8.0 and 8.4 at 37^{o}. For each pH value, a plot of reaction velocity against glycine concentration was drawn,

and the V_{max} at that particular pH value was determined from a double reciprocal plot.

The assay system used contained in a total volume of 1.0 ml: glycine in amounts varying from 0 - 100 µmole; succinyl-CoA, 500 mµmole; pyridoxal 5-phosphate, 0.4 µmole; enzyme in 0.05 M tris-HCl, 1 mM dithioerythritol, 0.01 M pyridoxal 5-phosphate, pH 7.6 (TDP buffer), 0.3 ml (40 units, specific activity 580 units/mg protein); and 200 µmole of tris buffer at the pH under test. Assays were preincubated at 37° for 3 min and reaction started by the addition of succinyl-CoA. Incubation was carried out for 20 min at 37°.

After the addition of 0.2 ml of cold 15% trichloroacetic acid solutions were allowed to stand for 10 min and centrifuged in a bench centrifuge. 1.0 ml of supernatant was mixed with 0.3 ml of 1 M sodium acetate to give pH 4.6, and 0.05 ml of acetylacetone and the mixture heated in a boiling waterbath for 15 min. After cooling, 1.3 ml of Ehrlich reagent was added to each assay and the optical density of the solution read at 552 mµ exactly 10 min after mixing.

Results obtained showed the V_{max} at pH 7.6 to be slightly greater than at 7.2, which in turn was greater than pH 8.0 and 8.4.

All subsequent assays were therefore carried out at pH 7.6 at 37°C.

(b) Effect of Enzyme Concentration on Observed ALA Synthetase Activity

Varying amounts of enzyme in TDP buffer were added to an assay mixture containing in a final volume of 1.0 ml: 50 µmole glycine, 100 mµmole succinyl-CoA, 50 µmole tris-HCl, pH 7.6 at 37°, and 0.4 µmole pyridoxal 5-phosphate. The mixture was incubated for 20 min at 37°, and ALA determined as described above.

As is seen in Fig. 6-1, the assay is linear up to an enzyme concentration of 40 units per assay.

(c) Determination of the Time Course of ALA Synthesis at 37°C

0.3 ml of enzyme (40 units) in TDP buffer was incubated for various times in an assay system of 1.0 ml containing 50 µmole glycine, 100 mµmole succincyl-CoA and 50 µmole tris-HCl, pH 7.6.

Fig. 6-2 shows ALA synthesis proceeds linearly for 15 min. After this time, the reaction stops very rapidly. This is probably due to hydrolysis of succinyl-CoA, which has a half-life of only 20 min under these conditions.

B. Some Kinetic Properties of Purified Rat Liver ALA Synthetase

For the determination of K_m values, the conditions chosen included 32 units of enzyme per assay and an incubation time of 15 min at 37°C.

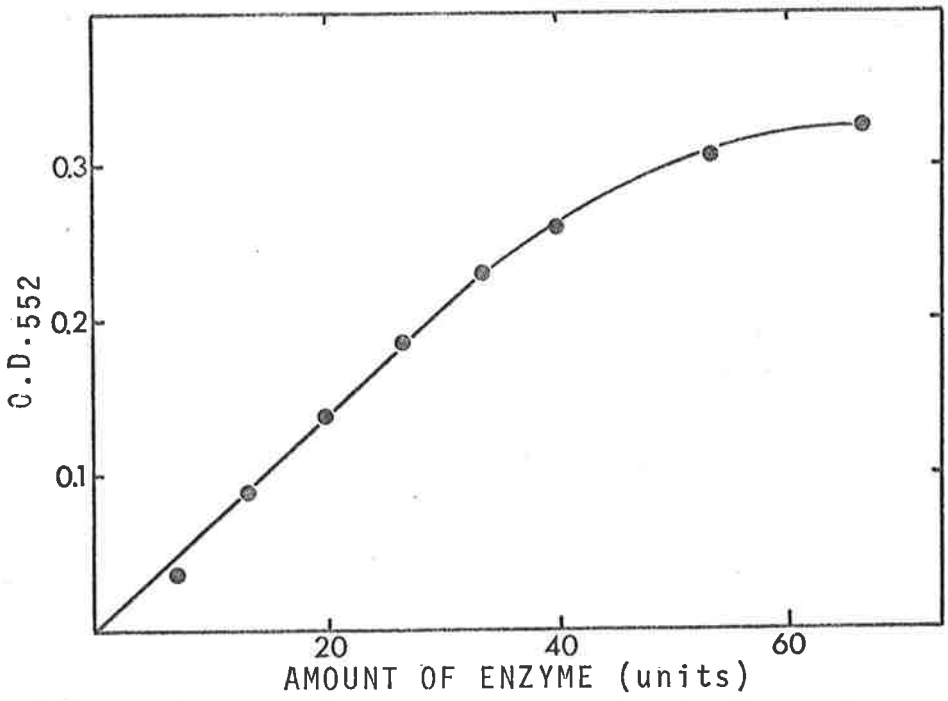


Fig. 6-1.
Effect of increasing enzyme concentration on ALA synthetase activity.
The assay procedure is described in the text.

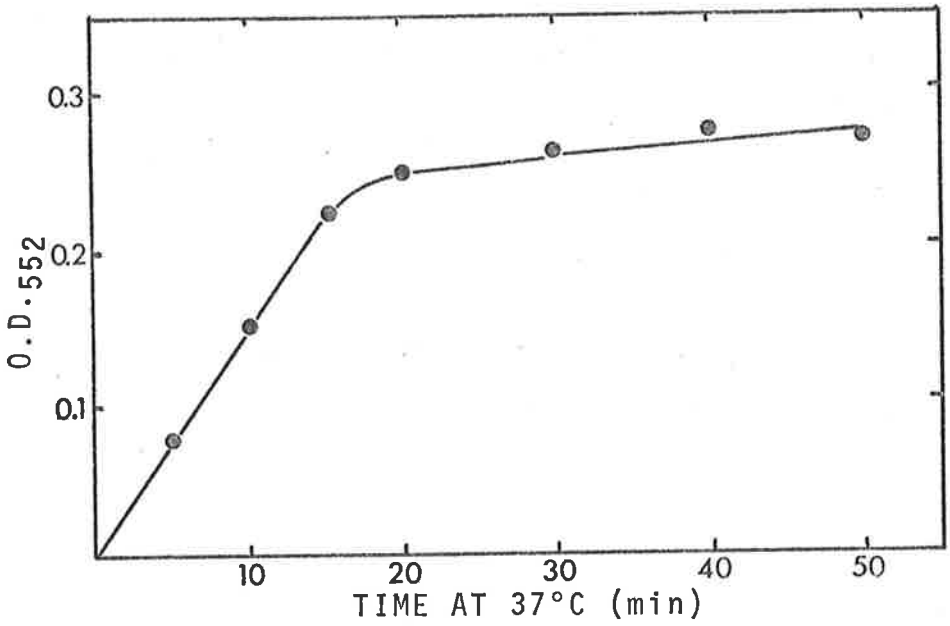


Fig. 6-2.
Time course of ALA synthesis at 37°C. The assay procedure is described in the text.

(a) Determination of K_m Values for Glycine and Succinyl-CoA

In the following experiments, pyridoxal 5-phosphate was contained in the enzyme buffer and was therefore added with the enzyme at a saturating level of 2.5 µmole per assay.

(i) K_m for Glycine

The assays performed contained in 1.0 ml, the following: tris-HCl buffer, pH 7.6 at 37°, 50 µmole; enzyme in TDP buffer, 32 units; glycine in amounts varying from 0 to 100 µmole, at fixed amounts of succinyl-CoA of 20, 30, 50 or 100 mµmoles.

After incubation, the reaction was stopped and ALA determined as described in Section 6.3A(a). Correction was made for background colour produced by the reaction of glycine and Ehrlich reagent.

The results are shown in Fig. 6-3 in the form of a double reciprocal plot.

(ii) K_m for Succinyl-CoA

Assays were carried out as above with varying amounts of succinyl-CoA from 0 to 100 mµmole at fixed glycine amounts of 4, 10, 20 and 30 µmole.

The results are shown in Fig. 6-4 in the form of a double reciprocal plot.

(iii) Secondary Plots

The series of parallel straight lines in Figs. 6-3 and 6-4 indicate a non-sequential reaction

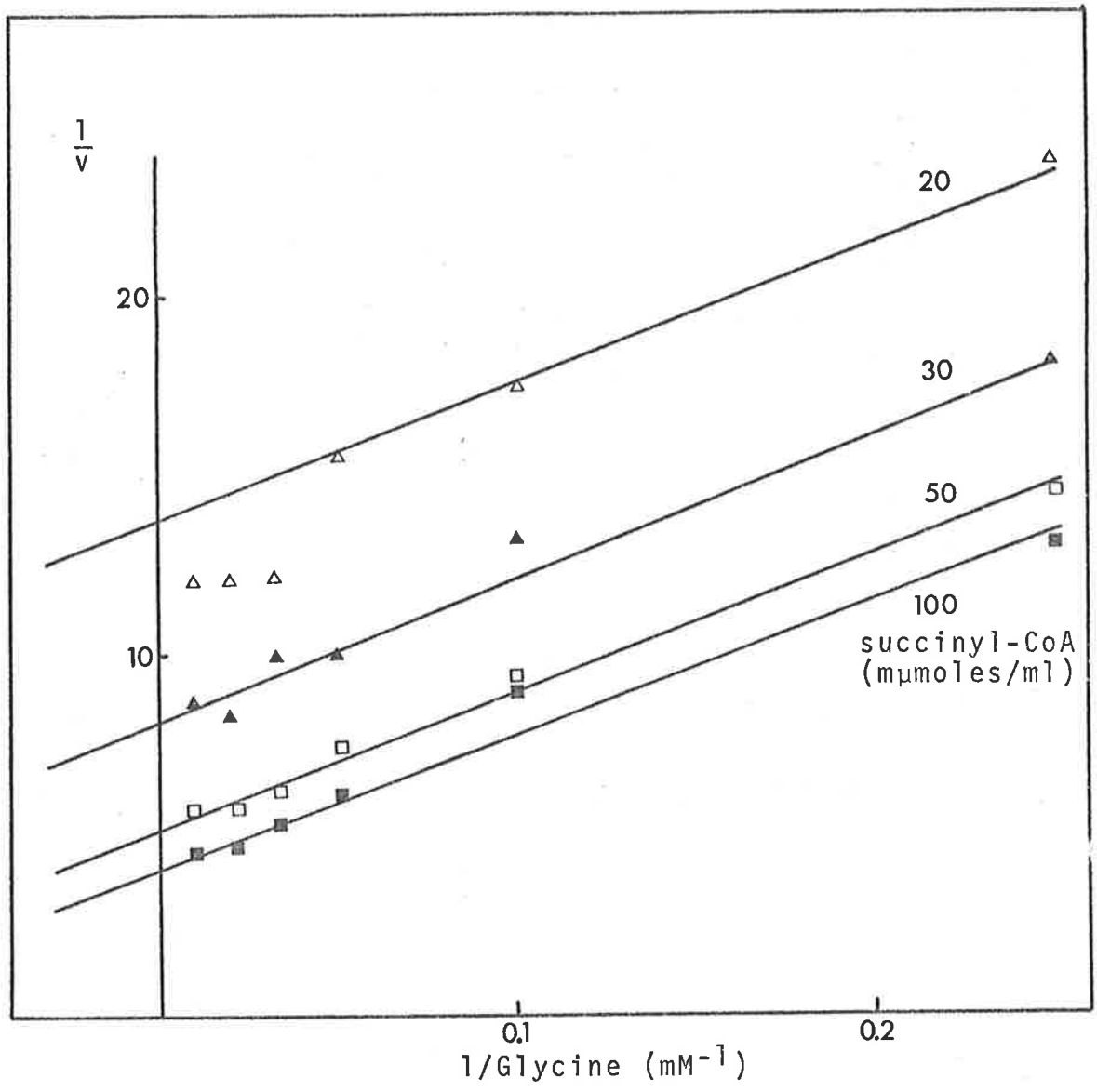


Fig. 6-3.

Double reciprocal plot of velocity versus glycine concentration, at fixed succinyl-CoA concentrations of 20, 30, 50 and 100 mμmoles/ml. The assay details are described in the text.

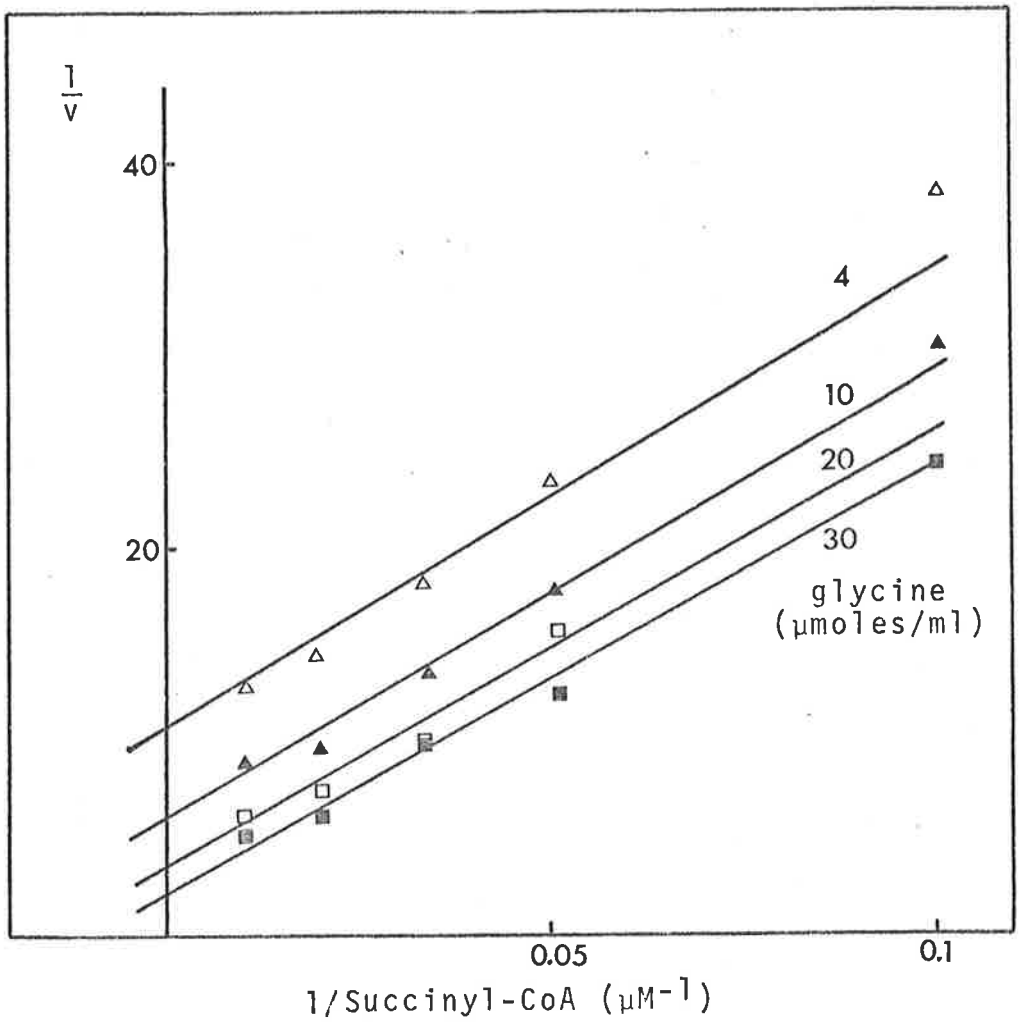


Fig. 6-4.

Double reciprocal plot of velocity versus succinyl-CoA concentration,

at fixed glycine concentrations of 4, 10, 20 and 30 µmoles/ml. The assay details are described in the text.

mechanism for the addition of glycine and succinyl-CoA to the enzyme surface.

For the generalised two substrate reaction $A + B \rightleftarrows P + Q$, the rate equation for the non-sequential reaction mechanism is:

$$v = \frac{VAB}{K_a B + K_b A + AB}$$

where K_a and K_b are the Michaelis constants for substrates A and B.

Rearranging this equation to the form of a straight line gives:

$$\frac{1}{v} = \frac{K_a}{V}\frac{1}{A} + \frac{1}{V}\left(\frac{K_b}{B} + 1\right).$$

The intercept on the ordinate for a double reciprocal plot is therefore

$$\text{intercept} = \frac{1}{V}\left(\frac{K_b}{B} + 1\right)$$

$$= \frac{K_b}{V}\frac{1}{B} + \frac{1}{V}.$$

A secondary plot of intercept against 1/B allows K_b to be calculated.

Figs. 6-5 and 6-6 show secondary plots using the data from Figs. 6-3 and 6-4 respectively. From these plots, the K_m for succinyl-CoA was calculated to be 2.0 x 10^{-4} M, and the K_m for glycine 1.9 x 10^{-2} M.

(b) Determination of the K_m for Pyridoxal 5-Phosphate

In an attempt to obtain enzyme free from pyridoxal 5-phosphate for this determination, ALA synthetase was eluted from an affinity chromatography column with 0.2 M NaCl instead of 0.01 M pyridoxal 5-phosphate. However, when this enzyme was assayed in the absence of pyridoxal 5-phosphate, activity was still detectable at a level of 20% that observed in the presence of 10^{-4} M pyridoxal 5-phosphate. Overnight dialysis of the enzyme to remove last amounts of pyridoxal 5-phosphate was not performed, due to the instability of the enzyme in its absence.

Thus, while an accurate value of the K_m for pyridoxal 5-phosphate could not be obtained, it was estimated to be in the range of 10^{-5} to 10^{-6} M.

(c) Effect of Hemin and Other Compounds on ALA Synthetase Activity

Using data from the previous experiments, the standard assay system used in this work was as follows: tris-HCl buffer, pH 7.6, 50 µmole; succinyl-CoA, 100 mµmole; glycine, pH 7.6, 100 µmole; enzyme in TDP buffer, 0.25 ml (20-30 units), and the compound under test, in a final volume of 1.0 ml.

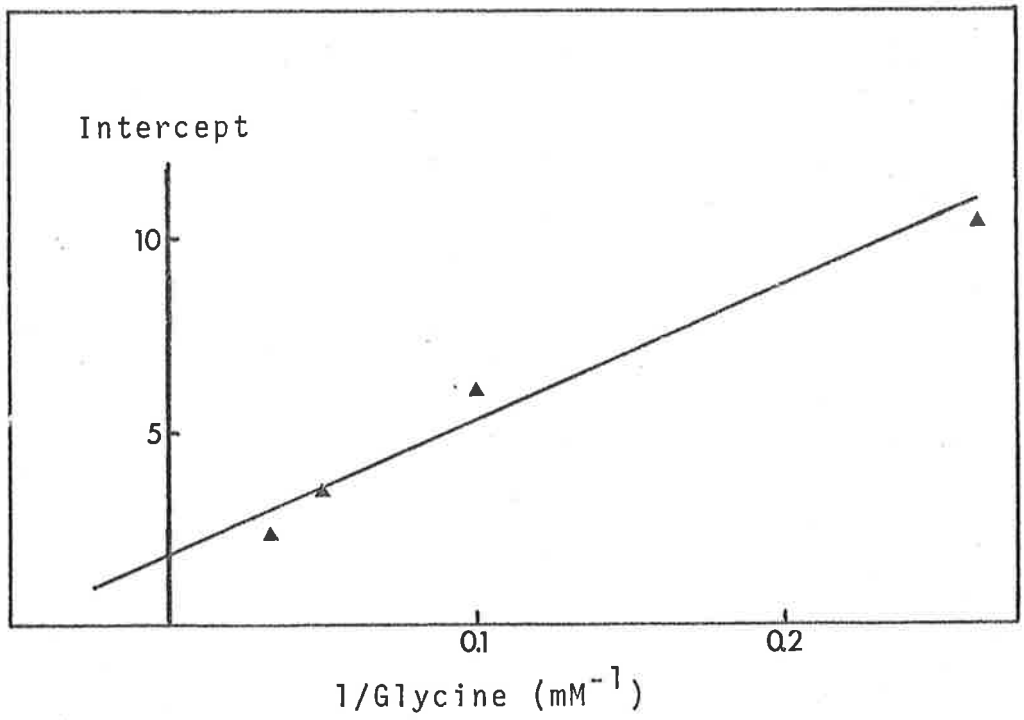


Fig. 6-5.
Secondary plot of intercept versus the reciprocal of succinyl-CoA concentration,
using the data in Fig. 6-3.

Slope = $\frac{K_m \text{ succinyl-CoA}}{V}$ = 0.24; Intercept = $\frac{1}{V}$ = 1.2;

K_m succinyl-CoA = 0.20 mM.

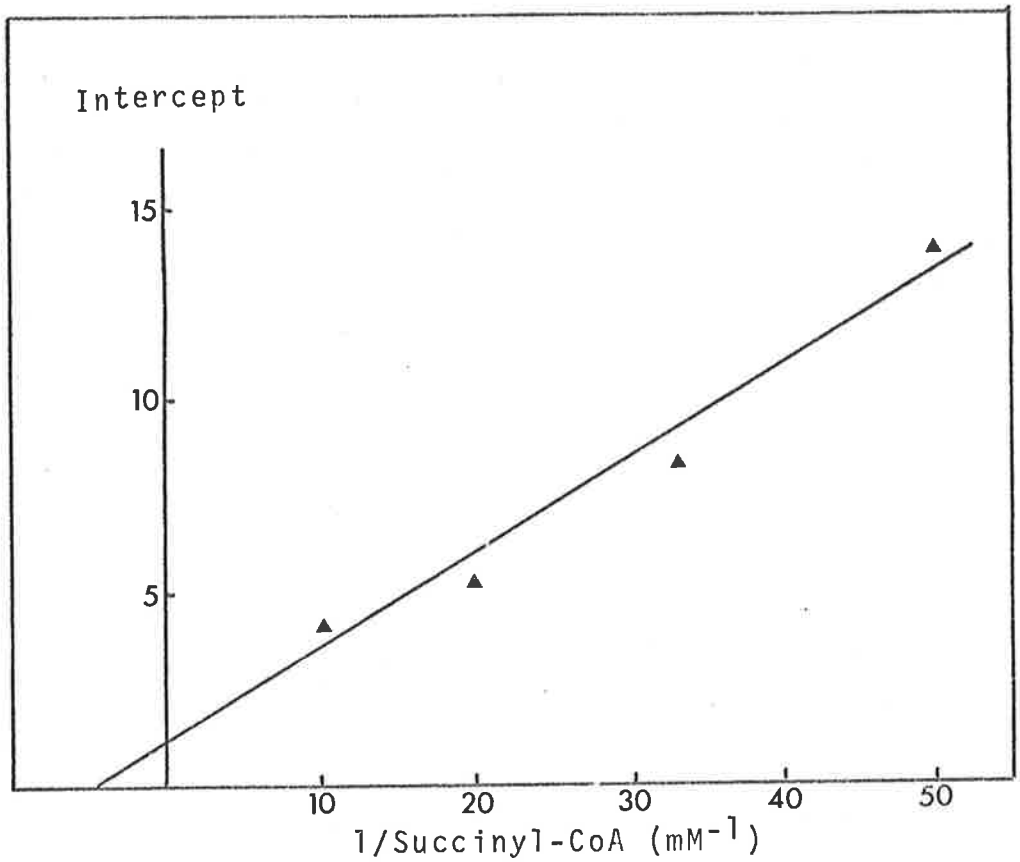


Fig. 6-6.
Secondary plot of intercept versus the reciprocal of glycine concentration,
using the data in Fig. 6-4.

Slope = $\frac{K_m \text{ glycine}}{V}$ = 35.0; Intercept = $\frac{1}{V}$ = 1.85;

K_m glycine = 19 mM.

Assays were pre-incubated for 2 min at 37°C and reactions started by the addition of succinyl-CoA. After incubation for 15 min, 0.2 ml of ice-cold 15% trichloroacetic acid was added to each assay, and the mixtures allowed to stand for 10 min.

The assay tubes were then centrifuged for 10 min, 1.0 ml of the supernatant was added to 0.3 ml of 1.0 M sodium acetate and 0.05 ml of acetylacetone to give a pH of 4.6, and the mixture heated in a boiling water-bath for 15 min.

After cooling in water, the assays were mixed with 1.3 ml of Ehrlich reagent, and the optical density read at 552 mμ exactly 10 min after mixing. Controls were carried out for each assay in which the trichloroacetic acid was added prior to the succinyl-CoA.

(i) Hemin

The effect of hemin on ALA synthetase activity is shown in Fig. 6-7. With enzyme of highest specific activity, 50% inhibition of activity was obtained at 10 μM hemin, while 95% inhibition occurred at a concentration of 40 μM.

However, for preparations of lower purity, inhibition was less and the kinetics of inhibition were different. Using the data in Fig. 6-7, the number of molecules of hemin involved in the inhibition was calculated by a Hill plot, as shown in Fig. 6-8. The slopes of the lines are 0.5 for enzyme of specific activity

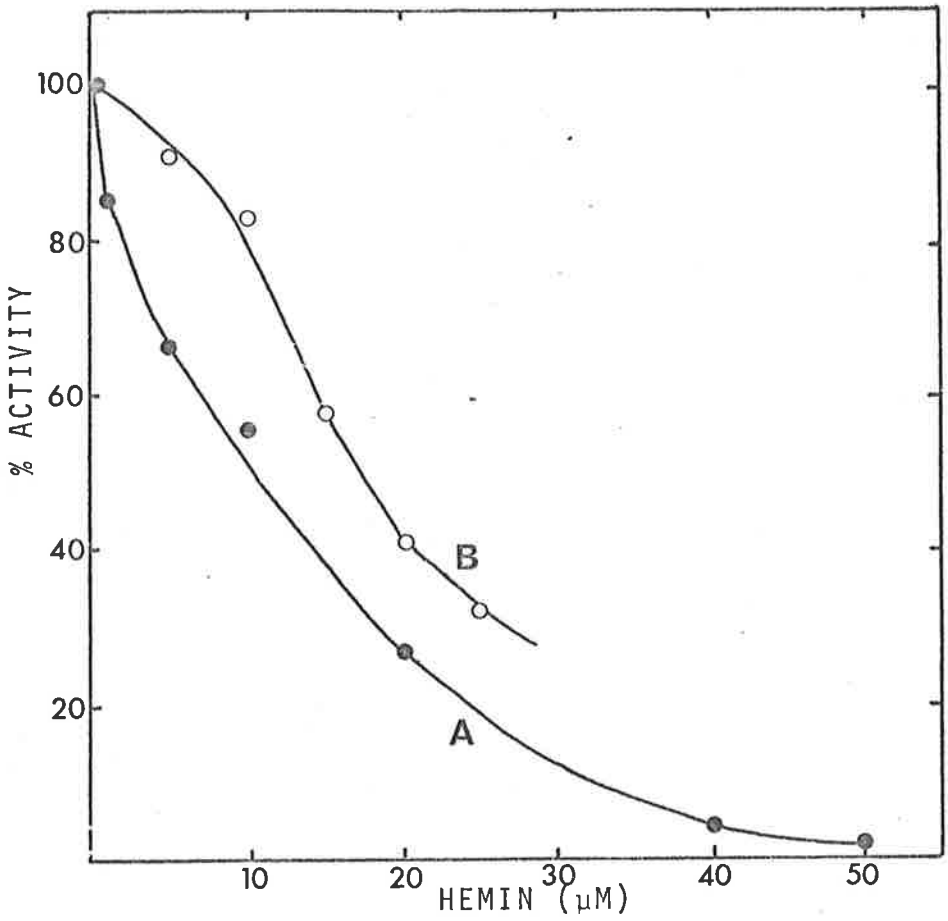


Fig. 6-7.

Inhibition of ALA synthetase by hemin.

A. Enzyme of specific activity 530 units/mg protein;
B. Enzyme of specific activity 200 units/mg protein.
The assay system is described in the text.

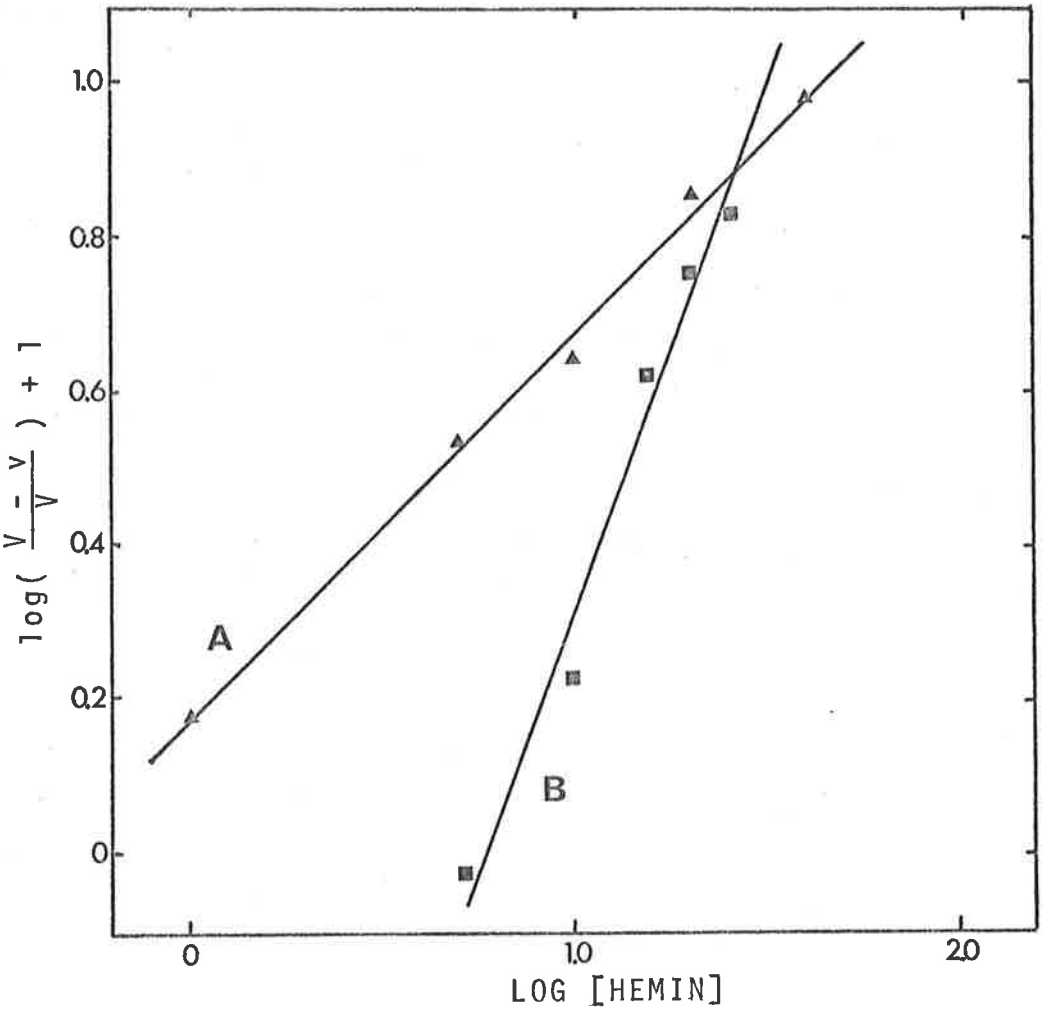


Fig. 6-8.

Hill plot,

using the data in Fig. 6-7. A. Enzyme of specific activity 530 units/mg protein; B. Enzyme of specific activity 200 units/mg protein. Slopes of lines are A = 0.50, B = 1.4.

530 units/mg protein and 1.4 for enzyme of specific activity 200 units/mg protein. The interpretation of this apparent discrepancy is discussed in Section 6.4.

A double reciprocal plot of velocity versus glycine concentration at different fixed levels of hemin, as seen in Fig. 6-9, shows that hemin is a mixed inhibitor with respect to glycine. Similar kinetic studies with succinyl-CoA could not distinguish between competitive, non-competitive or mixed inhibition, because the double reciprocal plot showed converging lines which intersected at a point very close to the point of intersection of the ordinate and the abscissa. The data were not sufficiently accurate to exactly place the intersection point.

(ii) Glycine and ALA Analogues

The effects of some analogues of glycine and ALA on ALA synthetase activity are shown in Table 6-1.

Serine and methylamine were completely ineffective in competing for the glycine binding site on the enzyme. Of the ALA analogues tested, α-aminoadipate, γ-aminobutyrate, and ε-aminocaproate were all ineffective at concentrations up to 10^{-2} M.

(iii) Miscellaneous

Table 6-2 shows the effect of a variety of compounds on ALA synthetase activity. The enzyme is sensitive to the sulfhydryl reagents p-hydroxymercuribenzoate, N-ethylmaleimide and lead acetate, and is stimulated by monovalent and divalent cations, as well as by ATP, AMP and CoA. Avidin, c-AMP, reducing agents and ferric

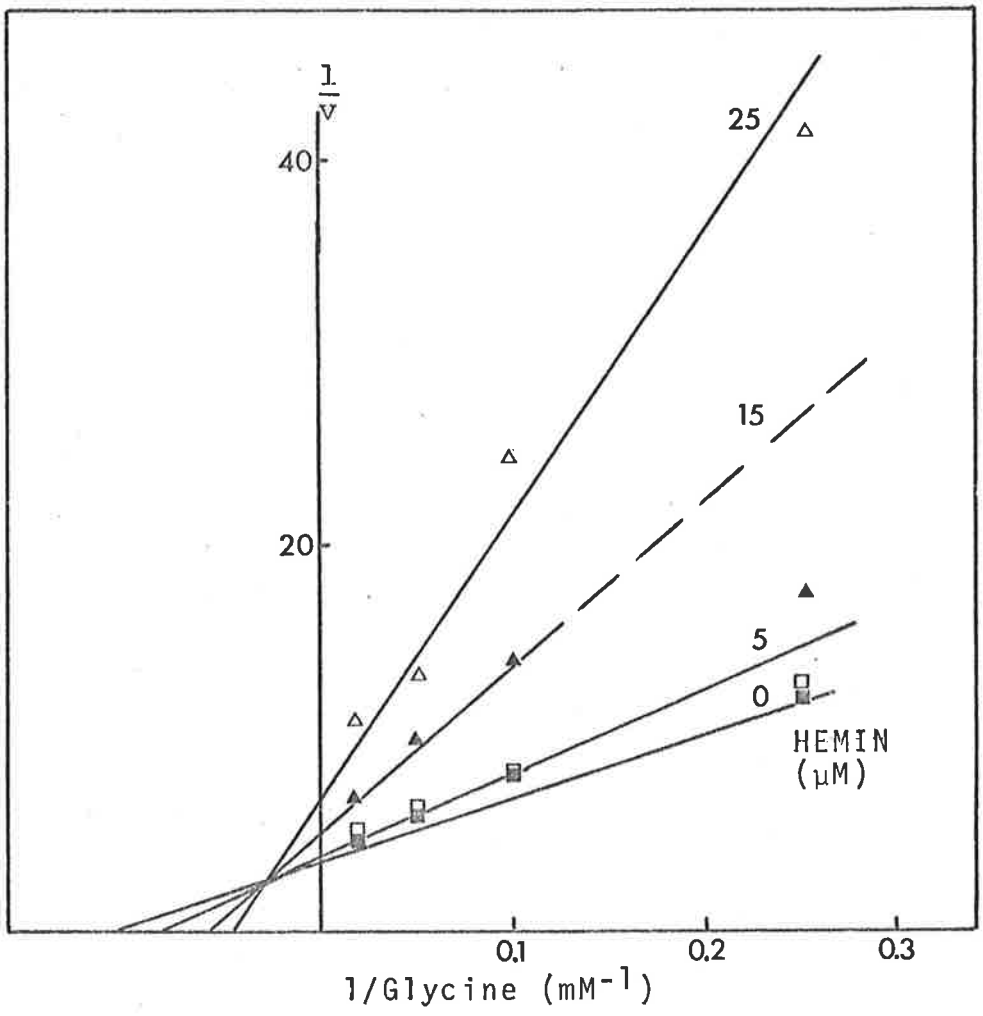


Fig. 6-9.

Double reciprocal plot of velocity versus glycine concentration, at fixed hemin concentrations

of 0, 5, 15 and 25 μM. Assay conditions were as described in Fig. 6-7.

Table 6-1

The Effect of Some Analogues of Glycine and ALA on ALA Synthetase Activity

All solutions of analogues were adjusted to pH 7.6 before addition to the assay mixture described in the text (Section 6.3B(c)).

Compound	Concentration (M)	Activity as % of Control
No addition		100
Glycine Analogues		
Serine	10^{-1}, 10^{-2}	92
Methylamine (hydrochloride)	10^{-2}	100
	10^{-3}	98
ALA Analogues		
α-aminoadipate	10^{-2}	97
	10^{-3}	103
	10^{-4}	107
δ-aminovalerate	10^{-2}	71
	10^{-3}, 10^{-4}	90
γ-aminobutyrate	10^{-2}	97
	10^{-3}, 10^{-4}	100
ε-aminocaproate	10^{-2}	100
	10^{-4}	98

Table 6-2

The Effect of Various Compounds and Inhibitors on ALA Synthetase Activity

Inhibitors were added to the assay system described in the text (Section 6.3B(c)).

Compound	Concentration (M)	Activity as % of Control
No addition		100
p-hydroxymercuribenzoate	10^{-3}, 10^{-4}	0
	10^{-5}	28
N-ethylmaleimide	10^{-3}	35
	10^{-4}	94
Lead acetate	10^{-3}	6
	10^{-4}	21
	10^{-5}	95
Ferric citrate	10^{-3}	89
	10^{-4}	91
Magnesium chloride	10^{-2}	116
	10^{-3}	100
NaCl	10^{-1}	119
	2.10^{-1}	135
EDTA	10^{-2}, 10^{-3}	107
Glutathione	4.10^{-3}	88
	4.10^{-4}	96
Dithioerythritol	10^{-3}	92
c-AMP	10^{-4}	107
	10^{-6}	100
ATP	10^{-2}	236
	10^{-3}	163
	10^{-4}	113
AMP	10^{-2}	164
	10^{-3}	122

Table 6-2 (Continued)

Compound	Concentration	Activity as % of Control
Coenzyme A	10^{-3}	154
	10^{-4}	129
	10^{-5}	98
Avidin	60 µg/ml	99
	600µg/ml	105

citrate had no effect at the concentrations used.

6.4. DISCUSSION

(a) K_m Values for Glycine and Succinyl-CoA

The true K_m values reported here of 19 mM for glycine and 200 μM for succinyl-CoA are of the same order as those obtained for ALA synthetase from other sources (see Table 6-3). The discrepancy between values obtained for the same enzyme by different workers may be related to the instability of succinyl-CoA. As the K_m values obtained for aggregated and solubilised mitochondrial ALA synthetase are of the same order, aggregation of the mitochondrial enzyme with other protein does not seem to greatly affect its kinetic properties. The cytosol enzyme also has K_m values of the same order as the mitochondrial enzyme, but no definite conclusion regarding the identity of these enzymes can be made, since enzymes from mitochondrial and cytosol fractions that are very similar kinetically may still be different immunochemically (e.g., ref. 253; also see Chapter 7).

(b) Hemin Inhibition Studies

The inhibition by hemin of the enzyme is the highest yet reported for the mammalian enzyme and is only slightly less than that reported for *R. spheroides* (see Table 6-4). As discussed by Scholnick *et al.* [8] for cytosol ALA synthetase, crude preparations of enzyme do not show maximum hemin inhibition. This is probably

Table 6-3

K_m Values for Glycine and Succinyl-CoA for ALA Synthetase from Various Sources

Source of Enzyme	K_m Glycine (mM)	K_m Succinyl-CoA (μM)	Reference
Rat liver mitochondria (Solubilised)	19	200	This thesis
Rat liver mitochondria (Aggregated)	2.5	200	38
Rat liver mitochondria	4	-	7
Guinea pig liver mito. (Aggregated)	33	-	208
Rat liver cytosol	10	70	45
Rat liver cytosol	4	-	7
Rabbit reticulocytes	10	60	39
R. spheroides	10	25	37
R. spheroides	5	5	40

Table 6-4

Hemin Inhibition of ALA Synthetase, Purified from Various Sources

Source of Enzyme	Purification (fold from mitochondria or cytosol)	Hemin Conc. (μM)	Inhibition (%)	Reference
Guinea pig mito. (Aggregated)	5-6	100 200	15 50	208
Rat liver mito. (Aggregated)	6	10 50 100	3 48 81	38
Guinea pig mito. (Solubilised)	10	100 200	22 53	254
Rat liver mito. (Solubilised)	40	1 5 10 40	15 34 50 96	This thesis
Rat liver cytosol	20	10 50 100	0 31 77	8
Rabbit reticulocytes	4400	10	40	39
R. spheroides	1300	0.1 1 5	13 33 57	37

due to other proteins in the preparation binding the inhibitor. The kinetics of inhibition seen in Fig. 6-7 can be explained on this basis. Less pure preparations of ALA synthetase (e.g., specific activity 200 units/mg protein, or less) show a sigmoid type of hemin inhibition which may be interpreted as allosteric. Indeed, a Hill plot of the data indicated two molecules of hemin per molecule of enzyme were involved in the inhibition. However, this effect was not seen with the purest samples, and a Hill plot showed only one molecule of hemin to be involved. The sigmoid-shaped curve could be produced by low amounts of hemin being bound by other proteins, decreasing ALA synthetase inhibition at low hemin concentrations.

Other data showed the inhibition was mixed with respect to glycine and either competitive, non-competitive, or mixed with respect to succinyl-CoA. Although heme repression of ALA synthetase is generally thought to be the mode of control of the heme biosynthetic pathway [28], end product inhibition may now also be a significant control mechanism. ALA synthetase and ferrochelatase, the first and last enzymes of the pathway, are located together inside the mitochondrion, while the second to fifth enzymes are cytoplasmic. Heme is therefore produced in the same cellular compartment as ALA synthetase, and could be readily available to regulate its activity.

(c) Other Properties of the Purified Enzyme

Some other properties of the enzyme were revealed by these studies. The enzyme is sensitive to sulfhydryl reagents, but is avidin insensitive and is therefore not a biotin enzyme. It was stimulated by NaCl or KCl, as was the cytosol enzyme [45], and also surprisingly by high concentrations of CoA, ATP, and less effectively AMP. The enzyme did not appear to require magnesium ions. EDTA, ferric citrate, glutathione and dithioerythritol had no significant effect on the enzyme.

The reported therapeutic effect of calcium EDTA administered to patients suffering from various forms of hepatic porphyria [255, 256] thus appears unconnected directly to ALA synthetase.

(d) The Mechanism of the ALA Synthetase Reaction

The overall mechanism for the ALA synthetase-catalysed reaction is generally accepted to be as shown in Fig. 6-10. Glycine and enzyme-bound pyridoxal 5-phosphate condense together in a Schiff's base with the formation of a stable carbanion. The electrophilic carboxyl carbon atom of succinyl-CoA then reacts with the carbanion to form α-amino-β-ketoadipic acid or ALA and carbon dioxide.

The results presented in Figs. 6-3 and 6-4 indicate the addition of glycine and succinyl-CoA to the enzyme-pyridoxal 5-phosphate complex is non-sequential. This suggests three possible reaction sequences, which are represented diagrammatically below.

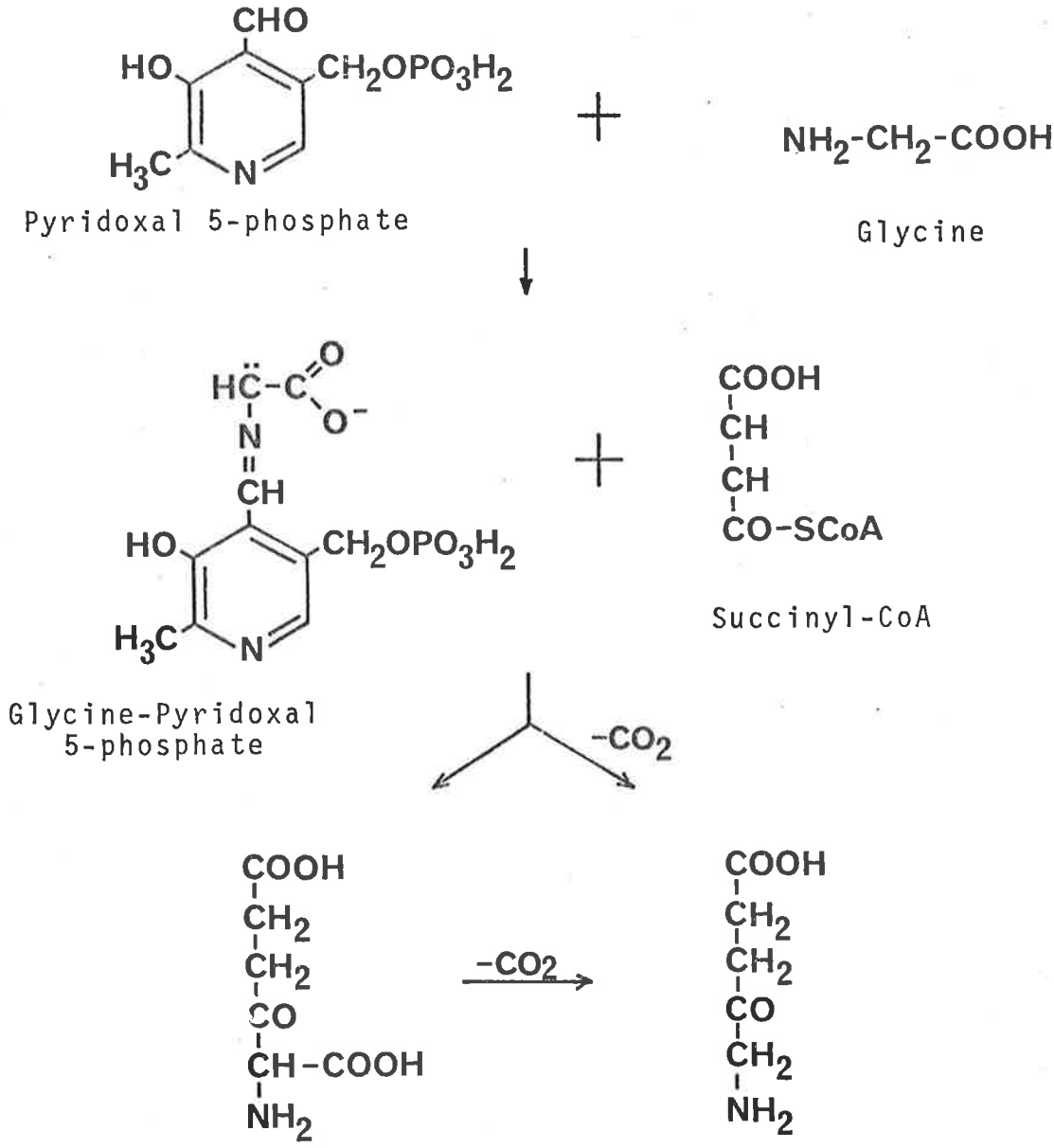
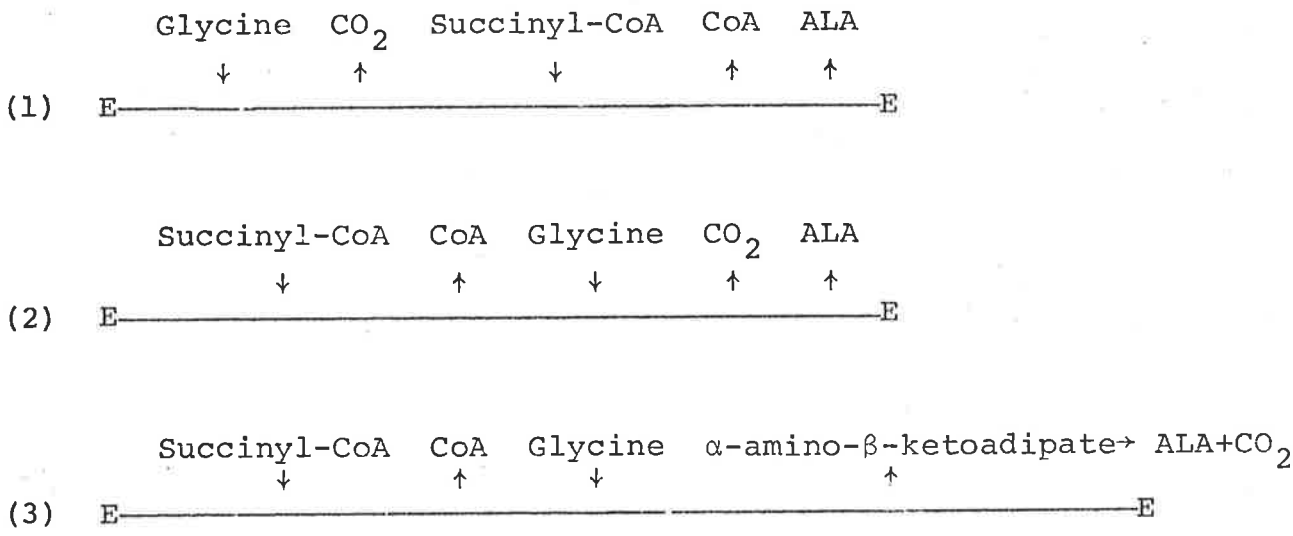


Fig. 6-10.
Proposed mechanism of formation of ALA by ALA synthetase.
(See text; also see Fig. 1-2.)



The α-amino-β-ketoadipate formed may decarboxylate on the enzyme surface as in (2), or spontaneously decarboxylate when released from the enzyme, as in (3).

It should be possible to distinguish between these alternatives by product inhibition studies, using α-amino-β-ketoadipate or ALA, and by isotope exchange studies between succinyl-CoA and CoA and glycine and carbon dioxide. Some product inhibition studies were performed, using α-aminoadipate as an α-amino-β-ketoadipate analogue, and δ-aminovalerate, γ-aminobutyrate and ε-aminocaproate as ALA analogues. No inhibition was seen with α-amino-adipate at levels up to 10^{-2} M (Table 6-1). This finding favours mechanisms (1) or (2) as the likely reaction sequence. Of the three ALA analogues tested, δ-aminovalerate did show 30% inhibition at 10^{-2}M. This analogue has the same carbon chain length as ALA, and this result also supports mechanism (1) or (2). Further

studies of this nature, and also isotope exchange studies, are needed to elucidate the complete reaction sequence.

CHAPTER 7

IMMUNOLOGICAL STUDIES OF RAT LIVER ALA SYNTHETASE

7.1. INTRODUCTION

The finding of a large difference in molecular weight between mitochondrial and cytosol forms of ALA synthetase from rat liver was described in Chapter 5. The relationship between the two forms of ALA synthetase is unknown, but it has been proposed on the basis of the kinetics of appearance of the two enzyme activities in rat liver following induction, that the cytosol enzyme is a precursor of the mitochondrial form and is transported into the mitochondrion [7,8,248].

The availability of a purified preparation of mitochondrial ALA synthetase permitted investigation into the relationship between the two enzymes by immunological techniques. Antibodies were prepared in rabbits against the mitochondrial enzyme and tested for cross-reaction with the cytosol enzyme. Experiments were also carried out with antibody to test for the presence of a precursor of ALA synthetase in cytosol and mitochondrial fractions of normal rat liver.

Finally, the specificity of the antibody preparation was investigated. A specific ALA synthetase antibody would allow quantitative measurements of ALA synthetase synthesis and degradation in labelling experiments after induction. Immunochemical techniques with labelled enzymes have been used successfully to measure rates of enzyme synthesis and degradation of other inducible enzymes such as tyrosine aminotransferase [257,258] and serine dehydratase [259], and serve as a valuable aid in the study of control

of enzyme production in animal cells.

7.2. METHODS

(a) Preparation of Antiserum to Rat Liver Mitochondrial ALA Synthetase

Rabbit antiserum was prepared by injection of the purified enzyme from rat liver, according to the following schedule.

Initially, 5 mg of purified enzyme (specific activity 450 units/mg protein) in 3 ml of buffer were emulsified in 1 ml of Freud's complete adjuvant by forcibly ejecting the mixture several times from a glass syringe. The solution was then injected into a rabbit subcutaneously at several periscapular sites. Two weeks later, 8 mg of purified enzyme (specific activity 600 units/mg protein) were injected without adjuvant in a similar fashion. After these injections approximately 50 ml of blood was removed on alternate days from the lateral ear vein of the rabbit, and the serum collected and stored at -15°C. Serum from control rabbits not injected with the enzyme was also collected.

Serum samples were tested for cross-reacting material by the Ouchterlony double diffusion test, as described below, and for ALA synthetase antibodies by titration against crude mitochondrial enzyme. Antibodies which inactivated ALA synthetase were detected 4 days after the second injections, and the maximum titre was obtained around 8-10 days.

Sera from both immunised and non-immunised rabbits were treated as described below to prepare the γ-globulin fractions. These fractions, at a concentration of 15 mg/ml in 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.2, were used in all the immunological experiments described in this chapter, unless otherwise indicated.

(b) Ouchterlony Double Diffusion Analysis in Agar

Thin layer Ouchterlony double diffusion precipitation analysis of ALA synthetase antibody was carried out on microscope slides. Approximately 4 ml of a solution containing 1% agar in 0.01 M sodium phosphate, 0.89% NaCl, pH 7.4 were layered onto a standard microscope slide. Centre and surrounding wells 4 mm in diameter were filled with 0.02 ml of either antibody to ALA synthetase or the mitochondrial or cytosol fraction under test. The slides were incubated for 24 hr at room temperature in a humidified chamber before being photographed.

(c) Isolation of the γ-Globulin Fraction from Serum

Both immune and non-immune sera were treated by a modification of the method of Goldstein *et al.* [260] to isolate and purify the γ-globulin fraction.

Cold saturated ammonium sulphate, pH 7.2, was added to 40 ml of serum. The solution was stirred for 1 hr at 4° and then centrifuged at 35,000 x g for 20 min. The precipitate was dissolved in glass distilled water to give a volume of 35 ml. The solution was then

dialysed against 0.0175 M potassium phosphate, pH 7.2, and applied to a column containing 100 ml of packed DEAE-cellulose equilibrated with the same buffer. The protein was eluted with the equilibrating buffer, fractions of 10 ml were collected, and those containing protein were pooled. The purified γ-globulin was concentrated by freeze-drying, and the protein redissolved in 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.2, and dialysed against this same buffer. The final protein concentration of the solution was adjusted to 15 mg/ml.

7.3. RESULTS

(a) Immunotitration of Solubilised and Aggregated Mitochondrial ALA Synthetases

This experiment was carried out to determine whether the physical state of ALA synthetase (i.e., aggregated or solubilised) markedly affects its immunological properties. Enzyme was extracted from freeze-dried mitochondria by suspending the powder in cold water and centrifuging the suspension at 105,000 x g for 60 min. The supernatant, containing ALA synthetase in an aggregated form, was divided into two samples, A and B, and 0.8 M NaCl and 1 mM dithioerythritol added to sample A to solubilise all enzymic activity. A fixed amount of enzyme (0.05 ml) was then titrated with increasing amounts of antibody (to 0.3 ml). The final volume of the solution was kept constant at 0.7 ml with buffer containing 0.05 M tris-HCl, 0.1 mM pyridoxal 5-phosphate, 0.5 M NaCl, 0.1 mM dithioerythritol, 0.1 mM EDTA, pH 8.0, for titrations of sample A and with TP buffer for titrations of sample B.

The results in Fig. 7-1 show the aggregated form of the enzyme does not require any more antibody than the solubilised form for complete inactivation, assuming the different salt concentrations in the incubation mixtures do not alter antigen-antibody interactions. Although the amount of enzyme in each titration was the same (0.05 ml) the activity measured in the presence of NaCl and dithioerythritol was much higher. This stimulation of enzyme activity by NaCl has previously been noted

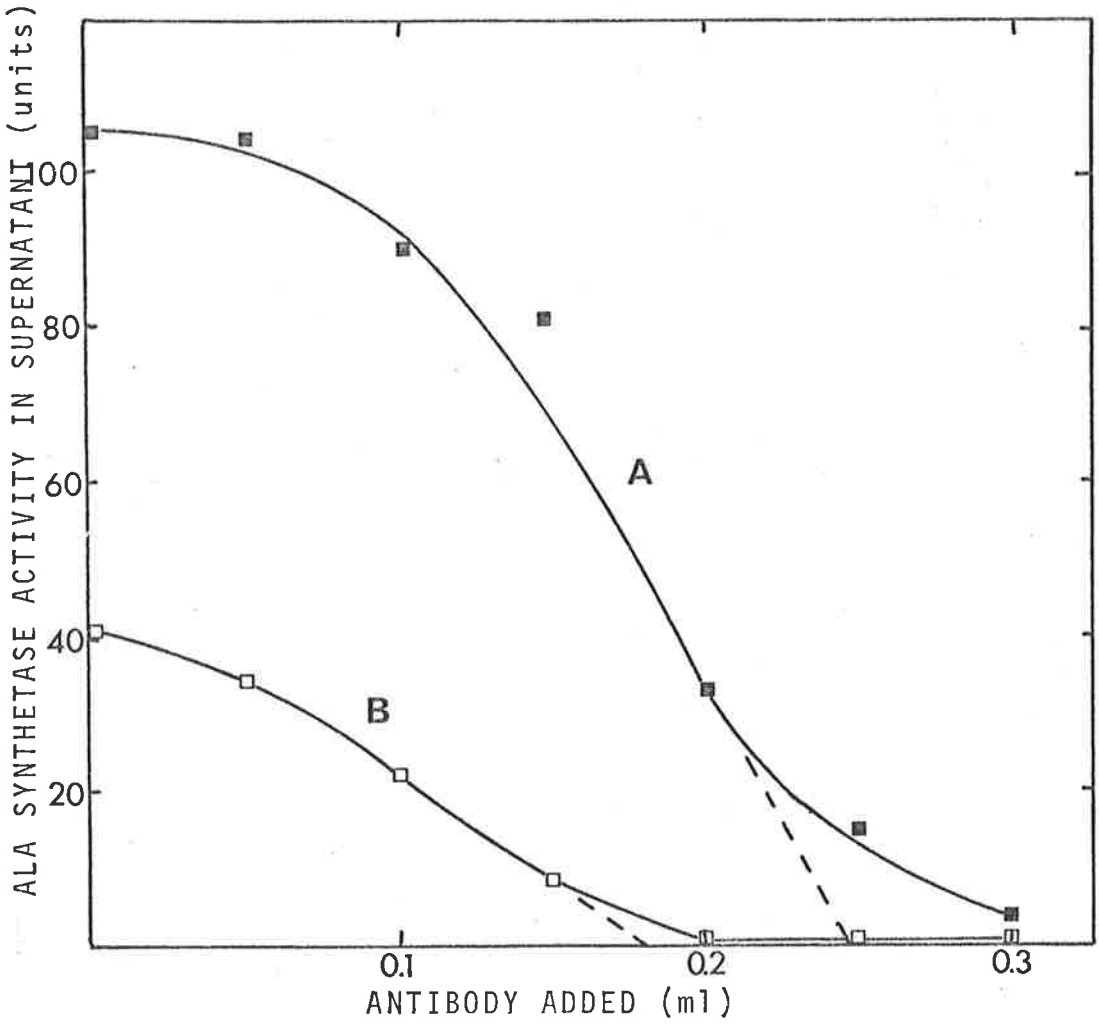


Fig. 7-1.

Immunotitration of solubilised (A) and aggregated (B) mitochondrial ALA synthetase.

Tubes containing enzyme, antibody and buffer were set up as described in the text, and incubated at room temperature for 30 min, and at 4°C overnight, The antigen-antibody complex was pelleted by centrifugation at 3,000 x g for 10 min, and supernatants assayed colorimetrically for ALA synthetase activity.

(see Sections 4.2(i) and 6.3B(c)).

(b) Immunotitration of Solubilised Mitochondrial and Cytosol ALA Synthetases.

Antibody to mitochondrial ALA synthetase completely inactivated crude cytosol ALA synthetase (see Fig. 7-2). The enzyme samples used in the titrations were purified mitochondrial enzyme (0.3 ml) from an affinity chromatography column, and crude cytosol enzyme (0.5 ml) prepared as in Section 5.2 and desalted on a Sephadex G-25 column to remove contaminating porphobilinogen. The final volumes of the titrations were maintained at 0.60 ml and 0.75 ml respectively with the corresponding enzyme buffers.

Titrations using the γ-globulin fraction from non-immunised rabbits are also shown in Fig. 7-2. The mitochondrial enzyme was inhibited to some extent, but no protein was precipitated and the inhibition was therefore regarded as non-specific.

Although the cytosol enzyme was totally inactivated by antibody to the mitochondrial enzyme, one unit of cytosol enzyme required 4-5 times more antibody to be completely precipitated. To ensure that this was due to an intrinsic difference in the enzymes, and not to the incubation conditions for immunotitrations, the following control experiments were carried out.

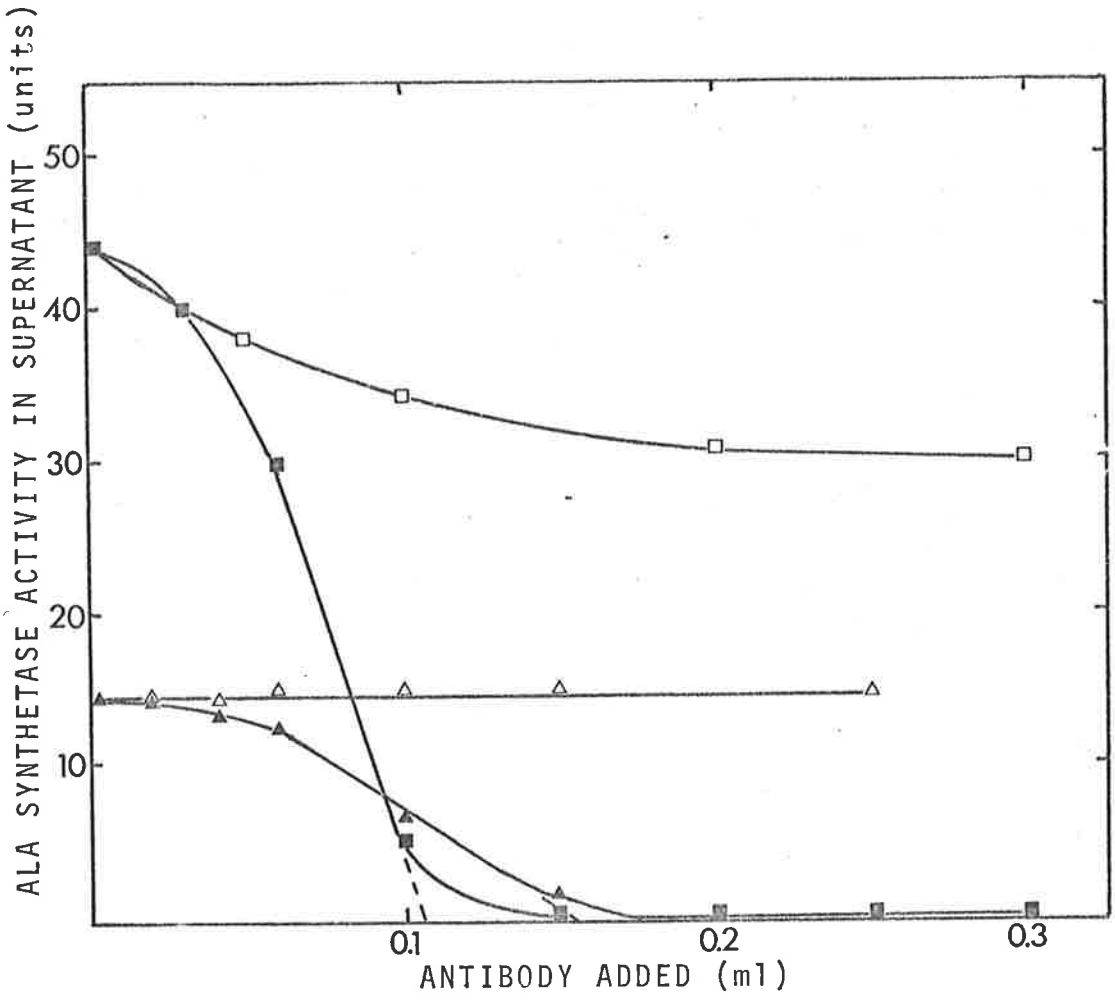


Fig. 7-2.

Immunotitration of solubilised mitochondrial and cytosol ALA synthetases.

Antibody was mixed with enzyme, as described in the text, and incubations carried out as in Fig. 7-1. After removal of antigen-antibody complexes by centrifugation, supernatants were assayed for ALA synthetase activity. Mitochondrial enzyme, test serum (■) and control serum (□); cytosol enzyme, test serum (▲) and control serum (△).

(i) Immunotitration of Mitochondrial and Cytosol ALA Synthetases in the Same Buffer Using the Same Incubation Volume

By passing samples through a Sephadex G-25 column, crude solubilised mitochondrial enzyme and crude cytosol enzyme were isolated in buffer containing 0.05 M sucrose, 0.01 M sodium phosphate, 0.3 M NaCl, 0.1 mM EDTA, 0.01 mM pyridoxal 5-phosphate, 0.1 mM dithioerythritol, pH 7.2.

Immunotitrations using the same incubation volume of 0.8 ml, still showed a marked difference in the amount of antibody needed to completely inactivate the same number of units of mitochondrial and cytosol enzymes.

(ii) Immunotitration of a Fixed Amount of Antibody with Increasing Amounts of Mitochondrial and Cytosol Enzymes

Titrations were carried out using a fixed amount of γ-globulin (0.1 ml), and adding increasing amounts of mitochondrial or cytosol enzymes, in the same buffer (prepared as in (i) above). Incubation volumes were kept constant at 0.8 ml with buffer. Fig. 7-3 shows the antibody precipitated approximately 5 units of cytosol enzyme, but 30 units of mitochondrial enzyme.

(iii) Immunotitration of Partially Purified Cytosol Enzyme

ALA synthetase from porphyric rat liver cytosol was partially purified to the calcium phosphate gel step of

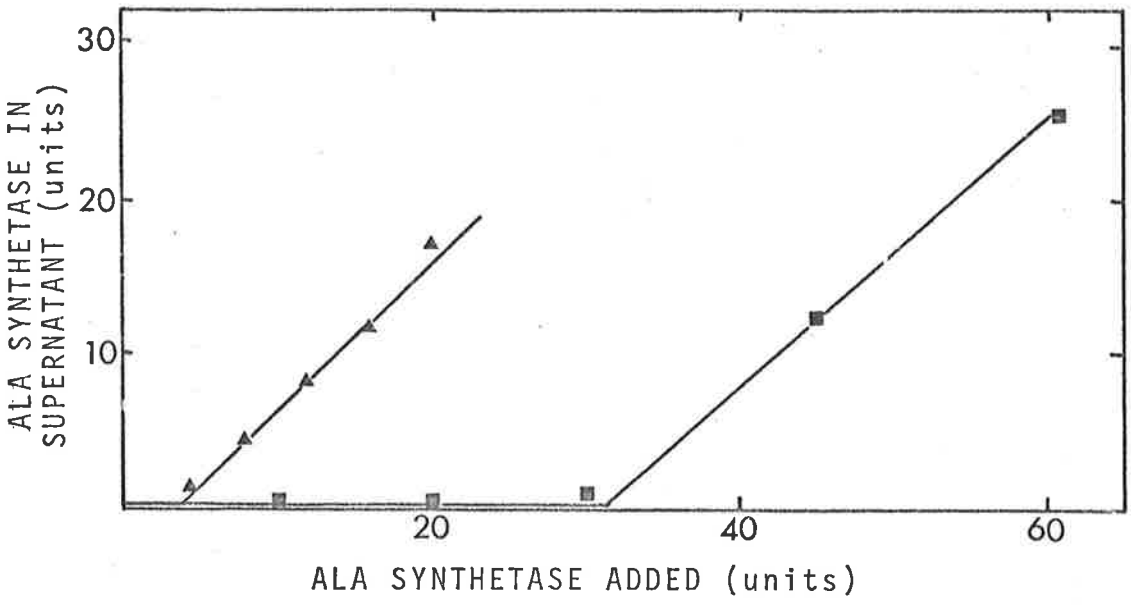


Fig. 7-3.

Immunotitration of a fixed amount of antibody with increasing amounts of mitochondrial and cytosol ALA synthetase.

Assays were set up as described in the text and incubated as in Fig. 7-1. After centrifugation, supernatants were assayed colorimetrically for ALA synthetase activity. Cytosol enzyme (▲); mitochondrial enzyme (■).

Scholnick *et al.* [8]. Fixed amounts of this enzyme were then titrated with increasing amounts of antibody in a total volume of 0.8 ml. The amount of antibody required to inactivate a unit of partially purified cytosol enzyme remained much greater than that needed for a unit of mitochondrial enzyme.

One additional experiment eliminated the possibility that other proteins present in the cytosol fraction were inhibiting antibody-antigen interactions. Samples of solubilised mitochondrial enzyme (0.5 ml, 2 mg protein) were titrated with and without the addition of normal cytosol protein (0.3 ml, 13 mg protein) which was prepared exactly as for porphyric cytosol protein (see Fig. 7-4). The final volume of the titrations was 0.6 ml. The amount of antibody that completely precipitated enzyme activity, as estimated by extrapolation of the linear portions of the curves, was proportional to the amount of enzyme activity present in the absence of antibody. Cytosol protein did not affect the inactivation of mitochondrial ALA synthetase by antibody.

Mitochondrial and cytosol forms of ALA synthetase are therefore immunologically different, although some antigenic sites are apparently common to both enzymes.

(c) Test for an Inactive Precursor of ALA Synthetase in Normal Rat Liver

Experiments were designed to test for the presence of an enzymically inactive, but immunologically reactive

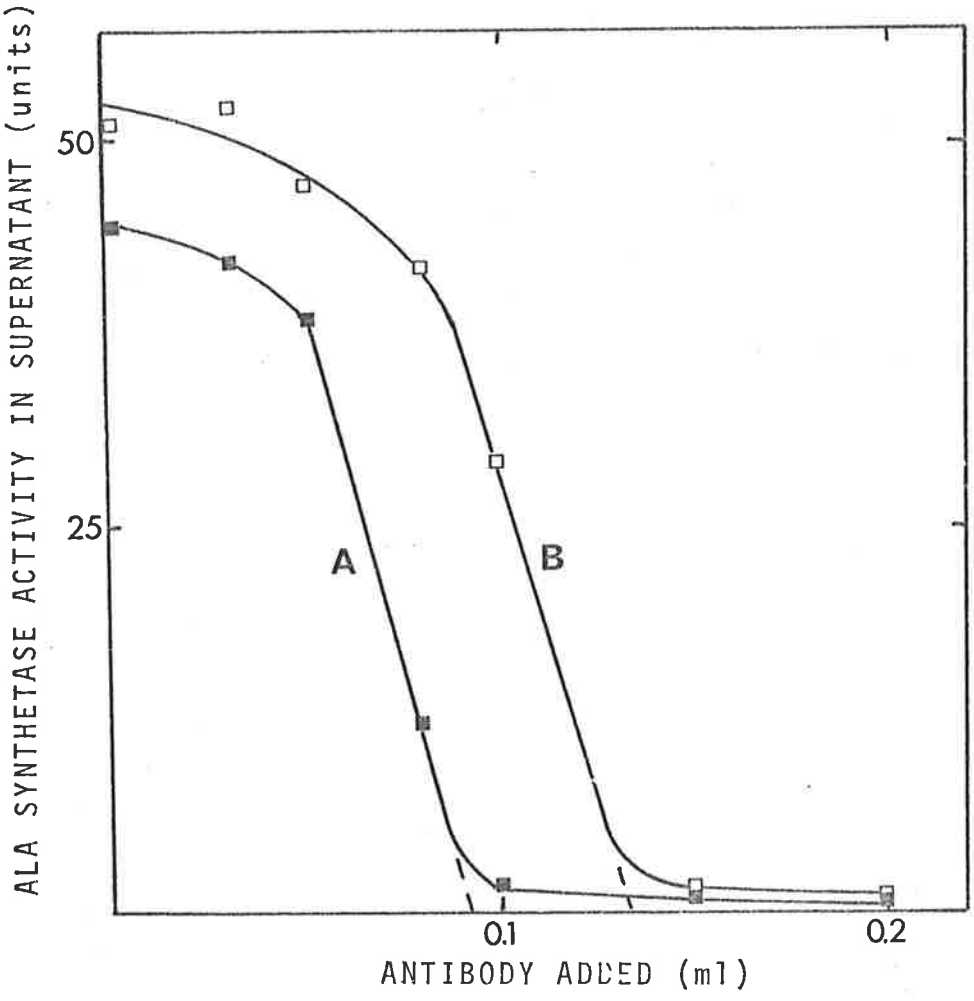


Fig. 7-4.

Effect of normal cytosol protein on the inactivation of mitochondrial ALA synthetase by antibody.

As described in the text, mitochondrial enzyme only (A) or mitochondrial enzyme plus normal cytosol protein (B) were titrated with increasing amounts of antibody. Incubations and assays were carried out as in Fig. 7-1.

precursor of ALA synthetase in liver fractions from rats that had not been treated with the drug, DDC. If such a precursor existed in normal liver, mixing an excess amount of normal liver protein with the identical fraction from porphyric liver should result in competition for any added antibody and extend markedly the amount of antibody required in immunotitrations to precipitate all enzymic activity from the porphyric fraction.

Fig. 7-5 shows such mixing experiments for mitochondrial and cytosol liver fractions. Mitochondria isolated from 8 g of porphyric rat liver were freeze-dried and resuspended in 5 ml of cold water. After centrifugation at 105,000 x g for 1 hr, fixed amounts of supernatant (0.04 ml) were mixed with increasing amounts of antibody and the volume made up to 1.0 ml with 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.6. Identical titrations were also set up containing 0.04 ml of porphyric mitochondrial protein plus 0.2 ml of normal mitochondrial protein which was prepared in an identical manner and contained a low level of ALA synthetase activity.

Porphyric cytosol was prepared and ALA synthetase partially purified, as described in (b)(iii) above. Porphyric cytosol protein (0.05 ml) was mixed with increasing amounts of antibody, in a final volume of 0.8 ml. Other titrations contained a mixture of 0.05 ml porphyric cytosol protein plus 0.1 ml of normal cytosol protein prepared in an identical manner, and were carried out as for porphyric cytosol alone.

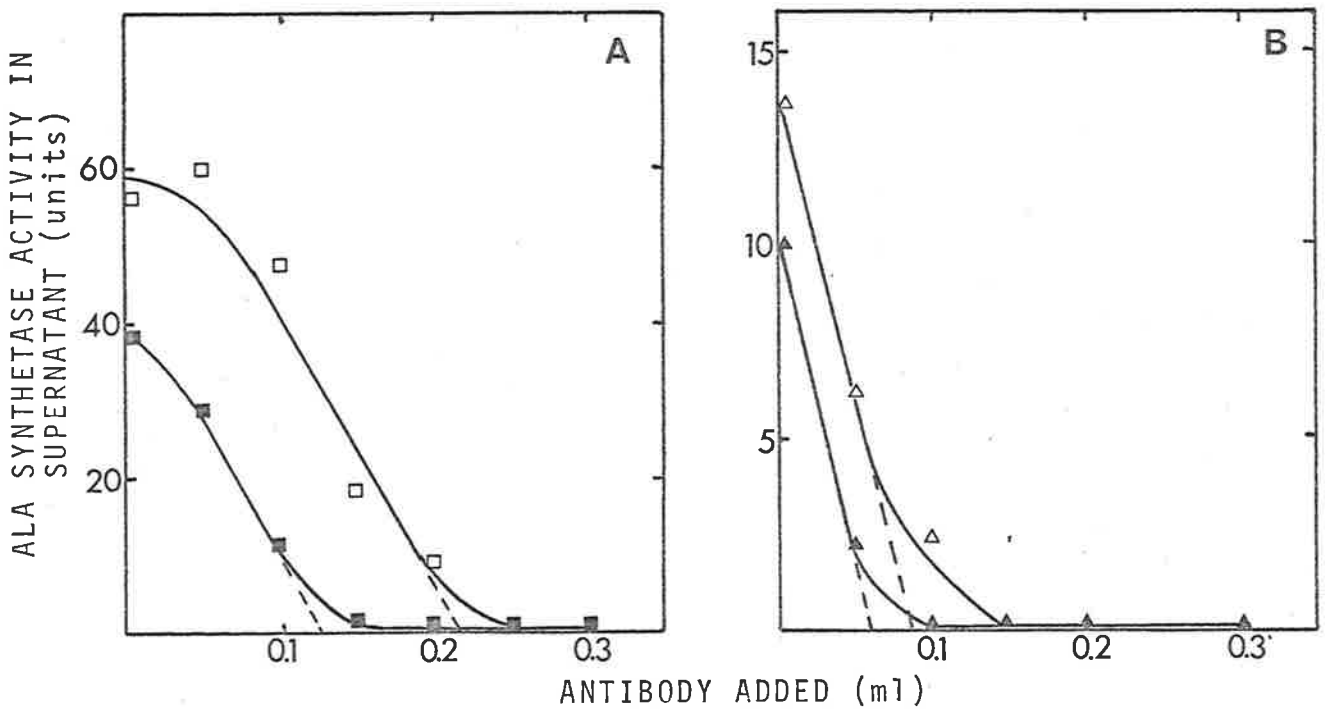


Fig. 7-5.

Immunotitration of ALA synthetase in porphyric liver fractions as compared with porphyric plus normal liver fractions.

Details are described in the text; incubation and assay conditions are described in Fig. 7-1.

A. Mitochondrial fractions: (■), porphyric; (□), porphyric plus normal.

B. Cytosol fractions: (▲), porphyric; (△), porphyric plus normal.

The titrations show that for both crude mitochondrial and partially purified cytosol fractions, no inactive precursor of ALA synthetase was present in normal liver, since the end points of all titrations were proportional to the enzyme activity present before addition of antibody. A similar result was obtained using crude cytosol fractions.

(d) Specificity of the Antiserum

Although the γ-globulin fraction from the serum of a rabbit injected with a purified preparation of ALA synthetase contained antibodies which inactivated ALA synthetase, other antibodies were also present. These were detected by the Ouchterlony double diffusion test, set up as described in Section 7.2, using purified γ-globulin and protein extracted from porphyric mitochondria. Two major precipitin lines were seen.

For comparison, the crude cytosol fraction from porphyric liver and crude mitochondrial and cytosol fractions from normal liver were diffused against the γ-globulin fraction. One major precipitin line was surprisingly observed in all fractions, and was identical to one of the precipitin lines detected in porphyric mitochondrial extract (see Fig. 7-6).

The implication from these findings is that ALA synthetase, purified as described in Chapter 5, contains at least two antigens, one of which is distributed in both mitochondrial and cytosol fractions prepared from rat liver

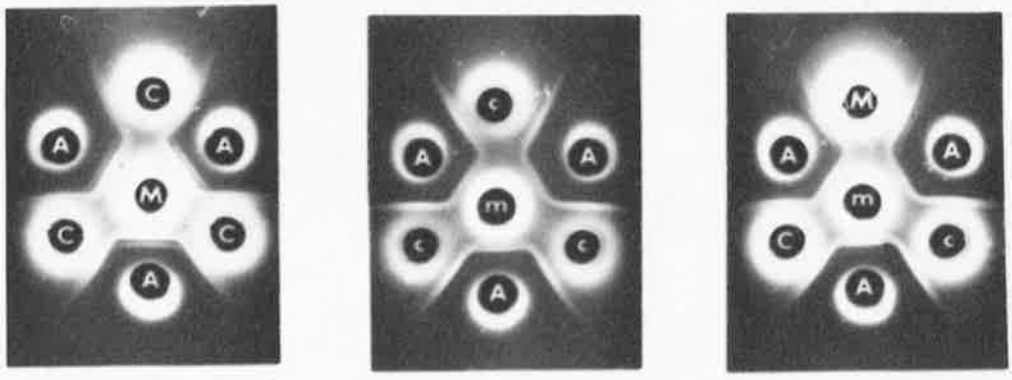


Fig. 7-6.

Ouchterlony immunodiffusion patterns, using the γ-globulin fraction from immune serum and various rat liver fractions. The symbols used are: A, antibody; M, porphyric mitochondrial extract; m, normal mitochondrial extract; C, porphyric cytosol extract; c, normal cytosol extract. The immunodiffusions were set up and incubated as described in Section 7.2. Wells were filled with 20 µl of the fractions indicated.

(component 2). The other major cross-reacting component which was observed only in extracts from porphyric mitochondria (component 1) may be ALA synthetase. Although component 1 was not detected in porphyric cytosol extracts, the amount of ALA synthetase present would be extremely small.

In an attempt to increase its specificity, the antiserum was mixed with protein extracted from freeze-dried mitochondria, isolated from normal, fed rats. This preparation had a very low ALA synthetase activity. After standing overnight at 4°C, a precipitate was obtained and removed by centrifugation. More normal mitochondrial protein was added and the step repeated. The γ-globulin fraction was then isolated from the mixture of serum and soluble mitochondrial protein, by the method described in Section 7.2, and tested for removal of antibodies to component 2, by the Ouchterlony test. However, no change was seen in diffusion patterns.

Another approach was used in an effort to remove antibodies to component 2. Protein extracted from normal mitochondria was converted into an insoluble gel derivative by polymerisation with glutaraldehyde, and used as an immunoadsorbent by mixing antiserum with the washed resuspended gel [261]. After stirring gently for 60 min at room temperature and removing the gel by centrifugation, the serum was again tested for antibodies to component 2 by the Ouchterlony test. Two major precipitin lines were still detected in extracts from porphyric mitochondria.

7.4. DISCUSSION

Experiments presented in this chapter have shown that mitochondrial and cytosol ALA synthetase are not identical immunologically, and differ in the extent to which they cross-react with antibodies prepared in rabbits against the purified mitochondrial enzyme. This is in keeping with the difference in the molecular sizes of the two enzymes, as reported in Chapter 5. However, since the two enzymes do have some antigenic sites in common, the results are still consistent with the suggestion that cytosol ALA synthetase is a precursor of mitochondrial ALA synthetase and is in transit to the mitochondrion [7,8,248]. If no cross-reaction at all was observed between the two forms of enzyme, a separate function for each in its respective cellular compartment might have been proposed. In fact other enzymes that are found in both intra- and extra-mitochondrial fractions in liver and other tissues, such as phosphoenolpyruvate carboxykinase [253], isocitrate dehydrogenase [262], malate dehydrogenase [263] and aspartate aminotransferase [264] have been reported to be immunologically distinct proteins, probably serving separate metabolic functions. Nevertheless, the results of the immunological studies do not *prove* that a relationship exists between the two enzymes, since even unrelated enzymes could cross react because of an active site structure obligatory to both enzymes.

Overall, the following evidence favours a precursor-product relationship between cytosol and mito-

chondrial ALA synthetases.

(a) The kinetics of the increase in ALA synthetase activity in cytosol and mitochondrial fractions from rat liver after induction by drugs are consistent with a transfer of enzyme from cytosol to mitochondria [7,248].

(b) A cytoplasmic form of ALA synthetase would probably not be functional, since the substrate succinyl-CoA has not been shown to occur extramitochondrially [8].

(c) The induction of ALA synthetase is blocked by cycloheximide [28], which inhibits protein synthesis on cytoplasmic ribosomes in mammalian cells. In contrast, chloramphenicol at levels which selectively inhibit mitochondrial protein synthesis, does not prevent induction of ALA synthetase in the cytosol fraction, although the increase in activity in the mitochondrial fraction is inhibited [248].

(d) The purified enzymes have similar kinetic properties. Michaelis constants determined for glycine and succinyl-CoA were of the same order, and both enzymes were inhibited 50% by hemin at concentrations of around 10^{-5}M.

(e) Both enzymes are completely inactivated by antibodies to the purified mitochondrial enzyme, as discussed above.

(f) ALA synthetase in aerobically grown yeast cells

is found principally in the mitochondria, but when grown under oxygen-free nitrogen, yeast cells contain only promitochondria, and approximately 90% of the ALA synthetase is in the cytosol [265].

The findings from previous chapters are summarised diagrammatically in Fig. 7-7. If cytosol ALA synthetase is a precursor of the mitochondrial enzyme some structural modifications of the enzyme must occur during transfer into the mitochondrion, since the molecular weight of the cytosol enzyme is 178,000 as compared with 77,000 for solubilised mitochondrial enzyme. The function of the additional material, presumably protein, making up the 100,000 in molecular weight of the cytosol enzyme is unknown. It could be synthesised on cytoplasmic ribosomes with the enzyme itself, and act as a "transport" protein, responsible for the penetration of enzyme through the mitochondrial membranes and its subsequent localisation within the mitochondrion.

Alternatively, the "transport" protein, may be synthesised on mitochondrial ribosomes and emerge into the cytoplasm, as studies by Beattie and Stuchell [248] with chloramphenicol suggest the entry of ALA synthetase into mitochondria requires mitochondrial protein synthesis. In yeast, the enzymes cytochrome oxidase and ATPase, which are synthesised on cytoplasmic ribosomes, are known to require both mitochondrial and cytoplasmic protein synthesis for localisation on the inner mitochondrial membrane as enzyme complexes [266,267].

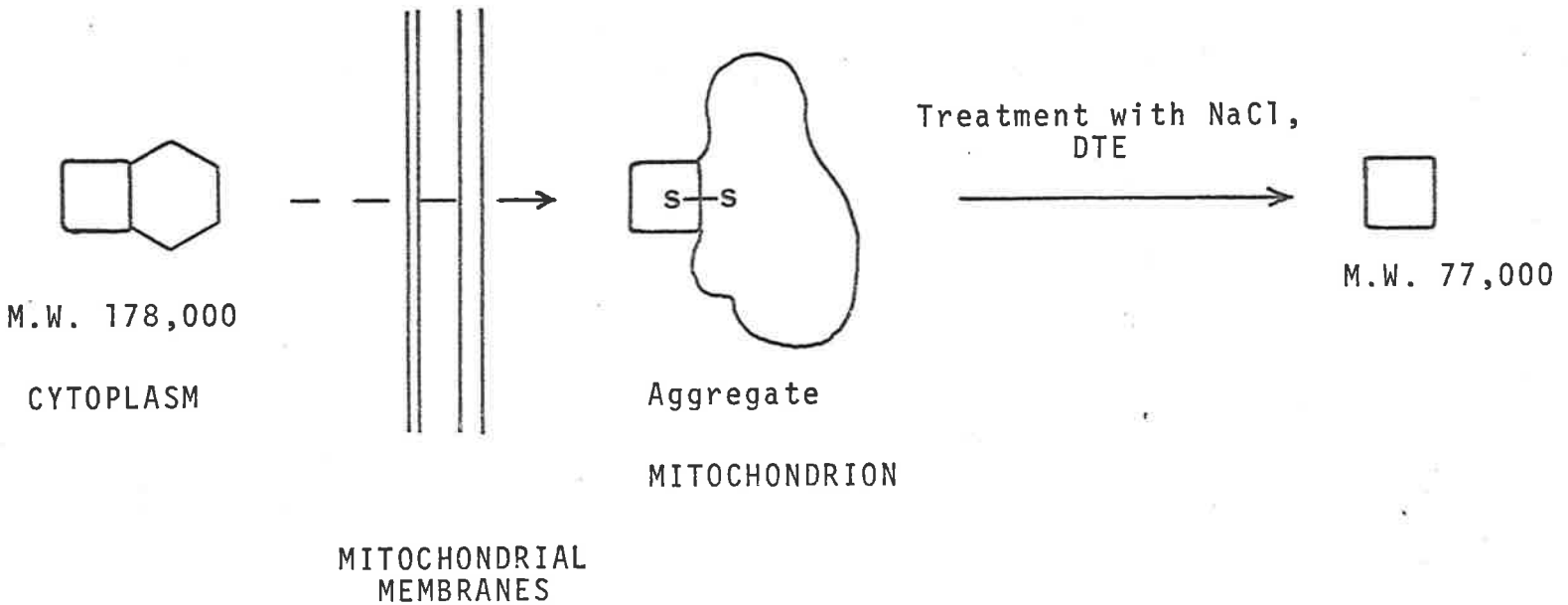


Fig. 7-7. Possible relationship between cytosol and mitochondrial forms of ALA synthetase.

If the ALA synthetase-containing aggregate extracted from disrupted mitochondria is a physiological entity and plays a role in the localisation of ALA synthetase within the mitochondrion as discussed in Section 4.3, part of the complex may be synthesised by the mitochondrion. The finding that cytosol ALA synthetase cannot be dissociated by treatment with NaCl and dithioerythritol as for the mitochondrial enzyme aggregate, however, indicates that if the additional 100,000 molecular weight protein of the cytosol enzyme is involved in ALA synthetase transport, it does not appear to become part of the mitochondrial enzyme aggregate.

Some more support for the enzyme aggregate extracted from mitochondria as having physiological significance was provided by these immunological studies. Since aggregated enzyme cross reacted with antibodies to the same extent as solubilised enzyme, all antigenic sites present on the soluble enzyme must still be exposed when the enzyme is aggregated with other components. This indicates a specific aggregation of molecular weight 77,000 enzyme with other material to form the aggregate complex, rather than a random association of proteins.

In this work it was not possible to detect an immunologically reactive precursor of ALA synthetase in normal rat liver. This implies the enzyme is synthesised *de novo* upon drug induction, and is consistent with the finding that increases in ALA synthetase activity are prevented by inhibitors of cytoplasmic protein synthesis [28,35]. An

activation process requiring protein synthesis as accounting for the increase in enzyme activity during induction is therefore ruled out.

The *direct* demonstration of new enzyme synthesis awaits the preparation of a specific ALA synthetase antibody to isolate the enzyme and show it can be labelled with radioactive amino acids after drug induction. Attempts at obtaining an antibody preparation which specifically precipitated ALA synthetase were unsuccessful. Although the antiserum contained antibodies to a protein present in normal liver mitochondria, mixing protein from normal mitochondria with antiserum did not totally remove the non-specific antibodies as an antigen-antibody precipitate. Unfortunately, time did not permit further attempts at removal of the non-specific antibodies.

CHAPTER 8

GENERAL DISCUSSION AND FURTHER RESEARCH

GENERAL DISCUSSION AND FURTHER RESEARCH

The topic of control of enzyme levels in animal tissues has been discussed in an excellent review by Schimke and Doyle [207]. Criteria proposed by these authors as necessary in a system for the study of enzyme regulation are:

(a) uniformity of cell population;

(b) a sufficient quantity of specific protein to allow purification and characterisation;

(c) wide fluctuations of enzyme content as affected by experimental variables;

(d) availability of mutations affecting both structure and content of an enzyme;

(e) well-defined agents or simple procedures for perturbing enzyme content;

(f) easy manipulation of isotopic tracers;

(g) demonstrable relationship between observations in the 'system' and events in the intact organism [207].

As discussed previously in this thesis, dramatic increases of hepatic ALA synthetase are produced by drugs and steroids in intact animals, but as yet, no suitable *in vitro* mammalian system which has many of the above properties has been developed for study. It is the aim of work in this laboratory to define such a system, and preliminary studies [92,93] have shown the isolated perfused rat liver may have potential in this respect. One other

report has recently appeared on the regulation of ALA synthetase in perfused rat liver [268].

The work described in this thesis has made considerable progress in fulfilling criterion (b) above. Liver mitochondrial ALA synthetase was not obtained in totally pure form, but major steps forward have been made in overcoming problems of enzyme aggregation and instability, and in purifying the enzyme, by the use of affinity chromatography. The way is now open to employ further techniques of protein fractionation and to isolate enzyme in an homogeneous form. As mentioned in Chapter 7, this will enable a specific antibody against mitochondrial ALA synthetase to be obtained, and thus should allow the measurement of rates of synthesis and degradation of ALA synthetase, using direct immunochemical methods for the first time.

A study of the induction of ALA synthetase in liver is now complicated by the occurrence of the enzyme in both mitochondrial and cytoplasmic cell compartments, since increases in activity occur in both enzymes after drug or steroid treatment. A possible precursor-product relationship between the two enzymes has been discussed at length in previous sections, the evidence being consistent with this hypothesis without conclusively proving it.

Experiments can be devised as extensions of work presented in this thesis to examine this hypothesis more directly. In theory, it should be possible to carry out a pulse-chase type of experiment to see if enzyme, labelled with a radioactive amino acid and isolated immunologically,

can be followed from the cytoplasm of the liver cell into the mitochondrion. However, some technical difficulties might be expected in such an experiment. For example, insufficient isotopic labelling of the enzyme, the different immunological reactivities of the two forms of enzyme and the many controls necessary to eliminate non-specific protein precipitation by antibody could all complicate any results obtained.

Another approach would be to try and obtain an *in vitro* system for the transport of cytosol ALA synthetase into mitochondria. Any requirements, such as mitochondrial protein synthesis, for this process could then be investigated with specific inhibitors such as chloramphenicol or erythromycin. The question of whether mitochondria from normal rats behave identically to mitochondria from porphyric rats could also be answered. A system such as this would be invaluable for studies of mitochondrial biogenesis in animal cells.

Lastly, the physical properties of purified cytosol ALA synthetase should be investigated more closely to examine its possible subunit structure for a protein of MW 77,000.

Further work is being carried out in this laboratory with an aim to answer some of these questions.

Another possibility for further research concerns the design and synthesis of an inhibitor of ALA synthetase for trial as a therapeutic agent for the hepatic porphyrias, especially acute intermittent porphyria. As described in

Section 1.4, acute intermittent porphyria is a genetically-inherited disease characterised biochemically by the production and excretion of excessive amounts of ALA and porphobilinogen, andclinically by severe neurological disturbances. Patients with the disease have been shown to have an elevated level of hepatic ALA synthetase [187, 188].

The relationship of an elevated level of ALA synthetase to the neurologic manifestations of the disease is not known. One suggestion is that high levels of ALA produced in the liver could result in the formation of ALA imines, which interfere with the metabolism in the brain of γ-aminobutyric acid, an inhibitory neurotransmitter [269]. Also, ALA and porphobilinogen have been shown to inhibit neuromuscular transmission in the rat hemidiaphragm [199], and the cerebrospinal fluid of an acute intermittent porphyria patient in relapse was shown to contain a considerable amount of porphobilinogen [270]. Although the significance of these observations is unknown, ALA and porphobilinogen are implicated as causative agents of the neurological symptoms by the general finding that excessive amounts of ALA and porphobilinogen are not produced in cutaneous porphyrias, where no neurological symptoms are observed, although excessive amounts of porphyrins are produced.

Theoretically then, inhibition of hepatic ALA synthetase activity should lower the production of ALA and porphobilinogen, and might eliminate any neurological

dysfunction. Indeed, Bonkowsky *et al.* [270] administered hemin intravenously to a patient suffering from an attack of acute intermittent porphyria in an effort to repress the level of hepatic ALA synthetase. The levels of ALA and porphobilinogen in serum and urine were decreased after this treatment, but unfortunately the patient had other serious complications, and died before any beneficial effects could be noted.

A rational approach to a possible therapy for the disease would be to design and synthesise a specific, irreversible enzyme inhibitor of ALA synthetase, for use as a chemotherapeutic agent, perhaps adopting the type of approach outlined by Baker [271]. An inhibitor would not only need to be effective in inhibiting the enzyme, but must also be transported into the liver cell and not be metabolised before reaching the required site of action. For any continued administration it would need to preferentially act on liver and not on immature red blood cells. A suitable analogue of ALA may provide the specificity required for such an inhibitor, and studies with the purified enzyme will aid greatly the search for an effective compound.

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