THE PURIFICATION OF ALA-SYNTHASE FROM AVIAN AND MAMMALIAN LIVER

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SUMMARY

1. Attempts to duplicate reported purification procedures for chick embryo ALA-synthase were unsuccessful.

2. A new rapid purification scheme for the purification of chick embryo liver mitochondrial ALA-synthase was developed resulting in the purification to homogeneity of ALA-synthase of specific activity 35,000 units/mg. *In vivo* labelling studies establish that this is the intact form of the enzyme.

3. The sensitivity of chick embryo liver ALA-synthase to breakdown by proteolytic degradation to smaller molecular weight forms which still retain complete catalytic activity indicates that previous workers isolated a breakdown product.

4. Rat liver mitochondrial ALA-synthase is purified in its intact form, having a molecular weight of 70,000, using the purification scheme developed for the chick embryo liver enzyme.

5. Translation of mRNA from the livers of drug-treated rats in a cell-free system showed that rat liver mitochondrial ALA-synthase is initially synthesised as a protein of molecular weight 76,000.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

In all of the experiments described in this thesis, the author was involved as the principal worker. However, in Chapters 4 to 7, Mr. Gopesh Srivastava collaborated in the work because of the time consuming nature of the repetitive purification procedures involved.

To the best of my knowledge and belief, this thesis contains no material previously published or written except where due reference is made in the text.

signed.

IAIN ANDREW BORTHWICK.

ABBREVIATIONS

The following abbreviations are used in this thesis:

AIA	:	allylisopropylacetamide
DDC	:	dihydrodicarbethoxycollidine
DTE	:	dithioerythritol
mRNA	:	messenger ribonucleic acid
PMSF	:	phenylmethylsulphonylfluoride
SDS	:	sodium dodecyl sulphate

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CHAPTER 1

GENERAL INTRODUCTION

I N T R O D U C T I O N

Haemoproteins fulfil a range of important functions in nature. In higher animals these include the transport of oxygen (haemoglobin and myoglobin), electron transport (mitochondrial cytochromes), activation of oxygen (cytochrome oxidase and cytochrome P-450) and those involved in hydrogen peroxide metabolism (peroxidases and catalases) (Granick and Gilder (1947)).

In humans the bone marrow is the major site of haem biosynthesis in the body (80%), being required for haemoglobin production. The liver is responsible for most of the synthesis of the rest of the body haem, (15%), the bulk of this being required for cytochrome P-450 synthesis. The biosynthesis of hepatic haem is thought to be rate-limited under normal conditions by the first enzyme in the pathway δ-aminolaevulinate synthase (ALA-synthase).

This thesis is concerned with the purification of ALA-synthase from avian and mammalian liver.

The purification of ALA-synthase is an essential step towards a complete understanding of the regulation of haem biosynthesis. This aim, the elucidation, at the molecular level, of the physiological regulation of haem synthesis, is an ultimate goal of the studies undertaken in this laboratory.

Many comprehensive reviews on haem, haemoproteins and haem metabolism have been published in recent years, (Granick and Sassa (1971), Marver and Schmid (1972),

Tschudy (1974), De Matteis and Aldridge (1978), Granick and Beale (1978), Meyer and Schmid (1978), Elder (1981), Sassa and Kappas (1981) and Romeo (1981) because of this no attempt will be made here to give a comprehensive treatise on the field and the reader is referred to the above works for a detailed coverage. This chapter will provide a summary of those areas which are relevant to the subject matter of the thesis.

THE HAEM BIOSYNTHETIC PATHWAY

The enzymes of the haem biosynthetic pathway are compartmentalised; the initial enzyme ALA-synthase functions physiologically in the mitochondrion, the next three enzymes are found in the cytosol and the last two enzymes are again located in the mitochondria.

ALA-synthase [succinyl CoA glycine C-succinyl transferase (decarboxylating)] the first enzyme in the haem biosynthetic pathway, catalyses the conversion of succinyl-CoA and glycine to give ALA. Pyridoxal phosphate is required as a cofactor in the reaction,

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

There have been proposals for the mechanism of the enzyme reaction (Neuberger (1961)), Jordan and Shemin (1972), Scholnick *et al* (1972)), but the complete details are yet to be elucidated. When one reviews the literature on ALA-synthase, it is clear that purification and study of this enzyme has

for a long time been plagued with difficulties and, as a consequence the reported findings add up to a somewhat confusing picture.

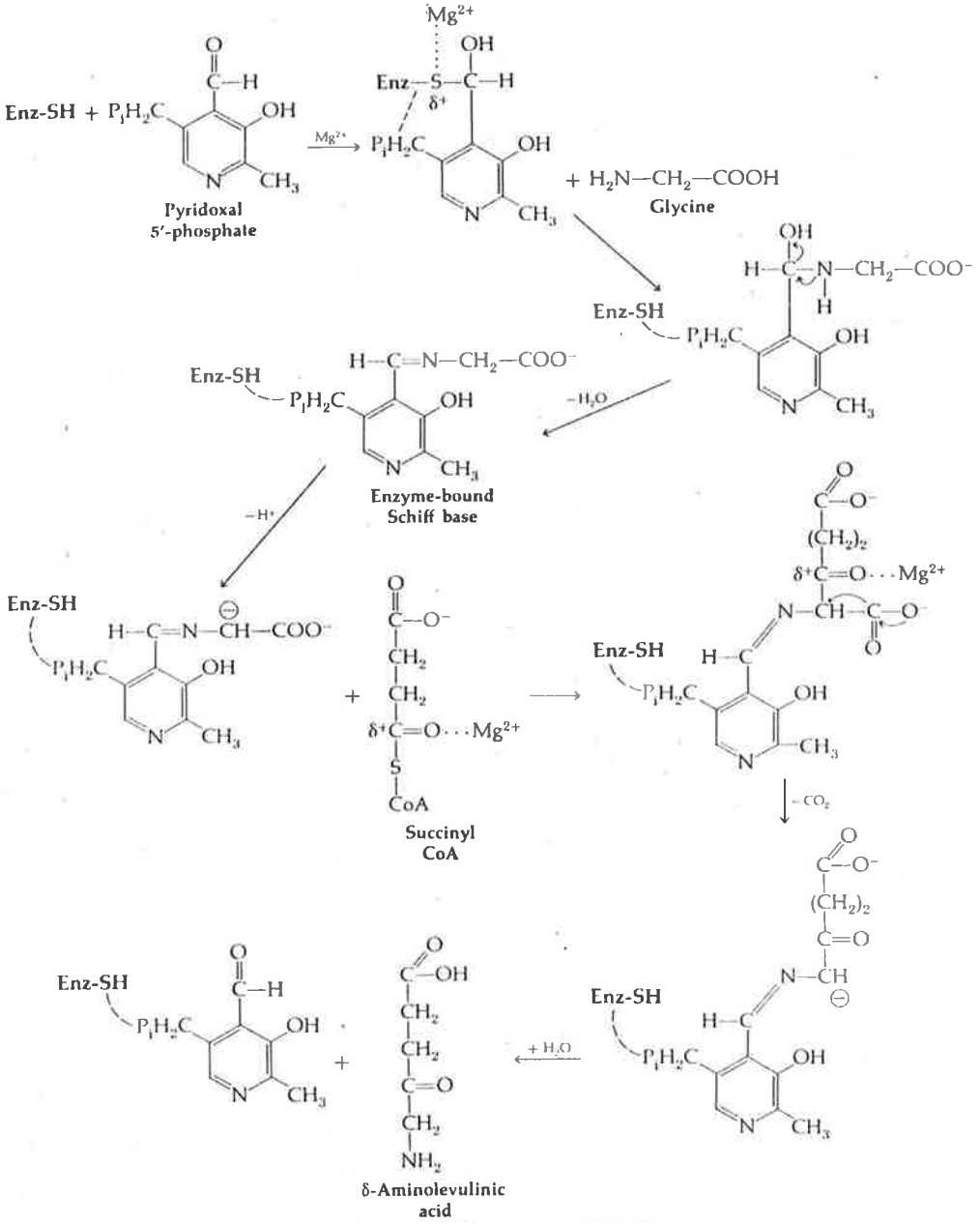
These difficulties in purifying the enzyme, which are described in detail later in this chapter, have limited mechanistic studies on the enzyme. However, the proposed reaction mechanism (Shemin (1972)) is as follows (outlined in Figure (1.1)); pyridoxal phosphate which binds first to the enzyme forms a Schiffs base with the amino group on glycine. Pyridoxal phosphate then transiminates with the amino group of glycine to form a stable carbanion, with the loss of a proton from the methylene carbon atom of glycine. The carbanion of glycine makes a nucleophilic attack on succinyl CoA, displacing CoA. The α-amino-β-ketoadipate formed spontaneously decarboxylates and ALA is released from the enzyme.

Under normal physiological conditions in avian and mammalian liver, ALA-synthase is found exclusively in the mitochondria. The low activities found in the cytosol, are probably due to leakage from mitochondria damaged during tissue fractionation (Patton and Beattie (1973)). By comparing the distribution of ALA-synthase in mitochondrial sub-fractions with that of marker enzymes ALA-synthase has been shown to exist free in the matrix or loosely bound to the inner mitochondrial membrane (Zuyderhout *et al* (1969),

FIGURE 1.1

PROPOSED MECHANISM OF ACTION OF ALA-SYNTHASE

Based on that of Scholnick *et al* (1972).
See text for details.


Enz-SH + P_iH_2C
Pyridoxal 5′-phosphate
Mg^{2+}
+ $H_2N—CH_2—COOH$
Glycine
Enzyme-bound Schiff base
$-H_2O$
$-H^+$
Succinyl CoA
$-CO_2$
+ H_2O
δ-Aminolevulinic acid

McKay *et al* (1969), Barnes *et al* (1971), Whiting (1973) and Patton and Beattie (1973)). However, in the livers of some animals (rats, guinea pigs, adult chickens), treated with porphyrinogenic drugs, where ALA-synthase is much higher than normal, a significant proportion of the total ALA-synthase activity is present in the cytoplasm. These levels are much higher than can be accounted for by mitochondrial damage. In addition, the molecular weight of the cytosol form has been claimed to be larger than the mitochondrial form (at least in rats), (Hayashi *et al* (1969)), leading to the proposal that the cytosol form of the enzyme undergoes modification on incorporation into the mitochondria (Whiting and Elliott (1972)).

ALA-synthase activity in avian and mammalian liver is normally very low, about 10-100 nmol ALA/hour/g liver tissue. This is one or more orders of magnitude less than the other enzymes in the pathway, with the possible exception of uroporphyrinogen I synthase which has been reported to have an activity equivalent to ALA-synthase in mouse liver (Hutton and Gross (1970)). In addition, ALA-synthase has a very short half-life, around 60-180 minutes (Tschudy *et al* (1965), Marver *et al* (1966), Sassa and Granick (1970). These two observations suggest that ALA-synthase is the rate-limiting enzyme in haem biosynthesis. The

FIGURE 1.2

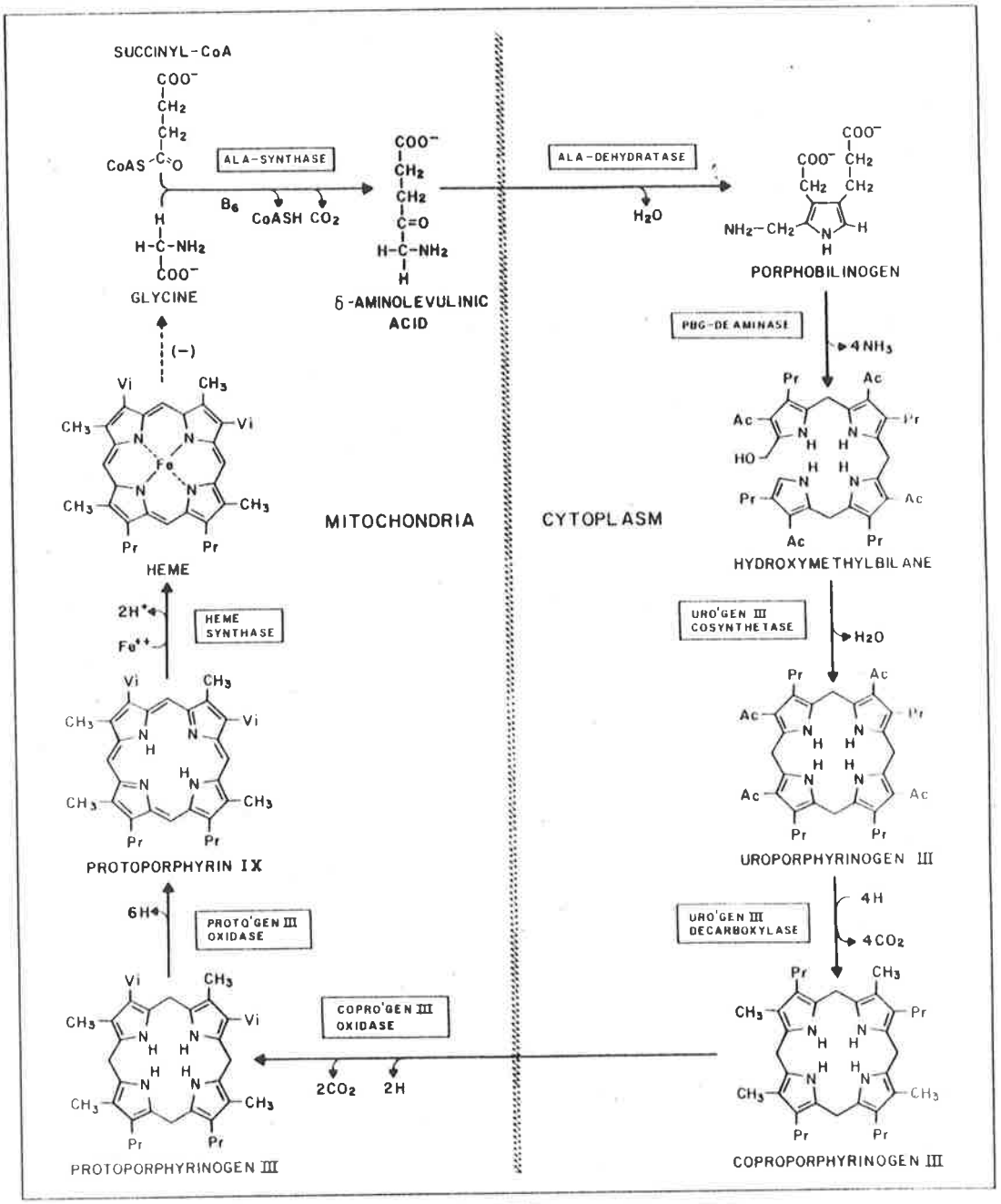
THE HAEM BIOSYNTHETIC PATHWAY

Abbreviations used in figure:

B6	=	pyridoxal 5' phosphate
ALA	=	δ aminolaevulinic acid
PBG	=	porphobilinogen
URO'GEN	=	uroporphyrinogen
COPRO'GEN	=	coproporphyrinogen
PROT'GEN	=	protoporphyrinogen

Ac = acetate Pr = propionate

Vi = vinyl


SUCCINYL-CoA
ALA-SYNTHASE
B6
CoASH CO2
GLYCINE
δ-AMINOLEVULINIC ACID
ALA-DEHYDRATASE
H2O
PORPHOBILINOGEN
PBG-DEAMINASE
4NH3
HYDROXYMETHYLBILANE
URO'GEN III COSYNTHETASE
H2O
UROPORPHYRINOGEN III
URO'GEN III DECARBOXYLASE
4H
4CO2
COPROPORPHYRINOGEN III
COPRO'GEN III OXIDASE
2CO2 2H
PROTOPORPHYRINOGEN III
PROTO'GEN III OXIDASE
6H
PROTOPORPHYRIN IX
HEME SYNTHASE
2H+
Fe++
HEME
(-)
MITOCHONDRIA
CYTOPLASM

regulation of haem biosynthesis through the control of ALA-synthase will be discussed in a later section in this chapter.

The other enzymes in the haem biosynthetic pathway and the reactions they catalyse are summarised in Figure 1:2. In general the other enzymes of the haem biosynthetic pathway are much better characterised than ALA-synthase because their purification from a wide variety of sources has been readily accomplished, thus facilitating their study. The possible exception to this is protoporphyrinogen oxidase, the enzyme catalysing the penultimate step in the pathway. Until recently the oxidation of protoporphyrin IX to protoporphyrin was thought to proceed non-enzymatically, but the work of Poulsen and Poulglase (1976), demonstrated the involvement of an enzyme when they isolated and purified protoporphyrinogen oxidase.

Newly synthesized haem moves out of the mitochondria, the bulk of it to the microsomes (Druyan and Kelly (1972)), where most of the apoproteins are synthesized, notably cytochrome P-450 which is reported to utilise 65% of hepatic haem (Estabrook *et al* (1970)).

REGULATION OF THE RATE OF HAEM BIOSYNTHESIS.

A. CHEMICAL PORPHYRIA AND THE HUMAN PORPHYRIA DISEASES

In experimental animals ALA-synthase levels can be greatly increased by administration of porphyrinogenic

drugs. The resulting condition termed "chemical porphyria" shows similar biochemical characteristics to the acute hepatic porphyria diseases in man in which one of the signs is an increase in ALA-synthase levels.

The chemical porphyrias provide an experimental system in which to study the modulation of haem synthesis by porphyrinogenic drugs; this should lead to an understanding of the normal regulatory mechanism and to an understanding of what contributes to the symptoms in genetic porphyrias. In turn, it is hoped that this might lead to an improved therapy for the acute hepatic porphyria diseases.

A brief account, therefore, will be included in this section of the human porphyria diseases and the chemically induced porphyrias in experimental animals.

The porphyrias are a group of diseases characterised by deficiencies in the levels of specific enzymes of the haem biosynthetic pathway. In normal conditions the intermediates of the haem biosynthetic pathway do not accumulate and appear in the urine only in very small amounts. In the porphyrias, there is an accummulation of large amounts of porphyrins and their precursors. The porphyria diseases are conveniently divided into the hepatic forms and the erythropoetic forms based on the tissue localisation of the disorder. The erythropoetic porphyrias are further subdivided into

TABLE 1.1

THE HUMAN PORPHYRIA DISEASES

TABLE 1.1

THE PORPHYRIA DISEASES

I ERYTHROPOETIC PORPHYRIAS	INHERITANCE	DEFECTIVE ENZYME
A. Congenital Erythropoetic Porphyria	autosomal recessive	Uro-III co synthetase
B. Erythropoetic Proto-porphyria	autosomal dominant	Ferrochel-atase
II HEPATIC PORPHYRIAS		
A. Acute Intermittent Porphyria	autosomal dominant	Uro-I synthase
B. Hereditary Copro-Porphyria	autosomal dominant	Copro oxidase
C. Variegate Porphyria	autosomal dominant	Proto oxidase
D. Porphyria Cutanea Tarda	autosomal dominant	Urodecarb-oxylase

congenital erythropoetic porphyria and erythropoetic protoporphyria which can be distinguished by the different type and amount of porphyrins which are produced. The hepatic porphyrias have also been classified into (four) groups based on the type and amount of porphyrin accumulated in the disease. Three of the hepatic porphyrias have been termed acute. These diseases are acute intermittent porphyria, hereditary coproporphyria and variegate porphyria. All the porphyrias so far mentioned are genetically determined diseases with the defect causing reduced amounts of one of the enzymes of the haem biosynthetic pathway. The fourth hepatic porphyria, porphyria cutanea tarda, can be acquired as well as inherited. Table 1:1 summarises the various porphyria diseases and the enzyme defect responsible.

It is the acute hepatic porphyrias which have received most of the attention of biochemical research workers. This is because an apparently similar condition can be brought about in experimental animals by administration of porphyrinogenic drugs.

The acute hepatic porphyrias have in common elevated levels of ALA-synthase and porphobilinogen in the urine. These diseases are manifested clinically as acute neurological disorders which are usually latent but may be precipitated by a wide variety of drugs (see footnote

FOOTNOTE The word 'drug' is used as a convenient term, here it is used to imply an organic chemical compound not normally found in the body or diet. It is commonly used in the literature on porphyria presumably because so many porphyrinogenic compounds are also pharmaceutical compounds.

including barbiturates, tranquillisers and anti-convulsants together with some naturally occurring steroids.

Many of these drugs are also used as porphyrinogenic compounds in generating the chemical porphyrias mentioned earlier in this section. It is possibly not surprising, then, that when a drug causes chemical porphyria the biochemical abnormality generated appears to be the same as in the acute hepatic porphyrias most notably a vast increase in ALA-synthase (De Matteis (1975), Granick (1966), Tomita *et al* (1974), Sassa and Kappas (1977)).

There have been attempts to classify the porphyrinogenic drugs based on some common structural feature (Marks *et al* (1965), Hirsch *et al* (1966), Schneck *et al* (1968), De Matteis (1971)); although no specific relationship was found most of the drugs inducing chemical porphyria share a high degree of lipid solubility (De Matteis (1971), Murphy *et al* (1975)).

More recently, it has been found that most of the drugs inducing ALA-synthase are substrates for the cytochrome P-450 system whose function it is to render compounds water soluble for excretion in the urine (Bock and Remmer (1978), De Matteis and Gibbs (1972), Padmanaban *et al* (1973). As will be discussed later, this may be a factor in controlling ALA-synthase levels.

B. CONTROL OF ALA-SYNTHASE

The regulation of the haem biosynthetic pathway in avian and mammalian liver is thought to occur through control of ALA-synthase, the first and rate-limiting enzyme. As mentioned earlier the short half-life and the low levels of the enzyme are in keeping with this. Further support for ALA-synthase being the rate-limiting enzyme in the pathway comes from the observation that an increased supply of ALA will increase both *in vivo* and *in vitro* the liver synthesis of porphyrins and haem while an increased supply of the precursors to ALA will not. (Granick (1966), Doss (1969), De Matteis and Gibbs (1972), Druyan and Kelly (1972)). Although there is general agreement that haem controls the production of ALA-synthase there is no consensus on the precise mechanism of this control.

There have been suggestions that haem can act at a number of levels, although even this varies from one experimental animal species studies to another. Since there is much conflict in this area and it is not directly related to the studies in this thesis, a brief summary only will be given. Transcriptional control was first put forward by Granick (1966) using chick embryo liver cells in culture as his model. Later Yamamoto *et al* (1982) provided evidence for transcriptional control from studies in rats *in vivo*. Post-transcriptional regulation by haem was proposed by Granick and

Sassa (1970) and Tyrrell and Marks (1972) from studies in chick embryo liver cells (in culture). More specifically Granick and Beale (1978) proposed haem might inhibit the processing of mRNA for ALA-synthase. Whiting (1976) and Srivastava (personal communication) in this laboratory have shown that haem at 10μM does not inhibit translation of ALA-synthase mRNA in both isolated liver polyribosomes and a wheat germ cell free system. Direct evidence of haem control on ALA-synthase mRNA, either at the point of transcription or subsequent processing of mRNA, awaits the availability of a complementary DNA probe to ALA-synthase mRNA in order to directly monitor message levels.

An alternative site for haem control of ALA-synthase is at the level of translocation of ALA-synthase from the cytosol into the mitochondria. This has been proposed to occur in adult chickens and rats (Ohashi and Kikuchi (1972), Hayashi *et al* (1972), Yamauchi *et al* (1980) but does not exist in chick embryos (Whiting and Granick (1976b).

Earlier experiments to investigate the direct effect of haem on ALA-synthase indicated that an inhibitory effect existed (Scholnick *et al* (1972), Whiting and Elliott (1972), Kaplan (1971). However. these studies utilised haem at (>10μM), levels considered unlikely to exist physiologically.

A more recent report (Paterniti and Beattie (1973)), showed that 10μM haem inhibited ALA-synthase activity in rats but this observation does not hold true for chick embryos (Whiting and Granick (1976b)) and Srivastava (personal communication). In addition Simpson and Beattie (1980) have reported the isolation of a protein that activates ALA-synthase in rats.

C. MECHANISM OF DRUG MEDIATED INCREASES IN ALA-SYNTHASE

The mechanism of increases in ALA-synthase levels by porphyrinogenic drugs in experimental animal systems has been investigated with the hope that the answer will help in determining the mechanism of control that operates physiologically. The current concept of drug action is that the drugs operate to relieve repression of ALA-synthase by haem by acting at one or more of the following levels; a) degrading haem directly; b) increasing the apoprotein cytochrome P-450 thereby creating a demand for more haem; c) inhibiting one of the enzymes of the haem biosynthetic pathway (Meyer (1982)). The two drugs, AIA and DDC, used as inducers of ALA-synthase activity for the studies in this thesis (and also widely used elsewhere) fit into this concept. AIA degrades haem (De Matteis (1970, 1971, 1973)) and increased cytochrome P-450 production (Correia and Meyer (1975)). DDC, on the other hand, not only degrades haem and increases cytochrome P-450 (Tephly *et al* (1979), De Matteis *et al* (1980), but in

addition also inhibits ferrochelatase (De Matteis and Abbritti (1973)) the final enzyme in the haem biosynthetic pathway.

The question of whether the drugs operate either to repress haem alone or have a dual role to (a) reduce haem and (b) induce ALA-synthase by some other action (presumably at the gene level) as proposed by Granick and Sassa (1971), is as yet unanswered. Srivistava *et al* (1980) provided evidence from studies on haem control of ALA-synthase in isolated chick embryo liver cells that supported the concept that variation of haem levels may be the sole control over ALA-synthase.

THE ISOLATION OF ALA-SYNTHASE

As already discussed the levels of ALA-synthase activity in the livers of experimental animals is very low, but administration of porphyrinogenic drugs can increase this in some cases several hundred fold. ALA-synthase is found in many tissues, spleen (Ebert *et al* (1970), bone marrow (Bottomley and Smithee (1968), brain (Barnes *et al* (1972), heart (Briggs *et al* (1976) and kidney (Barnes (1971). Apart from the kidney, induction of ALA-synthase by drugs is known to occur only in the liver. For this reason, most of the attempts to purify ALA-synthase have been from the liver of various animals and this section will be mainly concerned with the literature on the purification of hepatic ALA-synthases. Since the work described

in this thesis is primarily concerned with the purification of ALA-synthase, a detailed account will be given of attempts made by other workers to obtain homogeneous enzyme.

ADULT RAT LIVER MITOCHONDRIAL ALA-SYNTHASE

Early attempts to isolate ALA-synthase from experimental animals were hampered by the instability of the enzyme activity during purification. The first step toward a useful purification was made by Kaplan (1971). This author disrupted rat liver mitochondria with deoxycholate and made some progress toward purification using chromatography on calcium phosphate and hydroxylapatite gels. The enzyme was found to exist as a large aggregate of molecular weight greater than 500,000.

The propensity of ALA-synthase to aggregate has been a major stumbling block in the purification of ALA-synthase. Whiting (1973), working in this laboratory, found that treatment of rat enzyme extracted from mitochondria with 0.8M NaCl and 1mM DTE disaggregated the enzyme to give a form of molecular weight 77,000 as judged by gel permeation chromatography. This finding enabled a 40-fold purification of rat liver mitochondrial ALA-synthase to be made (Whiting and Elliott (1972). The purification scheme used was in sequence - Sephadex G-150 chromatography of a

mitochondrial extract, ammonium sulphate fractionation (0-50%) and pyridoxal phosphate affinity chromatography. The resultant enzyme had a specific activity of 1060 nmoles/ALA/hr/mg. More recently, Paterniti and Beattie (1979) reported the purification of mitochondrial ALA-synthase from non-induced rat liver mitochondria. The purification scheme used by these authors was detergent extraction of mitochondria, ammonium sulphate (33-40%), DEAE chromatography, Sephacryl S-200 chromatography and preparative gel electrophoresis. This procedure gave a specific activity of 2,100 nmols/ALA/hr/mg protein, but only realised a 0.01% yield. The protein was proposed to be a dimer of identical subunits of molecular weight 58,000.

FOETAL RAT LIVER MITOCHONDRIAL ALA-SYNTHASE

Foetal rat liver has a 10-fold higher constitutive level of ALA-synthase than that of adult rats. This enzyme is refractive to drug-mediated induction but levels decline shortly after birth. Woods and Murthy (1975) achieved a partial purification of the foetal enzyme using detergent extraction of mitochondria, Sephadex gel chromatography and affinity chromatography. The specific activity of the preparation was 180 nmol/ALA/hr/mg protein and the minimum molecular weight estimated at 47,000.

ADULT LIVER CYTOSOL ALA-SYNTHASE

In rats a cytosol form of ALA-synthase can be detected following drug treatment (Hayashi *et al* (1969). This can account for one half of the total cellular ALA-synthase and cannot be attributed to mitochondrial breakage. Scholnick *et al* (1972), reported a partial purification of the cytosol enzyme using a complex scheme based on a combination of molecular sieve chromatography on Sephadex gels and a range of ammonium sulphate fractionations. The enzyme was purified up to 200-fold to a specific activity of 1080 nmol/ALA/hr/mg protein. The molecular weight was 300,000 even in the presence of 0.3M NaCl indicating that the enzyme was still part of a large aggregate. Similarly, early studies by Ohashi and Kikuchi (1977) indicated that rat liver cytosol enzyme was an aggregate of molecular weight 320,000 but higher molecular weight complexes were formed when NaCl was omitted during molecular weight estimations. In a following report the same authors (Ohashi and Kikuchi (1978)) postulated the existence of a complex form of ALA-synthase in which a catalytically active protein of molecular weight 51,000 was associated with two catalytically inactive proteins of molecular weight 79,000 and 120,000 respectively. The purification of the stripped form (the catalytically native subunit)

of the enzyme was achieved through ammonium sulphate fractionation (35%), hydroxyapatite chromatography, Sephadex G-200 chromatography, ammonium sulphate fractionation (35%) and two sucrose density gradient centrifugations. The specific activity was 5,480 nmol/ALA/hr/mg protein. More recent work by the same group (Nakakuki *et al* (1980)), purified the catalytically active ALA-synthase protein to a specific activity of 73,000 nmol/ALA/hr/mg protein. The purification scheme was modified to comprise ammonium sulphate fractionation (35%), Sephacryl S-200 chromatography, papain digestion (to disaggregate the enzyme) a second Sephacryl S-200 chromatography, hydroxyapatite chromatography and phosphocellulose chromatography. The purified enzyme was proposed to be a dimer of identical subunits of molecular weight 51,000.

ADULT CHICKEN ALA-SYNTHASE

Both mitochondrial and cytosol forms of ALA-synthase have been reported in adult chicken livers (Ohashi and Kikuchi (1972)). The cytosol form has not been extensively purified but was found to exist as a high molecular weight aggregate (Ohashi and Kikuchi (1972)).

CHICK EMBRYO LIVER ALA-SYNTHASE

In contrast, in chick embryos only the mitochondrial form of ALA-synthase has been detected by colorimetric

assay techniques (Whiting and Granick (1976). These authors reported the purification of ALA-synthase from drug-treated chick embryo liver mitochondria to a specific activity of 20,500 nmol/ALA/hr/mg protein. The molecular weight of the native enzyme was 87,000 and the enzyme was proposed to be composed of two subunits of molecular weight 49,000 as electrophoresis of the protein on SDS polyacrylamide gels showed a single band at 49,000. This purification scheme will be described in detail in Chapter 3. In further studies in this laboratory Whiting (1976) and Brooker *et al* (1978) identified a small amount of a cytosolic form of ALA-synthase of molecular weight 70,000 using an antibody raised against the mitochondrial enzyme. This was considered to be a precursor to the mitochondrial enzyme.

In summary of the hepatic ALA-synthases a somewhat confusing picture emerges. It is apparent that the enzyme exists as an aggregate, at least during isolation attempts and this has made purification extremely difficult. The different values reported for the molecular weight of the enzyme cast some doubt on the reported work,but, it was generally concluded that in the rat and chick embryo, the minimum molecular weight of the enzyme was around 50,000 when the work in this thesis began. In addition, the finding of a putative precursor of mitochondrial ALA-synthase

TABLE 1.2

SUMMARY OF RECENT PURIFICATIONS OF ALA-SYNTHASES

FROM A VARIETY OF SOURCES

SOURCE	FOLD PURIFICATION	SPECIFIC ACTIVITY (nmol/hr/mg)	MOLECULAR WEIGHT NATIVE	MOLECULAR WEIGHT MINIMUM	AUTHORS
Drug treated rat liver mitochondria	11.2	38.4	large aggregate	N.D.	Kaplan (1971)
Drug treated rat liver mitochondria	40	1,060	77,000	N.D.	Whiting and Elliott (1972)
Untreated rat liver mitochondria	4,000	2,100	120,000	58,000	Paterniti and Beattie (1979)
Untreated foetal rat liver mitochondria	30	180	47,000	N.D.	Woods and Murthy (1975)
Drug treated rat liver cytosol	210	1,080	300,000	N.D.	Scholnick *et al* (1973)
Drug treated rat liver cytosol	3,500	73,000	110,000	51,000	Nakakuki *et al* (1980)
Drug treated chick embryo liver mitochondria	125	20,500	87,000	49,000	Whiting and Granick (1976)
Rabbit reticulocytes	4,400	18.7	200,000	N.D.	Aoki *et al* (19
R. Spheroides	3,000	130,000 - 170,000	62,000-68,000	49,000	Davies and Newberger (1979)
M. Denitrificans	788	37,000	68,000	68,000	Tait (1973)

leads to the problem of determining the exact nature of the relationship between the cytosolic and mitochondrial forms.

NON-HEPATIC ALA-SYNTHASES

ALA-synthase has been purified from rabbit reticulocytes (Aoki *et al* (1971). The molecular weight was 200,000 raising the possibility that it was still an aggregated form of the enzyme. ALA-synthase has been purified from a number of micro-organisms, notably Rhodopseudomonas Spheroides (Warnick and Burnham (1971), Fanica-Gaignier and Clement-Metral (1973), Nandi and Shemin (1977)), and most recently, by Davies and Neuberger (1979). These authors found a high activity form of the enzyme (specific activity 130-170,000 nmol/ALA/hr/mg protein). The apparent molecular weight was 62 - 68,000. Purification of ALA-synthase has also been made from Micrococcus denitrificans (Tait (1973)). The enzyme was found to have a molecular weight of 68,000 and existed as a monomer. The major findings of these studies are summarised in Table 1:2.

AIM OF THIS WORK

One of the major aims of work in this laboratory (and others) is to understand at the molecular level the mechanism by which haem regulates ALA-synthase. As mentioned previously this is being approached through

studies on chemical porphyria in an experimental animal system, the chick embryo.

In such an investigation, it is important to have as much information as possible about the enzyme whose regulation is being studied - its molecular size, physical properties, its site of synthesis, how it is transferred to its functional location and factors effecting its activity and stability. This type of information can only come from studies on the pure enzyme. Once the pure enzyme is obtained, an antibody can be produced and further progress can be made in the studies on the enzyme synthesis and on isolating and identifying mRNA coding for ALA-synthase enabling isolation of the ALA-synthase gene.

Prior to the commencement of work in this thesis it was believed that a method existed in the procedure of Whiting and Granick (1976) for the purification of ALA-synthase.

Attempts to duplicate this procedure were unsuccessful for a variety of reasons that will be described in the early part of this thesis. It, therefore, became necessary to develop a new procedure for the purification of ALA-synthase. The major aim of the work in this thesis, then, was the purification of ALA-synthase from avian and mammalian liver. The project was further developed to seek answers to the question of why previously reported

purifications of ALA-synthase led to molecular weights which differed widely even for the enzyme from the same tissue.

CHAPTER 2

MATERIALS AND METHODS

M A T E R I A L S

EXPERIMENTAL ANIMALS

Fertilised White Leghorn chicken eggs were obtained from the Department of Agriculture, Parafield Poultry Research Station, Adelaide, South Australia. The eggs were incubated in a humidified (70%) forced draught incubator at 37°C for 17 - 18 days prior to use. Male Albino Wistar rats (150 - 200g) were obtained from the Central Animal House, Waite Institute, Adelaide. Animals were maintained under natural light cycles and sacrificed under chloroform anaesthesia. Rats were fasted for 24 hours prior to drug administration but were permitted water *ad libitum*. When DDC was administered rats were lightly anaesthetised with ether prior to intragastric administration of drug as described in Chapter 7.

PORPHYRINOGENIC DRUGS

Allylisopropyl acetamide (AIA) was a gift from Hoffman-La Roche, Nutley, New Jersey, U.S.A. 1,4-dihydro-3,5 - dicarbethoxycollidine (DDC) was purchased from Eastman Organic Chemicals, Rochester, New York, U.S.A.

CHROMATOGRAPHIC MATERIALS

Sephadex G-150, Sephacryl S200, Activated Sepharose CH4B, Sephadex IEF Phenyl Sepharose DEAE, Sephadex A50, Polybuffer Exchanger 94, Polybuffer 96 and Poly (U)

Sepharose were obtained from Pharmacia, Sydney, New South Wales, Australia. CoA Agarose Type V and NAD^+Agarose was from P-L Biochemicals, Milwaukee, Wisconsin, U.S.A. Phosphocellulose Pll was purchased from Whatman and Hydroxylapatite was from Bio-Rad Laboratories, Richmond, California, U.S.A. All chromatographic columns were washed with Na azide (0.1%) to prevent bacterial contamination after use.

OTHER REAGENTS

Phenylmethylsulphonylfluoride (PMSF), 5' AMP, papain, Tris-HCl digitonin, pyridoxal phosphate, ATP, CoA, DTE, Triton X-100, cholic acid, guanidine HCl urea, acrylamide, bisacrylamide, Brij 58, SDS, 2 mercaptoethanol, creatine phosphate and creatine phosphate kinase were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Chymostatin and leupeptin were from Protein Research Foundation, Japan.

O-phenanthroline was obtained from By-Products and Chemicals, Melbourne, Australia.

Amino acids were obtained from Sigma Chemical Co., BDH Chemicals Ltd and Schwarz-Mann, Orangeburg, New York, U.S.A. Acetylacetone, cetyltrimethylammonium bromide, deoxycholic acid, ammonium persulphate were purchased from BDH Chemicals Ltd.

All other reagents were of A.R. grade and obtained from Ajax Chemicals Ltd., Sydney, Australia and BDH Chemicals Ltd.

METHODS

PREPARATION OF BACTERIAL SUCCINYL CoA SYNTHASE

This enzyme was prepared from E. Coli (Crookes strain) by the method of Ramaley *et al* (1967), but purification was carried out only until the ammonium sulphate fractionation step. The pellet from this stage was resuspended in 50mM potassium phosphate buffer pH 7.4 and dialysed overnight. The dialysate was clarified by centrifugation at 10,000 x g for 15 minutes and then stored at -15°C in 1ml aliquots. The resultant enzyme had a specific activity of 15µMoles succinichydroxamic acid/mg protein/30 min when assayed by the method of Kaufman (1955).

ESTIMATION OF PROTEIN

Protein obtained at each stage of the purification was estimated by the method of Lowry *et al* (1951) after precipitation with 10% trichloroacetic acid and solubilisation in 0.1N NaOH. Protein from the chromatofocusing column was measured directly by the method of Bradford (1976). Bovine serum albumin was used as a standard for both assays.

ASSAY FOR ALA-SYNTHASE

The assay used was that of Poland and Glover (1973). This assay determines colourimetrically the amount of ALA formed. The reaction mixture (100µl) contained 50mM Tris-HCl, pH 7.4, 100mM succinate, 0.25mM 50mM glycine

mM pyridoxal phosphate, 250mM NaCl, 15mM ATP, 20mM $MgCl_2$, 10mM EDTA and 2 units succinyl CoA synthase. Samples (50µl) containing ALA-synthase activity were added and incubated for 15 or 30 minutes at 37°C. Enzyme activity was terminated by the addition of 100µl of 10% trichloroacetic acid and protein precipitated by centrifugation at 5,000 x g for 10 minutes. The ALA-synthase content of the supernatant was determined by mixing 150µl of supernatant with 75µl of a 10:1 mixture of 1M sodium acetate pH5 acetylacetone and heating at 80°C for 10 minutes. After cooling, the solution was mixed with an equal volume of Ehrlichs reagent.

The pink pyrrole reaction colour was allowed to develop fully for 10 minutes and the absorbance read immediately at 555nm in a cell of 1cm optical length. The amount of ALA-synthase produced was calculated using a molar extinction coefficient of 58 $mM^{-1}cm^{-1}$. ALA-synthase activity was expressed as units/mg protein. One unit of ALA-synthase is defined as the amount of enzyme which catalyses the production of 1nmol of ALA-synthase in 60 minutes at 37°C. All assays were corrected for a blank to which trichloroacetic acid was added at zero incubation time.

When non-denaturing gel slices were assayed for ALA-synthase activity the assay volume was increased to 200µl in order to fully immerse the gel slice.

POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (1970). The separating gel contained 10% acrylamide, 0.2% bisacrylamide, 0.1% SDS, 375mM Tris-HCl pH 8.8 and the stacking gel 3% acrylamide, .08% bisacrylamide, 0.1% SDS, 125mM Tris-HCl pH 6.8. Gels were electrophoresed at 45mA, while the sample was in the stacking gel and at 120 volts while the sample was in the separating gel.

STAINING OF GELS

Following electrophoresis gels were stained 12 - 16 hours in 0.05% Coomassie Brilliant Blue, 10% acetic acid, 25% isopropanol then destained by successive washes in 10% acetic acid and 10% isopropanol until the stained bands were clearly visible.

FLUOROGRAPHY OF GELS

Fluorography was performed essentially as described by Bonner and Laskey (1974). Gels were washed in two successive changes of dimethylsulphoxide (DMSO) for 60 minutes, then soaked in 1.5% w/v 2.5 diphenyloxazole (PPO), 22% naphthalene w/v in DMSO for 2 hours. The gels were soaked for at least 60 minutes in water to precipitate the PPO/naphthalene in the gel after which they were dried onto 3MM Whatman paper using a slab-gel dryer (Model 224, Bio-Rad Labs) and autoradiographed.

NON-DENATURING GEL ELECTROPHORESIS

Non-denaturing gel electrophoresis was performed using the Tris-glycine system of Davis (1964). The separating gel contained 5% acrylamide 0.13% bisacrylamide .375M Tris-HCl pH 8.8 and the stacking gel 2.5% acrylamide, 0.75% bisacrylamide, 62.5mM Tris-HCl pH 6.6. Gels were electrophoresed at 100 volts for 10 - 12 hours at 4°C.

IN VIVO LABELLING STUDIES

a. CHICK EMBRYOS

Chick embryos were pulse-labelled with 100µCi of ^{35}S-methionine (specific activity 120 Ci/mmol) in 50µl 20mM potassium phosphate pH 7.6 150mM NaCl. The radioactive ^{35}S-methionine was layered over the yolk sac membrane via a small hole in the shell above the air space. Labelling was allowed to proceed for 45 minutes.

b. RATS

Rats were pulse-labelled with 300µCi of ^{35}S-methionine (120 Ci/mmol) in 100µl 20mM potassium phosphate, pH 7.6, 150mM NaCl injected intra-peritoneally. Labelling was allowed to proceed for 45 minutes.

PREPARATION OF ANTI ALA-SYNTHASE ANTISERUM

Antiserum to ALA-synthase was raised in sheep by

injection of 300µg of pure ALA-synthase in 1ml volume 0.9% saline mixed with an equal volume of Freunds complete adjuvant injected into sites in the inguinal area. Two weeks later, blood was collected (250ml) from the carotid artery. After allowing the blood to clot, the clot was allowed to contract for 12 hours at 4°C and the clear serum carefully collected.

SPECIFICITY OF SERUM

Serum was tested for its specificity against ALA-synthase by Ouchterlony double diffusion analysis. The antiserum was tested against pure ALA-synthase and mitochondrial extracts from drug-treated and untreated (control) chick embryo livers. A single precipitin line was obtained against the pure ALA-synthase and the mitochondrial extract from drug-treated chick embryo liver, but no precipitin line was observed with the untreated mitochondrial extract, (Figure 2.1)

The equivalence point of the antiserum was obtained by determining the inhibition of varying amounts of ALA-synthase activity by 100µl of antiserum. Pure ALA-synthase (0 - 100 units) was incubated with 100µl of antiserum for 60 minutes at 37°C and then the immune complexes were removed by centrifugation at 5,000 x g for 10 minutes. The supernatants were then assayed for ALA-synthase activity and compared with a control where no antiserum was added. The result (Figure 2.2) shows that 100µl of serum will

FIGURE 2.1

OUCHTERLONY DOUBLE DIFFUSION ANALYSIS OF CHICK-EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE AGAINST ANTI-CHICK EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE ANTISERUM

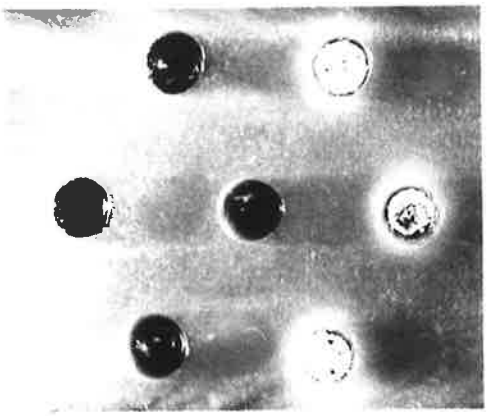
Mitochondrial extracts were prepared from drug-treated and untreated chick embryo livers as described in the text. Pure ALA-synthase was prepared as described in Chapters 4 and 5. These fractions were tested against non-immune serum and serum from sheep immunised with pure ALA-synthase as described in the text.

A. Immune serum (centre well) with untreated chick-embryo liver mitochondrial extract (3 right-hand wells).

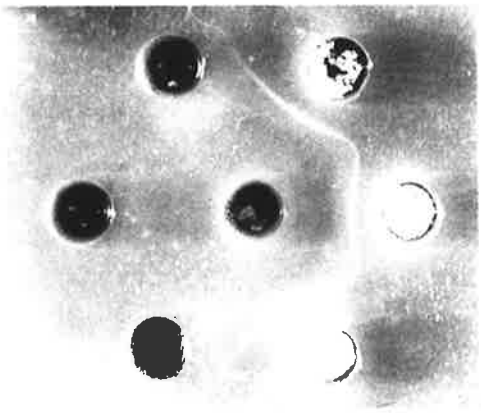
B. Immune serum (centre well) with drug-treated chick-embryo liver mitochondrial extract (3 right-hand wells).

C. Immune serum (centre well) with pure chick-embryo liver mitochondrial ALA-synthase (outer wells).

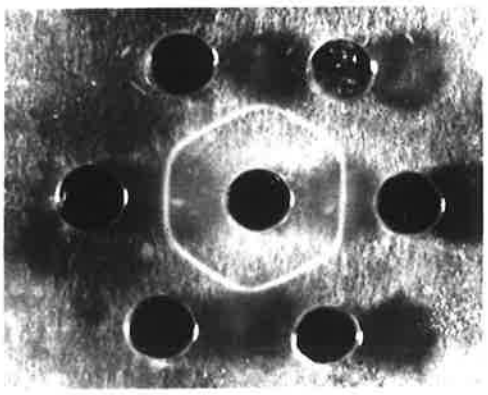
D. Non-immune serum (centre well) with pure chick-embryo liver ALA-synthase (outer wells).



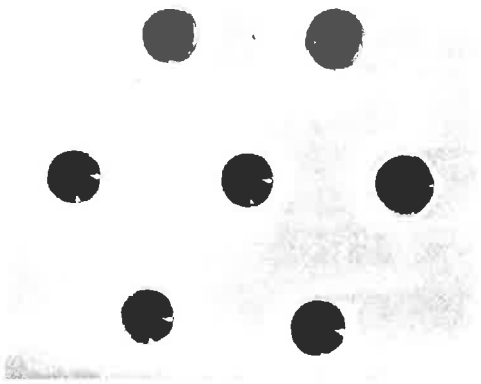
A



B



C

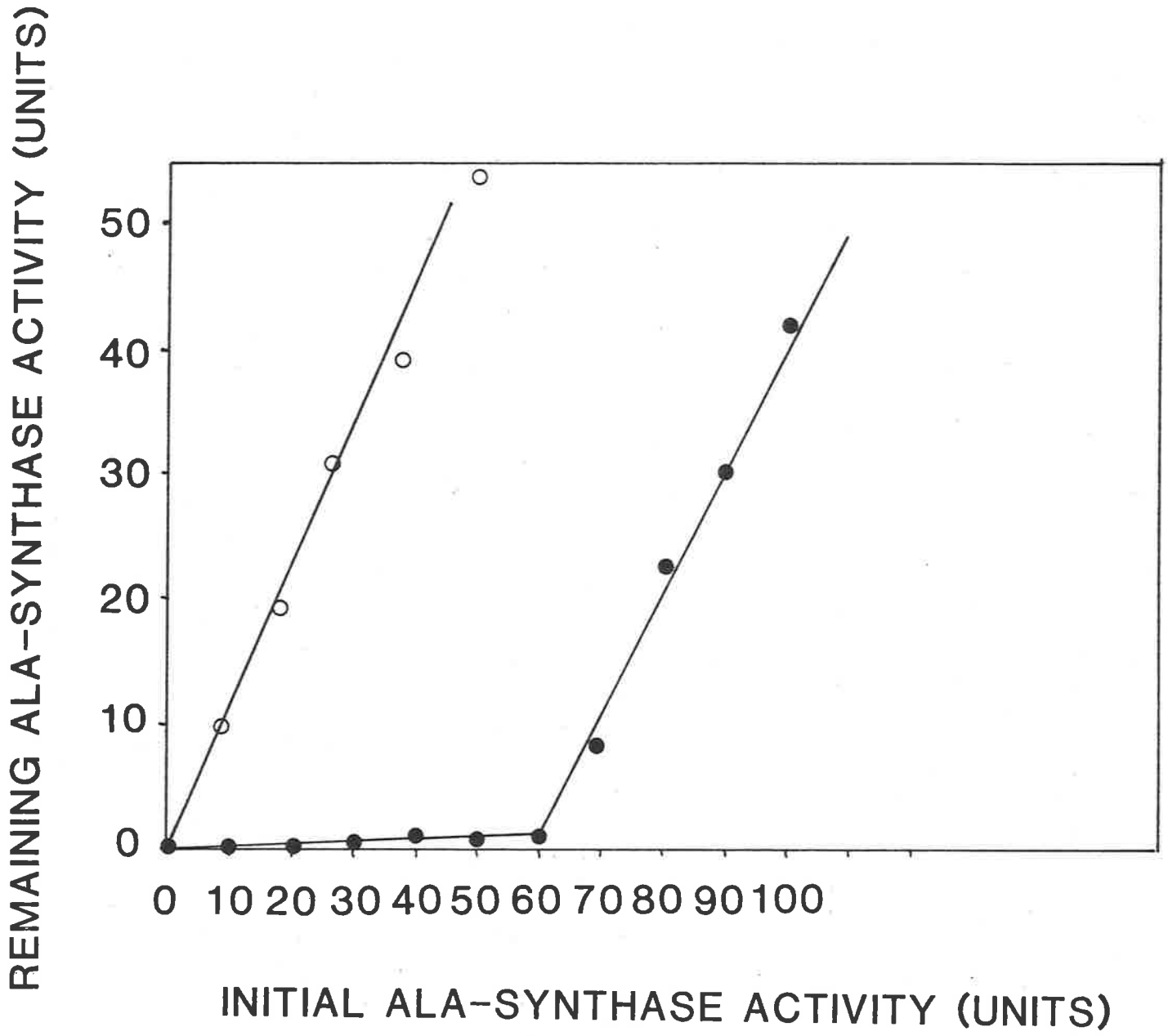


D

FIGURE 2.2

IMMUNOTITRATION OF CHICK EMBRYO LIVER ALA-SYNTHASE WITH ANTI-CHICK EMBRYO ALA-SYNTHASE ANTISERUM

Varying amounts of ALA-synthase (0 - 100 units) were incubated with 100µl of immune (● - ●) or non-immune (O - O) serum and the residual ALA-synthase activity determined as described in the text. 100µl of immune serum inhibits 50 units of ALA-synthase activity.


REMAINING ALA-SYNTHASE ACTIVITY (UNITS)
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
INITIAL ALA-SYNTHASE ACTIVITY (UNITS)

neutralise 50 units of ALA-synthase activity. Non-immune serum had no effect on ALA-synthase activity.

IMMUNOPRECIPITATION

a. *IN VIVO* LABELLED MITOCHONDRIA

Freeze-dried mitochondrial extracts were resuspended in water and diluted 1:10 with 10 x immunoprecipitation buffer (final concentration, 150mM NaCl, 50 mM Tris-HCl p.H 8, 1% Triton X-100, 1% deoxycholate) and anti-serum added (enough to precipitate all the ALA-synthase as measured by enzyme inhibition assays). The mixture was then incubated at 4°C for 12 - 16 hours prior to collection of immunoprecipitates with *S. Aureus* cells.

b. *IN VITRO* TRANSLATION PRODUCTS

Following translation in a wheat germ cell free system (see Chapter 7) the translation mixture (25µl) was diluted to 100µl with water and 20µl of 10% SDS added prior to heating at 100°C for 2 minutes. (Matsuura *et al* (1981). 20µl of this mixture was precipitated with 5 volumes of acetone for analysis of total translation products. The remaining 100µl was diluted 6:1 with immunoprecipitation buffer and 10µl anti-ALA-synthase antiserum added. The mixture was incubated at 4°C for 12 - 16 hours.

IMMUNOADSORPTION TO *S. AUREUS* CELLS

Immunoprecipitates were collected by adsorption to *S. Aureus* cells based on the method of Kessler (1975). *S. Aureus* cells (Cowan 1 strain) were purchased from CSL Laboratories, Melbourne, Australia and washed thoroughly in immunoprecipitation buffer prior to use. The washed cells were finally re-suspended as a 10% suspension and 100µl added to immunoprecipitation mixtures. Incubation was allowed to proceed for 1 hour at 4°C. The *S. Aureus* cells were precipitated by centrifugation at 10,000 x g for 3 minutes and washed twice in immunoprecipitation buffer. The pelleted cells were then resuspended in 50µl of SDS polyacrylamide gel loading buffer and heated at 100°C for 2 minutes. After centrifugation the supernatant was analysed by SDS polyacrylamide gel electrophoresis.

C H A P T E R 3 .

ATTEMPTS TO DUPLICATE THE WHITING AND GRANICK (1976) PROCEDURE

INTRODUCTION

As described in the introduction many attempts had been made in several laboratories to purify ALA-synthase but none were successful in obtaining a homogeneous preparation until the work of Whiting and Granick (1976) appeared. Based on work initiated in this laboratory, Whiting and Granick (1976) reported the purification of ALA-synthase and raised an antibody in rabbits which was apparently selective for ALA-synthase. Only a single batch of this antibody was ever prepared.

It was necessary to have a pure preparation of ALA-synthase to enable studies commenced in this laboratory to be completed. The obvious procedure, then, to follow was the method of Whiting and Granick (1976) for purifying ALA-synthase. However, this seemingly straightforward procedure proved to be an intractable problem. Despite exhaustive efforts to duplicate and later to modify the Whiting and Granick (1976) procedure, an homogeneous preparation of ALA-synthase was never obtained; nor indeed was a preparation of high specific activity prepared. Consequently, an antibody was not obtained that was specific for ALA-synthase as a result of following this procedure.

Since a great deal of effort and time went into duplicating the Whiting and Granick (1976) procedure this chapter will describe the problems encountered both in attempts to duplicate and later modify the procedure.

METHODS:

The Whiting and Granick (1976) procedure basically involves extraction of ALA-synthase from mitochondria by ultrasonication, chromatography on Sephadex G-150, ammonium sulphate precipitation, affinity chromatography for pyridoxal phosphate and isoelectric focusing in a sucrose gradient. The procedure as used in this laboratory will be described in detail to provide a background to the problems encountered.

DRUG TREATMENT OF EMBRYOS AND PREPARATION OF MITOCHONDRIA

Seventeen day old chick embryos were treated with the porphyrinogenic drugs AIA (2mg) and DDC (4mg in 0.1ml DMSO). These drugs raise the level of ALA-synthase 500-fold over the basal or constitutive level (Whiting and Granick (1976)). Induction of ALA-synthase was allowed to proceed for 18 hours at which time the livers were dissected from the embryos and placed in ice-cold buffered sucrose (0.1mM pyridoxal phosphate, 1mM EDTA, 0.25M sucrose, 5mM Tris-HCl pH 7.4-PEST buffer). The pooled livers were blotted, weighed and a 12.5% w/v homogenate prepared in PEST buffer using a Potter-Elvejhem glass homogeniser fitted with a motor driven teflon pestle. Mitochondria were prepared from the homogenate by differential centrifugation according to the method of Schneider and Hogeboom (1950). The isolated mitochondria were washed once and then suspended in 50mM Tris-HCl, pH 7.6, 0.1 mM pyridoxal phosphate, 1mM DTE (TPD buffer) to give a

protein concentration of 80 mg/ml. They were then rapidly frozen in dry ice/ethanol.

SONIC EXTRACTION OF ALA-SYNTHASE FROM MITOCHONDRIA

The thawed mitochondrial suspension was sonicated, while cooled on ice, four times for 20 seconds at 1 minute intervals using a Branson soniprobe fitted with microtip, at maximum power. Following centrifugation at 105,000 x g for 60 minutes the supernatant was collected and the loose pellet resuspended in TPD buffer to half the original volume. The sonication and centrifugation steps were repeated and the two supernatants combined. Solid NaCl (to 0.8M) and DTE (to 1mM) were added to the mitochondrial extract.

SEPHADEX G-150 CHROMATOGRAPHY

Half of the extract of mitochondrial protein was loaded onto a Sephadex G-150 column (5 x 80cm) equilibrated with buffer containing 50mM Tris-HCl, pH 7.6, 0.5M NaCl, 0.1mM pyridoxal phosphate, 1mM EDTA, 1mM DTE and eluted with the same buffer at a flow rate of 25ml/hour. ALA-synthase eluted as a single peak after the void volume. The most active fractions were pooled and fresh DTE (1mM) added. These fractions were stored at 4°C while the other half of the mitochondrial extract was chromatographed on the Sephadex G-150. The pooled active fractions from each half were then combined.

AMMONIUM SULPHATE FRACTIONATION

To the gently stirred enzyme solution solid $(NH_4)_2\ SO_4$ was added over a period of 15 minutes to a final concentration of 50% w/v. After standing for a further 15 minutes the precipitate was collected by centrifugation at 15,000 x g for 20 minutes and dissolved in a minimum volume of 20mM Tris-HCl pH 7.4, 80 mM NaCl, 0.1 mM EDTA, o.1% Triton X-100, 1mM DTE (TTEND buffer). The enzyme was dialysed against two successive changes of 2 litres of TTEND buffer, then given a clarifying centrifugation at 8,000 x g for 15 minutes to remove the large precipitate which formed during dialysis.

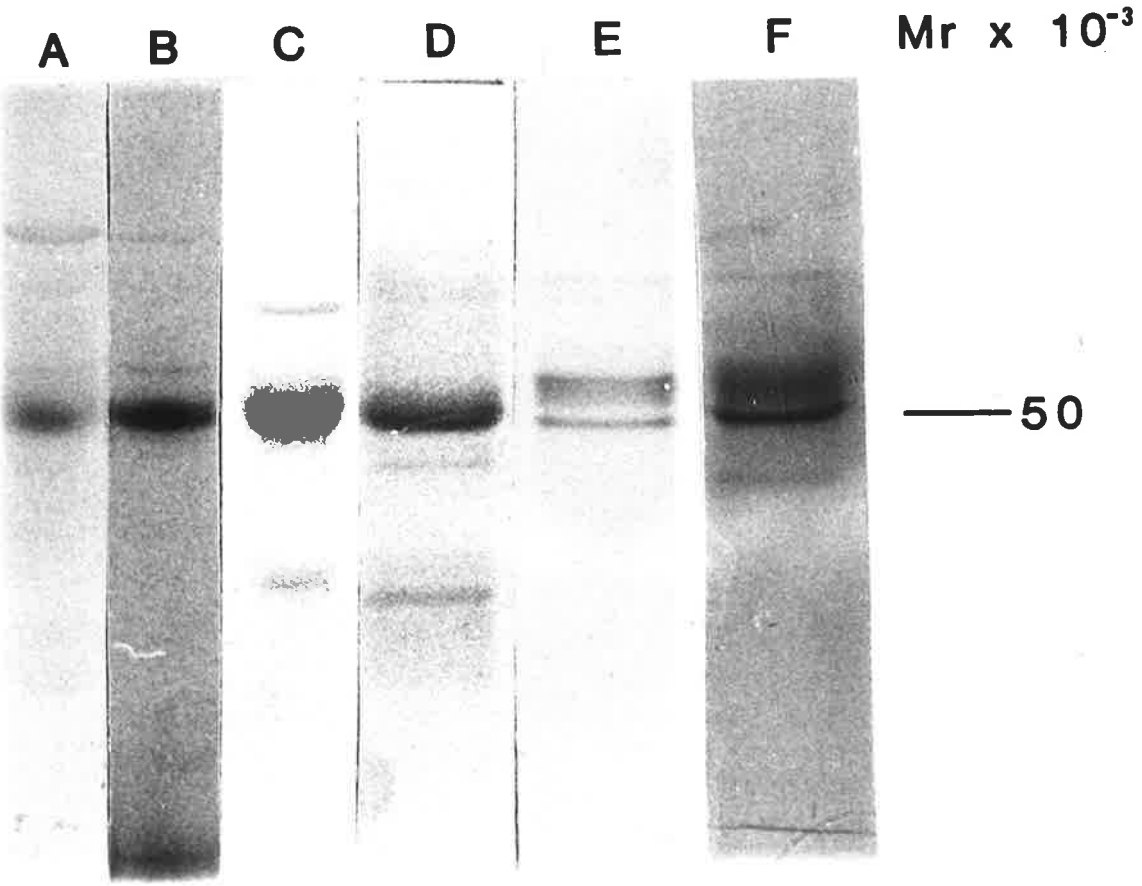
AFFINITY CHROMATOGRAPHY

An affinity gel of pyridoxamine phosphate coupled to Sepharose 4B via a six carbon arm spacer was prepared as recommended by the manufacturer and a column of affinity matrix (1.6 x 12cm) equilibrated in TTEND buffer. The dialysate resulting from the $(NH_4)_2SO_4$ fractionation was loaded onto the column. Unbound protein was washed through the column with TTEND buffer. Active fractions were pooled and concentrated by passage through a small column containing 2 ml hydroxylapatite equilibrated with 20 mM potassium phosphate buffer pH 7.6 containing 0.1 mM pyridoxal phosphate and 1 mM DTE. The enzyme activity was eluted from the column by 400 mM potassium phosphate pH 7.6 containing 1 mM DTE and dialysed exhaustively

FIGURE 3.1

SDS POLYACRYLAMIDE GEL ANALYSES OF THE PROTEIN OBTAINED AFTER FOLLOWING THE WHITING AND GRANICK (1976) PROCEDURE

Purification of chick embryo liver mitochondrial ALA-synthase was carried out as described in the text. The protein obtained after isoelectric focusing was analysed by SDS polyacrylamide gel electrophoresis. Lanes A-F show the end result of six separate purification attempts.


A
B
C
D
E
F
Mr x 10-3
50

against 20mM potassium phosphate pH 7.6, 0.1mM pyridoxal phosphate and 1mM DTE.

PREPARATIVE ISOELECTRIC FOCUSING

Isoelectric focusing was performed on a LKB 8101 electrofocusing column of 110ml volume at 4°C. A sucrose density gradient containing 1% ampholines pH 6 to 8 and 0.2mM DTE was formed, using a LKB gradient maker, between the electrode solutions with the enzyme from the hydroxylapatite column included in the centre fractions, After 27 hours at 600 volts 2.5ml fractions were collected from the column and assayed for ALA-synthase activity. The pooled active fractions were dialysed against 20mM potassium phosphate pH 7.6, 1mM DTE and 0.1 mM pyridoxal phosphate and concentrated on a small hydroxylapatite column in the same manner described earlier.

This procedure was reported by Whiting and Granick (1976) to purify ALA-synthase to a specific activity of 20,000 units/mg protein/hour. The SDS polyacrylamide gel analysis of the end product of several purifications is presented in Figure 3.1. Clearly, purification of a single protein species has not been achieved. It was assumed that the broad diffuse band of molecular weight around 50,000 was ALA-synthase. but each preparation has a variable amount of different contaminating species. The remainder of this chapter therefore, is concerned with discussion of the problems

TABLE 3.1

COMPARISON OF THE WHITING AND GRANICK (1976) RESULTS WITH THOSE OBTAINED IN DUPLICATION ATTEMPTS FOR PURIFYING ALA-SYNTHASE.

TABLE 3.1

COMPARISON OF WHITING AND GRANICK (1976) PUBLISHED RESULTS WITH WORK DESCRIBED IN THIS THESIS

	WHITING AND GRANICK (1976)		DUPLICATION ATTEMPTS	
STEP OR FRACTION	SPECIFIC ACTIVITY (units/mg/hr)	YIELD %	SPECIFIC ACTIVITY (units/mg/hr)	YIELD %
Mitochondria	166	100	155	100
Sonic Extract	322	103	305	99
Sephadex G-150	1230	97	1200	95
$(NH_4)_2SO_4$	1748	69	1725	65
Affinity Chromatography	7400	53	3500-5000	25-40
Isoelectric focusing and hydroxylapatite concentration	20,500	21	6000-8000	5-15

encountered in following the Whiting and Granick (1976) procedure including attempts to introduce useful modifications.

RESULTS AND DISCUSSION

A comparison of the results reported by Whiting and Granick (1976) with the results obtained in efforts to duplicate their procedure is presented in Table 3.1. It can be seen from these results that similar levels of purity were obtained up to the stage of ammonium sulphate fractionation. No further discussion of these stages will be made. The difficulties in achieving a purification comparable with Whiting and Granick (1976) arose in the last two steps of the procedure. The problems associated with these two steps, affinity chromatography for pyridoxal phosphate and isoelectric focusing will be dealt with in this chapter. In addition, modifications made to the purification scheme in efforts to obtain pure ALA-synthase will be described here.

AFFINITY CHROMATOGRAPHY

By following the Whiting and Granick (1976) procedure it was anticipated that following affinity chromatography approximately 75% of the enzyme activity would be recovered with a specific activity in excess of 7,000 units/mg. Despite the preparation of several affinity matrices by coupling pyridoxamine phosphate

TABLE 3.2

VARIABILITY OF PYRIDOXAL PHOSPHATE AFFINITY MATRICES USED IN THE PURIFICATION OF ALA-SYNTHASE

TABLE 3.2

PYRIDOXAL PHOSPHATE AFFINITY MATRICES

COLUMN	FOLD PURIFICATION	YIELD %
1	2.4	45
2	2.5	54
3	2.2	61
4	2.8	48

to activated Sepharose 4B this degree of purification was not achieved. Table 3.2 summarises the results obtained with affinity columns constructed using these matrices.

Whiting and Granick (1976) had remarked on the crucial importance of the composition of the equilibration buffer for their affinity column to allow a useful purification to be allowed. It seemed worthwhile, therefore, to investigate the conditions which might effect the binding of ALA-synthase. Three types of binding are likely to occur on the pyridoxamine phosphate affinity column, hydrophobic interactions with the six carbon arm spacer, ionic interaction with the phosphate groups and unsubstituted carbonyl groups and specific binding to the pyridoxamine phosphate groups. The first two types of binding are undesirable and measures taken to minimise them would be an advantage.

Triton X-100 had been incorporated in the column buffer at 0.1% to minimise hydrophobic interactions on theoretical grounds. To see whether Triton X-100 had a useful effect during affinity chromatography samples of ALA-synthase were chromatographed on an affinity column in the presence and absence of Triton X-100. The results (Table 3.3) show that when chromatography is performed in the presence of Triton X-100 the specific activity of

TABLE 3.3

THE EFFECT OF VARYING BUFFER COMPOSITION ON PURIFICATION OF ALA-SYNTHASE BY PYRIDOXAL PHOSPHATE AFFINITY CHROMATOGRAPHY

TABLE 3.3

EFFECT OF BUFFER COMPOSITION ON PERFORMANCE OF PYRIDOXAL PHOSPHATE AFFINITY COLUMN

BUFFER COMPOSITION	FOLD PURIFICATION
20mM Tris-HCl pH 7.6, 80mM NaCl 0.1mM EDTA, 0.1% Triton X-100 1mM DTE	2.5
20mM Tris-HCl pH 7.6, 80mM NaCl 0.1mM EDTA, 1mM DTE	1.7
20mM Tris-HCl pH 7.6, 100mM NaCl 0.1 mM EDTA, 0.1% Triton X-100, 1mM DTE	Nil
20mM Tris-HCl pH 7.6, 50mM NaCl, 0.1mM EDTA, 0.1% Triton X-100 1mM DTE	2.4
20mM Tris-HCl pH 7.6, 0.1mM EDTA 0.1% Triton X-100, 1mM DTE	Nil

ALA-synthase was increased 1.5 fold than when performed in its absence.

Attention was then focused on altering the ionic strength of the affinity column buffer. A series of experiments were performed varying the ionic strength of the column buffer (summarised in Table 3.3). An increase in ionic strength to 100mM NaCl and 20mM Tris-HCl pH 7.6 did not cause ALA-synthase to bind tightly. Although the enzyme was retarded, eventually all the ALA-synthase was washed out with the column buffer. Reduction of the ionic strength to 50mM NaCl and 20mM Tris-HCl pH 7.6 did not have any apparent effect on the specific activity by comparison with the standard buffer conditions. Omitting NaCl from the buffer allowed the majority of the protein to bind but the ALA-synthase was not eluted by 10mM pyridoxal phosphate. 0.5M NaCl was required for elution of ALA-synthase together with most of the other protein resulting in no improvement in specific activity. It was clear that under these conditions the pyridoxal phosphate - Sepharose was not acting as an affinity column.

These results are very similar to those reported by Whiting and Granick (1976) for their pyridoxal phosphate affinity column. It was concluded that no improvement in this type of column would come from a prolonged investigation of the composition of the equilibration buffer of the affinity column.

Recent studies in this laboratory (Parslow (1978)), had also failed to successfully reproduce pyridoxal phosphate affinity chromatography for purification of rat liver mitochondrial ALA-synthase.

Thus, although the purity achieved by Whiting and Granick (1976) was not attained at this stage, it was decided to complete the purification scheme using the material prepared up to this stage.

ISOELECTRIC FOCUSING

As used by Whiting and Granick (1976) isoelectric focusing gave an increase in specific activity slightly greater than two-fold to complete their purification. When the preparations from pyridoxal phosphate affinity chromatography of specific activity described in the previous section were subjected to isoelectric focusing a purification of 1.6 fold (see Table 3.1) was obtained. This low level of purification was disappointing and may have been due to the relative impurity of the starting material. However, several problems were encountered with isoelectric focusing in sucrose gradients. Precipitation of the protein at its isoelectric point with subsequent mixing and mixing of protein upon elution resulted in a broad spread of ALA-synthase activity over the column. Several runs were performed where activity was detectable in greater than half the column volume.

By far the most serious problem associated with the isoelectric focusing step were the huge losses of

enzyme activity incurred during the procedure. These losses were up to 75% of the ALA-synthase activity. Particular care was taken to ensure that the chromatographic materials, at all stages, were free from proteases derived from bacterial contamination, but the losses of enzyme activity were still incurred.

Thus the attempts to purify ALA-synthase by the method of Whiting and Granick (1976) resulted only in the preparation of partially pure protein assumed to be ALA-synthase (Figure 3.1) with a specific activity around 8,000 units/mg.

MODIFICATION TO THE WHITING AND GRANICK (1976)PROCEDURE

Since many attempts to duplicate the Whiting and Granick (1976) procedure had failed to yield a pure enzyme it was possible that the introduction of additional purification steps might have effected a complete purification. Several techniques were tried for their efficacy in purifying ALA-synthase in order to determine whether they may prove useful in purification of the enzyme.

DEAE SEPHADEX A-50

A glass column (10 x 1.6cm) was packed with DEAE Sephadex A-50 and equilibrated in 50mM Tris-HCl pH 7.8, 0.1mM pyridoxal phosphate and 1mM DTE. ALA-synthase, eluted from Sephadex G-150 as described earlier, was equilibrated in the same buffer (by dialysis in an Amicon ultrafiltration cell) and was loaded on the column. The column was washed with 4 volumes

of equilibration buffer and then eluted with a linear gradient of 50-500mM NaCl. Enzyme activity was eluted as a broad peak halfway through the gradient. Measurement of specific activity before and after passage of the enzyme through the column showed a two-fold purification had been achieved with a 60% recovery of enzyme activity.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

As ALA-synthase is located in the matrix of the mitochondria (McKay *et al* (1969)), (Zuyderhout *et al* (1969)),or associated with the inner membrane, it was likely that some parts of the molecule were hydrophobic in nature, therefore hydrophobic chromatography seemed potentially useful. Phenyl Sepharose was equilibrated in 1M NaCl, 1mM DTE, 0.1mM pyridoxal phosphate and 20mM Tris-HCl, pH 7.6 and packed in a column (12 x 1.6cm). Enzyme active fractions from Sephadex G-150 were adjusted to 1M NaCl and loaded onto the column. The ALA-synthase activity was retained on the column and after washing with 4 volumes column buffer a decreasing linear gradient of 1M-50mM NaCl was applied to the column. No ALA-synthase activity was released by this procedure and less than 10% of the applied protein was eluted. Washing with 1mM Tris-HCl, pH 7.6, 0.1mM pyridoxal phosphate and 1mM DTE also failed to release ALA-synthase. Elution of enzyme activity was accomplished

by washing the column with 1% Triton X-100 in 20 mM Tris-HCl pH 7.6, 0.1 mM pyridoxal phosphate, 1mM DTE. Measurement of specific activity before and after passage through the Phenyl Sepharose showed a 1.5 fold increase in specific activity. Recovery was 85-90%. Attempts to improve theresolution of the column by eluting with linear gradients of either 0-1% Triton X-100 or 0.25% ethylene glycol proved to be ineffective.

PHOSPHOCELLULOSE

Phosphocellulose (Whatman P11) was prepared according to the manufacturers instructions. A column (12 x 1.6cm) was packed with phosphocellulose and equilibrated in 50mM KPO_4 pH 7.0. Fractions containing ALA-synthase from the Sephadex G-150 column were equilibrated in the same buffer and loaded on the column. The ALA-synthase did not bind to the column and no enrichment of ALA-synthase was noted. Attempts to induce binding to the phosphocellulose by lowering the pH to 6.4 proved ineffective.

ISOELECTRIC FOCUSING

Although Whiting and Granick (1976) used sucrose gradients for isoelectric focusing other techniques available were using acrylamide and Sephadex as supporting media. These two alternatives have the potential to overcome the inherent disadvantages of sucrose gradient IEF, namely, mixing of protein on elution, mixing due to

convection currents and precipitation of protein at its isoelectric point. Therefore isoelectric focusing was performed in pre-cast acrylamide slabs on an LKB IEF apparatus according to manufacturers instructions. Although the technique was working satisfactorily as judged by monitoring control protein from a red blood cell lysate, it did not prove useful because of the small capacity of the gels and the inability to recover active enzyme from the gel. Isoelectric focusing in Sephadex was performed in a Pharmacia IEF apparatus designed for Sephadex IEF and used according to the manufacturers instructions. The specific activity of ALA-synthase following isoelectric focusing conducted in Sephadex IEF proved to be similar to that obtained after isoelectric focusing sucrose gradients, 1.6 fold, and similar losses of enzyme activity were also incurred.

NEGATIVE CHROMATOGRAPHY

The possibility of using chromatographic procedures known to bind specific mitochondrial enzymes was examined briefly. The rationale behind this approach was that passage of an extract containing ALA-synthase through a column removing specific protein (s) would enrich the eluted ALA-synthase, thus providing a purification. One such chromatographic material tested was NAD^+ agarose, an affinity absorbent for a number of dehydrogenases (Leissing N., McGuiness E. (1978)) of

the mitochondrial matrix. When ALA-synthase was passaged through an NAD^+ agarose column under conditions established to bind glutamate dehydrogenase (Goss, (1977)), most of the ALA-synthase was also retained on the column. ALA-synthase was eluted under the same conditions required to elute glutamate dehydrogenase giving a two-fold increase in specific activity. Most likely this was due to the NAD^+ agarose acting as an ion-exchange column. The negative chromatography technique was not pursued as it was more complex than at first envisaged.

MODIFIED PURIFICATION SCHEMES

Having evaluated the potential of several purification procedures, some of them were tried in combination with the Whiting and Granick (1976) procedure. Purification schemes such as (1) sonic extraction of mitochondria, Sephadex G-150 chromatography, ammonium sulphate fractionation, DEAE chromatography, pyridoxal phosphate affinity chromatography and isoelectric focusing; (2) sonic extraction of mitochondria, Sephadex G-150 chromatography, ammonium sulphate fractionation, hydrophobic interaction chromatography, NAD^+ agarose chromatography, pyridoxal phosphate affinity chromatography and DEAE Sephadex chromatography were tried. These combinations proved to be no better than the basic Whiting and Granick (1976) purification and further enrichment of ALA-synthase

beyond that achieved by that procedure was not attained.

CONCLUDING REMARKS

Attempts to duplicate the purification scheme of Whiting and Granick (1976) for chick embryo liver mitochondrial ALA-synthase had not been successful. Specific activities up to 8,000 units/mg protein had been obtained which is only about one third of the purity reported by Whiting and Granick (1976). SDS polyacrylamide gel analysis clearly showed that several contaminating proteins were present. The major band of molecular weight 50,000 was assumed to be ALA-synthase, but this was always disconcertingly diffuse. It appeared as an ill-defined smeared zone on gels and never as a sharply defined band. This was in contrast to the sharpness of the other protein bands (contaminants). The most serious deficiency of the procedure was the large loss of ALA-synthase throughout the purification particularly in the latter stages. It was suspected that these losses were due to endogenous protease attack as apparent breakdown products of low molecular weight could be seen following SDS polyacrylamide gel electrophoresis of some preparations. A second serious obstacle in this work was the fact that the Whiting and Granick (1976) procedure took about 10 - 12 days. This coupled with the large number of chick embryos used in a single preparation (150-200) meant that testing modifications in an overall purification procedure took an

unacceptable length of time. It became evident that an approach to purification based on the Whiting and Granick (1976) procedure was impractical. Therefore, it was decided to make a completely new start.

When the work described in this thesis was near completion a report was published by Ades and Harpe (1981) in part attempting to duplicate the Whiting and Granick (1976) procedure. These authors were also unable to purify ALA-synthase to homogeneity, reaching only a specific activity of 3,300 units/mg protein. SDS polyacrylamide gel analysis of their protein showed a major protein of molecular weight around 50,000 as well as several contaminating proteins. They also came to the conclusion that ALA-synthase was subject to degradation during purification.

C H A P T E R 4

PRELIMINARY STEPS IN THE PURIFICATION OF CHICK EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE

INTRODUCTION

In the previous chapter, it was described that using the method of Whiting and Granick (1976), it was not possible to obtain pure ALA-synthase. It was evident that a new procedure would be required to purify the enzyme.

As a first step in a purification protocol, it was decided that a more rigorous approach to the preparation of a mitochondrial extract would be of benefit before attempting further chromatographic procedures. This would have the advantage of reducing the number of contaminating proteins to be separated by the later purification steps. A number of possible approaches were investigated. Firstly, the collection of mitochondria by differential centrifugation was modified to reduce the amount of rapidly sedimenting endoplasmic reticulum. Secondly, the conversion of mitochondria to mitoplasts (mitochondria devoid of their outer membrane) was undertaken. In addition to removing contaminating proteins, a second benefit was envisaged to occur from the preparation of mitoplasts, this was the removal of lysosomes. Lysosomes are known to be disrupted at concentrations of digitonin lower than those required for preparing mitoplasts (Loewenstein *et al* (1970)). This will result in the removal of one potential source of proteolytic activity, lysomal proteases.

The next step investigated in this chapter is the release of ALA-synthase from mitoplasts and its subsequent disaggregation. A number of procedures exist to disrupt the inner mitochondrial membrane and allow extraction of the contents. These include sonication, the use of various detergents, conditions of high ionic strength, freeze-thawing and freeze-drying. These techniques vary in the severity of the membrane disruption achieved. Since ALA-synthase is loosely associated with the inner mitochondrial membrane (McKay *et al* (1969), Zuyderhout *et al* (1969)), it was decided that a disruption technique which would minimise release of membrane proteins would be the most desirable. It was considered, therefore, that freeze-thawing and freeze-drying would be the most suitable methods to investigate for disruption of mitoplasts.

All previous attempts to purify mitochondrial ALA-synthase from a variety of animal sources have encountered the problem of enzyme aggregation. ALA-synthase appears to occur as large aggregates following release from mitochondria. Earlier work by Whiting (1973) in this laboratory established that treatment of crude rat liver mitochondrial ALA-synthase with 0.8M NaCl and 1mM DTE resulted in "solubilisation" of the enzyme. This treatment was also applied to the chick embryo enzyme by Whiting and Granick (1976)

It was uncertain, however, whether this procedure had completely disaggregated the chick mitochondrial ALA-synthase or if further disaggregation could be achieved by alternative procedures. An investigation into the disaggregation of the enzyme following its release from mitoplasts was undertaken.

This chapter, therefore, describes the isolation of mitochondria and their conversion into mitoplasts and following this the release and disaggregation of ALA-synthase from the mitoplasts.

RESULTS

PREPARATION OF MITOCHONDRIA

Livers were dissected from 17 or 18 day old drug-treated chick embryos (as described in Chapter 3) and placed in buffered sucrose (0.1mM pyridoxal phosphate, 0.25M sucrose, 5mM Tris-HCl pH 7.6 - PST buffer). The livers were rinsed, blotted and weighed and a 20% homogenate prepared by five passes of a motor driven teflon pestle (500 rpm) in a Potter-Elvejhem glass homogeniser. Mitochondria were prepared by differential centrifugation as follows. The homogenate was centrifuged at 500 x g for 5 minutes and the supernatant carefully decanted. The pellet was resuspended in half the original volume of PST buffer and briefly homogenised to break up the pellet before recentrifugation at 500 x g for

5 minutes once more to remove any contaminating nuclei. The pellet resulting from the centrifugation was resuspended in PST buffer and assayed for ALA-synthase activity; none was detected. The mitochondria were pelleted from this supernatant by centrifugation at 4,300 x g for 5 minutes then immediately at 12,000 x g for 5 minutes to pack the mitochondrial pellet more tightly. This procedure results in a visibly more turbid post-mitochondrial supernatant by comparison with centrifugation at 12,000 x g for 10 minutes alone. ALA-synthase assays of the post-mitochondrial supernatant showed no significant ALA-synthase activity. The specific activity at this point was 90 - 110 units/mg protein.

PREPARATION OF MITOPLASTS

The preparation of mitoplasts from mitochondria was accomplished according to the method of Schnaitman and Greenawalt (1968). The procedure was optimised for use with chick embryo liver mitochondria. The protein : detergent ratio was determined by monitoring release of ALA-synthase as a marker for mitoplast integrity. The ratio finally selected (6.6mg protein : 1mg digitonin) was chosen as lower protein : detergent ratios resulted in loss of ALA-synthase from the mitoplasts under the incubation conditions used. The procedure, therefore, continued as follows. The fluffy layer was decanted from the

mitochondrial pellet and the mitochondria were resuspended to a protein concentration of 80 mg/ml in PST buffer. Mitoplasts were formed by incubation of the resuspended mitochondria, at 0°C for 15 minutes with an equal volume of 1.2% digitonin in a gyrotory shaking water bath at 150 cycles per minute. The detergent action was terminated by diluting the mixture four fold with PST buffer. Mitoplasts were collected by centrifugation at 18,000 x g for 15 minutes. The fluffy layer was discarded and the mitoplasts resuspended in the same volume of PST buffer leaving behind a small dark brown pellet presumed to be peroxisomes (A. Snoswell, personal communication). The mitoplasts were washed to remove any remaining digitonin and collected by centrifugation at 18,000 x g for 15 minutes. When the mitoplast pellet and supernatant were assayed for ALA-synthase less than 5% of the total ALA-synthase was present in the supernatant.

EXTRACTION OF ALA-SYNTHASE ACTIVITY FROM MITOPLASTS

Freeze-thawing and freeze-drying were investigated for their ability to release ALA-synthase and minimise release of other mitochondrial proteins. The effects of freeze-thawing once, freeze-thawing twice and of freeze-drying were examined. The pelleted mitoplasts were resuspended in 5mM Tris-HCl pH 7.6,

TABLE 4.1

EXTRACTION OF ALA-SYNTHASE FROM MITOPLASTS

Mitoplasts were prepared from chick embryo liver mitochondria as described in the text. Samples were either freeze-thawed once or twice, or freeze-dried and resuspended to the original volume with water. The suspensions were then centrifuged at 105,000 x g for 60 minutes and the supernatant assayed for ALA-synthase activity and protein content.

TABLE 4.1

COMPARISON OF MITOPLAST EXTRACTION

PROCEDURES

METHOD	% ALA-SYNTHASE IN SUPERNATNANT	SPECIFIC ACTIVITY
Freeze thawing once	25-30	600
Freeze thawing twice	45-50	680
Freeze drying	85-90	550

1mM DTE, 0.1mM pyridoxal phosphate at 2 ml/g original liver weight for treatment. Following freeze-thawing the mitoplast extracts were centrifuged at 105,000 x g for 60 minutes and the supernatants and pellets were assayed for ALA-synthase activity. The freeze-dried mitoplasts were resuspended to the original volume in distilled water prior to being centrifuged and assayed as for the freeze-thawed extracts. The results are shown in Table 4.1. Although freeze-thawing twice released ALA-synthase of a higher specific activity than the other treatments only 45-50% of the total enzyme activity was released. Freeze-drying released 85% of the enzyme. The specific activity of this enzyme fraction was twice that (160 U/mg) obtained by Whiting and Granick (1976) from a sonicated mitochondrial extract. Freeze-drying was selected as the most suitable method of extraction of ALA-synthase from the mitoplasts. A second advantage of freeze-drying was that the volume of the sample after resuspension could be kept small without adversely affecting recovery of the enzyme. Subsequently, when purifying ALA-synthase from 25-30g liver, this resuspension volume of the freeze-dried mitoplasts was kept to a maximum of 7 mls to facilitate direct loading of the extract on a Sephacryl S200 column.

DISAGGREGATION OF ALA-SYNTHASE

Treatment of mitochondrial extracts of chick

embryo ALA-synthase with 0.8M NaCl and 1mM DTE had been reported by Whiting and Granick (1976) to solubilise the enzyme as judged by retardation of the treated enzyme when determined by gel exclusion chromatography. These workers obtained molecular weight estimate for ALA-synthase of 87,000 which was by far the lowest reported for the native chick enzyme but it was not clear if disaggregation of the enzyme had been completely achieved.

Therefore, an investigation was undertaken to determine whether the disaggregation could be increased beyond this. The ability of a number of compounds to disaggregate ALA-synthase was assessed by comparing the chromatographic profile of ALA-synthase on Sephacryl S-200 equilibrated in buffer containing the compound under test with that profile obtained after treatment with 0.8M NaCl and 1mM DTE. A retardation beyond that reached by 0.8M NaCl and 1mM DTE would be taken to indicate an enhanced disaggregating effect.

A number of compounds were tested, all in conjunction with 1mM DTE, as listed in Table 4.2. Non-ionic detergents (Triton X-100, Nonidet P40, Tween 20) did not produce any disaggregating effect even at high concentrations. Of the ionic detergents tested all except cholic acid inhibited enzyme activity, but this compound proved to be ineffective in causing disaggregation.

TABLE 4.2

COMPOUNDS TESTED FOR THEIR ABILITY TO DISAGGREGATE CHICK EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE.

Compounds were first tested for their effect on ALA-synthase activity. Those not inhibiting activity were tested for their ability to disaggregate ALA-synthase by comparison of the elution profile on Sephacryl S-200 with that obtained after chromatography in 0.8M NaCl 1mM DTE in Sephacryl S-200.

TABLE 4.2

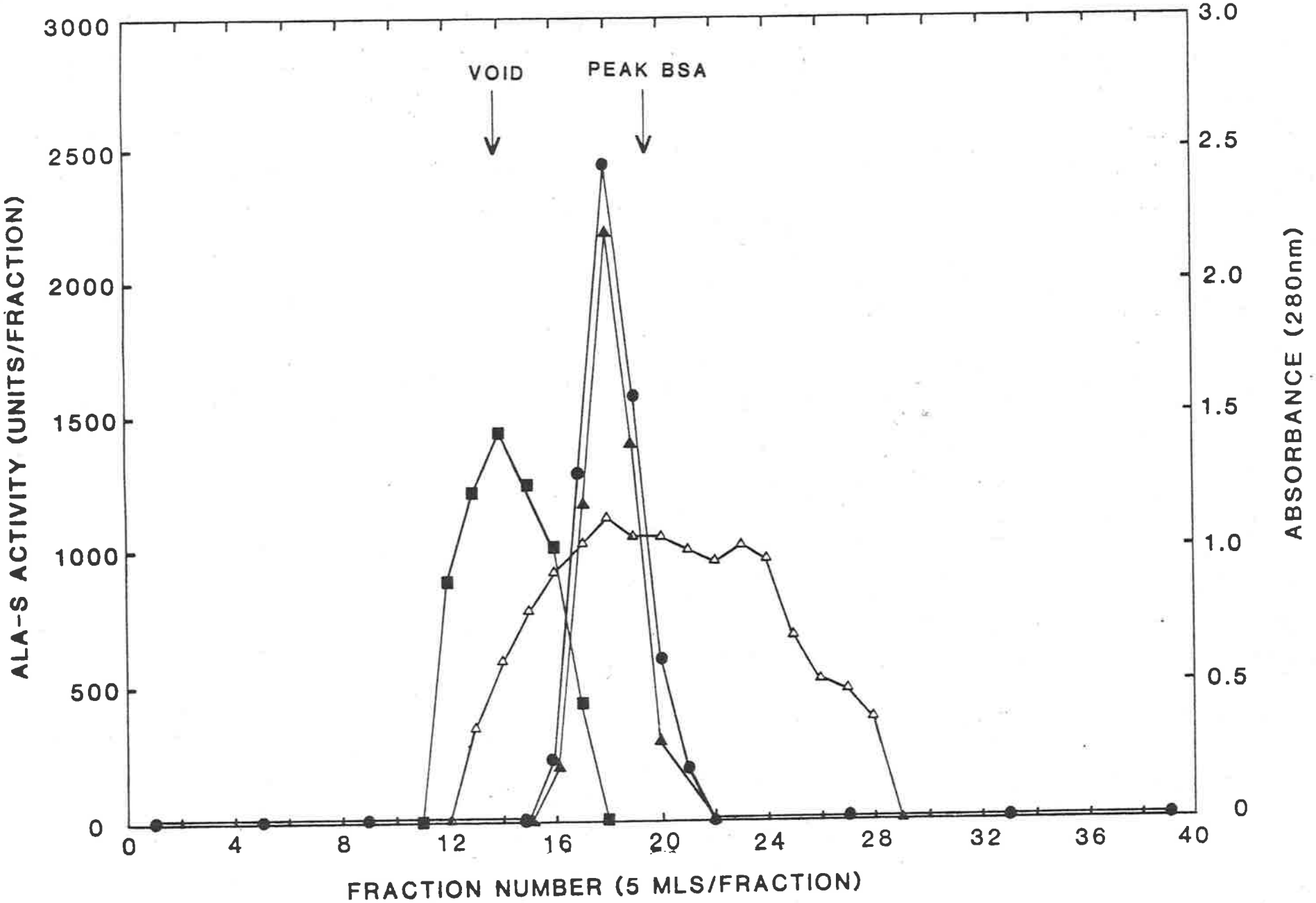
COMPOUNDS TESTED FOR ABILITY TO DISAGGREGATE ALA-SYNTHASE

COMPOUND		EFFECT ON ALA-SYNTHASE ACTIVITY	DISAGGREGATION EFFECT
Triton X-100	1% & 5%	nil	nil
Tween 20	1%	nil	nil
Nonidet P40	1%	nil	nil
Brij 58	1%	nil	nil
Octyl pyranoside	2%	nil	-
Cholic acid	1%	nil	nil
Deoxycholic acid	0.25%	60% inhibition	-
SDS	0.1% & 0.4%	100% inhibition	-
Cetyl trimethylammonium bromide	0.25%	65% inhibition	-
Guanidine HCl	0.25 mM-10 mM	70% inhibition	-
Urea	0.5M	100% inhibition	-
Na^{+}	0.8M	50% stimulation	standard
Mg^{++}	5mM	nil	nil
Mg^{++}	50mM	50% stimulation	= Na^{+}
Mn^{++}	5mM & 50 mM	nil	nil
Ca^{++}	50mM	10% stimulation	-
Fe^{++}	0.5mM	>90% inhibition	-
Fe^{+++}	0.25mM	>90% inhibition	-
Spermidine	50mM	nil	nil
Glycine	0.5mM	nil	nil

FIGURE 4.1

SEPHACRYL S-200 CHROMATOGRAPHY OF CHICK EMBRYO LIVER ALA-SYNTHASE

ALA-synthase was disaggregated with either NaCl/dithioerythritol (▲ - ▲) or $MgSO_4$/dithioerythritol (● - ●). Details are given in the text. The enzyme was chromatographed on a Sephacryl S-200 column (76 x 1.6cm) equilibrated in 50 mM Tris-HCl, pH 7.6, 0.1 mM pyridoxal phosphate, 1 mM DTE and either 0.8 M NaCl or 50 mM $MgSO_4$. The protein elution profile for the $MgSO_4$ disaggregated enzyme is shown by (Δ - Δ). The elution profile of aggregated ALA-synthase chromatographed in low ionic strength buffer (■ - ■) containing 20 mM Tris-HCl, pH 7.6, 0.1 mM pyridoxal phosphate is shown for comparison. The arrow indicates position of void volume.


VOID
PEAK BSA
ALA-S ACTIVITY (UNITS/FRACTION)
ABSORBANCE (280nm)
FRACTION NUMBER (5 MLS/FRACTION)
3000
2500
2000
1500
1000
500
0
3.0
2.5
2.0
1.5
1.0
0.5
0
0
4
8
12
16
20
24
28
32
36
40

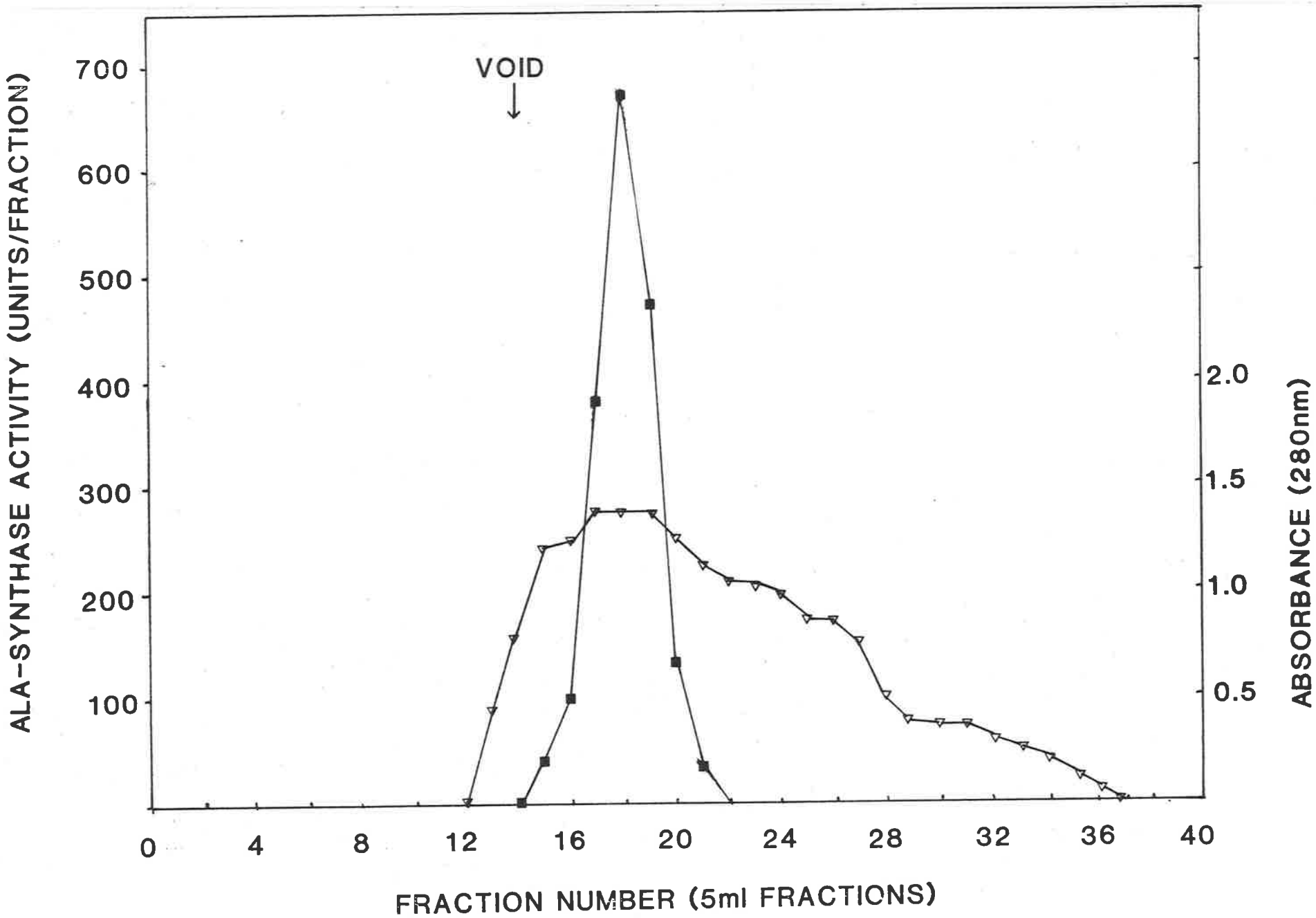
Urea and guanidine HCl also inhibited enzyme activity and were unsuitable for use. Several divalent cations were tested as alternatives to Na^+, two of which Mn^{++} and Fe^{++}, inhibited enzyme activity. However, $MgCl_2$ or $MgSO_4$ at a concentration of 50mM proved to be equally effective as 0.8M NaCl (Figure 4.1); apparently the anion is not important. Interestingly, these two cations (Mg^{++} and Na^+) both stimulate ALA-synthase activity 50% at the concentration used for disaggregation (Whiting and Granick (1976)). Other compounds such as the polycation spermidine and the substrate glycine at 0.5M did not have any disaggregating effect. Further investigations using combinations of the effective cations (Mg^{++} or Na^+) with a non-ionic detergent or cholic acid failed to produce any further improvement in disaggregation. At this point in the investigation it seemed unlikely that further disaggregation of the enzyme could be achieved. For reasons which will be discussed later 50mM $MgSO_4$ and 1mM DTE were selected as the means to obtain disaggregation of ALA-synthase.

Most importantly, it was also found that once the enzyme had been disaggregated by 50mM $MgSO_4$ and 1mM DTE and chromatographed through Sephacryl S-200 the Mg^{++} concentration could be lowered without reaggregation occuring as determined by subsequent chromatographic profiles on Sephacryl S-200 equilibrated in 5mM Mg^{++}

FIGURE 4.2

ALA-SYNTHASE REMAINS DISAGGREGATED AFTER TREATMENT WITH HIGH IONIC STRENGTH BUFFER

ALA-synthase was chromatographed on Sephacryl S-200 in 50mM $MgSO_4$ as described in the text. The pooled active fractions were concentrated and adjusted to 5mM $MgSO_4$ in an Amicon ultrafiltration cell and rechromatographed on a Sephacryl S-200 (76 x 1.6cm) column equilibrated in 5mM $MgSO_4$, 50mM Tris-HCl, pH 7.6, o.1mM pyridoxal phosphate and 1mM DTE. The elution profile for ALA-synthase rechromatographed in 5mM $MgSO_4$ is shown (▲-▲) and that of ALA-synthase chromatographed in 50mM $MgSO_4$ (■-■).


VOID
700
600
500
400
300
200
100
ALA-SYNTHASE ACTIVITY (UNITS/FRACTION)
2.0
1.5
1.0
0.5
ABSORBANCE (280nm)
0
4
8
12
16
20
24
28
32
36
40
FRACTION NUMBER (5ml FRACTIONS)

(Figure 4.2). This effect is similar to that reported by Whiting and Granick (1976) following treatment of ALA-synthase with 0.8M NaCl and 1mM DTE. The procedure adopted as a result of these investigations, (and subsequently incorporated into the final purification scheme) was to resuspend the freeze-dried mitoplast extract in 7 mls distilled water and centrifuge at 105,000 x g for 60 minutes. The supernatant was collected and adjusted to 50mM Tris-HCl pH 7.6, 50mM $MgSO_4$ and 1mM DTE before being chromatographed on a Sephacryl S-200 column (76 x 1.6cm) equilibrated in 50mM Tris-HCl pH 7.6, 50mM $MgSO_4$, 0.1mM pyridoxal phosphate and 1mM DTE. The specific activity of the ALA-synthase following the procedure described in this chapter was 1200 units/mg which was almost twice that of the Whiting and Granick (1976) procedure at the comparable stage.

INCORPORATION OF PROTEASE INHIBITORS INTO THE PURIFICATION

As a further precaution against proteolytic degradation during the ALA-synthase preparation protease inhibitors were added to the PST buffers used in preparation of mitochondria and again added to the TPD buffer used to resuspend the mitoplast fraction prior to freeze-drying. A mixture of four protease inhibitors were selected; these were phenylmethylsulphonylfluoride at 1mM final concentration, o-phenanthroline 1mM,

TABLE 4.3

PROTEASE INHIBITORS USED IN THE PURIFICATION OF ALA-SYNTHASE

TABLE 4.3

PROTEASE INHIBITORS USED IN PURIFICATION

INHIBITOR	SOLVENT	CONCENTRATION IN BUFFER	KNOWN SPECIFICITY
Phenylmethylsulphonylfluoride	95% Ethanol	1mM	Serine proteases, lysosomal proteases, cathepsins B and L
O-Phenanthroline	95% Ethanol	1mM	Metalloendopeptidases
Chymostatin	Dimethylsulphoxide	10µg/ml	Chymotrypsin, papain cathepsin B
Leupeptin	95% Ethanol	10µg/ml	Plasmin, trypsin, papain, cathepsin B

chymostatin 10µg/ml and leupeptin 10µg/ml. The inhibitors were dispensed into the buffer solutions from concentrated stocks. The proteolytic enzymes these inhibitors are known to inhibit are presented in Table 4.3. It can be seen that a broad spectrum of inhibition was catered for and in this way it was hoped that any proteolytic activity associated with the enzyme purification would be inhibited. The effect of using protease inhibitors in the purification of ALA-synthase is more appropriately dealt with in a later chapter.

DISCUSSION

As a result of the investigations described in this chapter a disaggregated fraction of ALA-synthase of high specific activity was obtained. The specific activity reached of 1200 units/mg is the highest obtained for chick embryo ALA-synthase without using chromatographic procedures. In addition this part of the purification could be achieved in less than two days, a significant improvement over the (Whiting and Granick (1976)) method, (a factor which as described later is important). The increase in specific activity was obtained through improvements in several areas of the preparation. Firstly, manipulation of the differential centrifugation speeds during isolation of the mitochondria prevented co-sedimentation of membranous material as evidenced by a visibly more

turbid post mitochondrial supernatant. Secondly, digitonin treatment stripped away most of the outer mitochondrial membrane as shown by electron microscopy. In addition it was expected that lysosomes would have been removed by this treatment as demonstrated by other investigators (Loewenstein *et al* (1970)), although no direct measurement of this was undertaken. Since lysosomes are a rich source of proteolytic enzymes a decrease in these organelles would be beneficial. As further protection against proteolytic breakdown the use of protease inhibitors was incorporated into the purification scheme. The use of inhibitors was important in obtaining homogeneous intact ALA-synthase as breakdown of ALA-synthase does occur during purification in their absence. However, as mentioned earlier their role is better understood once the full purification scheme is described. The usefulness of the inhibitors will be discussed more fully in Chapter 6 when the susceptibility of ALA-synthase to protease attack is described.

Freeze-drying proved to be an effective method for extracting ALA-synthase from mitoplasts and it produced a higher specific activity of ALA-synthase than the sonication procedure described by Whiting and Granick (1976).

The inability to further disaggregate ALA-synthase beyond that attained by Whiting and Granick (1976) using a number of compounds does not indicate that the enzyme

is truly solubilised by $MgSO_4$ or NaCl in the presence of DTE. However, this disaggregation was sufficient to permit further successful purification of ALA-synthase which will be described in the next chapter.

It is not known whether the physical nature of the ALA-synthase aggregate is entirely protein or if it contains membrane material. Previous reports have speculated on this question with Kaplan (1971) supporting the idea of a group of proteins held together by hydrophobic interactions. On the other hand, Whiting (1973) supported the idea that a membrane fragment was involved largely based on the difficulties he encountered in disaggregating the enzyme. No information was directly sought on this question during the course of the work described here. However, as non-ionic detergents are known to be ineffective in breaking protein-protein interactions (Helenius *et al* (1979)) their failure to cause disaggregation of ALA-synthase might suggest that the nature of the aggregate is of the protein-protein type (but this is speculative).

The finding that the enzyme did not reaggregate after disaggregation in high ionic strength buffer was very important as many protein purification techniques cannot be performed under conditions of high ionic strength; this would have severely limited the options available for further purification.

C H A P T E R 5

PURIFICATION OF CHICK EMBRYO LIVER

MITOCHONDRIAL ALA-SYNTHASE

INTRODUCTION

As described in the previous chapter a procedure had been determined for obtaining apparently disaggregated ALA-synthase from drug induced chick embryo liver mitochondria. The work in this chapter describes the purification to homogeneity of ALA-synthase by two chromatographic procedures, chromatofocusing and CoA-agarose affinity chromatography.

Chromatofocusing, a relatively new technique combines the techniques of ion exchange chromatography and isoelectric focusing. The procedure takes advantage of the buffering action of a charged group on an ion exchange column. When a buffer of one pH is run through an ion exchange column previously equilibrated at a different pH, a linear gradient is formed on the column. As the eluting buffer titrates the ion exchange groups the pH approaches the isoelectric point of a bound protein and that protein is eluted as a sharp focused band.

Although chromatography based on the affinity of ALA-synthase for its co-factor pyridoxal phosphate had been used by Whiting and Granick (1976) with some success this had not been the case in attempts to duplicate their work. However, the opportunity existed to attempt affinity chromatography for either of the two substrates glycine or succinyl-CoA. Several workers (Scholnick *et al* (1972), Whiting (1973), Paterniti and

Beattie (1979)) using crude preparations of ALA-synthase have reported that the Km for glycine is of the order of 10mM. This value indicates that glycine has a low affinity for the enzyme and probably would not be a good affinity ligand. Succinyl-CoA, on the other hand was reported (by the same workers) as having a much smaller Km, around 1-10μM, and would be more suitable as an affinity ligand. Unfortunately, succinyl-CoA is very unstable and cannot itself be used as an affinity ligand. CoA linked through a thioester bond and a hexane spacer to agarose is an analogue of succinyl-CoA and was used for affinity chromatography. Bishop *et al* (1981) had reported the use of CoA agarose affinity chromatography in studies on guinea pig ALA-synthase although these workers did not purify the enzyme to homogeneity. Since a number of other mitochondrial enzymes might also be expected to bind to a CoA ligand (acyl-CoA decarboxylases and acyl-CoA dehydrogenases). CoA-agarose affinity chromatography was left until late in the purification. By this stage, it was felt that any such proteins might have been removed by previous purification steps.

R E S U L T S

CHROMATOFOCUSING

Preliminary experiments were undertaken to determine the operating pH range of the chromato-

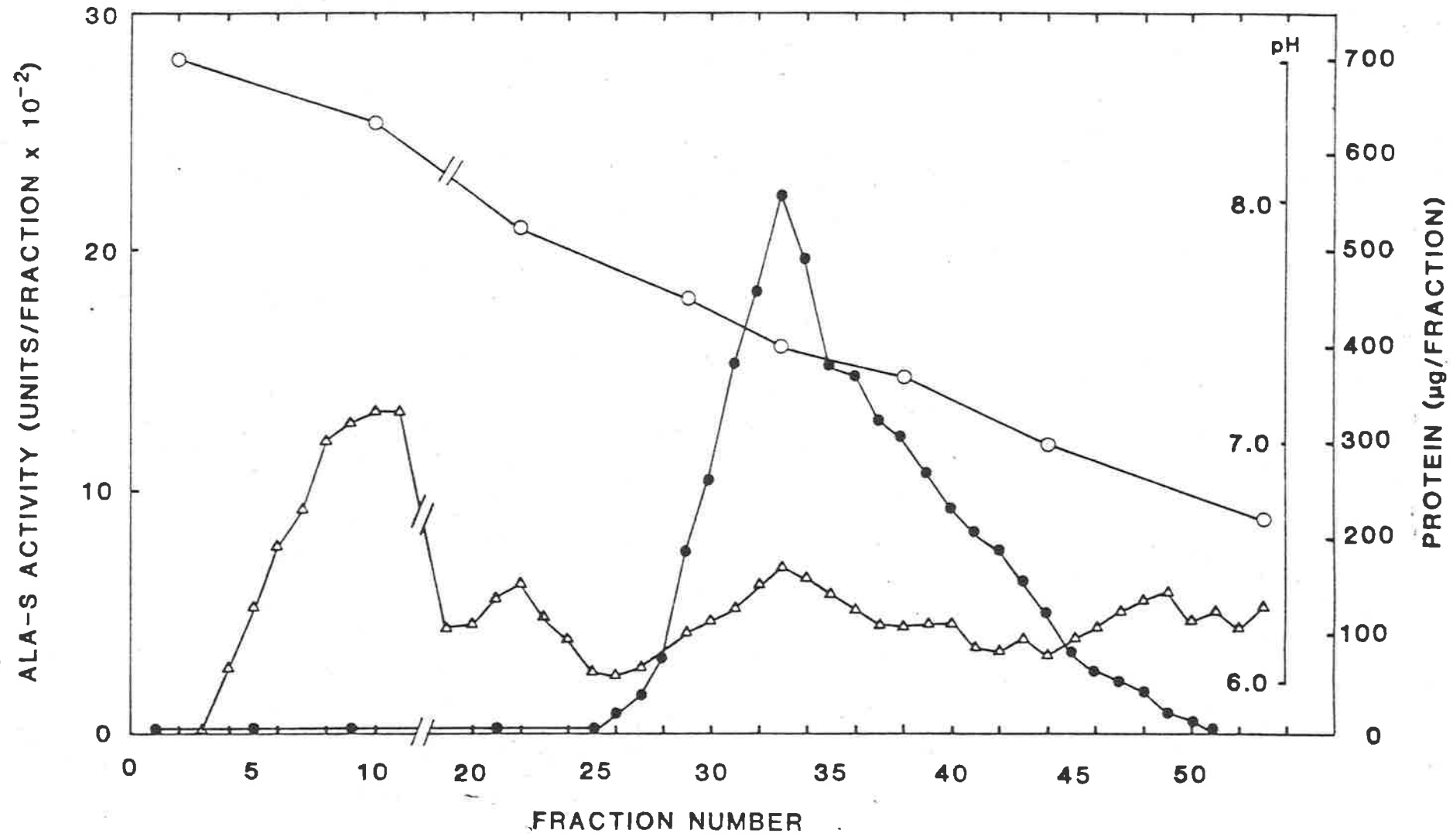
focusing column for ALA-synthase. Using Polybuffer Exchanger 94 it was found that ALA-synthase would bind when the Polybuffer Exchanger was equilibrated at pH 9 and that elution with polybuffer at pH 6 removed the enzyme. Under these conditions ALA-synthase eluted approximately halfway thgrough the gradient. It was also discovered that the amount of ALA-synthase loaded on the column had to be greater than 10,000 units of enzyme in order to obtain a reasonable recovery. With lesser amounts of enzyme (2,000-3,000 units) recovery of ALA-synthase decreased to only about 5%. With greater than 10,000 units of ALA-synthase loaded corresponding to 6mgs of protein, recoveries of 80% of ALA-synthase activity were obtained.

Once the elution pH of ALA-synthase was established the pH range of the chromatofocusing gradient was altered to the narrower range of pH 8.6 - pH 6.6 in order to improve purification. The conditions established for the use of the chromatofocusing column (25 x 0.8cm) containing Polybuffer Exchanger 94 was equilibrated in 25mM Tris-HCl pH 8.6 and 1mM DTE. The ALA-synthase active fractions from the Sephacryl S-200 were pooled and reduced in volume to 3mls in an Amicon ultrafiltration cell (model 52) fitted with a YMIO membrane. The concentrated extract was then diluted four fold with 5mM Tris-HCl pH 10. This had the dual effect of adjusting the pH to 8.6 and reducing the

FIGURE 5.1

CHROMATOFOCUSING OF CHICK EMBRYO LIVER ALA-SYNTHASE

ALA-synthase was chromatofocused as described in the text. Fractions (5ml) were collected and assayed for ALA-synthase activity (● - ●) as described in Materials and Methods and protein (Δ - Δ) by the method of Bradford (1976). The pH of each fraction, at 4°C was also measured (O - O).


ALA-S ACTIVITY (UNITS/FRACTION x 10^{-2})
PROTEIN (µg/FRACTION)
pH
FRACTION NUMBER
30
20
10
0
700
600
500
400
300
200
100
0
8.0
7.0
6.0
0
5
10
20
25
30
35
40
45
50

concentration of the $MgSO_4$ to 12.5mM. The extract was then loaded on the column and eluted with polybuffer pH6.6 at a flow rate of 12mls/hr. The ALA-synthase eluted after 15 hours, at approximate pH of 7.3. Figure 5.1 shows the result of chromato-focusing 12,000 units of ALA-synthase on the column as described. This single step results in a 16-fold purification with an increase in specific activity from 1200 units/mg to 17,500 units/mg. Approximately 80% of the ALA-synthase which was loaded on the column could be recovered and the most enzymatically active fractions, comprising 75% of the recovered activity was collected for further purification. The selected active fractions from the chromatofocusing column were pooled (45ml) adjusted to 50mM Tris-HCl, pH 7.6, 100mM glycine, 0.1mM pyridoxal phosphate and fresh protease inhibitors added as described in the previous chapter. The ionic strength of the buffer was increased after chromatofocusing in order to stabilise the enzyme as investigation had shown that enzyme activity was lost when the enzyme was kept in conditions of low ionic strength. The glycine was conveniently added at this point in preparation for the final step of the purification, CoA-agarose affinity chromatography.

AGAROSE CoA AFFINITY CHROMATOGRAPHY

At this point in the purification the ALA-synthase

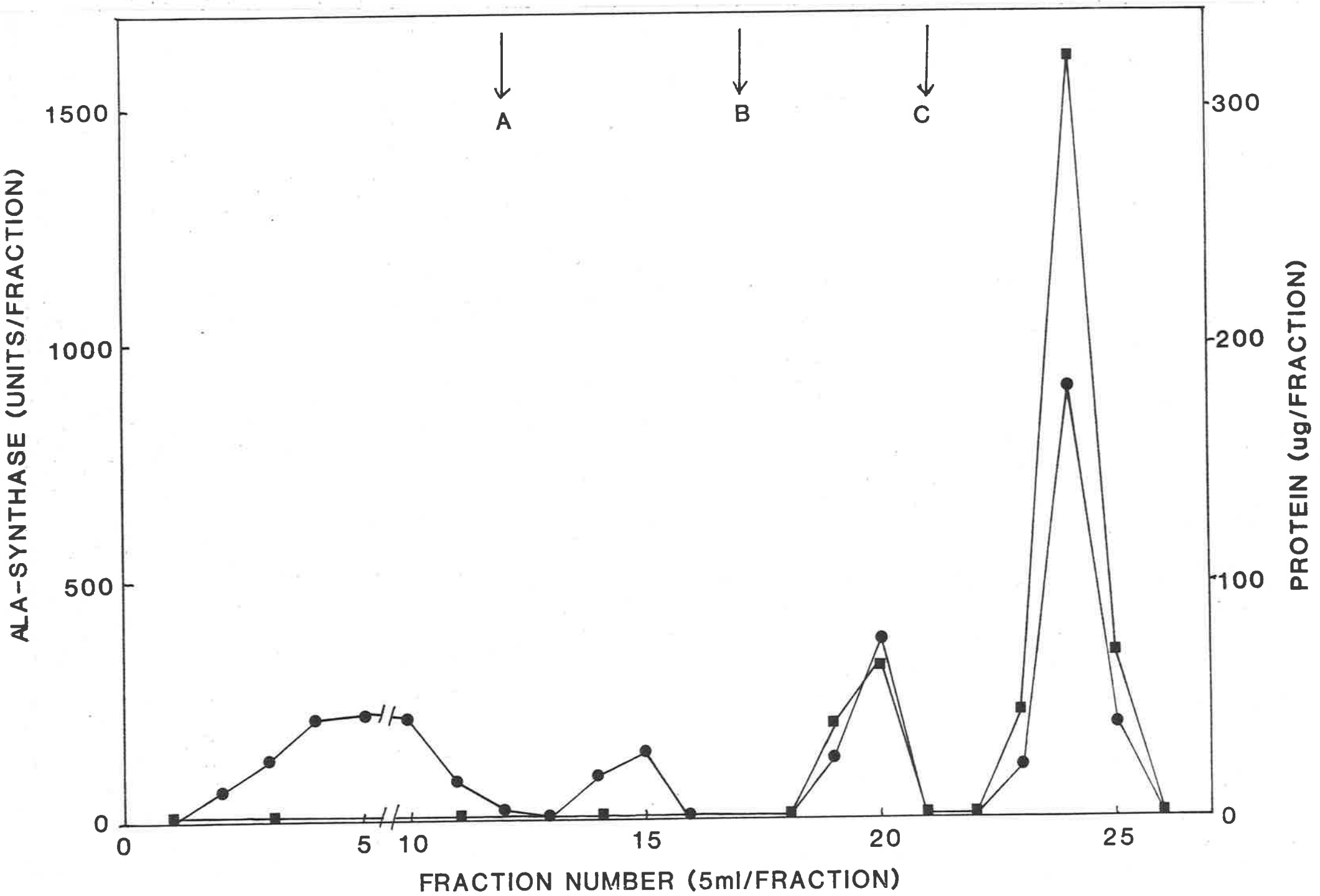
had been purified to almost the same level as previously reported by Whiting and Granick (1976) yet was still only 50% pure as judged by SDS polyacrylamide gel electrophoresis (see Figure 5.3, lane e). With the ALA-synthase being close to purity CoA-agarose affinity chromatography was introduced with the expectation of completing the purification.

The enzymically active fraction from the chromatofocusing step was loaded onto a CoA-agarose affinity column (0.8cm x 5cm) equilibrated in 50mM Tris-HCl pH 7.6, 100mM glycine, 0.1mM pyridoxal phosphate and 1mM DTE (TGPD buffer) and washed in with 3 column volumes of TGPD buffer. The ALA-synthase remained bound to the column. However, when glycine was omitted from the buffer the ALA-synthase was not retarded on the column but passed straight through. This result is similar to the report of Bishop *et al* (1981) for adult guinea pig liver ALA-synthase. In the presence of glycine, the ALA-synthase was eluted from the column as a sharp peak by the CoA analogue 5' AMP at a concentration of 50mM. Although a 50% increase in specific activity was obtained,analysis by SDS polyacrylamide gel electrophoresis showed the presence of a major protein band together with several minor contaminating proteins. The CoA-agarose affinity chromatography procedure was therefore modified to improve the purification. After the loading and washing of the enzymically active fractions, the

FIGURE 5.2

CoA-AGAROSE AFFINITY CHROMATOGRAPHY OF CHICK EMBRYO LIVER ALA-SYNTHASE

ALA-synthase was chromatographed on CoA-agarose as described in the text. The arrows indicate where each of the following buffers were used: A = 0.5 M glycine in buffer B; B = 20 mM 5' AMP in buffer B; C = 50 mM 5' AMP in buffer B, ALA-synthase activity was measured in each fraction (5 ml) (■-■). Protein content (●-●) was estimated by the method of Bradford, (1976).


A
B
C
1500
1000
500
0
0
5
10
15
20
25
300
200
100
0
ALA-SYNTHASE (UNITS/FRACTION)
PROTEIN (ug/FRACTION)
FRACTION NUMBER (5ml/FRACTION)

column was washed with 0.5M glycine. This step was routinely included in the procedure to remove non-specifically bound proteins. No ALA-synthase was eluted by the wash. The column was then eluted by a two step gradient of 5' AMP. The first step of the gradient was 20mM 5'AMP in TGPD buffer and the second 50mM 5'AMP in TGPD buffer. The first step resulted in the elution of 10-15% of the ALA-synthase activity. The remainder of the recoverable activity was eluted by the second step. The elution profile from this procedure is presented in Figure 5.2. The procedure resulted in a recovery of 50% of the applied ALA-synthase in the 50mM 5'AMP elution step. Further washing of the column with increased 5'AMP or 1M NaCl failed to release further ALA-synthase activity.

Analysis by SDS polyacrylamide gel electrophoresis of the ALA-synthase eluted by the 50mM 5'AMP showed only a single protein band. The specific activity of the purified enzyme was 35,000 units/mg. Figure 5.3 shows the analysis by SDS polyacrylamide gel electrophoresis at each stage of the purification. It is apparent that chromatofocusing is the most powerful step in the purification. Table 5.1 summarises the details of the purification starting with 22gm of liver from drug treated chick embryos. The yield of ALA-synthase upon completion of the purification is 20 - 25%.

FIGURE 5.3

ANALYSIS OF PROTEIN CONTENT AT EACH STAGE OF THE PURIFICATION OF CHICK EMBRYO LIVER ALA-SYNTHASE.

SDS polyacrylamide gel analysis was performed as described in Materials and Methods. Equivalent amounts of ALA-synthase activity were analysed from each stage of the purification. Lane a = mitochondria; lane b = mitoplasts; lane c = freeze-dried extract from mitoplasts; lane d = Sephacryl S-200; lane e = chromatofocusing; lane f = CoA-agarose; lane g shows the analysis of an enzyme active gel slice from a non-denaturing gel as described in the text. Values for molecular weight markers are shown.

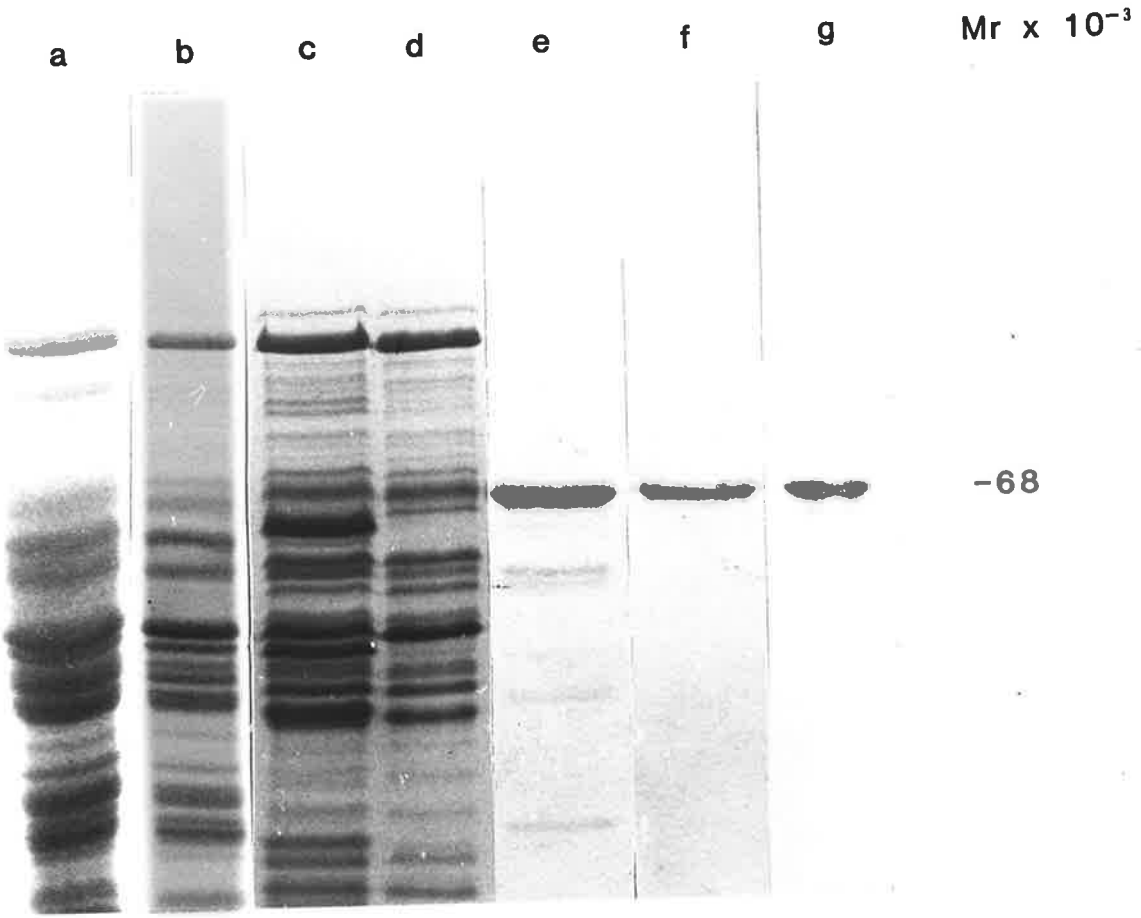

a
b
c
d
e
f
g
Mr x 10⁻³
-68

TABLE 5.1

PURIFICATION OF δ-ALA-SYNTHASE FROM CHICK EMBRYO LIVER MITOCHONDRIA

TABLE 5.1

PURIFICATION OF δ-ALA-SYNTHASE FROM CHICK EMBRYO LIVER MITOCHONDRIA

STEP AND FRACTION	VOLUME ml	TOTAL PROTEIN mg	TOTAL ACTIVITY units	SPECIFIC ACTIVITY units/mg/protein	YIELD %
Mitochondria (from 22 g of liver)	6.5	456.0	72,966	160	100
Mitoplasts	20.0	231.0	69,318	305	95
Freeze-dried supernatant	6.0	123.0	67,944	550	93
Sephacryl S-200 chromatography	16.0	31.0	59,480	1,920	88
Chromatofocusing	55.0	1.93	35,800	18,500	53
CoA-agarose affinity chromatography	10.6	0.43	14,870	35,000	22

POSITIVE IDENTIFICATION OF ALA-SYNTHASE

The ALA-synthase purified as described in this thesis migrated as a single band on an SDS polyacrylamide gel and had a molecular weight of 68,000. In order to eliminate any doubts that this protein is ALA-synthase, confirmation was sought directly associating the purified protein species with ALA-synthase activity. This can be achieved when the enzyme is isolated on a non-denaturing gel permitting assays for enzyme activity to be carried out. The following experiment was therefore carried out. Three thousand units of ALA-synthase obtained from the 50mM 5' AMP eluate fraction off the CoA-agarose column on a non-denaturing Tris-glycine polyacrylamide of Davis (1964) as described in Chapter 2. Some difficulty was encountered in preparing samples of pure ALA-synthase for electrophoresis on these gels because the buffer containing the enzyme had to be reduced in ionic strength to allow electrophoresis which resulted in loss of enzyme activity. This difficulty was overcome by exchanging the elution buffer from CoA-agarose (50mM 5' AMP in TGPD buffer) for 100mM triethylamine, pH 7.6 by dialysis followed by lyophilisation to remove the volatile buffer. By this procedure the ionic strength of the buffer was maintained until the sample was freeze-dried allowing sufficient ALA-synthase to remain active following

electrophoresis to allow detection. The lyophilised sample was dissolved in 100µl 20% glycerol and loaded on a 5% acrylamide slab gel. Electrophoresis was performed at pH 9 rather than pH 9.5 because experiments had shown that the enzyme was inactivated above pH 9. Upon completion of electrophoresis the gel track was excised from the gel and cut into 2mm slices. Alternate slices were assayed directly for ALA-synthase activity in a 200µl assay volume. Intermediate slices were equilibrated in 50mM Tris-HCl pH 6.8 for 30 minutes at room temperature (25°C) and then set in the well of an SDS polyacrylamide gel using molten 1% agarose prior to electrophoresis. Enzyme assays of the alternate slices showed that the ALA-synthase activity was located as a broad peak encompassing several gel slices approximately halfway down the gel track. When the intermediate slices were stained with Coomassie Blue after SDS polyacrylamide gel electrophoresis, they showed the presence of a major band of molecular weight 68,000., (Figure 5.3, lane g). The presence of trace amounts of a smaller molecular weight protein was presumed to be due to breakdown incurred during sample preparation or electrophoresis since the original sample migrated as a single band.

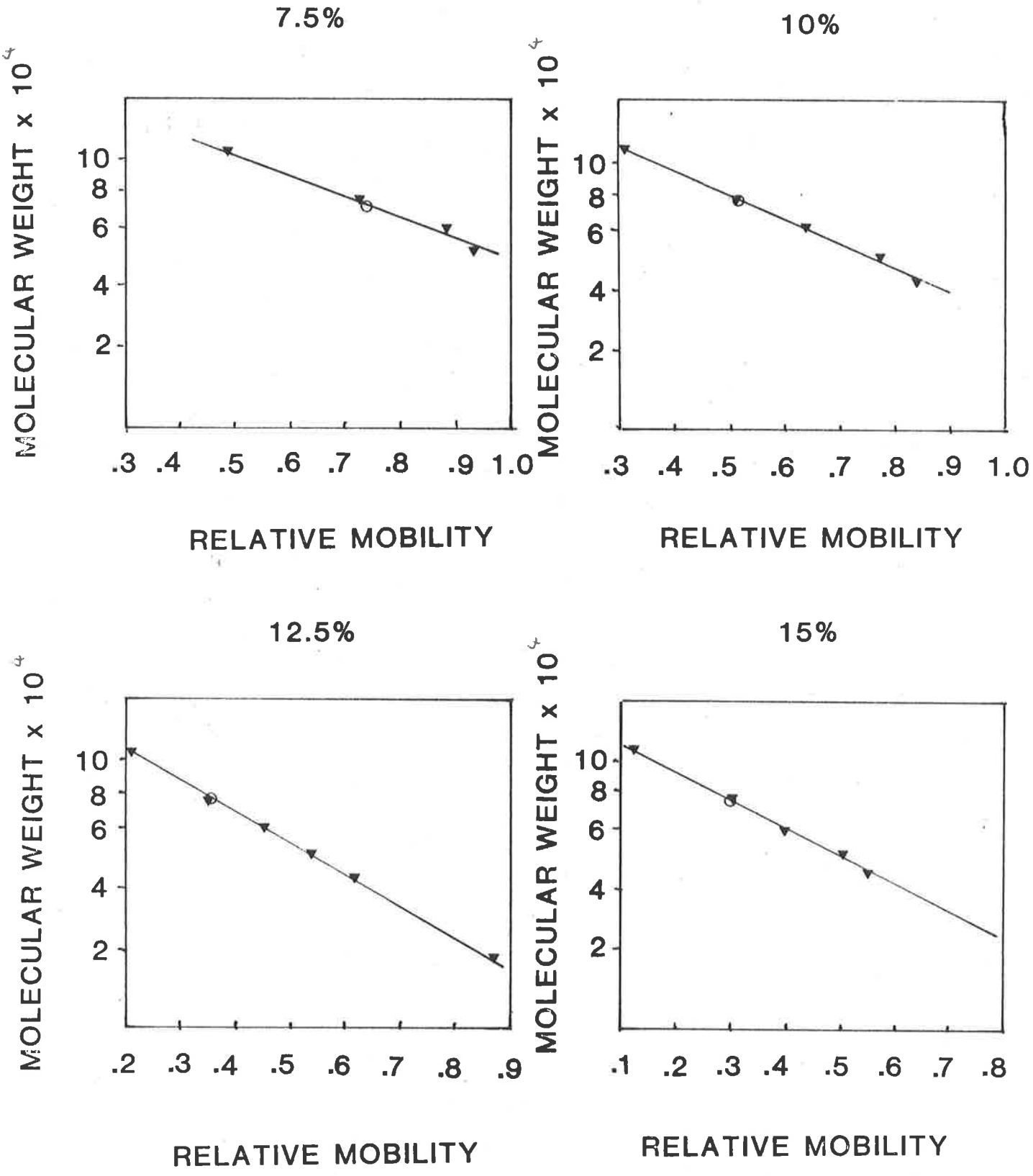
MOLECULAR WEIGHT ESTIMATE

The minimum molecular weight estimate of ALA-synthase was determined by subjecting purified

FIGURE 5.4

MINIMUM MOLECULAR WEIGHT ESTIMATE OF PURIFIED CHICK EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE

Pure ALA-synthase was electrophoresed in SDS polyacrylamide gels, at four concentrations of acrylamide, in the presence of proteins of known molecular weight. The molecular weight of ALA-synthase was estimated from a plot of molecular weight vs. the relative mobility of the marker proteins. The proteins of known molecular weight were, phosphorylase b, 94,000; bovine serum albumen, 68,000; glutamate dehydrogenase, 53,000; ovalbumen, 43,000; glucose 3 phosphate dehydrogenase, 36,5000; lactoglobulin, 18,400. The marker proteins are indicated by (▼) and the position of ALA-synthase by (O).


7.5%
10%
12.5%
15%
MOLECULAR WEIGHT x 10⁴
10
8
6
4
2
.3 .4 .5 .6 .7 .8 .9 1.0
.2 .3 .4 .5 .6 .7 .8 .9
.1 .2 .3 .4 .5 .6 .7 .8
RELATIVE MOBILITY

ALA-synthase to electrophoresis on SDS polyacrylamide gels at 7.5%, 10%, 12.5% and 15% acrylamide in the presence of protein markers of known molecular weight. The result is shown in Figure 5.4. At 15%, 12.5% and 10% acrylamide the apparent molecular weight was 68,000 and at 7.5% acrylamide it was apparently 66,000. The molecular weight value of 68,000 was therefore taken as the molecular weight of the purified enzyme.

AMINO ACID COMPOSITION AND N-TERMINAL SEQUENCE OF ALA-SYNTHASE

The amino acid composition of ALA-synthase was determined using a Beckman Model 120C amino acid analyser, kindly performed by Mr. M. Calder. Preparation of pure ALA-synthase for analysis was as follows. Pure ALA-synthase was precipitated with 10% trichloroacetic acid and washed in ethanol and ether. The ALA-synthase was hydrolysed for 16 hours in 0.1N HCl at 100°C. Results were obtained for all amino acids except tryptophan and cysteine/cystine. Glutamine/glutamic acid and asparagine/aspartic acid were not differentiated. The results are presented in Table 5.2. There is a high proportion of non-polar (34%) and neutral (29%) amino acids perhaps reflecting the hydrophobic nature of the location of functional ALA-synthase. In addition the molecule may be slightly acidic as there are a large number of glutamate and aspartate residues.

TABLE 5.2

AMINO ACID COMPOSITION OF CHICK EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE.

The amino acid composition was determined on a Beckman Amino-Acid Analyzer Model 120C. Tryptophan and cystine/cysteine were not determined.

TABLE 5.2

AMINO ACID COMPOSITION OF CHICK EMBRYO LIVER ALA-SYNTHASE

RESIDUE	MOLES %	NO. RESIDUES PER MOLECULE
ALANINE	4.48	44
ARGININE	2.71	26
ASPARTATE	8.45	83
GLUTAMATE	5.04	49
GLYCINE	5.15	50
HISTIDINE	2.17	21
ISOLEUCINE	1.85	18
LEUCINE	4.34	42
LYSINE	3.35	33
METHIONINE	0.59	6
PHENYLALANINE	2.01	20
PROLINE	3.46	34
SERINE	3.59	35
THREONINE	2.59	26
TYROSINE	1.27	13
VALINE	3.16	31
		531

The N-terminal amino acid sequence of ALA-synthase was determined. This analysis was performed by Professor P.G. Martin of the Botany Department of the University of Adelaide. The first ten amino acids were determined, these were glycine-serine-valine-glutamic acid-glutamic acid-threonine-proline-alanine-alanine-threonine.

DISCUSSION

The results in this chapter completed the description for the purification of ALA-synthase from chick embryo liver mitochondria. The purified enzyme has a minimum molecular weight of 68,000, some 19,000 daltons larger than the size reported by Whiting and Granick (1976). The enzyme is homogeneous as judged by SDS polyacrylamide gel analysis and has a specific activity of 35,000 units/mg protein. This is the highest specific activity recorded for ALA-synthase purified from chick embryos. The successful purification was made possible through the use of chromatofocusing and CoA-agarose affinity chromatography. The power of the chromatofocusing step is dramatically illustrated by comparing Figure 5.3, lanes d and e. This single step gives a 16-fold purification and is the backbone of the purification scheme.

The effect of glycine on the binding of ALA-synthase to the CoA-agarose affinity column can possibly be explained by consideration of the proposed reaction mechanism for ALA-synthase (Shemin (1972)).

In this scheme pyridoxal phosphate binds to the enzyme first, glycine then forms a Schiffs base with the enzyme pyridoxal phosphate then allowing succinyl CoA to bind. If this is the case then glycine would be required in order to allow CoA binding to occur. Future studies in this laboratory, using purified ALA-synthase may shed more light on the mechanism of enzyme action.

The procedure described here for purifying ALA-synthase has two significant advantages over the Whiting and Granick (1976) procedure. Most importantly, it gives an homogeneous enzyme preparation of high specific activity. Secondly, it is considerably more rapid than the Whiting and Granick (1976) method ($2\frac{1}{2}$ days compared with at least 10 days) and this aspect is of particular importance in preventing proteolytic breakdown of the ALA-synthase (see Chapter 6). Moreover, in practical terms the number of manipulative steps performed to achieve purification are significantly less (five compared to eight).

C H A P T E R 6

SUSCEPTIBILITY OF ALA-SYNTHASE TO PROTEOLYTIC DEGRADATION

INTRODUCTION

In the previous chapter the isolation of ALA-synthase of minimum molecular weight 68,000 (as measured by SDS polyacrylamide gel electrophoresis) was described. In view of the fact that Whiting and Granick (1976) reported that the molecular weight was 49,000 daltons, two questions immediatley arise. Firstly how was it possible that a smaller form of ALA-synthase was identified by Whiting and Granick (1976)?

Secondly, is the form of the enzyme purified as reported here the true native form of ALA-synthase? This chapter will describe experiments undertaken to provide answers to these two questions.

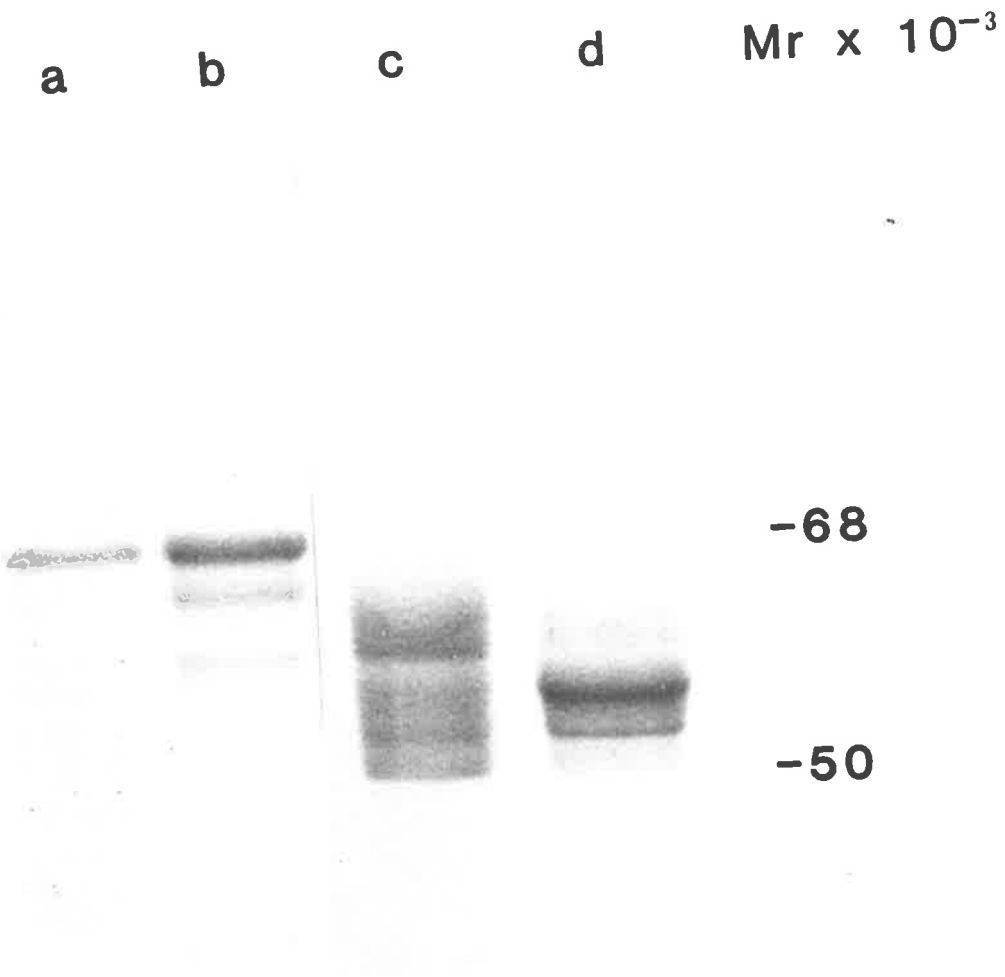
DEGRADATION OF ALA-SYNTHASE BY ENDOGENOUS PROTEOLYTIC ACTIVITY

As described briefly in Chapter 4 a combination of four protease inhibitors were routinely used throughout the purification of ALA-synthase. Once the purification procedure was established, a comparison was made between a purification carried out in the presence of these four protease inhibitors and one carried out in their absence. The final product of both purifications were analysed by SDS polyacrylamide gel electrophoresis. The results of these analyses are presented in Figure 6.1. The protein purified in the presence of protease inhibitors migrated as a single band, (Figure 6.1, lane a), but the preparation carried out in the absence

FIGURE 6.1

ANALYSIS OF CHICK EMBRYO LIVER ALA-SYNTHASE BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS AFTER MODIFICATIONS TO THE BASIC PROCEDURE.

Details of the modifications to the purification procedure are described in the text. Lane a = basic procedure; lane b = purified in the absence of protease inhibitors; lanes c and d = two separate purifications after a delay of 5 days for both at the freeze dried extract stage.


a
b
c
d
Mr x 10^{-3}
-68
-50

of inhibitors showed three protein bands (Figure 6.1, lane b). The major band was ALA-synthase of molecular weight 68,000 with the two minor bands having molecular weights of 64,000 and 58,000 respectively. These three proteins were subjected to the partial proteolytic mapping technique of Cleveland *et al* (1977). Very similar peptide maps were obtained indicating that these three proteins are related (data not shown). This result indicates that breakdown of ALA-synthase to smaller forms does occur during purification of the enzyme in the absence of protease inhibitors. Interestingly, there was no trace of a protein of molecular weight 49,000 in these preparations.

The purification procedure of Whiting and Granick (1976) took about 10 days compared to the $2\frac{1}{2}$ days required for the purification described in this thesis. It seemed likely that breakdown of ALA-synthase during purification would be more severe over this longer time span. To ascertain the effect of a prolonged purification procedure on the size of the purified enzyme a crude extract from freeze-dried mitoplasts was left at 4°C for 5 days in the absence of protease inhibitors before completing the purification. The purified product was then analysed by SDS polyacrylamide gel electrophoresis. The results of two such experiments are shown in Figure 6.1, lanes c and d. A broad smear of protein is seen in the molecular weight range of 65,000 to 49,000. Strikingly, there

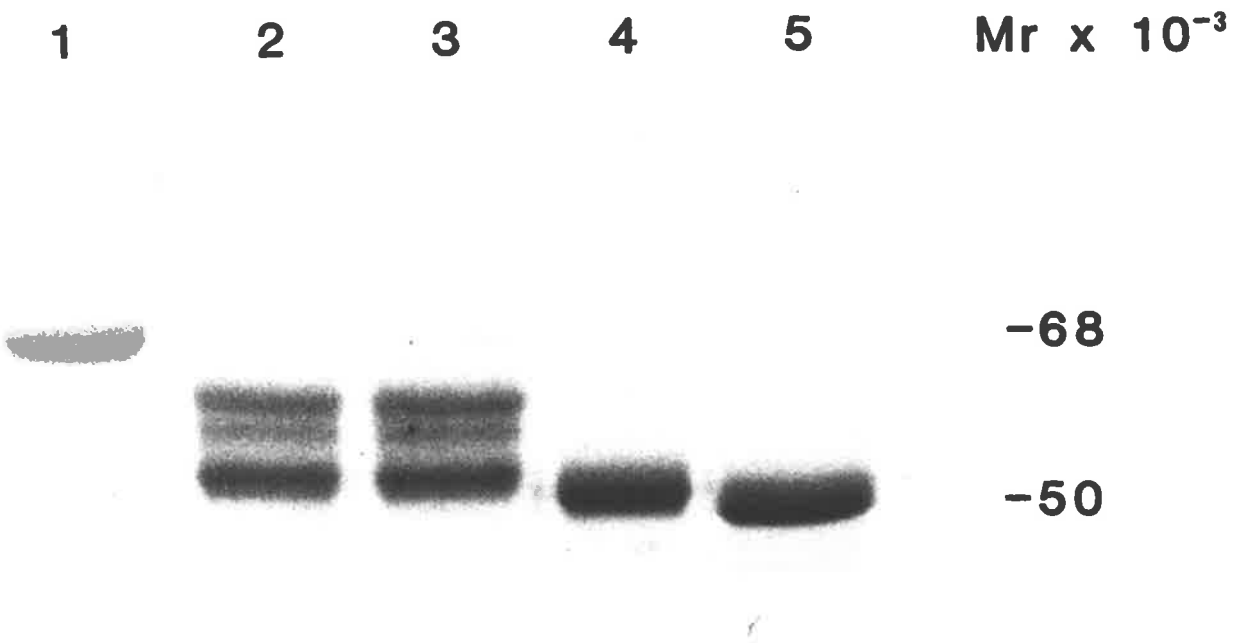
is a distinct cut off at 49,000 molecular weight in both preparations although there are differences in the intensities of the protein bands in the smeared area. When assayed for ALA-synthase activity these preparations proved they have the same levels of ALA-synthase activity as enzyme purified by the rapid procedure described in this thesis. This shows that the breakdown of ALA-synthase results in several components which apparently retain full catalytic activity. The results also suggest that the long time taken by Whiting and Granick (1976) for their purification allowed the enzyme to degrade to a molecular weight 49,000.

To further investigate the proteolytic sensitivity of ALA-synthase, the effect of adding an endogenous protease papain to the pure enzyme was investigated. Aliquots of pure ALA-synthase (30µg) were incubated with increasing amounts of papain for 10 minutes at 37°C as described in the legend to Figure 6.2. After the incubation period the papain digestion was terminated by the addition of a 20 times molar excess of antipain. Aliquots were assayed for ALA-synthase activity and the remainder was analysed by SDS polyacrylamide gel electrophoresis. Assays for ALA-synthase activity showed that all the test fractions retained full catalytic activity following papain digestion as compared with the control incubated in the absence of papain. Analysis by SDS polyacryl-

FIGURE 6.2

PAPAIN TREATMENT OF PURIFIED CHICK EMBRYO LIVER ALA-SYNTHASE

Aliquots of purified enzyme (40μg) were digested with varying amounts of papain at 37°C for 10 minutes. Antipain was then added in a 20 molar excess over papain. Samples were assayed for ALA-synthase activity. The remainder was analysed by SDS polyacrylamide gel electrophoresis as follows: lane 1 = untreated; lane 2 = 1 μg papain; lane 3 = 2 μg papain; lane 4 = 20 μg papain; lane 5 = 100 μg papain.


1
2
3
4
5
Mr x 10⁻³
-68
-50

amide gels (Figure 6.2) showed that the enzyme had been progressively degraded, by increasing amounts of papain, through several intermediate forms to a molecular weight of 49,000 daltons.

Taken together the results of the above experiments show that ALA-synthase is sensitive to protease digestion but only till a 49,000 molecular weight form is reached which is apparently stable and retains full catalytic activity.

IS THE PURIFIED ALA-SYNTHASE THE INTACT FORM?

In view of the sensitivity of ALA-synthase to proteolytic attack, it was important to establish whether or not the 68,000 molecular weight form of ALA-synthase was a breakdown product. An attempt was made to answer this question by *in vivo* pulse labelling a drug-treated chick embryo and indentifying ALA-synthase under conditions minimising proteolytic degradation. Two chick embryos, one drug-treated and one untreated were pulse labelled with 100μCi S^{35} methionine as described in Chapter 2. Following a 45 minute labelling period the embryo liver mitochondria were prepared as described for the purification procedure. The mitochondria were freeze-dried and the resulting supernatant from the resuspended extract used for immunoprecipitation by anti-ALA-synthase antiserum in the presence of protease inhibitors as used previously. Total and immunoprecipitated

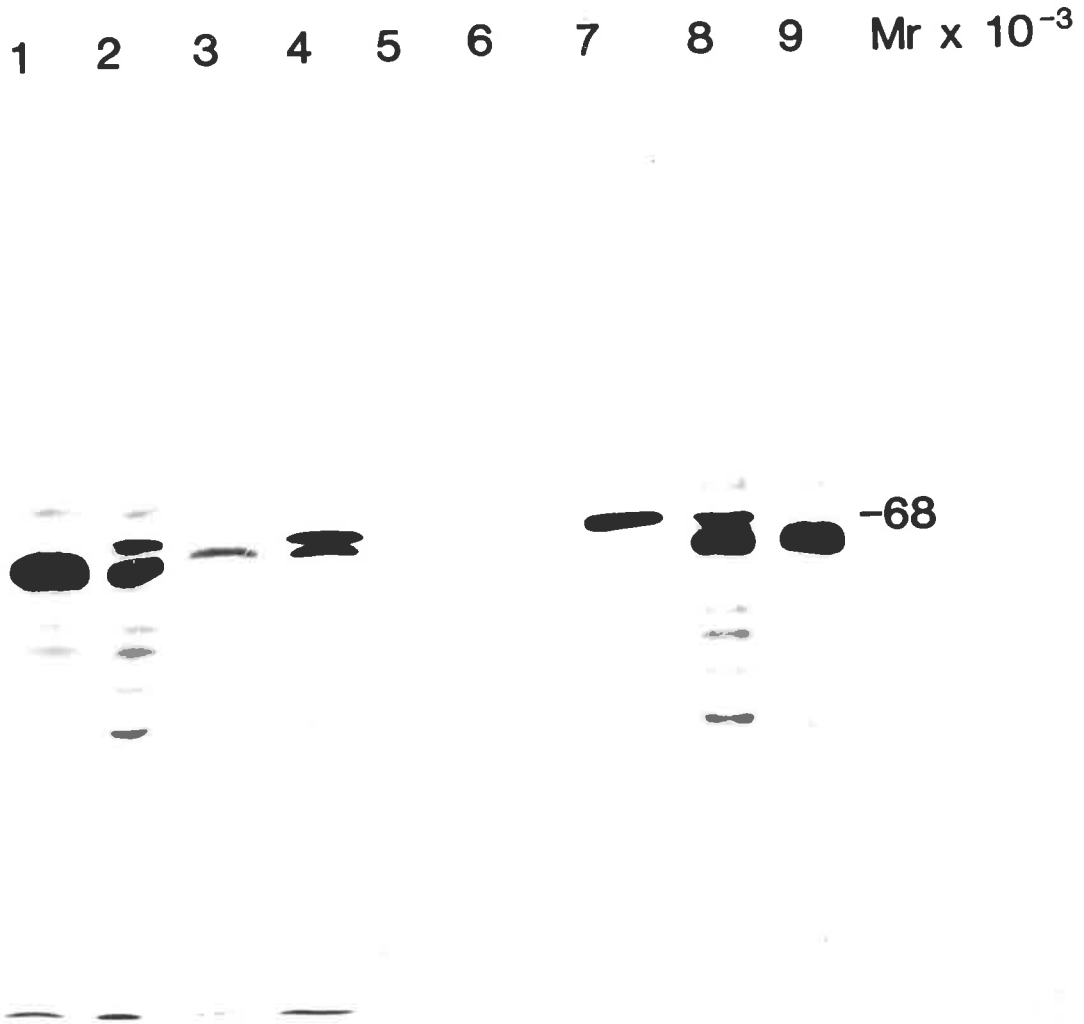
products were analysed by SDS polyacrylamide gel electrophoresis. The result is shown in Figure 6.3. A comparison of the labelled mitochondrial proteins from drug-treated and untreated chick embryos (Figure 6.3, lanes 1 and 2) shows the presence of an extra radioactively labelled protein band of molecular weight 68,000 in the proteins from the drug-treated liver mitochondrial extract. This protein was specifically immunoprecipitated by the anti-ALA-synthase antiserum (Figure 6.3, lane 7). No radioactively labelled immunoprecipitable protein was detected in the extract from the untreated chick embryo (Figure 6.3, lane 6).

These experiments were extended to examine the labelled mitochondrial proteins under conditions more stringent in preventing proteolytic attack. Freshly prepared radioactively labelled mitochondria were boiled in 3% SDS in the presence of protease inhibitors and loaded directly on an SDS polyacrylamide gel. The result was identical to that described for the freeze-dried mitochondrial extract (Figure 6.3, lanes 8 and 9), an induced protein of molecular weight 68,000 is present only in the mitochondria from drug-treated chick embryos. This result strongly suggests that the form of the enzyme purified by the procedure described in this thesis is the same native form of ALA-synthase.

FIGURE 6.3

RADIOISOTOPE LABELLING OF CHICK EMBRYO LIVERS AND IMMUNOCHEMICAL IDENTIFICATION OF ALA-SYNTHASE.

Drug treated and untreated chick embryos were labelled with ^{35}S methionine as described in Materials and Methods and mitochondrial or mitoplast extracts prepared as described in the text. Samples of the extract were subjected to immunoprecipitation and analysis of total products and immunoprecipitated products by SDS polyacrylamide gel electrophoresis was performed as described in Materials and Methods. Lane 1 = untreated mitochondria; lane 2 = drug treated mitochondria; lane 3 = untreated mitoplasts; lane 4 = drug treated mitoplasts; lane 5 = non-immune serum with drug treated mitochondria extract; lane 6 = immune serum with untreated mitochondria extract; lane 7 = immune serum with drug treated mitochondria extract; lane 8 = drug treated mitochondrial extract prepared under conditions minimising proteolysis; lane 9 = untreated mitochondrial extract prepared under conditions minimising proteolysis.


1 2 3 4 5 6 7 8 9 Mr x 10^{-3}
-68

THE BREAKDOWN OF ALA-SYNTHASE FOLLOWING SONICATION OF MITOCHONDRIA.

Although an investigation determining the origin of the degradative enzymes responsible for breaking down ALA-synthase was not within the scope of this thesis an interesting result was obtained when freeze-drying and sonication were compared for their ability to release ALA-synthase for use in immunoprecipitation of ALA-synthase in *in vivo* pulse labelling experiments. The experiment was carried out as follows. Two drug-treated chick embryos were pulse labelled with ^{35}S-methionine and mitochondrial fractions prepared as described earlier in this chapter. The mitochondria from one liver were disrupted by freeze-drying and the other disrupted by sonication, (using a Branson Soniprobe at full power for 4 x 20 second bursts, on ice). The freeze-dried mitochondria were resuspended in water and both extracts were centrifuged at 105,000 x g for 60 minutes before being subjected to immunoprecipitation with anti-ALA-synthase as described in Chapter 2. The immunoprecipitates were then analysed by SDS-polyacrylamide gel electrophoresis, (Figure 6.4). The immunoprecipitate from the freeze-dried mitochondria shows a single radioactively labelled band of molecular weight 68,000. In contrast, the extract from the sonicated mitochondria showed no protein of 68,000 but instead had a band of molecular weight 49,000. This result makes it very likely that sonication of a crude mitochondrial extract, as used by Whiting and Granick (1976) in their

FIGURE 6.4

COMPARISON OF FREEZE-DRYING vs. SONICATION FOR DISRUPTING MITOCHONDRIA.

Two chick-embryos were pulse labelled with ^{35}S-methionine and mitochondria prepared as described in the text. Labelled proteins were released from the mitochondria of one embryo by freeze-drying and from the other by sonication. The released proteins were immunoprecipitated with anti ALA-synthase antiserum and the proteins analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

Lane A - Immunoprecipitated protein from sonicated mitochondria.

Lane B - Immunoprecipitated protein from freeze-dried mitochondria.

A B Mr x 10^{-3}

-68

-49

purification caused the release of an endogenous protease which degraded the ALA-synthase to a molecular weight of 49,000.

DISCUSSION

The work described in this chapter clearly establishes that ALA-synthase is readily modified by proteolytic activity. The evidence for this comes from two experiments. Firstly, when a freeze-dried extract from mitoplasts was stored at 4°C for 5 days before completing the purification of ALA-synthase considerable breakdown of ALA-synthase occurred as judged by SDS polyacrylamide gel electrophoretic analysis of the finally purified proteins. A broad range of products was observed with a sharp cut off at molecular weight 49,000; significantly there was no protein of molecular weight 68,000. These degraded forms of ALA-synthase are apparently enzymically active as the expected catalytic activity was retained. Secondly, treatment of the purified native ALA-synthase with papain showed directly that breakdown forms of ALA-synthase could be generated which retained full catalytic activity. ALA-synthase appears to be relatively resistant to further protease digestion in the 49,000 molecular weight form. Despite the rapidity of the purification procedure described in this thesis ($2\frac{1}{2}$ days), it is still necessary to maintain the presence of protease inhibitors if

degradation of the enzyme is to be avoided since it was found that significant degradation was seen if the purification was performed in the absence of protease inhibitors.

In view of the sensitivity of ALA-synthase to degradation, it was important to establish that the 68,000 molecular weight form of the enzyme was the intact form. Evidence to support this came from the experiment where drug-treated chick embryos were pulse labelled with S^{35}-methionine for 45 minutes and a mitochondrial extract prepared under conditions minimising proteolytic activity. Under these conditions ALA-synthase could not be detected in the extract in a form larger than 68,000 molecular weight by immunoprecipitation.

It was of interest that sonication of a mitochondrial extract greatly exacerbated the breakdown of ALA-synthase. Precisely how sonication leads to this is unknown. Nevertheless, it is very likely that sonic disruption of mitochondria and the long time taken by Whiting and Granick (1976) were two major factors in their procedure producing a 49,000 molecular weight form of ALA-synthase. Presumably this form is the same as the relatively resistant form of ALA-synthase generated by papain or endogenous protease activity.

It will be interesting to determine which part of the ALA-synthase molecule is susceptible to

proteolytic attack. Comparison of the N-terminal amino acid sequence of the intact and breakdown forms of the enzyme would indicate at which end of the molecule breakdown occurs.

C H A P T E R 7

PURIFICATION OF RAT LIVER MITOCHONDRIAL ALA-SYNTHASE AND IDENTIFICATION OF ITS PRIMARY TRANSLATION PRODUCT

INTRODUCTION

The purification of rat liver mitochondrial ALA-synthase has been attempted by several workers (Kaplan (1971), Patton and Beattie (1973), Whiting and Elliott (1974), Woods and Murthy (1975), Paterniti and Beattie (1979)). The preparation of homogeneous enzyme has proven to be extremely difficult, and the reported information on its molecular weight correspondingly confusing. Paterniti and Beattie (1979) purified the enzyme with a minimum molecular weight of 58,000 from non-induced rats. Nakakuki *et al* (1980) purified rat cytosol ALA-synthase, using papain to disaggregate the enzyme, resulting in a molecular weight of 51,000. An antibody raised to this protein was used to immunochemically identify the mitochondrial ALA-synthase as having a molecular weight of 45,000.

With the knowledge that the chick embryo mitochondrial ALA-synthase was very susceptible to degradation, and the availability of a new and rapid purification procedure it was decided to investigate rat liver mitochondrial ALA-synthase in an attempt to clarify the conflicting reports on its molecular weight. This chapter will describe the purification of rat liver mitochondrial ALA-synthase.

In addition the possibility that the enzyme is initially synthesised as a larger precursor will be examined. This is particularly relevant since chick

embryo liver ALA-synthase is known to be synthesised in the cytoplasm on free polyribosomes as a larger molecular weight precursor which is apparently transferred to the mitochondrion (Brooker *et al* (1979)). The initial translation product for the chick embryo liver ALA-synthase is of molecular 74,000 daltons, some 6,000 daltons larger than the mitochondrial enzyme. In rats the cytosolic form of ALA-synthase has been proposed to be a precursor of larger molecular weight *en route* to the mitochondrion (Hayashi *et al* (1969), Whiting and Elliott (1972), Nakakuki *et al* (1980)). It was therefore of considerable interest to compare the size of the primary translation product of rat liver ALA-synthase with that of the mitochondrial enzyme.

METHODS

1. DRUG TREATMENT OF RATS

Levels of mitochondrial ALA-synthase were increased using DDC. Earlier work in this laboratory (Parslow (1978)) had established that administration of DDC alone achieved optimum induction of mitochondrial ALA-synthase. Male Wistar rats, weighing 150 - 200g were starved for 24 hours prior to drug treatment to improve induction. The DDC was administered in 4ml as a suspension in 5% w/v tragacanth mucilage via a stomach tube and the induction of ALA-synthase was allowed to proceed for 15 - 20 hours.

2. PURIFICATION OF RAT LIVER MITOCHONDRIAL ALA-SYNTHASE

Livers from ten drug treated rats were used as the starting material for purification. The purification was exactly as described for the chick embryo enzyme except that the rat livers were finely minced with scissors prior to homogenisation to facilitate disruption.

3. RNA ISOLATION AND FRACTIONATION

Total RNA was isolated from the livers of drug-treated and untreated rats by the guanidine-HCl method of Brooker *et al* (1979). Livers were rapidly removed from the rats and immediately homogenised in 10ml/g of a solution of 6M guanidine HCl, 0.2M sodium acetate, pH5.2, 1mM 2 mercaptoethanol (added freshly) at 4°C using a Dounce homogeniser. The homogenate was passed through a French pressure cell at 1200 psi and 4°C, and RNA was precipitated by the addition of 0.5 volume 95% ethanol. The suspension was held at -20°C for 1 hour then centrifuged at 12,000 x g for 20 minutes. The RNA pellet was resuspended in 5ml/g (original liver weight) of a solution of 6M guanidine HCl, 0.2M sodium acetate, pH 5.2, 10mM EDTA and reprecipitated at -20°C for 1 hour with 0.5 volume 95% ethanol. After centrifugation the RNA pellet was resuspended by homogenisation in 2.5ml/g original liver weight of a solution of 7M urea, 0.1M Tris-HCl pH 8.5, 0.1mM

EDTA, 0.1% SDS, then phenol/chloroform (1:1) extracted and ether washed. RNA was precipitated with 100mM potassium acetate pH5 and 2.5 volumes ethanol. After centrifugation the RNA was washed firstly with 2M LiCl, to remove tRNA and low molecular weight DNA, and then 70% ethanol/30% 0.1M potassium acetate. Poly (A) rich RNA was isolated by poly (U) sepharose chromatography. The poly (A) rich RNA was fractionated on 5-25% sucrose density gradient containing 70% formamide, 1mM Tris-HCl pH 7.6, 1mM EDTA (buffer A) after heat denaturation (45°C,5 min) in buffer A plus 0.1% SDS. RNA fractions were ethanol precipitated and fractions containing poly (A) rich RNA of size greater than 18S by comparison with ribosomal RNA markers were collected. The RNA was precipitated three times to remove formamide. A yield of 60µg of fractionated poly (A) rich RNA per gram liver was obtained. This RNA was used for cell free translation.

4. CELL FREE PROTEIN SYNTHESIS

Translation of fractionated poly (A) rich RNA was carried out in a wheat germ cell free system as described by Brooker *et al* (1979). Immunoprecipitation of the cell free translation products was carried out by the procedure of Matsuura *et al* (1980) as described in Chapter 2. The antigen-antibody complex was precipitated by the addition of Staphlococcus Aureus cells as described in Chapter 2. Total and immunoprecipitated

proteins were analysed by SDS polyacrylamide gel electrophoresis and fluorography.

RESULTS

PURIFICATION AND IDENTIFICATION OF RAT LIVER MITOCHONDRIAL ALA-SYNTHASE

Rat liver mitochondrial ALA-synthase behaved in a similar manner to chick embryo liver mitochondrial ALA-synthase when subjected to the purification procedure described in this thesis. The chromatographic profiles on Sephacryl S-200 chromatofocusing and CoA-agarose are identical to those of the chick embryo (Figures 7.1, 7.2 and 7.3 respectively). Table 7.I summarises the purification scheme starting with 86g liver. The enzyme was purified 750-fold to a specific activity of 9790 units/mg protein, the highest yet reported for rat liver mitochondrial enzyme. Figure 7.4 shows the protein profile at each stage of the purification as analysed by SDS polyacrylamide gel electrophoresis. A major protein of molecular weight 70,000 was purified together with three other proteins of molecular weight of around 27,000 (Figure 7.4, lane f). Significantly, there is no protein in the molecular weight range 45,000 - 60,000.

To confirm that the protein of molecular weight 70,000 was ALA-synthase, a sample of enzyme from the CoA-agarose column was dialysed against 0.1M triethylamine pH 7.6 and, after freeze-drying, was subjected to non-denaturing gel electrophoresis as described for

FIGURE 7.1

SEPHACRYL S-200 CHROMATOGRAPHY OF RAT LIVER MITOCHONDRIAL ALA-SYNTHASE

ALA-synthase was disaggregated with 50mM $MgSO_4$ and 1mM DTE as described in the text. The enzyme was chromatographed on a Sephacryl S-200 column (76 x 1.6cm) equilibrated in 50mM Tris-HCl, pH 7.6, 0.1mM pyridoxal phosphate, 1mM DTE and 50mM $MgSO_4$ The elution profile for the ALA-synthase is shown (■-■) and the protein profile (▽-▽). The position of the void volume is indicated by the arrow.

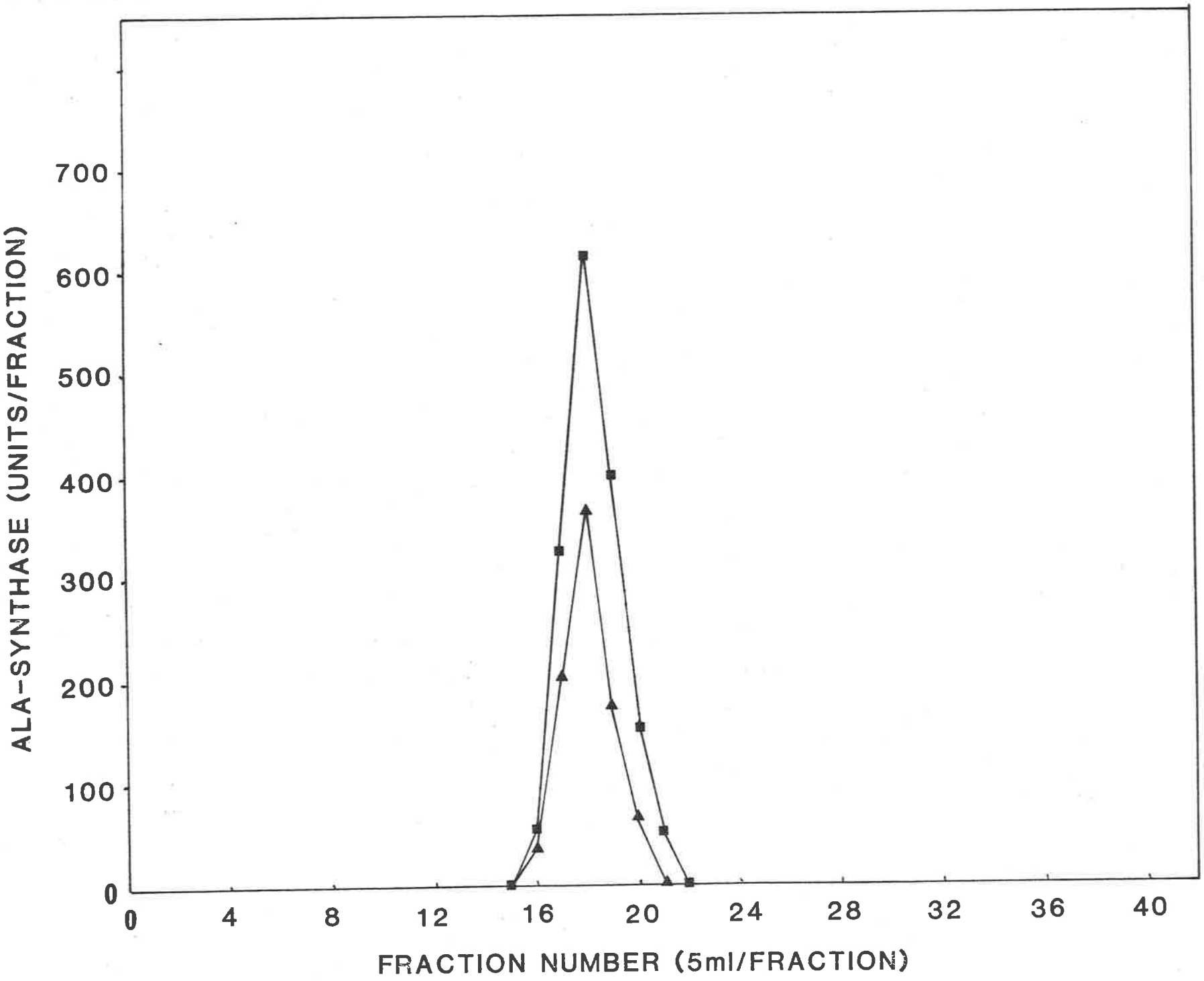

ALA-SYNTHASE (UNITS/FRACTION)
700
600
500
400
300
200
100
0
0
4
8
12
16
20
24
28
32
36
40
FRACTION NUMBER (5ml/FRACTION)

FIGURE 7.2

CHROMATOFOCUSING OF RAT LIVER ALA-SYNTHASE

ALA-synthase was chromatofocused as described in the text. Fractions (5ml) were collected and assayed for ALA-synthase activity (■ - ■) as described in Materials and Methods and protein (▽ - ▽) by the method of Bradford (1976). The pH of each fraction, at 4°C was also measured (O - O).

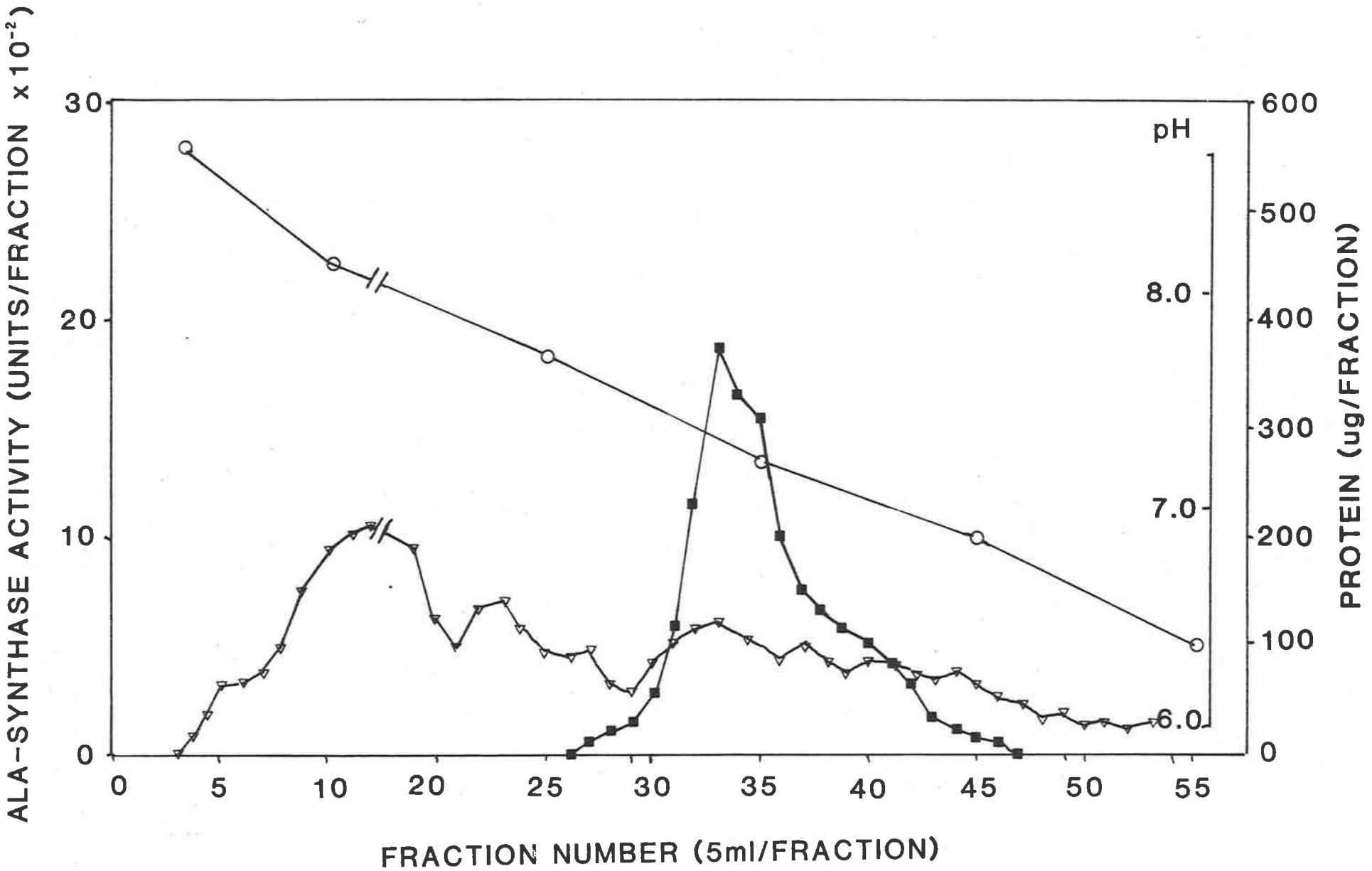

ALA-SYNTHASE ACTIVITY (UNITS/FRACTION x10⁻²)
PROTEIN (ug/FRACTION)
pH
FRACTION NUMBER (5ml/FRACTION)
0
10
20
30
0
100
200
300
400
500
600
6.0
7.0
8.0
0
5
10
20
25
30
35
40
45
50
55

FIGURE 7.3

CoA-AGAROSE AFFINITY CHROMATOGRAPHY OF RAT LIVER ALA-SYNTHASE

ALA-synthase was chromatographed on CoA-agarose as described in the text. The arrows indicate where each of the following buffers were used: A = 0.5 M glycine in buffer B; B = 20mM 5' AMP in buffer B; C = 50 mM 5' AMP in buffer B. ALA-synthase activity was measured in each fraction (5 ml) (■ - ■). Protein content (▽ - ▽) was estimated by the method of Bradford, (1976).

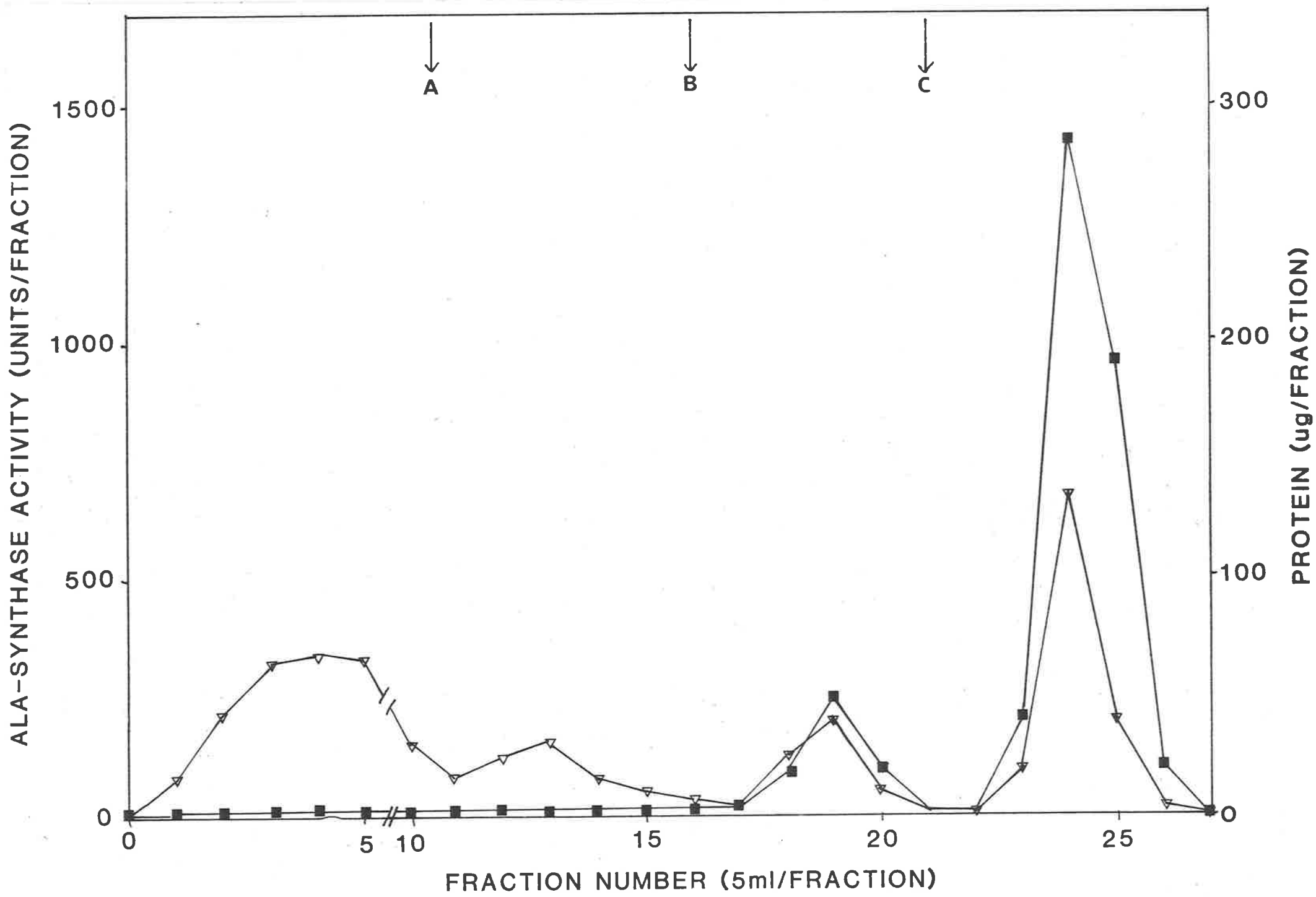

A
B
C
1500
1000
500
0
300
200
100
0
0
5
10
15
20
25
ALA-SYNTHASE ACTIVITY (UNITS/FRACTION)
PROTEIN (ug/FRACTION)
FRACTION NUMBER (5ml/FRACTION)

TABLE 7.1

PURIFICATION OF δ-AMINOLAEVULINATE SYNTHASE FROM RAT LIVER MITOCHONDRIA

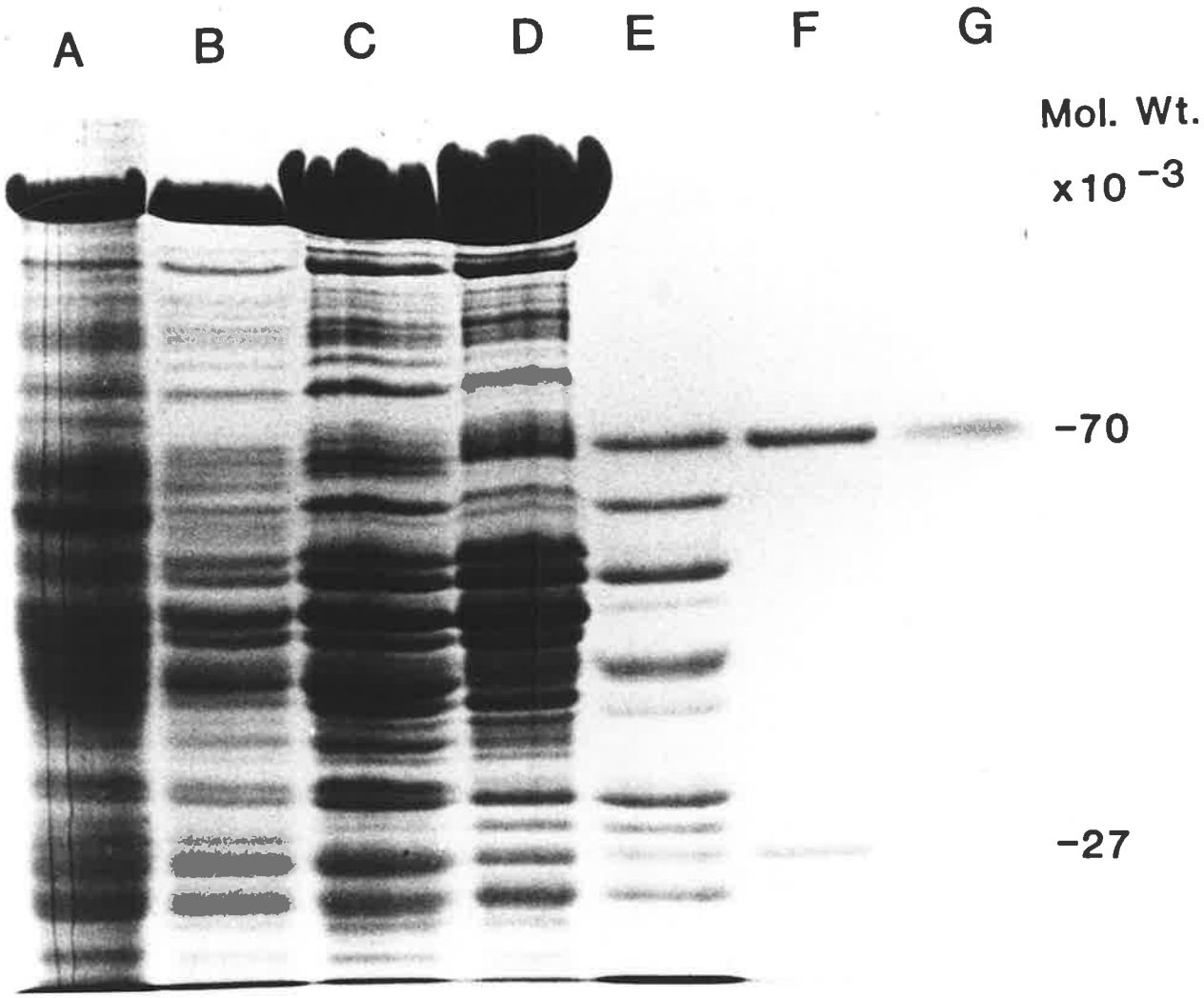
TABLE 7.1 PURIFICATION OF δ-AMINOLAEVULINATE SYNTHASE FROM RAT LIVER MITOCHONDRIA

Step and Fraction	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Yield %
Mitochondria (from 86 g liver)	20	2320	30,238	13	100
Mitoplasts	40	514	28,044	54	93
Extract from freeze-dried mitoplasts	12	294	25,496	87	84
Sephacryl S-200 chromatograph	41.6	102	22,694	223	75
Chromatofocusing	37	3.2	21,710	6,800	72
CoA-agarose affinity chromatography	17.3	0.94	9,200	9,790	30

FIGURE 7.4

PROTEIN PROFILE AT EACH STAGE OF THE PURIFICATION OF RAT LIVER ALA-SYNTHASE AS ANALYSED BY SDS-POLY-ACRYLAMIDE GEL ELECTROPHORESIS

Equivalent amounts of ALA-synthase activity were analysed from each stage of the purification. Lane a = mitochondria; lane b = mitoplasts; lane c = freeze-dried extract from mitoplasts; lane d = Sephacryl S-200 chromatography; lane e = chromatofocusing; lane f = CoA-agarose affinity chromatography; lane g = analyses of an enzyme active gel slice from a non-denaturing gel as described in text.


A
B
C
D
E
F
G
Mol. Wt.
x10 -3
-70
-27

chick embryo mitochondrial ALA-synthase using the Tris-glycine system of Davis (1964) in a slab gel apparatus. On completion of electrophoresis the gel track was cut into slices which were assayed directly for ALA-synthase activity and those slices containing activity were analysed directly by SDS polyacrylamide gel electrophoresis (in contrast to the similar experiment with chick embryo ALA-synthase where alternate slices were assayed for enzyme activity and the intermediate slices analysed by gel electrophoresis). These slices were shown to contain a single protein of molecular weight 70,000 (Figure 7.4, lane g).

CHICK EMBRYO LIVER ALA-SYNTHASE ANTI-SERUM RECOGNISES RAT LIVER ALA-SYNTHASE

It was useful to determine if the antibody raised against chick embryo liver ALA-synthase would recognise rat liver ALA-synthase. This would remove the necessity to raise a separate antiserum to the rat enzyme. The antiserum to chick embryo ALA-synthase was therefore tested against the rat liver ALA-synthase by Ouchterlony double diffusion analysis. Purified enzyme and mitochondrial extracts from both induced rats and control (uninduced) rats were tested. A single precipitin line was observed when the purified enzyme and crude extract from drug treated rats was tested but no precipitin line was observed with the control extract, (Figure 7.5)

FIGURE 7.5

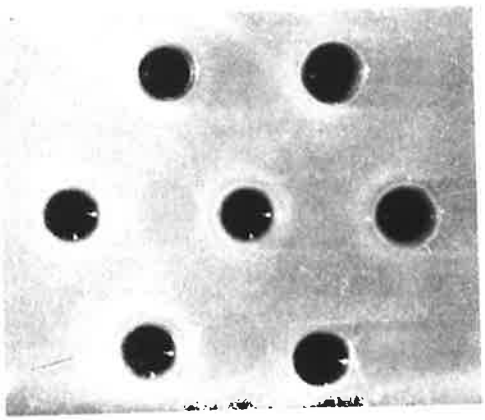
OUCHTERLONY DOUBLE DIFFUSION ANALYSIS OF RAT LIVER MITOCHONDRIAL ALA-SYNTHASE AGAINST ANTI-CHICK EMBRYO LIVER MITOCHONDRIAL ALA-SYNTHASE ANTISERUM.

Mitochondrial extracts were prepared from drug-treated and untreated rat liver, and tested against anti-chick embryo liver mitochondrial ALA-synthase antiserum and non-immune serum.

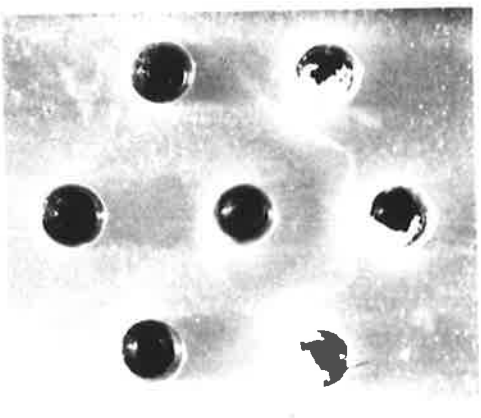
A. Non-immune serum (centre well) with untreated rat liver mitochondrial extract (3 left-hand side wells) and drug-treated rat liver mitochondrial extract (3 right-hand side wells).

B. Anti-chick embryo liver mitochondrial ALA-synthase mitochondrial antiserum (centre well) with untreated rat liver mitochondrial extract (3 left-hand side wells) and drug-treated rat liver mitochondrial extract (3 right-hand side wells).

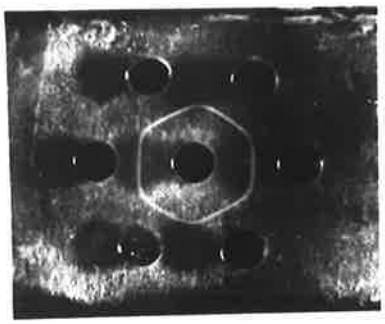
C. Anti-chick embryo liver mitochondrial ALA-synthase antiserum (centre well) with purified rat liver mitochondrial ALA-synthase (outer wells).



A



B



C

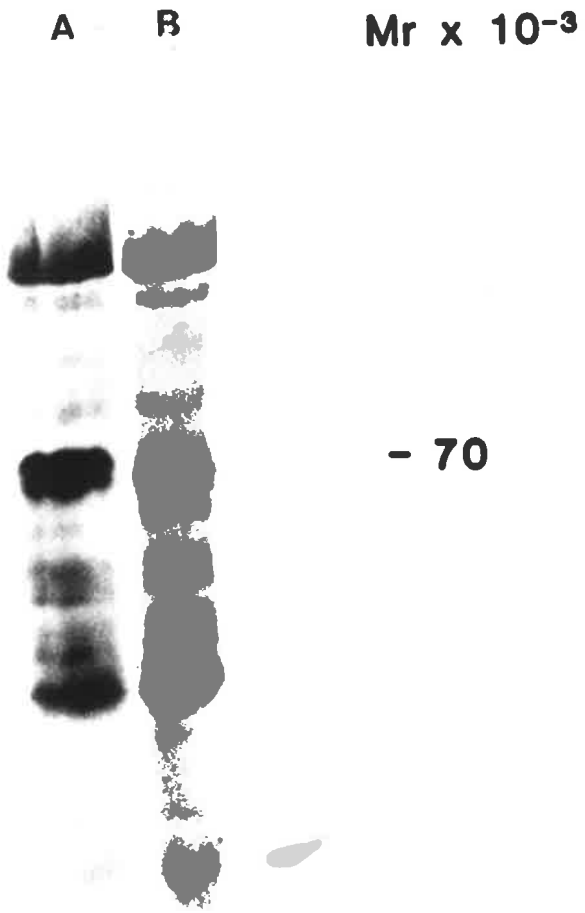
FIGURE 7.6

RADIOISOTOPIC LABELLING OF RATS

A drug treated and untreated rat were labelled with ^{35}S-methionine as described in Chapter 2 and mitochondrial extracts prepared under conditions minimising proteolytic degradation. Samples of each extract were analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

Lane A - mitochondrial extract from drug-treated rat

Lane B - mitochondrial extract from untreated rat.


A
B
Mr x 10-3
- 70

IS THE PURIFIED ENZYME THE INTACT FORM?

Experiments were then undertaken to determine if the purified 70,000 daltons form was the intact form of the enzyme, and not a degradation product. A drug-treated and untreated rat were labelled with ^{35}S methionine for 45 minutes. Liver mitochondria were prepared as described for the purification and immediately boiled in 3% SDS to minimise proteolytic action. The proteins were then analysed by SDS polyacrylamide gel electrophoresis and fluorography. The result (fig. 7.6) shows a DDC-inducible mitochondrial protein of molecular weight 70,000.

This experiment establishes that the minimum molecular weight of the enzyme is 70,000 daltons and is considerably larger than previously reported.

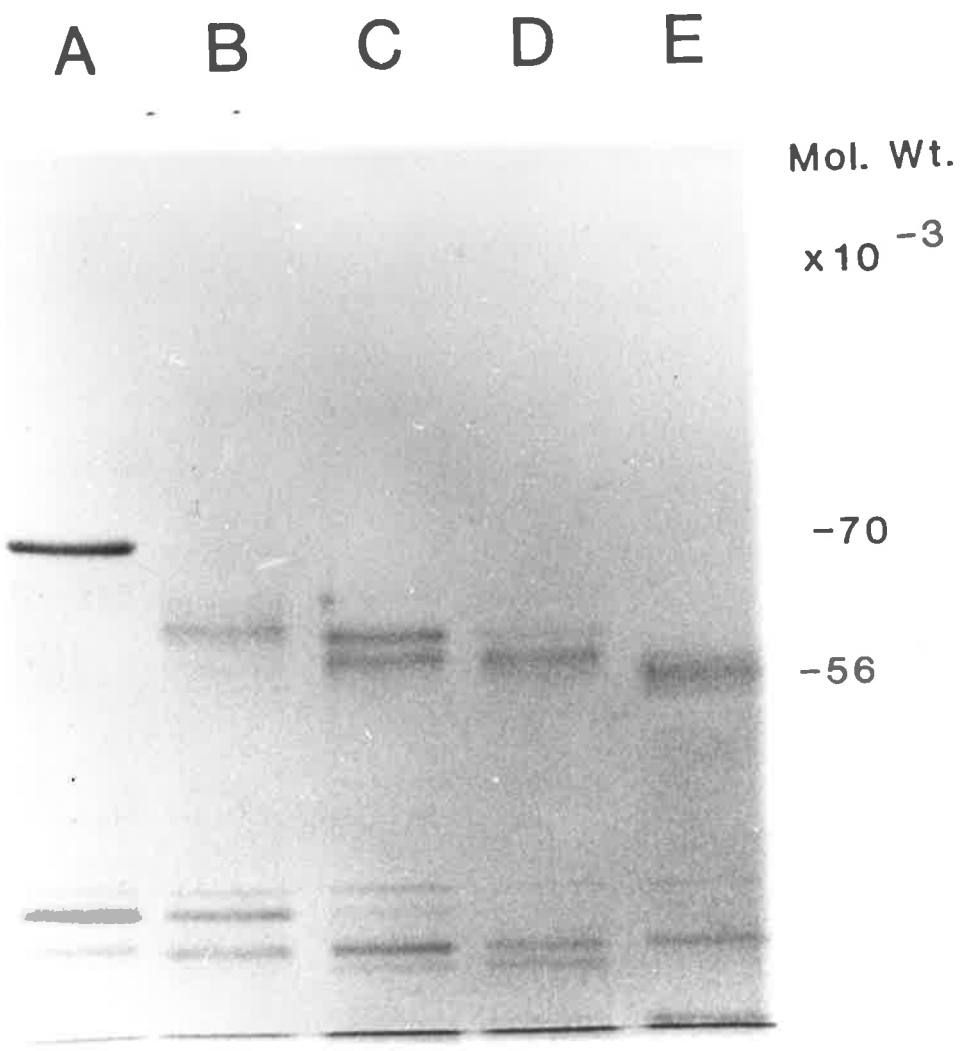
SUSCEPTIBILITY TO DEGRADATION

As described in the last chapter chick embryo ALA-synthase could be degraded to smaller forms whilst still retaining full catalytic activity. An investigation was carried out to determine if the same situation existed in the rat. Purified enzyme from the CoA-agarose was digested with papain as described in the legend to Figure 7.7. The digested proteins were analysed by SDS polyacrylamide gel electrophoresis and visualised by staining with Coomassie Blue. Figure 7.7 shows that the ALA-synthase of molecular weight 70,000 was progressively degraded to smaller forms

FIGURE 7.7

PAPAIN TREATMENT OF PURIFIED RAT LIVER ALA-SYNTHASE

Aliquots (10μg) of purified enzyme were incubated with varying amounts of papain at 37°C for 10 minutes. Antipain was added in a 20 molar excess over papain and samples were assayed for ALA-synthase activity. The remainder was analysed by SDS-polyacrylamide gel electrophoresis: Lane a = untreated; Lane b = 1 μg papain; lane c = 2 μg papain; lane d = 20 μg papain and lane e = 100 μg papain.


A
B
C
D
E
Mol. Wt.
x10 -3
-70
-56

of molecular weight ranging down to 56,000 daltons. No forms lower than 56,000 daltons were detected. (The lower molecular weight bands of about 27,000 daltons were also digested). However, there was no loss of enzyme activity following treatment of the enzyme with any of the concentrations of papain used.

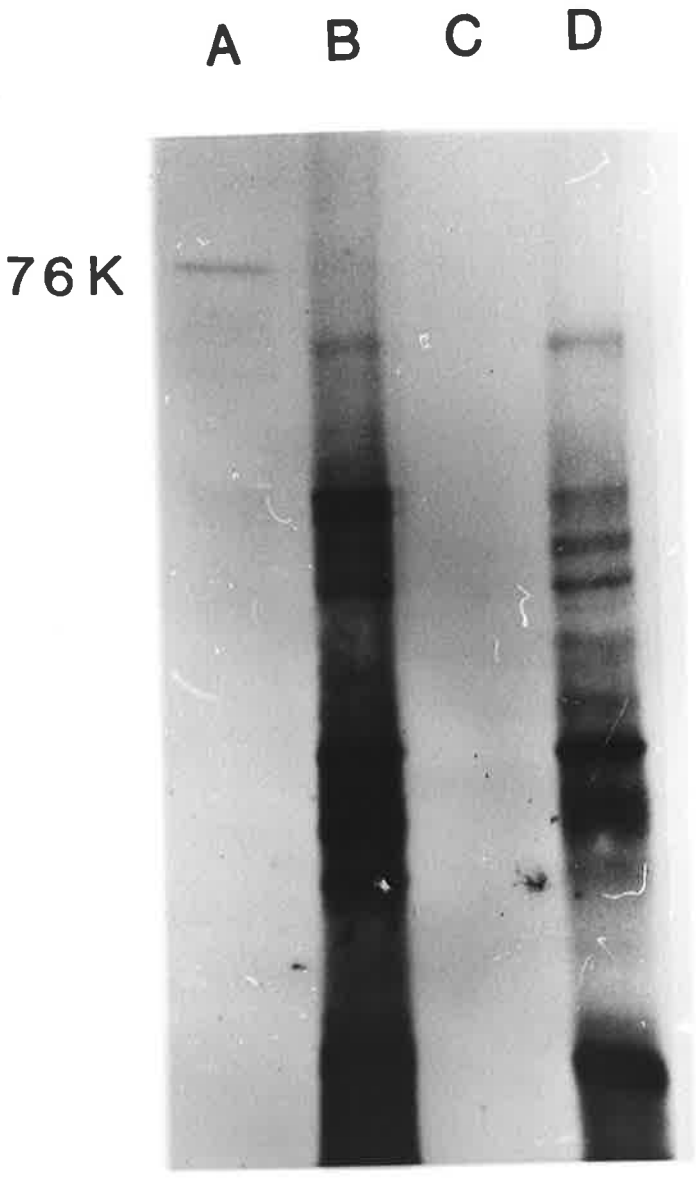
PRIMARY TRANSLATION PRODUCT OF RAT LIVER ALA-SYNTHASE mRNA

Since chick embryo liver ALA-synthase is initially synthesised in the cytosol as a larger molecular protein an investigation was made to determine whether rat liver ALA-synthase was also synthesised initially as a larger molecular weight form. Fractionated total poly (A) mRNA was extracted from drug treated and untreated (control) rat livers by the guanidine HCl method and translated in a wheat germ cell free system aa described in the methods to this chapter. Aliquots of the translation products were subjected to immuno-precipitation with antibody to chick embryo ALA-synthase. Total and immunoprecipitated products were analysed by SDS gel electrophoresis and fluorography as described in Chapter 2. The result (Figure 7.8) shows that a major immunoprecipitated protein of molecular weight 76,000 was observed when poly (A) mRNA from drug treated rats was translated (Figure 7.8, lane A) but not when untreated poly (A) mRNA was translated (Figure 7.8, lane C). This result suggests that the rat mitochondria

FIGURE 7.8

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PRODUCTS FROM THE TRANSLATION OF POLY (A)-RICH RNA FROM RAT LIVER IN A WHEAT GERM CELL-FREE SYSTEM

Poly(A)-rich RNA (0.5 g) from induced and non-induced livers was translated *in vitro* in the presence of 0.5 μCi of [^{35}S]methionine (specific activity = 1200 Ci/nmol). Products were immuno-precipitated using chick anti-δ-amino-laevulinate synthase antibody. Lane a = immunoprecipitate from induced RNA; lane b = total translation products from induced RNA; lane c = immunoprecip-itate from non-induced RNA; lane d = total translation products from non-induced RNA.


A
B
C
D
76K

ALA-synthase is initially synthesised as a precursor some 6,000 daltons larger than the mature enzyme.

DISCUSSION

The work described in this chapter shows that rat liver mitochondrial ALA-synthase has a minimum molecular weight of 70,000. This size is considerably larger than estimates previously reported, and is very close to the size determined for pure chick embryo liver mitochondrial ALA-synthase.

In common with the chick embryo enzyme, evidence is presented to show the susceptibility of rat ALA-synthase to proteolysis. The enzyme can be degraded to a number of smaller catalytically active forms ranging in molecular weight from 70,000 to 56,000. This observation makes it very likely that previous reports on the purification (Paterniti and Beattie (1979)) and identification (Nakakuki *et al* (1980)) of rat liver mitochondria ALA-synthase were describing degradation products of the enzyme. It is particularly noteworthy that Nakakuki *et al* (1980) reported the use of papain as a disaggregating agent in their purification scheme for the cytosol form of rat liver ALA-synthase of molecular weight 51,000. As with the chick embryo enzyme it was important to know that the purified rat ALA-synthase was the intact form of the enzyme. The pulse labelling studies on drug treated and untreated rats showed that when mitochondrial extracts were prepared under conditions designed to

minimise proteolytic activity only ALA-synthase of molecular weight 70,000 was detected.

The similarities in purification between the rat enzyme and the chick embryo enzyme were striking and unexpected, (in view of the previously reported differences in the literature). Both enzymes require high ionic strength conditions for disaggregation and are retarded to a similar extent on Sephacryl S-200- indeed the same fractions were collected for further purification. The pH range required to elute the two enzymes from the chromatofocusing column were pH 7.3 - 7.6 suggesting that they have similar isoelectric points. However, when peptide mapping (Cleveland *et al* (1977)) was performed on the two purified enzymes (result not shown) it was evident that the two ALA-synthases were not identical.

Since it was clearly established by the direct analysis of enzyme active slices from a non-denaturing gel that the rat liver mitochondrial ALA-synthase was of molecular weight 70,000 further attempts to chromatographically purify the enzyme to homogeneity were not attempted.

Recently, Brooker *et al* (1979) have shown that chick embryo ALA-synthase is synthesised as a 74,000 molecular weight precursor. This enzyme is proposed to be synthesised as a precursor on free polyribosomes before transfer to the mitochondria. The finding that

rat liver mitochondrial ALA-synthase is first synthesised as a 76,000 molecular weight precursor further emphasises the similarities between the rat and chick embryo enzymes. Indeed both enzymes conform to the findings for the majority of mitochondrial enzymes investigated so far (Conboy, J.G. and Rosenberg, L.E. (1981), Schmelzer, E. and Heinrich P.C. (1980), Raymond Y. and Shore G.C. (1981)), which are synthesised as larger cytoplasmic precursors that are processed at some point during or after translocation into the mitochondria. It will now be of interest to determine if the rat cytosol form is in fact the same size as the precursor of molecular weight 76,000.

Shortly after this work was completed, Hayashi and Kikuchi (1981) revised their estimate of the molecular weight of rat mitochondrial ALA-synthase to 66,000. Although no details of their purification were published, it appears these authors have not completely overcome the problem of proteolytic degradation.

CHAPTER 8

CONCLUSION

CONCLUSION

The successful purification of ALA-synthase from avian and mammalian liver mitochondria has resolved the confusion arising from previously published reports. Not only has the purification of ALA-synthase from these sources been complicated by difficulties in stabilising and solubilising the enzyme but it is now apparent that a third problem was the sensitivity of ALA-synthase to degradation.

As a result of the work in this thesis, it can be seen that there are several similar features between the chick embryo and rat liver enzymes. The chick embryo liver mitochondrial ALA-synthase has a molecular weight of 68,000 and the rat liver mitochondrial enzyme, a molecular weight of 70,000. Both enzymes are very susceptible to proteolytic degradation and in each case a fragment or fragments of 15,000 - 20,000 molecular weight are removed. The rat liver and chick embryo enzymes (Whiting, (1976), Brooker *et al* (1979)), are first synthesized as a larger precursor. This feature is shared with most other mitochondrial enzymes that are first synthesized in the cytoplasm.

The ability to purify native intact ALA-synthase now makes it possible to further investigations concerning the regulation of ALA-synthase.

Firstly, the physical properties and kinetics of chick embryo liver mitochondrial ALA-synthase

can be studied using a pure preparation of the enzyme. Form these, it should be possible to elucidate details on the mechanism of enzyme action opening the possibility of designing and synthesizing an inhibitor of ALA-synthase for trial as a therapeutic agent for the acute hepatic porphyrias.

Secondly, the availability of a specific antibody to ALA-synthase will enable studies on the transport of ALA-synthase from the cytosol to the mitochondria both *in vivo* and in an *in vitro* system. It is now possible to investigate the phenomenon of the cytosolic form of the enzyme and to determine, immunologically, the relationship between the mitochondrial and cytosolic forms. Further, the proposed regulatory role of haem in the translocation of ALA-synthase can be studied in such a system.

Thirdly, the knowledge of the true size of ALA-synthase will facilitate the cloning of a complementary DNA probe to ALA-synthase for use in studies on the regulation of ALA-synthase in tissue culture and other expression systems. Until the present work a mRNA fraction, from liver cells, coding for a protein of molecular weight around 50,000 had been sought as a template for cDNA synthesis. It is now obvious that the mRNA as well as the protein is larger than first anticipated.

The current work in this laboratory is concerned with furthering progress in these three areas.

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