STUDIES ON δ-AMINOLAEVULINIC ACID SYNTHETASE INDUCED IN GUINEA PIG LIVER BY 3,5-DICARBETHOXY-1,4-DIHYDROCOLLIDINE

Thesis submitted for the degree

of

Doctor of Philosophy

by

Elizabeth Anne Irving, M.Sc.

from

The Department of Biochemistry,

University of Adelaide.

SEPTEMBER, 1969.

STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in this or any other University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

Elizabeth A. Irving

PUBLICATIONS

Irving, E.A. and Elliott, W.H. (1968). Studies on δ-aminolaevulinic acid synthetase in the livers of normal and porphyric guinea pigs. Proc. Aust. Biochem. Soc., p.62.

Irving, E.A. and Elliott, W.H. (1969). A sensitive radiochemical assay method for δ-aminolevulinic acid synthetase. J. Biol. Chem. 244: 60.

Irving, E.A. and Elliott, W.H. (1969). δ-Aminolaevulinic acid (ALA) synthetase, a control point; its purification and properties. Proc. Aust. Biochem. Soc. p.85.

Irving, E.A. and Elliott, W.H. (1969). δ-Aminolevulinic acid synthetase (Assay). Methods in Enzymology, 14, in press.

Porra, R.J. and Irving, E.A. (1969). The distribution and properties of δ-aminolaevulic acid synthetase. In preparation.

ACKNOWLEDGEMENTS

I thank the Australian Wool Board whose generous financial assistance made this study possible, and also the Council of the Institute of Medical and Veterinary Science for further financial assistance and for allowing the extended period of study leave needed to carry out the work presented in this thesis.

The guidance, patience and kindness of my supervisor, Professor W.H. Elliott is most gratefully acknowledged; I also thank him for allowing me to work in his Department.

During part of the time, the salary of a technical assistant, Miss Patricia Dunnear, was provided by the National Health and Medical Research Council; I gratefully acknowledge the financial assistance and the skilled technical assistance of Miss Dunnear.

The studies on plant organelles and yeast cells were carried out at the Division of Plant Industry, C.S.I.R.O., Canberra, in collaboration with Dr. R.J. Porra, and I thank Dr. J.E. Falk, Chief of the Division of Plant Industry for making this possible.

I also thank Miss P. Dyer of the Department of Biochemistry, University of Adelaide, for electron microscopy.

Discussions with many members of the Department of Biochemistry, Universityof Adelaide, were of great assistance to me during my time in the Department.

I appreciate very much the meticulous work of Mrs. Kim Warnaby who typed the final draft of this thesis.

INDEX

ABBREVIATIONS

AA	aminoacetone
AA pyrrole	2,4-dimethyl-3-acetyl pyrrole
AIA	allyl isopropyl acetamide
ALA	δ-aminolaevulinic acid
ALA pyrrole	2-methyl-3-acetyl-4-propionic acid pyrrole
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cm	centimeter
CoA	coenzyme A
cpm	the number of counts or pulses/minute as recorded by a gas flow or scintillation counter
DDC	3,5-dicarbethoxy-1,4-dihydro-collidine
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid, disodium salt
g	gram
GSH	L-glutathione, reduced
GTP	guanosine triphosphate
M	molar
mg	milligram
ml	millilitre
mM	millimolar
μC	microcurie
μg	microgram
μmole	micromole
NAD	nicotine adenine dinucleotide
NADH	nicotine adenine dinucleotide, reduced
PBG	porphobilinogen
RNA	ribose nucleic acid
tris	Tris (hydroxy methyl) aminomethane
UDP	uridine diphosphate

SUMMARY

1. A sensitive radiochemical assay procedure for the determination of δ-aminolaevulinic acid (ALA) synthetase activity is described. The method depends on the measurement of the amount of ^{14}C-succinate incorporated into ALA; with crude enzyme preparations it is necessary to restrict the metabolism of succinate to ALA synthesis by means of Krebs cycle inhibitors.

2. The method was applied to a variety of biological systems but, with the exception of photosynthetic bacteria and reticulocytes, ALA synthetase activity could not be detected in any of the normal tissues studied.

3. A factor present in the insoluble fraction of ultrasonicated mitochondria inhibits ALA synthetase activity. The nature of the inhibitor could not be determined.

4. ALA synthetase in crude extracts of Rhodopseudomonas spheroides and in mitochondria isolated from the livers of guinea pigs treated with 3,5-dicarbethoxy-1, 4-dihydrocollidine (DDC) is stabilised by pyridoxal 5-phosphate.

5. ALA synthetase from the livers of DDC treated guinea pigs was solubilised and partially purified. The enzyme

in purified preparations is very unstable, even in the presence of pyridoxal 5-phosphate.

6. Preliminary studies of the properties of the purified liver enzyme were carried out and the requirement for substrates and cofactors examined.

7. ALA synthesis by both crude and purified preparations of the liver enzyme is inhibited by haem; the possibility of feedback inhibition of ALA synthetase activity in liver is discussed.

8. ALA synthesis is inhibited by EDTA, a compound used in the treatment of hereditary hepatic porphyrias; this effect may be mediated through inhibition of succinyl CoA synthetase activity.

9. Aminoacetone (AA) synthetase was also partially purified from the livers of normal and DDC treated guinea pigs and some of its properties studied. Two peaks of AA synthetase activity are eluted from Sephadex G-100; the first of these peaks is associated with the peak of ALA synthetase activity.

CHAPTER 1

INTRODUCTION

The role of the enzyme δ-aminolaevulinic acid (ALA) synthetase in the regulation of tetrapyrrole biosynthesis has recently been the subject of three major reviews (Lascelles, 1964; Granick and Levere, 1964; de Matteis, 1967). While these reviews cover the whole field of tetrapyrrole biosynthesis, a different aspect of the subject has been emphasised in each. Thus, in her monograph, Lascelles (1964) concentrated mainly on porphyrin and bacteriochlorophyll production by photosynthetic bacteria; whereas Granick and Levere (1964) discussed in detail haem and haemoglobin synthesis in differentiating and maturing erythroid cells and de Matteis (1967) paid particular attention to the disturbances of porphyrin metabolism in liver cells resulting from genetic abnormalities or the administration of drugs.

ALA synthetase is an enzyme of particular interest, not only because it is the first enzyme specific to the haem biosynthetic pathway, but also because the level of this enzyme can be rapidly and greatly altered under certain conditions. It is currently regarded as a repressible enzyme, subject to feed back inhibition of its activity (at least in bacteria) and to end product repression of its synthesis.

The following sections describe published work leading

to our present concepts of the mechanisms whereby activity and synthesis of this enzyme may be controlled in a variety of biological systems.

1. MECHANISM OF HAEM BIOSYNTHESIS

A. Metabolic Pathway

From their studies of haem biosynthesis in avian erythrocytes, Shemin and his co-workers showed not only that δ-aminolaevulinic acid (ALA) was derived from succinate and glycine (Shemin and Russell, 1953; Shemin, Abramsky and Russell, 1954), but also that of the 34 carbon atoms in haem, 8 were derived from glycine (Shemin and Wittenberg, 1951), and 26 from succinate (Shemin and Kumin, 1952). Further work (Shemin, Russell and Abramsky, 1955) which demonstrated that two molecules of ALA condensed to form the basic monopyrrole subunit, firmly established the role of ALA in tetrapyrrole biosynthesis.

At the same time Lascelles (1956) showed that a similar sequence of events occurred in the synthesis of porphyrins by the photosynthetic bacterium, Rhodopseudomonas spheroides (R. spheroides). By varying the constituents of the growth medium she found that either ALA itself, or glycine and succinate had to be present in the medium for porphyrin synthesis to occur; furthermore, α-oxoglutarate could replace

succinate in the medium.

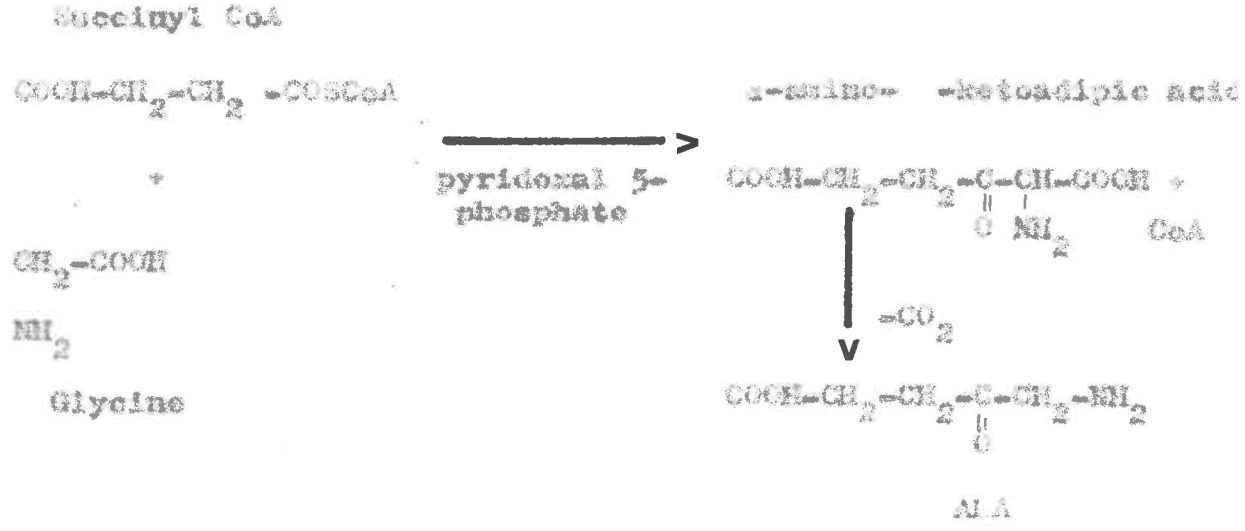
B. Enzymic Synthesis of ALA

The enzymic synthesis of ALA was simultaneously reported by Shemin, Kikuchi and Bachman (1958) who used extracts prepared from R. spheroides, and by Gibson, Laver and Neuberger (1958a) who used particles, presumably mitochondria, isolated from immature erythrocytes. In both the bacterial system (Kikuchi et al. (1958)) and the reticulocyte system (Laver, Neuberger and Udenfriend (1958)), the synthesis of ALA from glycine and succinate was enhanced by pyridoxal 5-phosphate and CoA; ALA synthesis also occurred when α-oxoglutarate replaced succinate in the reaction.

On the basis of early studies, Shemin and Kumin (1952) had concluded that ALA was formed from glycine and an "activated" succinate derivative which, on available evidence, was presumed to be a succinyl coenzyme compound, formed either from succinate or α-oxoglutarate. Evidence that this compound was succinyl CoA was obtained by Kikuchi et al. (1958) who measured ALA synthesised from succinyl CoA, glycine and pyridoxal 5-phosphate in extracts of R. spheroides. Freshly isolated reticulocyte particles (Laver, Neuberger and Udenfriend, 1958) could not however, use synthetic succinyl CoA, but were able to do so after the structural integrity of these particles

had been destroyed by freeze-drying (Gibson, Laver and Neuberger, 1958b). It was concluded that the enzymic synthesis of ALA from α-oxoglutarate in whole particles depended on the formation of succinyl CoA by the action of α-oxoglutarate dehydrogenase and that permeability barriers were responsible for the inability of whole particles to form ALA from succinyl CoA and glycine. It was further concluded that pyridoxal 5-phosphate was an essential co-factor in the reaction (Kikuchi et al., 1958).

The overall reaction catalysed by ALA synthetase, which involves spontaneous decarboxylation of an intermediate product, α-amino-β-ketoadipic acid (Shemin and Russell, 1953), is shown below.



The pathway of biosynthesis of haem from succinyl CoA and glycine is shown in Fig. 1.1 (reproduced from de Matteis (1967)). The formation of haem from ALA will not be further discussed here except to note that the first and last enzymes of the entire sequence (ALA synthetase and ferrochelatase) are located within the mitochondria.

2. ABNORMALITIES RELATED TO HAEM BIOSYNTHESIS

The regulation of haem biosynthesis is such that under normal physiological conditions only traces of ALA, PBG and non-functional porphyrins can be detected. In the group of metabolic disorders known as the porphyrias, this regulation is disturbed and the conditions are characterised by excessive production of non-functional porphyrins and porphyrin precursors.

A. Porphyria in Man

The classification of porphyrias is based broadly on whether the condition is of erythropoietic or hepatic origin. Many reviews on the clinical symptoms and classification of the various forms of porphyria are available (Watson, 1960; Goldberg and Rimington, 1962; Tschudy, 1965; Schmid, 1966; Waldenström and Haeger-Aronsen, 1967; Roman, 1967) and

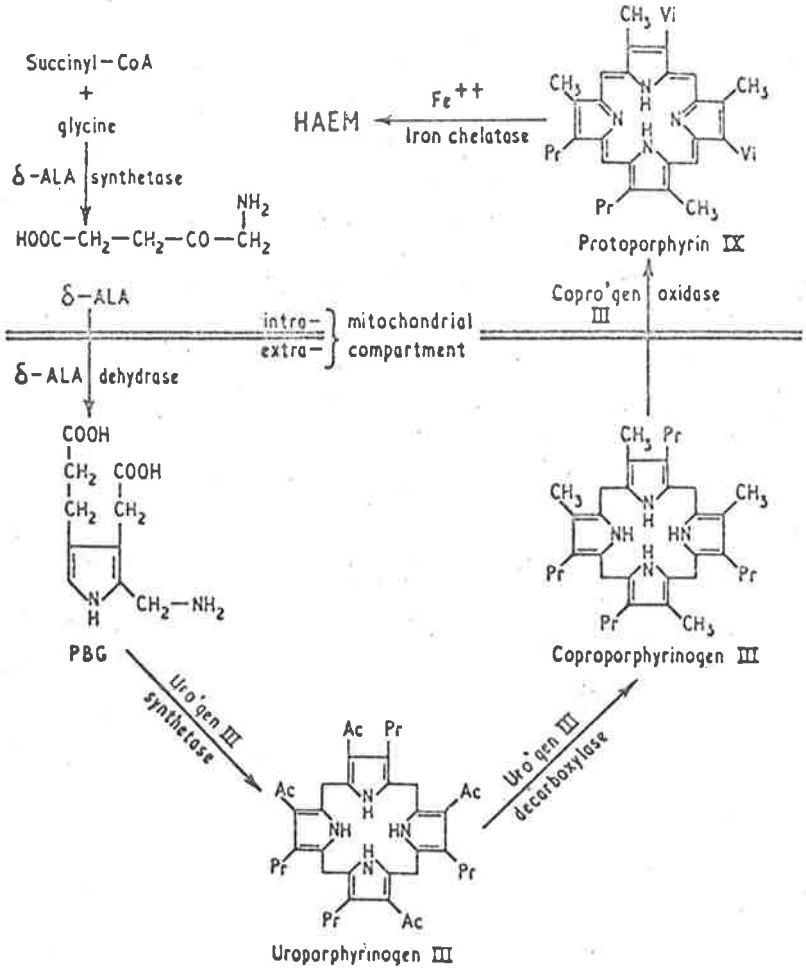


Fig. 1.1. (reproduced from de Matteis, 1967)
The intracellular distribution of the enzymes of the haem biosynthetic pathway.

Ac, $-CH_2.COOH$; Pr, $-CH_2.CH_2.COOH$; Vi, $-CH{:}CH_2$; Uro'gen, uroporphyrinogen; copro'gen, coproporphyrinogen.

no attempt will be made to review these here.

The condition of most relevance to the present work is acute intermittent porphyria which is transmitted as an autosomal dominant trait. This disease, which rarely manifests itself before puberty and is more common in females than in males, is characterised by acute attacks followed by periods of remission. The clinical symptoms include severe abdominal pain, neurological and psychotic disturbances and are accompanied by excessive excretion of ALA and porphobilinogen (PBG) in the urine. Porphyrin accumulation, as occurs with other forms of porphyria, is not a feature of acute intermittent porphyria, and the condition can be both precipitated and aggravated by certain drugs (see below).

It is worth noting however, that in addition to the specific genetically determined conditions, porphyria may be acquired. Thus Schmid (1960) reported the occurrence of porphyria following accidental hexachlorbenzene poisoning of populations in Turkey.

B. Experimental Porphyria

(a) Nature of drugs which induce porphyria

A porphyria resembling the inherited hepatic condition can be induced in experimental animals by a wide variety of chemicals. Thus the hypnotic drug Sedormid (allyl isopropyl

acetyl carbamide) causes raised PBG and porphyrin levels in the urine and liver of rabbits (Schmid and Schwartz, 1952). Allylisopropylacetamide (AIA), a drug structurally related to Sedormid but with no hypnotic properties, also produces symptoms of porphyria (Goldberg and Rimington, 1955). The porphyrinogenic effect of barbiturates was studied by Goldberg (1954) who found that only the diallyl- and allylisopropyl- barbituric acids were effective in rabbits.

The concept that porphyria inducing drugs might possess some structural feature in common was questioned however, when a different type of compound, 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC), was found to raise hepatic and urinary coproporphyrin levels (and protoporphyrin in liver) in mice (Solomon and Figge, 1959, 1960; Onisawa and Labbe, 1963) and other experimental animals (de Matteis and Prior, 1962, Granick and Urata, 1963).

Other classes of chemicals, including the antifungal agent griseofulvin (de Matteis and Rimington, 1963) and the insecticide hexachlorbenzene (Ockner and Schmid, 1961; de Matteis, Prior and Rimington, 1961) are now known to be capable of causing symptoms of porphyria in animals. Porphyrinogenic agents are dealt with in detail by de Matteis (1967) and Waldenström and Haeger-Aronsen (1967).

(b) Overproduction of ALA

At the same time that the role of ALA in haem biosynthesis was established (Shemin, Russell and Abramsky, 1955), Granick and vanden Schriek (1955) demonstrated the presence of excessive amounts of the aminoketone in the urine of patients suffering from acute intermittent porphyria. Following publication of a method of assay for ALA which eliminated interference from PBG (Mauzerall and Granick, 1956), this finding was confirmed both for acute intermittent porphyria (Ackner et al., 1961) and for experimental porphyria (Abbott and Rudolph, 1961). At the time it was thought that some of the symptoms of acute intermittent porphyria might be due to ALA, but rats injected with ALA suffered no distress (Granick and vanden Schriek, 1955); nor was the amount of ALA excreted in urine any indication of the severity of an attack of acute intermittent porphyria (Ackner et al., 1961).

Many possibilities to account for the accumulation of ALA in the various types of porphyria have been investigated. Those considered were based mainly on the supposition that ALA accumulated due to a block in its utilisation. These hypotheses could not however, be

supported by experimental evidence. In the first place, Gibson (1955) found ALA dehydrase activity in liver and kidney was increased following administration of Sedormid; secondly, the rate of conversion of PBG to porphyrins was the same in liver homogenates of normal rats (Schwartz and Watson, 1957) and in liver homogenates of rats treated with Sedormid (Merchante, Wajchenberg and Schwartz, 1957).

Shemin, Russell and Abramsky (1955) proposed that ALA is also metebolised in a glycine oxidation cycle, though there is little evidence that such a pathway either exists or has physiological significance. Tschudy et al. (1962) investigated the utilisation of ALA through the postulated cycle in the livers of normal rats and of rats treated with AIA. They found no difference in the rate of oxidation of ^{14}C-4-ALA to $^{14}CO_2$ in liver slice preparations of normal and porphyric rats. These authors also reported that no difference existed between the livers of control and AIA treated rats in their capacity for glycolysis, or ability to oxidise the alpha carbon atom of pyruvate or acetate.

In addition to increased ALA levels, another consistent feature of experimental porphyria is the lowered catalase actitty in the livers of porphyric animals (Schmid, Figen and Schwartz, 1955; de Matteis and Prior, 1962; Tschudy et al. 1962), and it has been suggested that a block in the synthesis of this haem protein might be responsible for the accumulation of porphyrins in the liver. However, Schmid, Figen and Schwartz (1955) considered that under-utilisation of ALA and other porphyrin precursors was not sufficient to account for the massive excretion of these compounds.

In summary therefore, all attempts to demonstrate a 'metabolic block' which could account for the excessive accumulation and excretion of porphyrins and their precursors in the various forms of porphyria were unsuccessful.

Granick and Urata (1963) made the key observation that this excessive accumulation and excretion of porphyrins and porphyrin precursors in experimental porphyria is due, not to a metabolic block but to overproduction of ALA. These workers found that while the synthesis of ALA in normal liver was barely detectable, in the livers of guinea pigs treated with DDC the synthesis of ALA was greatly elevated. This was followed by a report that ALA synthesis in normal human liver was only 10% of that in the liver (obtained post mortem)

of a patient who had died of acute intermittent porphyria (Tschudy et al., 1963). Since normal liver is capable of rapidly forming porphyrins and haem from ALA, it is apparent that the synthesis of ALA is the rate limiting step in this biosynthetic pathway.

The control of ALA synthetase levels will now be considered in some detail.

3. CONTROL OF ALA SYNTHETASE LEVELS

"The enzyme, ALA synthetase must be presumed to be present in all cells containing haem proteins and chlorophyll" (Gibson et al., 1961). "All aerobic cells, bacterial as well as plant and animal, have the ability to synthesise haem" (Granick and Levere, 1964). "Bacteria must be able to synthesise some ALA under all conditions of growth in order to make haematin compounds such as cytochromes" (Lascelles, 1960). Statements such as the above show the general uncertainty which has been expressed regarding the mechanism whereby ALA synthetase is so strictly controlled that although it must be presumed to be present it is barely detectable in some cell systems (Granick and Urata, 1963), at least by existing methods of assay.

The total amount of enzyme activity in a cell can be

controlled by a number of different mechanisms, including feedback inhibition of enzyme activity (Moyed and Umbarger, 1962), endproduct repression of synthesis of an enzyme (Monod, Changeux and Jacob, 1963), or by induction of synthesis of an enzyme (Halvorsen, 1960). Which, if any, of these mechanisms is operative in the control of ALA synthetase has been extensively investigated in both bacterial and mammalian systems.

A. CONTROL OF ALA SYNTHETASE IN BACTERIAL SYSTEMS

(a) Pigment formation in Rhodopseudomonas spheroides and levels of ALA synthetase

R. spheroides, a member of the group of photosynthetic bacteria which can grow either under anaerobic conditions in the light or under aerobic conditions in the dark, is the organism most frequently used for studies of ALA synthetase in bacterial systems. ALA synthetase activity has been shown to be present in crude extracts of the organism adapted either to anaerobic growth in the light (Kikuchi et al., 1958) or to aerobic growth in the dark (Gibson, 1958). In growing cultures, the further metabolism of ALA is directed, through protoporphyrin, on the one hand towards the synthesis of

haem and haem proteins and on the other, towards bacteriochlorophyll synthesis (Lascelles, 1960).

Since growth under anaerobic conditions occurs only in the light and is dependent on photosynthetic pigments, adaptation of the organism from non-photosynthetic to photosynthetic growth results in increased demands and hence production of bacteriochlorophyll (Cohen-Bazire, Sistrom and Stanier, 1957). Such cells are highly pigmented and the bacteriochlorophyll content is approximately 40 to 100 times that of non-pigmented cells. During adaptation of these cells to photosynthetic growth, the concentration of haemproteins increases in parallel with that of bacteriochlorophyll production, although the actual amount of haemproteins formed is only approximately 1% of the amount of bacteriochlorophyll (Porra and Lascelles, 1965). The total amount of haemproteins in cell free extracts of pigmented cells is approximately twice that of non-pigmented cells (Porra and Lascelles, 1965).

Lascelles (1959) reported that bacteriochlorophyll synthesis could also be induced in cultures adapted to grow in the dark, provided the percentage of oxygen in the gas phase is extremely low. She also found that synthesis of bacteriochlorophyll was preceded by a rise in ALA synthetase activity. Both the synthesis of bacteriochlorophyll and the increase in

ALA synthetase activity were prevented by chloramphenicol, although enzymes unrelated to tetrapyrrole biosynthesis were not affected. These findings were the first real indication that bacteriochlorophyll synthesis might depend on prior production of ALA synthetase. At the same time it was demonstrated that induced bacteriochlorophyll synthesis was prevented by p-fluorophenylalanine and 8-azaguanine (Lascelles, 1959).

Subsequent studies with a variety of inhibitors of protein synthesis (Gibson, Neuberger and Tait, 1962a,b; Bull and Lascelles, 1963; Higuchi et al., 1965) indicated that while the induced synthesis of photosynthetic pigments, in particular bacteriochlorophyll, requires synthesis, not only of RNA and protein, but also of DNA, synthesis of DNA is not necessary for the increased formation of ALA synthetase.

(b) Repression of synthesis of ALA synthetase

(i) Effect of haem* on ALA synthetase formation

Lascelles (1959) found no evidence of substrate induction of synthesis of ALA synthetase and the alternate

---

*Experimental work dealing with the effects of haem (ferrous protoporphyrin) on ALA synthetase has been mainly carried out using haemin (ferric protoporphyrin). In this text, no distinction has been made, and references to the use of haem include that of haemin.

mechanism of endproduct repression of synthesis was therefore investigated (Lascelles, 1960; Goto et al., 1967). The finding that the addition of haem to cultures of R. spheroides actively synthesising ALA synthetase resulted in an immediate decrease in the rate of its production indicated that synthesis of ALA synthetase was repressed by the end product of the pathway (haem).* The duration of the lowered rate of production depends on the amount of haem added to the cultures (Goto et al., 1967). At very low concentrations, the original rate of enzyme synthesis was resumed as the added haem was used up by growth of the organism (Lascelles, 1960).

Although ALA synthetase production is inhibited by ALA, it is not certain whether ALA itself is responsible for the repression of enzyme synthesis or whether it serves merely as a precursor for an active tetrapyrrole, presumably haem (Lascelles, 1960; Goto et al., 1967) since protoporphyrin and magnesium protoporphyrin do not appear to affect the synthesis of the enzyme (Lascelles, 1960).*

(ii) Effect of oxygen and light of ALA synthetase formation

The requirement of R. spheroides for photosynthetic pigments is dependent on its growth conditions, and in

*See Appendix I

particular on the oxygen tension and the light intensity (Cohen-Bazire, Sistrom and Stanier, 1957). As already mentioned (p.13) synthesis of bacteriochlorophyll is accompanied by increased formation of ALA synthetase. The reverse situation has also been examined (i.e. the effect of light and oxygen on the production of ALA synthetase by organisms which are actively synthesising both enzyme and bacteriochlorophyll). It was demonstrated that inhibition of pigment formation in both anaerobic-light (Lascelles, 1960) and semi-anaerobic-dark grown cultures (Goto et al., 1967) by increased oxygen tension produced by vigorous aeration is in fact, accompanied by inhibition of enzyme synthesis. On withdrawal of oxygen from the gas phase, synthesis of both enzyme and pigment is resumed at an even faster rate (Lascelles, 1960). Both enzyme and pigment synthesis are also affected by light intensity, the rate being decreased by increasing the light intensity (Lascelles, 1960).

Marriott, Neuberger and Tait (1969) reported that during the first hour after disruption of cells, ALA synthetase in extracts of R. sphaeroides in which synthesis of the enzyme had been induced by low aeration, was spontaneously activated on standing at 4°. No such activation of the enzyme occurred

in extracts of the organism harvested from cultures which had been vigorously aerated. Furthermore, a heat stable, low molecular weight factor, capable of activating ALA synthetase, was isolated from extracts of the organism grown under the former conditions; whereas after vigorous aeration of cultures, a factor capable of inhibiting the activation process was isolated from cell free extracts of the organism. In vitro, the effect of the inhibitor could be counteracted by the addition of the activator. These authors therefore consider that the effect of oxygen on bacteriochlorphyll synthesis might be mediated through changes in the relative concentrations of an activator and an inhibitor of ALA synthetase.

It has also been suggested that changes in the oxido-reductive state of one or more carriers in the electron transport chain may be controlled by light and oxygen in the environment and that such changes are reflected by changes in the rate of bacteriochlorphyll synthesis (Cohen-Bazire, Sistrom and Stanier, 1957). The observation that exposure to light, as well as oxygen, results in oxidation of reduced cytochromes and other electron transport carriers of photo-synthetic bacteria supports this suggestion (Nishimura and Chance, 1963).

de Matteis (1967) pointed out that two different mechanisms appear to operate in the control of ALA synthetase levels in photosynthetic bacteria, one intrinsic to the haem biosynthetic pathway (end product repression of synthesis by haem) and the other extrinsic, integrating the whole haem biosynthetic pathway with the energy producing reactions of the cell.

On the other hand, Lascelles (1964, 1966) has postulated that photosynthetic bacteria might synthesise two distinct ALA synthetases, one for haem biosynthesis subject to repression by haem, and the other for bacteriochlorophyll synthesis, possibly controlled by oxygen.* This suggestion is supported by the finding that the synthesis of ALA synthetase is definitely repressed by haem while the synthesis of bacteriochlorphyll is barely affected (Goto et al., 1967). It has also been supported by Higuchi et al. (1968). They found that ALA synthetase in crude extracts of organisms, grown aerobically in the absence of light, was more stable to temperature and storage than the enzyme extracted from organisms in which the synthesis of the enzyme had been induced either in the light by anaerobiosis or in the dark by low oxygen tension. Moreover, in vivo levels of the enzyme in aerobic-dark grown cultures were barely affected

*See Lascelles, J.

by chloramphenicol or increased aeration, both of which caused a marked reduction in enzyme level in cultures of the organism in which synthesis of the enzyme had already been induced. On the basis of their results, the authors discussed the possible existence of two ALA synthetases. They concluded that *in vivo* the stability of the induced enzyme is markedly affected by a change in the intracellular oxidation-reduction state but that the other is not.

The title of a more recent publication by Kikuchi's group of workers* suggests however, that these latter results may be explained on the basis of findings similar to those of Marriott, Neuberger and Tait (1969). The work of Marriott, Neuberger and Tait (1969), has not only done much to elucidate some of the anomalies reported in studies of *R. sphaeroides* ALA synthetase (e.g. the early discovery of Kikuchi *et al.* (1958) that ALA synthetase activity in anaerobic-light grown organisms is inhibited by extracts of aerobic dark grown organisms and the unsuccessful attempts by Lascelles (1959) to reproduce this work) but it has also shown a means whereby the synthesis of bacteriochlorophyll could be efficiently controlled.

*See Appendix III.

B. Hepatic ALA Synthetase Levels in Experimental

Porphyria

(a) Effect of porphyrinogenic drugs on enzyme levels

The original observation of Granick and Urata (1963), that ALA synthetase activity in the livers of guinea pigs dosed with DDC was greatly elevated, was followed by reports that administration of other drugs (AIA (Tschudy et al., 1964; Marver et al., 1966b) and ethanol (Shanley, Zail and Joubert, 1968)) had similar effects.

As in the case of the reticulocyte enzyme (Laver, Neuberger and Udenfriend, 1958), Granick and Urata (1963) established that hepatic ALA synthetase is localised in the mitochondria. They also found that in intact liver mitochondria, in the presence of pyridoxal 5-phosphate, ALA is generated from added glycine and α-oxoglutarate (other intermediates of the tricarboxylic acid cycle can replace α-oxoglutarate). Provided that th mitochondria were first rendered permeable by freezing and thawing, ALA could be formed from added glycine and synthetic succinyl CoA.

While the activity of ALA synthetase is barely detectable in normal liver mitochondria, the activity of amino acetone (AA) synthetase is readily demonstrated. The latter enzyme

condenses glycine and acetyl CoA to form the amino ketone, amino acetone (AA) (Urata and Granick, 1963). Because of the similarity of the reactions catalysed by the two enzymes, the possibility was considered that the level of AA synthetase activity in liver might also be affected; however, this was not so and it was concluded that the action of porphyrinogenic drugs is rather more specific than this (Granick, 1966).

One of the features of DDC poisoning is the red fluorescent appearance of the liver (Solomon and Nigge, 1959) due to increased synthesis of porphyrins. Granick and Urata (1963) noted that the red fluorescent granules which accumulated in parenchymal liver cells contained mainly protoporphyrin. On the basis of this observation Granick (1963a,b) evolved a technique whereby the synthesis of porphyrins in cultured chick embryo liver cells could be estimated by fluorescence intensity. Quantitative measurements of protoporphyrin indicated that the fluorescence intensity is a reasonable guide to the amount of porphyrins synthesised by the cultured liver cells. Since it was also shown that ALA supply limits porphyrin synthesis in such cells (Granick, 1966) the fluorescence is also an indirect measure of the ALA synthetase level. (Existing methods of assay for ALA synthetase activity were not sufficiently sensitive for direct measurement of enzyme levels in such a system.)

(b) Factors which affect the drug induced synthesis of ALA synthetase

(i) Effect of inhibition of protein synthesis

The important observation that the drug induced synthesis of porphyrins in cultured chick embryo liver cells could be prevented by inhibitors of RNA and protein synthesis (Granick, 1963b, 1964, 1965, 1966) led to the conclusion that the increased hepatic ALA synthetase activity found *in vivo* following administration of DDC to guinea pigs (Granick and Urata, 1963) could be attributed to increased synthesis of the enzyme (Granick, 1966). Direct measurements of ALA synthetase levels in liver confirmed that the *in vitro* inhibition of porphyrin production by inhibitors of RNA and protein synthesis was in fact paralleled *in vivo* by their effect on the AIA mediated increase of ALA synthetase levels. AIA induced synthesis of the enzyme was inhibited by actinomycin D or puromycin, or prevented altogether when either of these compounds was administered simultaneously with the drug (Tschudy, Marver and Collins, 1965; Marver *et al.*, 1966c). The possibility that continuous synthesis and decay of ALA synthetase occurs and that inducing drugs prevent decay was ruled out by Granick (1966) since decay

of 'induced' enzyme occurred at the same rate whether caused by removal of drug or by addition of acetoxycycloheximide in the presence of the drug.

Narisawa and Kikuchi (1966) found that when ALA was administered to rats at 12 hourly intervals over a period of three days, the induced synthesis of ALA synthetase occurred in two stages; an initial small increase in the level of the enzyme was followed by a second phase in which the rate of synthesis was greatly increased. These workers also demonstrated that only during the second phase was enzyme synthesis prevented by mitomycin C, an inhibitor of DNA synthesis; the ALA induced synthesis of enzymes unrelated to haem biosynthesis was not affected by mitomycin C. Narisawa and Kikuchi (1966) suggested that the enzyme formed during the second stage of the induction process might represent mitochondrial synthesis of the enzyme and furthermore that it might be an isoenzyme of the enzyme formed during the first stage. More recently however, Hayashi, Yoda and Kikuchi (1969) have found that ALA synthetase also accumulates in the cytoplasm following drug administration and that the

properties of this enzyme differ in certain respects from those of the intramitochondrial enzyme*.

The half lives of ALA synthetase and of the mRNA involved in its synthesis have been calculated from the rate of decay of enzyme activity following administration of actinomycin D or puromycin to porphyric rats. It was found to be approximately 70 minutes for both the enzyme and the mRNA (Tschudy, Marver and Collins, 1965). Since the major portion of rat liver mRNA is thought to be stable for at least 40 hours, these workers considered that the unusually short half life of this particular mRNA allowed for rapid changes in the level of ALA synthetase in response to a requirement for haem synthesis. A similar investigation in cultured chick embryo liver cells indicated a longer half life for ALA synthetase of between 4 and 6 hours (Granick, 1966).

---

*See Appendix IV

(ii) Effect of haem on formation of ALA synthetase in liver

The possibility that haem itself may be involved in the repression mechanism is supported by experimental evidence. Granick (1966) reported that stimulation of *in vitro* porphyrin synthesis in cultured chick liver cells is decreased when haem is added to the culture medium at the same time as the inducing chemical. Hayashi *et al.* (1968) found that hepatic ALA synthetase levels were lower in rats treated with both haem and ALA than in rats treated with ALA alone. In a similar series of experiments (Waxman *et al.* 1966) it was shown that over a period of time, the level of ALA synthetase oscillated following the injection of haem to normal or ALA treated rats. The authors considered that the fluctuating levels of ALA synthetase were due to variations in the intracellular level of free haem and that as the free haem was metabolised to bilirubin or was combined with haem apoproteins, so the synthesis of ALA synthetase was derepressed.

As an example of such a situation, studies of the haem enzyme tryptophan pyrrolase (Marver *et al.*, 1965, 1966d) indicated that the increased binding of haem to the apoenzyme, such as occurs following the administration of tryptophan

(Feigelson and Greengard, 1962) results in decreased availability of haem for the repression of ALA synthetase and as a consequence, induced synthesis of the latter enzyme.

There is no evidence that drugs such as AIA and DDC cause elevation of ALA synthetase in erythroid cells. Levere and Granick (1965, 1967) have proposed that the production of ALA is an important regulatory factor in haemoglobin production by chick embryo blood island cells. A good deal of work has been carried out which indicates that haem may control the rate of haemoglobin synthesis (Zucker and Schulman, 1968; Adamson, Herbert and Godchaux, 1968; Adamson, Herbert and Kemp, 1969). However, the role of haem in this process is the subject of intensive interest at the present time and is regarded as being outside the scope of this thesis.

(ii) Effect of hormones on ALA synthetase formation in liver

Hydrocortisone (Marver, Collins and Tschudy, 1966; Matsuoka, Yoda and Kikuchi, 1968) and triiodothyronine (Matsuoka, Yoda and Kikuchi, 1968) have both been found to stimulate the ALA induced synthesis of ALA synthetase in the livers of normal and adrenalectomised rats; no increase in

ALA synthetase levels occurred however, when either of these compounds was administered in the absence of ALA.

On the other hand, sex steroids were found to stimulate porphyrin synthesis in cultured chick embryo liver cells in the absence of an inducing drug (Granick, 1966). In ovariectomised rats, a single dose of the naturally secreted oestrogen, oestradiol, gives rise to a series of oscillations in the level of ALA synthetase in the livers of both normal and ALA treated rats (Tschudy, Waxman and Collins, 1967), the amplitude of the oscillations being greater in the livers of the latter. The oscillations can continue for as long as 90 hours after administration of the oestrogen and the authors consider that they may reflect the influence of hormones at some stage of the circuit controlling the synthesis of ALA synthetase. (This is further discussed on p. 33.)

C. ALA Synthetase Levels in Hereditary Hepatic Porphyrias

As already mentioned (p. 11), the synthesis of ALA in autopsy liver obtained from patients who had died in an attack of acute intermittent porphyria was found to be increased approximately tenfold above normal (Tschudy et al., 1965;

Nakao et al., 1966). While these findings indicate that the basal level of hepatic ALA synthetase is higher than normal in acute intermittent porphyria and, as has been recently reported (Dowdle et al., 1967), also in porphyria variegata and symptomatic porphyria, there is, as yet, no real evidence to indicate that there is a causal relationship between high levels of the enzyme in liver and manifestation of the disease. Nevertheless, the similarity of the disturbances of porphyrin metabolism which occur both in hereditary and experimental porphyria, coupled with the fact that hepatic ALA synthetase levels are also raised in the latter condition, has constituted circumstantial evidence for the proposal that in inherited forms of the disease a genetic lesion leads to overproduction of ALA synthetase (Perloth et al., 1966).

D. Mechanism of Control of ALA Synthetase Formation

(a) Haem repression hypothesis

Granick and his co-workers have postulated a mechanism which could account for the increased synthesis of ALA synthetase which occurs under the influence of a number of drugs, sex steroids and miscellaneous compounds (Granick,

1966; Granick and Kappas, 1967a,b; Kappas and Granick, 1968; Kappas et al., 1968). This is shown in Fig. 1.2.

A repressor-operator control of the structural gene for ALA synthetase is proposed (Granick, 1966) in which the inactive aporepressor protein is activated by combination with haem. Thus the intracellular concentration of free haem (Warman et al., 1966), or of haem loosely bound to protein (Burnham and Lascelles, 1963) is the factor which determines whether or not production of ALA synthetase occurs. It is postulated that the operator gene for the ALA synthetase gene is normally blocked by the repressor and that porphyrinogenic drugs, by displacing haem from its binding site on the aporepressor, derepress the synthesis of ALA synthetase.

Although no evidence that the activity of ALA synthetase in liver is controlled by feedback inhibition by haem has been obtained (see p.40), this possibility is shown in Granick's scheme.

A secondary consideration was the possibility that the intracellular concentration of free haem might be lowered due to the haem requirement of liver enzymes (e.g. cytochrome P-450) involved in the detoxification of foreign chemicals. Fig. 1.2 also shows the relationship of microsomal cytochrome synthesis to the remainder of the scheme. In a comprehensive

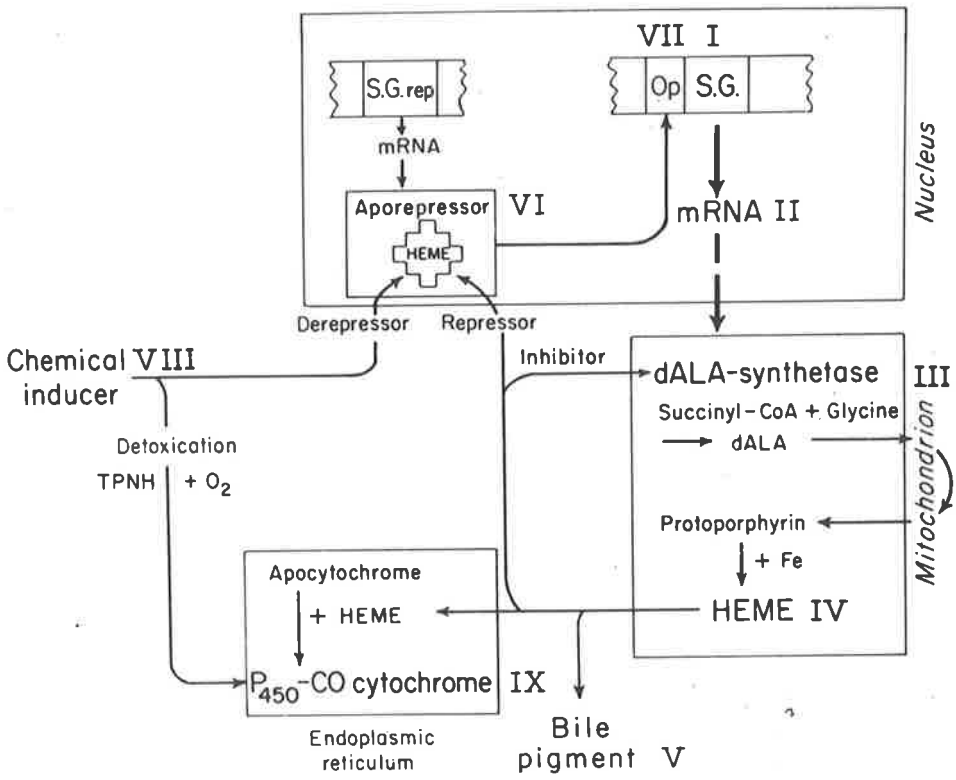


Fig. 1.2. (reproduced from Granick, 1966). Mechanism for the detoxification of chemical inducers in liver by derepression of the repressor control on the synthesis of ALA synthetase.

study of a series of porphyrinogenic drugs and various derivatives thereof. Schmeck et al. (1968) found that porphyria inducing ability was dependent on a sterically hindered ester or amide group. These authors suggested that drugs which cannot be metabolised by a hydrolytic mechanism because of such steric factors are oxidatively metabolised and, as already indicated by Granick (1966), increased haem formation is required for this process. It has recently been demonstrated that in general porphyrinogenic drugs induce the synthesis of cytochromes P-450 and b_5 (Kuntzman et al., 1968) whereas haem represses this synthesis (Marver, Schmid and Schützel, 1968).

One of the seeming drawbacks to Granick's hypothesis was the widely varying, and apparently unrelated, chemical structures of the inducing compounds. However, Granick (1966) pointed out that, in fact, a large number of these could be shown to contain one of five basic chemical groups, each of which had been found individually to be capable of inducing experimental porphyria, and certain of these may be regarded as structural analogues of haem. The justification for the proposal of a single corepressor site is based on the observation that the simultaneous addition of AIA and DDC in amounts which when given alone produced maximum fluorescence,

did not increase the rate of porphyrin synthesis. That the inducing drug and haem compete for the same binding site was suggested by the decreased fluorescence observed when both haem and ALA were added to the culture medium. The decreased synthesis of ALA synthetase *in vivo* in the presence of added haem has been demonstrated by direct measurements of the activity of this enzyme (Waxman *et al.*, 1966; Hayashi *et al.*, 1968).

With the stipulation that the controlling mechanism is associated with nuclear DNA, Granick (1966) does not exclude the possibility that the structural gene for ALA synthetase is associated with mitochondrial DNA and that synthesis of the enzyme could occur within the mitochondrion. As already mentioned (p.23) this possibility was discussed by Narisawa and Kikuchi (1966). It is known that all the DNA (Borst, 1966) and RNA (Winterberger, 1966) species necessary for protein synthesis are present within mammalian mitochondria. While the mitochondrial synthesis of some mammalian enzymes is inhibited by chloramphenicol (Firkin and Linnane, 1968), Granick (1966) found this compound increased porphyrin synthesis in the cultured chick embryo liver cells, possibly due to the fact that its chemical structure includes two of the essential

inducing groups.

The inheritance of the porphyrias and the type of genetic lesions which could be responsible have been extensively reviewed by Watson et al. (1964). It was considered that a defect in the operator controlling a structural gene could be responsible for the dominant character of the hereditary hepatic porphyrias. This assumption is compatible with Granick's (1966) theory which postulates that the resulting limited efficiency of the repressor mechanism could be responsible for the manifestation of latent porphyria caused by very small doses of inducing drugs.

Alternatively, as Perloth et al. (1966) pointed out, induced synthesis of ALA synthetase could also occur if a genetic defect led to decreased levels of "repressor" haem within the liver cell. For example, Rechcigl and Heston (1967) reported that in mice there are genetic differences in the rates of degradation of haem enzymes.

The process of induction of porphyrin synthesis in cultured liver cells by steroids (Granick and Kappas, 1967b) so closely resembled that of the synthesis induced by drugs that the original hypothesis (Granick, 1966) was extended to

include a mechanism whereby haem biosynthesis might be controlled under normal physiological conditions (Kappas and Granick, 1968). Of the many steroid compounds examined for their effect on porphyrin synthesis (Granick and Kappas, 1967a,b), the most potent inducers were normal metabolites of hormones such as progesterone and testosterone. Moreover, the induction of porphyrin synthesis by these steroids was prevented by inhibitors of protein synthesis and by haem (Kappas and Granick, 1968). The characteristics of porphyrin induction by steroids fulfilled the requirements necessary for considering the effects to be physiologically meaningful. Firstly, the chemical structure was sharply specific; it was found that only a limited number of steroids with 5β-H configuration were potent inducers. Secondly, very small amounts of these compounds were required; porphyrin synthesis was induced by concentrations as low as 10^{-8}M. Thirdly, the inducing compounds were physiological products and were also capable of stimulating haem biosynthesis in erythroid tissue (Granick and Kappas, 1967a). Furthermore, natural hormones are known to aggravate symptoms of acute intermittent porphyria (Goldberg and Rimington, 1962; Welland et al., 1964b).

Kappas and Granick (1968) proposed a scheme whereby

such steroid compounds might operate to induce the synthesis of ALA synthetase. This is shown in Fig. 1.3 and is based on a number of experimental observations. (The role of haem in the repression mechanism has already been discussed on p.29 .) Firstly, it was found that the glucuronides of even the most potent of the steroid inducers were comparatively inactive inducers of porphyrin synthesis. Secondly, addition of uridine diphosphate glucuronic acid (UDP-glucuronic acid) to the cultured cells inhibited steroid induced porphyrin synthesis. Kappas and Granick (1968) considered this inhibition to be a reflection of the conversion of free steroid inducer to inactive glucuronide and moreover, that the induction of porphyrin synthesis is dependent on the amount of unconjugated steroid present in the cell at any particular time. The activity of UDP-glucuronyltransferase, the enzyme responsible for the transfer of glucuronic acid to steroids, is enhanced by UDP-glucuronic acid and inhibited by 5β-H steroids. Alternatively, active steroids could accumulate in a situation such as starvation or lack of glucose when the hydrolysis of glucuronides is enhanced. Thus although a mechanism for maintaining a balance between the amount of active and inactive steroid exists within the cell, this mechanism can be disturbed relatively easily.

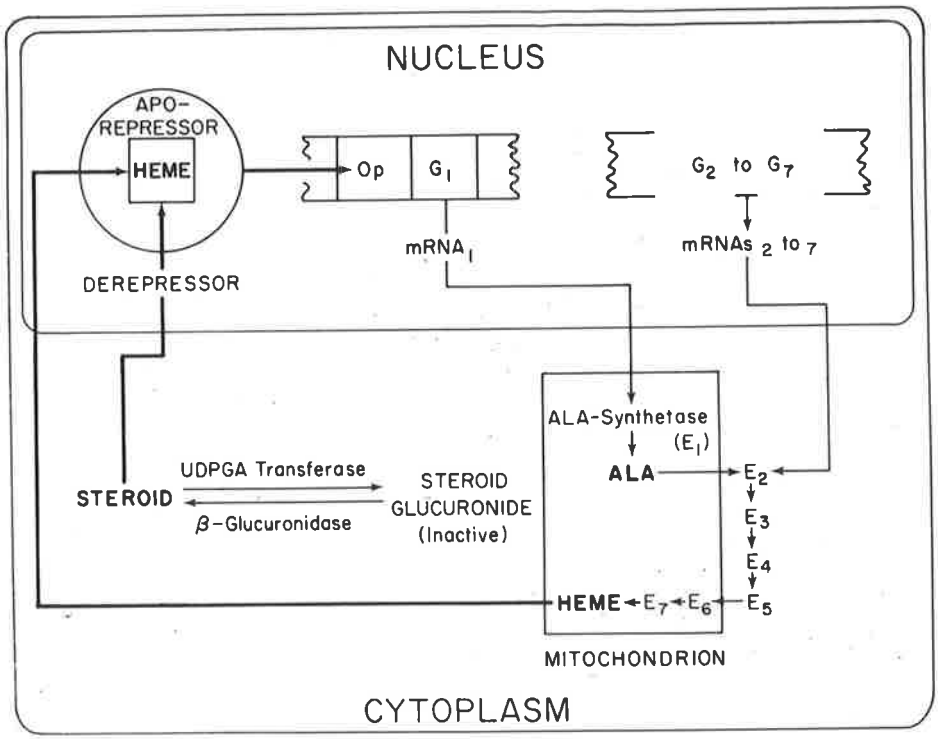


Fig. 1.3. (reproduced from Kappas and Granick, 1968).

Mechanism for steroid control of porphyrin and haem biosynthesis.

Kappas and Granick (1968) discussed the repression of ALA synthetase production by glucose (Tschudy et al., 1964; Marver et al., 1966c) as the possible result of such a disturbance. In the scheme proposed by Kappas and Granick (Fig. 1.3), the relative rates of glucuronidation and glucuronide hydrolysis determine the intracellular concentration of free active steroid which in turn competes with haem for the corepressor site on the aporepressor protein. Thus the relative concentration of each can control the rate of haem biosynthesis in a normally functioning cell.

(b) Alternative hypotheses on mechanism of control of ALA synthetase formation and porphyrin synthesis

Alternative hypotheses for control of ALA synthetase have been proposed. The evidence for these will be summarised briefly. Labbe (1967) has postulated that drug induced porphyrin synthesis can be explained by inhibition of terminal oxidation by drugs which results in accumulation of NADH. This in turn results in reduction of fumarate to succinate and increase of succinyl-CoA. The latter compound is postulated to act as a substrate-inducer of ALA synthetase formation. The evidence that succinyl CoA does accumulate is indirect;

the claim (Labbe, Kurumada and Onisawa, 1965) that the rise in ALA synthetase is preceded by the appearance of a new succinyl CoA synthetase has not been reproducible either in this laboratory (R.L. Walsh and W.H. Elliott, personal communication) or by Natsuoka, Yoda and Kikuchi (1968). However, this does not affect the main argument presented by Labbe (1967) that porphyrin synthesis is secondary to inhibition of terminal oxidation. Labbe's hypothesis alsopostulates that natural porphyrias are due to a genetic defect in the terminal oxidase system, and the mechanism of the postulated system is described fully by Labbe (1967).

If terminal oxidation is defective in the presence of porphyrinogenic drugs, then defective ATP synthesis might be expected. The school of thought led by Gajdos postulates that it is the ATP level which controls porphyrin biosynthesis. Support for this idea was that malonate reduced the ATP level in liver of rats and stimulated porphyrin production (Gajdos, 1966). Administration of inosine (to raise the ATP level of liver) was said to reduce the liver porphyrin content by 80% (Lottsfeldt, Labbe and Aldrich, 1961). Sedormid intoxication lowers the ATP level and recent evidence that porphyrin production caused by drugs is associated

primarily with lowered ATP rather than with increased NADH level was presented by Gajdos and Gajdos-Török (1968).

It is clear that the mechanism of control of haem synthesis and its derangement, either naturally or by drugs, is not fully understood. It is perhaps worth noting that any postulated explanation of acute intermittent porphyria needs to cover the neurological symptoms as well as the raising of ALA synthetase levels.

4. CONTROL OF ALA SYNTHETASE ACTIVITY

A. Control of Enzyme Activity in R. spheroides

In addition to repression of the synthesis of ALA synthetase by haem, it has been shown that the activity of ALA synthetase can also be controlled by haem (Gibson et al., 1961; Burnham and Lascelles, 1963; Marriott, Neuberger and Tait, 1969). In partially purified enzyme preparations obtained from crude extracts of R. spheroides (Burnham and Lascelles, 1963), the addition of haem results in inhibition of ALA synthetase activity. The enzyme was found to be extremely sensitive to this compound, 40% inhibition being obtained with a concentration of haem at 0.1 μM. However, Marriott, Neuberger and Tait (1969) found that while the activity of the activated form of the enzyme was markedly inhibited by haem, the non-activated form was barely affected.

In a study of the kinetics of haem inhibition of ALA synthetase activity, Burnham and Lascelles (1963) found the inhibition to be non-competitive with respect to glycine and

dilution experiments established that the inhibition is reversible. Although a Lineweaver-Burk plot of the kinetic data indicates pure non-competitive inhibition, Burnham and Lascelles (1963) draw attention to the non-linear relationship between the amount of added haem and the degree of inhibition of ALA synthetase and they suggest that the inhibition is not the result of a simple stoichiometric reaction between haem and a catalytic site on the enzyme. In discussing the nature of the inhibition, Granick (1966) and de Matteis (1967) suggest since the inhibitor bears no steric resemblance to the substrate, that it is an example of "allosteric" inhibition in which it is thought that the inhibitor alters the conformation of the enzyme so that its affinity for the substrate is reduced (Monod, Changeux and Jacob, 1963; Stadtman, 1965).

A number of iron tetrapyrroles and metal porphyrins also inhibit ALA synthetase, but are not nearly as effective as haem itself. Of haem proteins tested, only haemoglobin and myoglobin were found to be inhibitory and much higher concentrations were required than of free haem (Burnham and Lascelles, 1963).

The only situation in which non-functional porphyrins accumulate during growth of R. spheroides occurs when the

growth medium is deficient in iron (Lascelles, 1955); at the same time the synthesis of haem is reduced (Lascelles, 1956). While no evidence has been obtained which indicates that the overproduction of porphyrins by such cells is due to increased levels of ALA synthetase (Lascelles, 1960, Goto et al., 1967) it was found, in intact cells, that exogenous haem inhibited the synthesis of porphyrins from glycine and α-oxoglutarate, but not from ALA (Burnham and Lascelles, 1963). These findings indicate that ALA synthetase is the site for negative feedback control of porphyrin biosynthesis by haem.

In intact cells porphyrin synthesis from glycine and α-oxoglutarate, but not from ALA was found to be inhibited by purine nucleotides (Gajdos and Gajdos-Török, 1963). While it is not considered that the action of these compounds is directly on ALA synthetase, it is suggested that the reduced synthesis of ALA is due to the formation of an "unknown" inhibitor which favours the use of succinate for metabolic pathways other than ALA synthesis, and thus limiting the supply of adequate amounts of substrate (Gajdos and Gajdos-Török, 1965). The penetration of intact cells by nucleotides would not be expected to occur

readily and this consideration makes interpretation of this work more difficult.

B. Control of ALA Synthetase Activity in Animal Systems

That a similar controlling mechanism to that in R. spheroides exists in rabbit reticulocytes is indicated, although indirectly only, by the work of London, Bruns and Kariban (1964) and Kariban and London (1965). These workers found that the incorporation of labelled glycine into haem was markedly reduced by exogenous haem, whereas the incorporation of labelled ALA into haem was barely affected.

There is no evidence that the activity of hepatic ALA synthetase can be controlled by feedback inhibition. Granick (1966) found that haem and a number of other metal porphyrins had no effect on the synthesis of ALA from citrate and glycine in mitochondria isolated from livers of guinea pigs treated with DDC, and Marver et al. (1966d) were unable to demonstrate inhibition of ALA synthetase activity in homogenates of liver from ALA treated rats using physiological concentrations of haem (0.05 mM).

5. THERAPY OF THE HEPATIC PORPHYRIAS

To alleviate the symptoms of acute intermittent porphyria avoidance of porphyrinogenic compounds such as barbiturates is obviously important (Goldberg, 1959). Diet is also important since a high carbohydrate content alleviates the clinical condition (Welland et al., 1964a). Moreover, glucose "represses" ALA synthetase formation in experimental porphyria (Tschudy et al., 1964; Marver et al., 1966c). In the case of the hereditary condition, it has been suggested (Granick and Kappas, 1967a; Kappas and Granick, 1968) that this is due to high glucose causing efficient removal of inducing steroids by glucuronidation.

Massive dosing of patients with CaEDTA is clinically effective in therapy of acute intermittent and other porphyrias (Peters et al., 1958; Woods et al., 1961; Peters et al., 1966; Donald et al., 1969). Originally based on the concept that heavy metal accumulation may be responsible for the disease, the treatment now has no obvious rational basis. Claims that intravenous injection of cytochrome C (Lang et al., 1968), AMP

(Gajdos, Plainfosse and Scringe, 1967) or pyridoxal 5-phosphate (Petres *et al.*, 1968) alleviates porphyrias are difficult to interpret in view of the uncertainty about the effectiveness of cellular penetration by these compounds.

The difficulty in assessing the clinical effectiveness of treatments for a disease which may undergo spontaneous remission is obvious. Goldberg (1968) summarised the present position by saying that "there is still no rational method for a fundamental approach to the treatment of porphyrias, but a great deal has been done already in lowering the mortality rate".

6. AIMS OF THE WORK IN THIS THESIS

Despite the very great progress which has been made towards understanding both genetically determined and drug induced porphyria, the preceding account makes it clear that the final answers are not unequivocably established and that much work remains to be done on ALA-synthetase control.

There appeared to be at least two areas of work which might contribute ultimately to the understanding of ALA-synthetase control. The first of these was the development of a method of assay, more sensitive than existing ones, which might permit direct quantitative measurements of ALA synthetase in normal tissues in those experimental systems such as cultured chick embryo cells which the Rockefeller group has used so successfully. The direct quantitative measurement of activity in such systems might be expected to enable finer details of the induction to be studied. It might also help with problems such as where ALA synthetase is actually made, inside or outside the mitochondria.

The second area of work that seemed desirable was the study of the animal ALA synthetase itself. A knowledge of

the molecular nature of ALA synthetase, its properties, its control characteristics and whether only a single enzyme exists seems a useful basis for considering control of its synthesis. A knowledge of its active site could also well facilitate development of specific inhibitors as therapeutic agents.

It perhaps should be noted that initially at least, the above two aims seemed to be inter-related. It had been intended that with an accurate measurement of ALA synthetase of normal liver, possible isolation of the enzyme from a source such as ox liver, available in almost unlimited quantities, might be contemplated. The unexpectedly low levels found in normal livers frustrated this idea.

This thesis reports the development of a sensitive radio-chemical assay for ALA synthetase (Irving and Elliott, 1969) and its application to micro-organisms and plant and animal tissues. In addition, the enzyme has been partially purified from the livers of guinea pigs made porphyric with DDC and preliminary studies of the enzyme in partially purified preparations made.

Because of the extreme instability of ALA-synthetase in mitochondrial preparations obtained from the livers of porphyric guinea pigs, initial studies relating to the radiochemical

assay, to stability and purification procedures, were carried out on soluble preparations of the enzyme obtained from *R. sphaeroides*.

CHAPTER 2
MATERIALS AND METHODS

1. Chemicals

ATP (disodium salt, 99% pure), CoA (85% pure), GSH, and pyridoxal 5-phosphate were Sigma products. Solutions of these compounds were adjusted to pH 7.4 with tris base and were stored at -15°. ^{14}C-1,4-succinic acid and ^{14}C-2-glycine were obtained from the Radiochemical Centre, Amersham. ALA-HCl was obtained from Calbiochem and AA was prepared as the p-toluene sulphonic salt as described by Elliott (1960a). ALA and AA pyrroles were prepared from ALA-HCl and the p-toluene sulphonic acid salt of AA respectively, by condensation with acetyl acetone (Urata and Granick, 1963). Acetyl phosphate was prepared from acetic anhydride, K_2HPO_4 and lithium hydroxide and twice recrystallised (Avison, 1955). Solutions of acetyl phosphate were stored frozen at -15°. Haem (ferric protoporphyrin) and haem proteins were Sigma products. Solutions of haem were freshly prepared when required as described by Burnham and Lascelles (1963). ALA was donated by Roche Products, Basle, Switzerland, and DDC was obtained from Aldrich or Eastman. The DDC was twice re-crystallised from ethanol. Tris base (Eastman) was twice re-crystallised from ethanol (Sutherland and Wasilait, 1956). Other

chemicals were, wherever available, of Analytical Reagent grade.

Buffers.

Phosphate buffers were prepared from KH_2PO_4 and K_2HPO_4, and acetate buffers from acetic acid and sodium acetate. Pyridine-acetate buffer was prepared by adjusting the pH of solutions of pyridine with glacial acetic acid. All other buffers were prepared by adjusting the pH of solutions with HCl or KOH.

Ehrlich reagent.

(a) For colorimetric assays of AA and A A, the reagent was freshly prepared when required as a 2% (w/v) solution of p-dimethylamino-benzaldehyde in glacial acetic acid containing 16% (v/v) of 70% perchloric acid and 0.3% (w/v) of $HgCl_3$ (Urata and Granick, 1963).

(b) For detection of AA and A A pyrroles on chromatograms, the reagent was prepared as a 1% (w/v) solution of p-dimethylamino-benzaldehyde in 1N HCl which was diluted with an equal volume of acetone (Dent, 1948).

Scintillation fluid.

This was a solution of 0.3% (w/v) 2,5 diphenyloxazole (PPO) and 0.03% (w/v) 1,4-bis [2-(4-methyl-5-phenyloxazolyl)]

benzene (dimethyl POPOP) in toluene. Both PPO and POPOP were obtained from Packard Instrument Co.

Thixotropic gel.

2.5% (w/v) Cabo-O-sil (Packard Instrument Co.) was suspended in scintillation fluid and the mixture homogenised in a blendor for 4 minutes.

2. Chromatographic adsorbents and gels.

DEAE-cellulose (Whatman) was washed with 1N NaOH until the washings were colourless, then with water followed by 1N HCl and water again. It was then washed with 0.1N NaOH, followed by water until the washings were neutral. It was stored at 4° as a suspension in water. Before use, it was equilibrated in the column with the required buffer.

DEAE-sephadex (Pharmacia, Uppsala, Sweden) was equilibrated in the required buffer for at least 24 hours before use.

Dowex 50 (H^+) (AG-50 x 8) Biorad, California) was prepared for use by washing with 5N HCl, followed by water until the washings were neutral.

Kieselgel G 7731 (Merck) was prepared as a slurry with twice its weight of water and shaken for 90 seconds before spreading on glass plates. The thin layer chromatography plates were

dried at 105° and stored at room temperature.

Sephadex G-100 (medium) and G-200 (medium) (Pharmacia, Uppsala, Sweden) were allowed to stand in water for at least 3 days before use. The gels were equilibrated in columns with required buffers.

3. Animals.

All animals were obtained from the Institute of Medical and Veterinary Science, Adelaide, South Australia.

Guinea pigs, unless otherwise stated, were males (5-6 months old) weighing 450 - 500 g.

Rats were albino males, weighing between 150 - 200 g.

Elevation of ALA synthetase in mammalian liver.

DDC treatment of guinea pigs. The animals were fasted for 48 hours prior to treatment. 2 g of DDC were suspended in water and administered orally. After 24 hours the animals were killed by a blow on the head.

ALA treatment of rats. The animals were fasted for 48 hours before a solution of ALA (30 mg/ml) was injected subcutaneously. ALA was administered at the rate of 400 mg/kilo body weight. After 24 hours the animals were killed by a blow on the head.

Normal controls, either rats or guinea pigs, were starved for 48 hours before killing.

Isolation of liver mitochondria.

After killing the animals, the livers were removed and immediately cooled on ice. Mitochondria were isolated by differential centrifugation (Schneider and Hogeboom, 1950) and after washing in 0.25M sucrose were finally resuspended in 0.25M sucrose containing 10^{-3}M Tris-HCl and 5×10^{-4} EDTA (pH, 7.4) using a Potter-Elvejhem homogeniser. Protein concentrations of mitochondrial preparations ranged between 20 and 80 mg/ml, although final volumes of the suspensions were adjusted to contain mitochondria from a constant weight of liver (8 g wet weight of liver/ml of suspension). The mitochondrial suspensions were dispensed in small vials, rapidly frozen and stored at -15°. Unless otherwise stated, all preparations used were once frozen and thawed.

Liver homogenates were prepared as described by Tschudy et al. (1965).

4. Avian reticulocytes.

Adult chickens were made anaemic by continued injection of phenylhydrazine (Bertles and Beck, 1962). Packed cells, obtained from the blood of these birds were frozen to disrupt cell membranes. Cells, fractionated by equilibrium centrifugation on a bovine serum albumin density gradient (Kabat and Attardi, 1967), were provided by Mr. A.F. Williams of the Department of Biochemistry, University of Adelaide. Fractions from the gradient, diluted to contain the same number of cells (2×10^9) per ml were frozen to disrupt cell membranes.

5. Plant material.

Spinach (Spinacia oleracea) plants were grown in liquid culture as described by Spencer and Possingham (1960) for tomato seedlings except that the spinach seeds were germinated and grown in vermiculite for 11 days before transfer into the nutrient solution of Tsui (1948). Leaves (1-2 inches long) were harvested when the seedlings were 3-4 weeks old.
Bean (Phaseolus vulgaris var Brown Beauty) plants were grown in vermiculite at 25° for 11 days either in the light as described by Boardman and Anderson (1964) or in darkness.

Preparation of cell-free fractions of plant tissues.

Spinach leaf fractions.

Washed chloroplast fraction. A cell free homogenate of spinach leaves (20 g) was prepared by the hand chopping method of Spencer and Wildman (1964) in a buffer (50 ml) containing 0.4M sucrose, 0.01M KCl, 0.01M $MgCl_2$, 4 mM 2-mercaptoethanol and 0.05M tris-HCl buffer, pH 7.8; the crude homogenate was filtered through three layers of Miracloth (Chicopee Mills Inc., New York, N.Y., U.S.A.). Subsequent fractions were prepared at 4° in the same buffer. The filtered homogenate was centrifuged at 1,000 x g for 10 minutes and the pellet resuspended in the same buffer (final volume 30 ml) with gentle shaking and stirring. Much of the mitochondrial contamination was removed from this suspension by centrifuging again as above. The pellet was resuspended in the same buffer from which the 2-mercapto-ethanol was omitted (final volume 3 ml) in a Potter-Elvejhem homogeniser.

Mitochondrial fraction. The two supernatants from the above centrifugations were combined and centrifuged at 5,000 x g for 10 minutes to remove many of the chloroplast fragments, and the pellet was discarded. The supernatant was centrifuged at 15,000 x g for 20 minutes and the pellet

resuspended in buffer (final volume 3 ml) in a Potter-Elvejhem homogeniser.

Bean primary-leaf fractions.

Washed chloroplast fraction and washed proplastid fraction were prepared from the primary leaves of light grown and dark grown (etiolated) seedlings respectively by the procedure used to prepare the washed chloroplast fraction from spinach leaves.

6. Micro-organisms.

Torulopsis utilis was supplied by Dr. Frank Moss, Department of Biochemistry, University of New South Wales.

Saccharomyces cervisiae (Strain M) was supplied by Professor A.W. Linnane, Department of Biochemistry, Monash University, Clayton, Victoria.

Euglena gracilis (Strain Z) was supplied by Dr. R.M. Smillie, Plant Physiology Unit, C.S.I.R.O., University of Sydney.

Maintenance T. utilis and S. cervisiae were maintained on slopes of malt yeast-agar. E. gracilis was maintained in sloppy agar slopes of the following composition: 0.25% (w/v) agar, 0.2% (w/v) yeast extract, 0.05% (w/v) Bacto broth and vitamin 0.004% (w/v).

Growth of organisms. T. utilis was grown anaerobically for 18 hours at 30° under N_2 in a medium similar to that described by Ephrussi and Slonimski (1950); the concentration of Difco yeast extract was lowered from 1% to 0.5% and ergosterol 0.002% (w/v) and Tween 80 0.5% (w/v) were added. S. cerevisiae was grown in the same medium for 18 hours at 30° both under N_2 and in vigorously shaken aerobic cultures. Lactate grown cells of S. cerevisiae were grown in vigorously shaken aerobic conditions for 18 hours at 30° in the medium of Ephrussi and Slonimski (1950) in which the glucose was replaced by 3% sodium lactate; again, the Difco yeast extract was lowered from 1% to 0.5%. E. gracilis was grown heterotrophically in the light (700 ft. candles) at 25° for 4 days in the medium of Hutner et al. (1956); or at 25° in the dark for 4 days, followed by 4 hours illumination at 125 ft. candles.

Preparation of cell-free extracts.

Crude extracts. After being harvested and washed in 0.02M tris-HCl buffer (pH 7.4) the organisms were suspended in fresh buffer to give a final concentration of approximately 150 mg dry weight/ml and then disrupted in the following manner: T. utilis and S. cerevisiae cells were broken by passing the cell suspension three times through the pressure

cell described by Milner *et al.* (1950) at 15,000 lb/in^2. Whole organisms and debris were removed from broken cell suspensions by centrifugation at 6,000 x g for 15 minutes at 4°. These extracts contained 20 - 30 mg protein/ml. *E. gracilis* cells were harvested and washed in 0.02M tris-HCl buffer (pH 7.4). A cell suspension in the same buffer was passed once through the high pressure cell at 1,125 lb/in^2. Cell debris and paramylum granules were removed by centrifuging at 200 x g for 5 minutes.

7. Preparation of enzymes.

Bacterial ALA synthetase.

Rhodopseudomonas spheroides (N.C.I.B. 8253) was maintained as stab cultures (Lascelles, 1956). Inoculum cultures (30 ml) were grown in dark semi-anaerobic conditions in Medium S containing 0.2% yeast extract (Lascelles, 1956). 10 ml of this were inoculated into 1l batches of Medium S and grown in dark semi-anaerobic conditions according to Higuchi *et al.* (1965) for 36 hours. Cells were harvested and washed as described by Lascelles (1956). Crude enzyme extracts were prepared from freeze-dried organisms by the method of Kikuchi *et al.* (1958), using 0.005M phosphate buffer, pH 6.9. The

100,000 x g supernatant was dialysed against the same buffer, changed twice, for three to four hours. The enzyme was stored frozen in small vials at -15°.

Bacterial succinyl CoA synthetase

Purified preparations of the enzyme were obtained from Escherichia coli (Crookes strain) as described by Ramaley et al. (1967). The enzyme was purified to the stage of first elution from DEAE-Sephadex; column fractions were pooled and the enzyme precipitated by ammonium sulphate. Phosphate was removed from the redissolved protein precipitate by dialysis against 0.05M tris-HCl (pH 7.2) containing 0.01M KCl. The enzyme was stored frozen in small vials at -15°, and diluted with 0.1M tris-HCl (pH 7.2) as required. Dilution was such that the activity was approximately 10 µmoles of succinhydroxamic acid produced/30 minutes/ml of enzyme solution, as determined by the method of Kaufman (1955).

Bacterial phosphotransacetylase

Crude extracts of the enzyme were prepared from Escherichia coli (strain B), grown overnight in nutrient broth. The harvested and washed organisms, resuspended in 1.5 to 2 volumes of 0.1M tris-HCl (pH 7.8) containing 0.14M magnesium acetate and 0.06M KCl, were disrupted by ultra-sonication. The resulting suspension was centrifuged for

30 minutes at 100,000 x g and the precipitate discarded. The supernatant, dialysed overnight against the same buffer, was stored frozen in small vials at -15°. The average protein content of these extracts was 4.0 mg/ml and the activity (measured by the method of Stadtman, 1955) in 0.05 ml was 5 μmoles of acetyl phosphate degraded/15 minutes.

8. Determinations

Estimation of ALA

ALA in trichloracetic acid supernatants of incubations or in solutions of synthetic ALA-HCl was converted to ALA pyrrole by the procedure described in this chapter (p. 46). After cooling, colour was developed by the addition of an equal volume of Ehrlich reagent. The amount of ALA originally present was calculated from the molar absorbancy of this solution at 552 mμ as described by Urata and Granick (1963).

Estimation of AA

AA in trichloracetic acid supernatants of incubations or in solutions of the p-toluene sulphonic acid salt of AA was converted to AA pyrrole as described in this chapter (p46). The same procedure described above for the

estimation of ALA was then applied and the amount of AA originally present was calculated from the molar absorbancy at 552 mμ as described by Urata and Granick (1963).

Estimation of ALA and AA in mixtures of both

When ALA and AA were to be determined separately in mixtures of both, the ether extraction procedure of Granick (1966) was used (modifications are described in the relevant sections).

Estimation of protein

The concentration of protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine plasma albumin (Armour Laboratories) as the standard.

Estimation of haem

Total haem content of crude extracts was determined from the difference spectra of reduced and oxidised pyridine haemochromagen as described by Porra and Jones (1963).

Estimation of chlorophyll

Total chlorophyll content of plant cell fractions was determined by the method of Arnon (1949).

CHAPTER 3

PRELIMINARY STUDIES ON ALA SYNTHETASE FROM RHODOPSEUDOMONAS SPHEROIDES

As already described (p. 43) the overall aim of the research project, of which the work in this thesis is the first part, was to further study ALA synthetase of liver mitochondria and its control, and also to investigate possible therapeutic measures for treatment of acute intermittent porphyria. As a first approach to this it was decided that a more sensitive assay method for ALA synthetase was needed so that normal tissues and small amounts of tissue could be quantitatively investigated. Secondly, it was decided that a study of the liver ALA synthetase was necessary since very little is known about it as an enzyme.

In view of these aims, initial work on a bacterial enzyme may seem paradoxical. However, first attempts to develop a new assay method for the liver enzyme ran into the serious difficulty of enzyme instability. The fact that even storage at -15° gave large and unpredictable losses, coupled with the necessity to obtain the enzyme from drug treated guinea pigs, made initial work on the animal enzyme

very difficult. As a result of this, and despite the risk that the animal and bacterial enzymes could prove to be quite different, it was decided to carry out initial preliminary studies on R. spheroides extracts in the hope that information and experience gained would be useful as a guide to the study of the animal enzyme. To a reasonable extent this proved to be the case.

The work in this chapter describes (a) the colorimetric assay of R. spheroides ALA synthetase based on existing procedures, (b) a study of the stability of the enzyme and (c) a partial purification of the enzyme. It perhaps should be emphasised that the work was strictly of a preliminary nature and no attempt was made to investigate the enzyme in any detail. The work is described however, despite this since it is relevant to later parts of the thesis.

1. RESULTS

A. Colorimetric Assay of ALA Synthetase of Crude R. spheroides Extracts

R. spheroides cells were grown semi-anaerobically in the dark and extracts prepared as described in Materials and Methods (p. 55). These soluble, dialysed extracts were used

in all subsequent work described in this chapter.

The incubation system for assay of enzyme activity (described in full in Fig. 3.1) contained, in addition to other components, purified E. coli succinyl CoA synthetase to generate succinyl CoA. Although Lascelles (1959) has reported the presence of this enzyme in crude extracts of R. sphaeroides, the E. coli enzyme was found to be essential; very little ALA synthetase activity could be detected in its absence. It was also determined that optimal activity was obtained in this system at pH 7.4 in tris-HCl buffer. Separate controls showed that the E. coli preparation contained no ALA (or AA) synthetase activities.

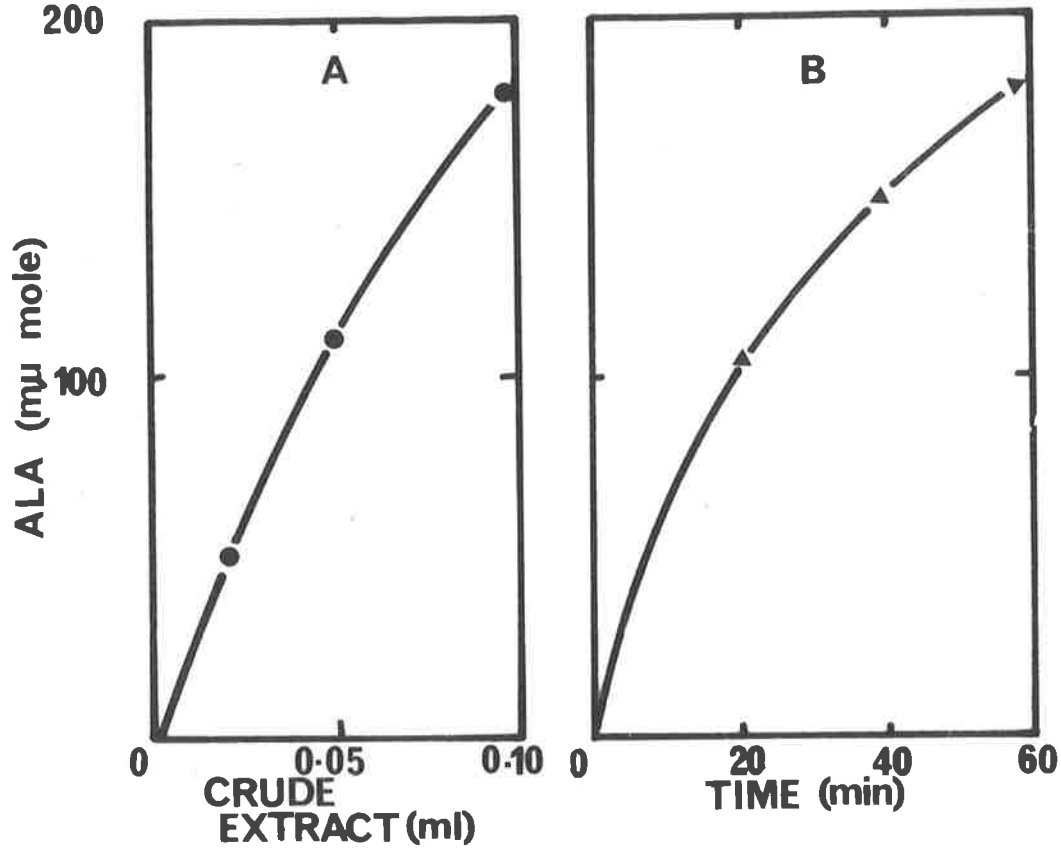
The crude extracts of R. sphaeroides produced AA; this was shown by conversion of the aminoketones to pyrroles and extraction of the solution at pH 7.0 with ether (Granick, 1966). The ether extract was found by thin layer chromatography to contain AA pyrrole. For this reason the ether extraction procedure of Granick (1966), slightly modified as described in Fig. 3.1, was used for all assays. The results in Fig. 3.1A show that the method gives reasonable proportionality between the amount of ALA formed and enzyme concentration. The time course of the reaction (Fig. 3.1B) which is linear for approximately 20 minutes, is also shown. Additional

Fig. 3.1. Colorimetric assay of ALA synthetase activity in crude extracts of R. spheroides.

A. Effect of enzyme concentration.

B. Time course of enzymic reaction.

Incubation mixtures contained in μmoles (unless otherwise stated); tris-HCl, pH 7.4, 100; glycine, 100; potassium succinate, 100; $MgCl_2$, 40; CoA, 0.25; pyridoxal 5-phosphate, 2; ATP, 10; GSH, 4; succinyl CoA synthetase, 0.2 ml; R. spheroides extract and water to a final volume of 2.0 ml. Tubes were incubated at 37° in a shaking water bath for 60 minutes and the reactions were stopped with 2 ml of 0.3M trichloracetic acid. After centrifugation, 2.5 ml of the supernatants were mixed with 0.75 ml of 1.0M sodium acetate and 0.1 ml of acetyl acetone added. These solutions were heated at 100° for 15 minutes and after cooling, 1.0 ml of each was treated with 1 ml of Ehrlich reagent and total pyrrole present measured. The remainder (2.35 ml) was adjusted to pH 7.0 with 0.05 ml of 0.5M Na_2HPO_4 and 0.15 ml of 1.0M NaOH and AA pyrrole extracted into ether (Granick, 1966). ALA pyrrole in the aqueous layer was measured and corrected for AA pyrrole remaining unextracted according to the formula of Granick (1966). (This latter correction formula was adjusted to allow for the altered volumes used here.) Values given refer to ALA in the complete incubation mixtures.


200
100
ALA (mμ mole)
A
B
0
0·05
0·10
CRUDE
EXTRACT (ml)
0
20
40
60
TIME (min)

experiments in which the trichloracetic acid supernatants were directly treated with Ehrlich reagent showed no detectable PBG formation by the crude extracts.

The standard procedure of assay described in Fig. 3.1 was therefore adopted routinely for all of the work reported in this chapter and assays were adjusted to give not more than 100 nmoles of ALA so as to be on the virtually linear part of the curve.

B. Studies on the Stability of ALA Synthetase in Crude Extracts of R. spheroides

(a) Effect of temperature on enzyme stability

Samples of crude extract were held at different temperatures for varying times up to 3 hours. Fig. 3.2 shows that the enzyme lost, over this period, 8% of its activity at 4°, 17% at 20° and 80% at 30°. 30° was chosen therefore as a suitable temperature for subsequent stability tests.

(b) Effect of pH on enzyme stability

Samples of the enzyme were incubated at different pH's at 30° and the activity remaining at the end of 180 minutes measured. The enzyme was most stable between pH 7.0 and 7.7 and the stability decreased sharply on both sides of this

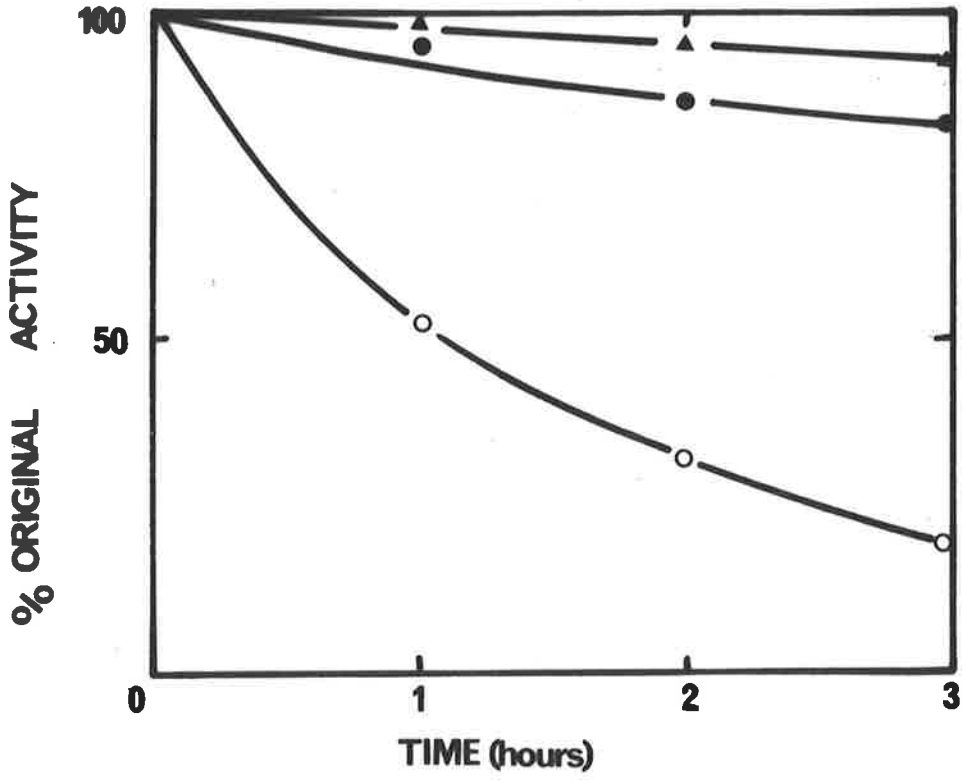


Fig. 3.2. The effect of temperature on the stability of ALA synthetase in crude extracts of R. spheroides.

Samples of crude extract (13.3 mg of protein/ml) were preincubated at 4°, 20° and 30°. ALA synthetase activity was determined, as described in Fig. 3.1, in 0.1 ml samples withdrawn at hourly intervals from each solution. Results are expressed as the percentage remaining of the activity of the original untreated extract (46.5 mμmole ALA produced/mg of protein/hr).

▲——▲, 0°; ●——●, 20°; ○——○, 30°.

range (Fig. 3.3).

(c) Effect of GSH, succinate and glycine on enzyme stability

Kikuchi, Kumar and Shemin (1959) concluded from inhibition and reactivation experiments that R. sphaeroides ALA synthetase is an SH enzyme; (the experimental evidence for this has however, not been published). By contrast, Burnham and Lascelles (1963) found that thiol compounds stabilised the enzyme only when a specific iron chelating compound was also present.

In the present work GSH and substrates were examined for stabilising ability. Separate experiments had established that R. sphaeroides ALA synthetase activity was enhanced by approximately 40% when incubations contained 2mM GSH but that it was inhibited when the concentration of GSH was greater than 4mM. (The effect of GSH on the activity of the enzyme is shown in Fig. 3.4.) As shown in Table 3.1, GSH had a deleterious effect on the stability of the enzyme. After standing for 90 minutes at either 4° or 30° in the presence of GSH, the enzyme activity remaining was 17% less than that remaining in the comparable controls (no additions).

The table also shows that glycine and succinate had little or no significant effect on the stability of the enzyme.

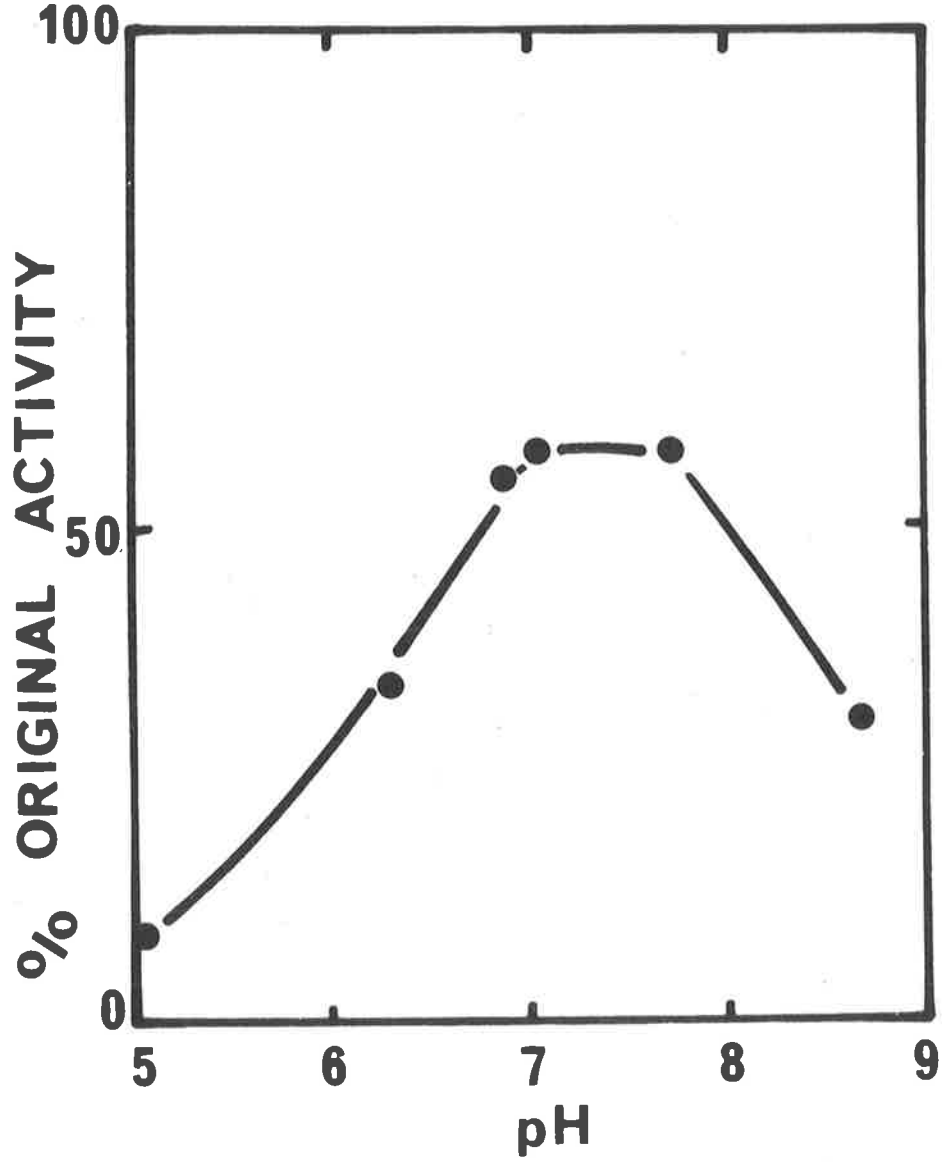

100
50
0
% ORIGINAL ACTIVITY
5
6
7
8
9
pH

Fig. 3.3. The effect of pH on the stability of ALA synthetase in crude extracts of R. spheroides.

Samples of crude extract (14.7 mg of protein/ml) were mixed with buffers (described below) and the pH of each checked on a glass electrode. The final concentration of buffer in each solution was 10 mM. The buffers used and the pH values obtained were as follows: succinate, 5.1; cacodylate, 6.3; phosphate, 6.8; N-ethylmorpholine, 7.0; tris-HCl, 7.7 and 8.65. After 2½ hours at 30°, 0.1 ml samples of each solution were removed and assayed for ALA synthetase activity as described in Fig. 3.1. (The amount of buffer (1 μmole) in 0.1 ml of enzyme solution was insufficient to interfere with the pH of the incubation mixture.) Results are expressed as a percentage of the activity in the original untreated extract (45.3 μmole ALA produced/mg of protein/hr).

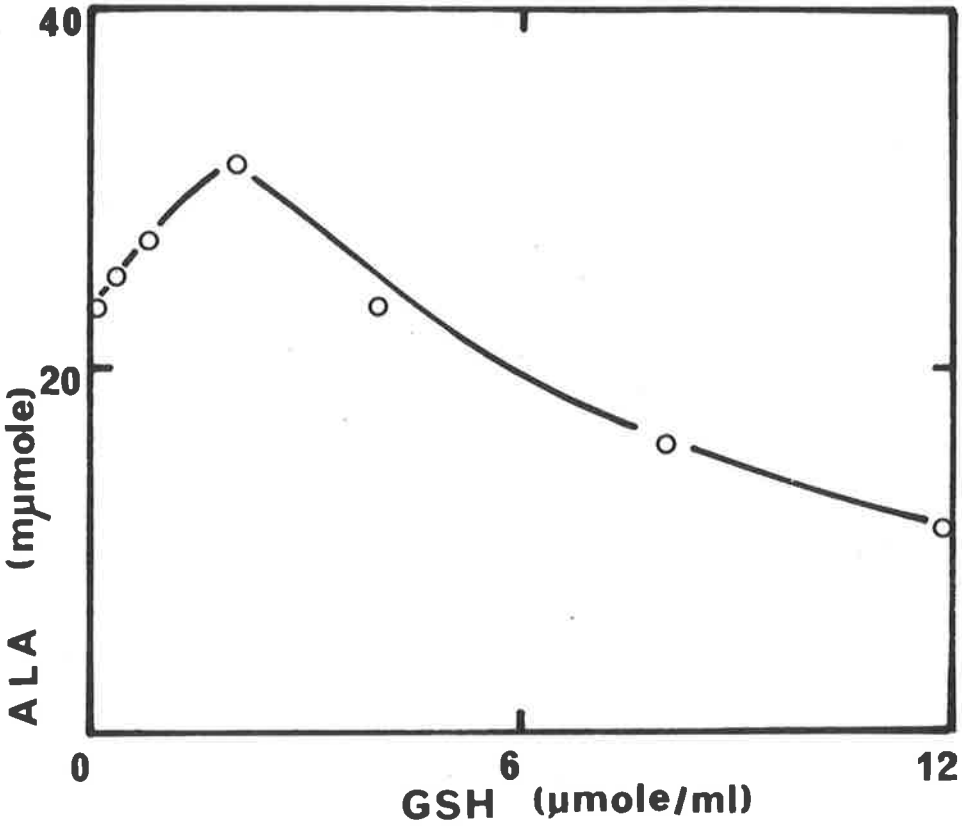


Fig. 3.4. The effect of GSH on the activity of ALA synthetase in crude extracts of R. spheroides.

Incubation conditions and method of assay were as described in Fig. 3.1, except that the concentration of GSH was varied. Values given refer to ALA produced in the complete incubation mixtures.

TABLE 3.1

THE EFFECT OF GSH, SUCCINATE AND GLYCINE ON THE STABILITY OF ALA SYNTHETASE IN CRUDE EXTRACTS OF R. SPHEROIDES

Addition	Concentration (mM)	ALA synthetase (% original activity) 4°	30°
None	-	97	53
GSH	10	82	35
Potassium succinate	100	-	55
Glycine	100	88	41

All solutions, adjusted to pH 7.4, were added to samples of crude extract (13.3 mg of protein/ml) to give the final concentrations shown. The enzyme solutions were then divided, one half being maintained at 4° and the other at 30° for 90 minutes. ALA synthetase activity in 0.1 ml samples (equivalent to 0.09 ml of the untreated extract) of each solution was determined as described in Fig. 3.1. Results are expressed as the percentage remaining of the activity of the original untreated extracts (33.7 mμmole ALA produced/mg of protein/hr).

(d) Effect of pyridoxal 5-phosphate on enzyme stability

Pyridoxal 5-phosphate exerted a considerable stabilising effect on the enzyme. The effect of this compound over a four-hour period is clearly shown in Fig. 3.5. After 4 hours at 30°, 60% of the activity present initially remained, compared with only 40% in the controls (no additions).

(e) Effect of EDTA on enzyme stability

Fig. 3.5 also shows that EDTA had a deleterious effect on the stability of the enzyme; activity remaining at the end of 4 hours was 15% less than that of the control (no additions). In accordance with the observation of Marver et al. 1966b), that ALA synthetase activity in liver homogenates was almost doubled when 1 mM EDTA was included in incubation mixtures, ALA synthetase activity in crude extracts of R. spheroides was 25% higher in the presence of 1 mM EDTA.

(f) Effect of metal ions on enzyme stability

Since the stability of ALA synthetase in crude extracts of R. spheroides was decreased in the presence of the chelating agent EDTA, the effect of metal ions on the

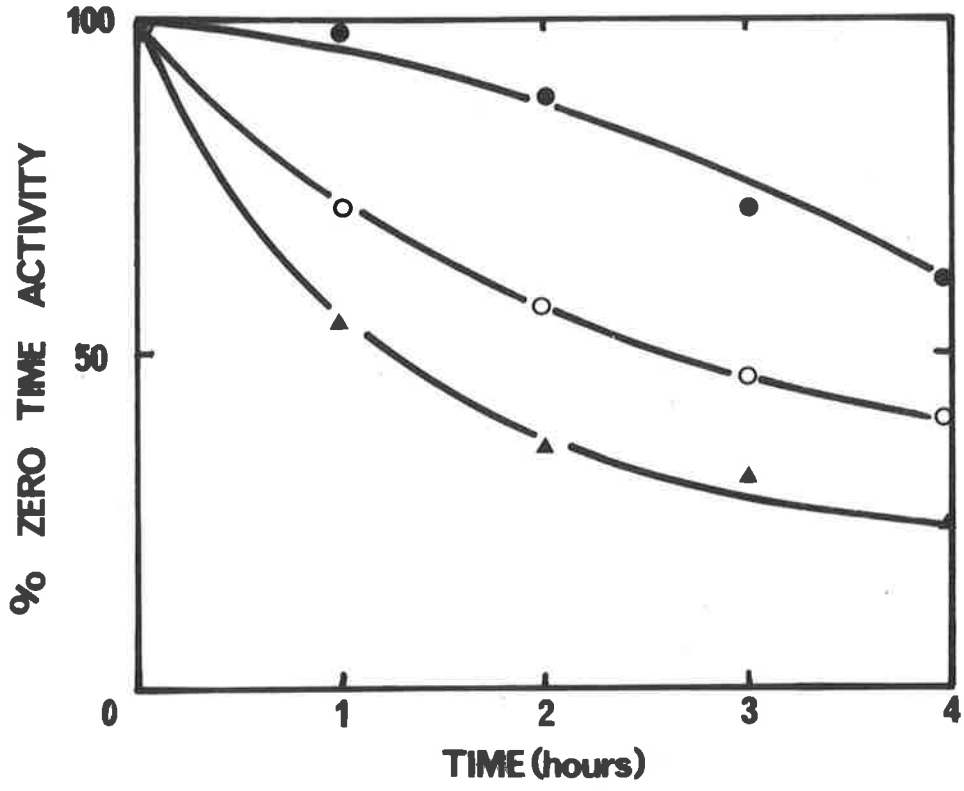


Fig. 3.5. The effect of pyridoxal 5-phosphate and EDTA on the stability of ALA synthetase in crude extracts of R. spheroides.

Pyridoxal 5-phosphate and EDTA, adjusted to pH 7.4, were added to separate samples of crude extract (14.0 mg of protein/ml) to give final concentrations of 10 and 1mM respectively. 0.1 ml samples of these enzyme solutions, together with a control (without additions) were immediately assayed for ALA synthetase activity as described in Fig. 3.1. The remaining portion of each of these solutions was maintained at 30°. At hourly intervals, 0.1 ml samples were withdrawn and ALA synthetase activity determined. Results are expressed as a percentage of the enzyme activity present in each solution, when assayed at zero time. Activity of the original untreated extract was 42.7 mμmole of ALA produced/mg ofprotein/hr.

○— ○ , no additions to preincubation;
●— ● , pyridoxal 5-phosphate present;
▲— ▲ , EDTA present.

stability of the enzyme was examined. Magnesium was chosen as one of the metals to be tested since a number of workers have included $MgCl_2$ in buffers used in the preparation of extracts of the bacterial enzyme (Burnham and Lascelles, 1963; Higuchi et al., 1965) and suspensions of liver mitochondria (Granick and Urata, 1963). The effect of zinc was also investigated because it has been suggested that zinc may be involved in the overproduction of porphyrins in man (Peters, 1957).

The results in Fig. 3.6A show that while Mg^{++} had a slight stabilising effect on the enzyme Zn^{++} had none. However, in the presence of pyridoxal 5-phosphate and either Mg^{++} or Zn^{++}, no activity was lost during the first hour at 30°. After 4 hours, 75% of the initial activity remained when both pyridoxal 5-phosphate and Mg^{++} were present, 64% in the presence of pyridoxal 5-phosphate and Zn^{++} and 60% when pyridoxal 5-phosphate alone was added.

C. Partial Purification of the Bacterial ALA Synthetase

Burnham and Lascelles (1963) purified the enzyme from R. sphaeroides approximately 10 fold. These authors however,

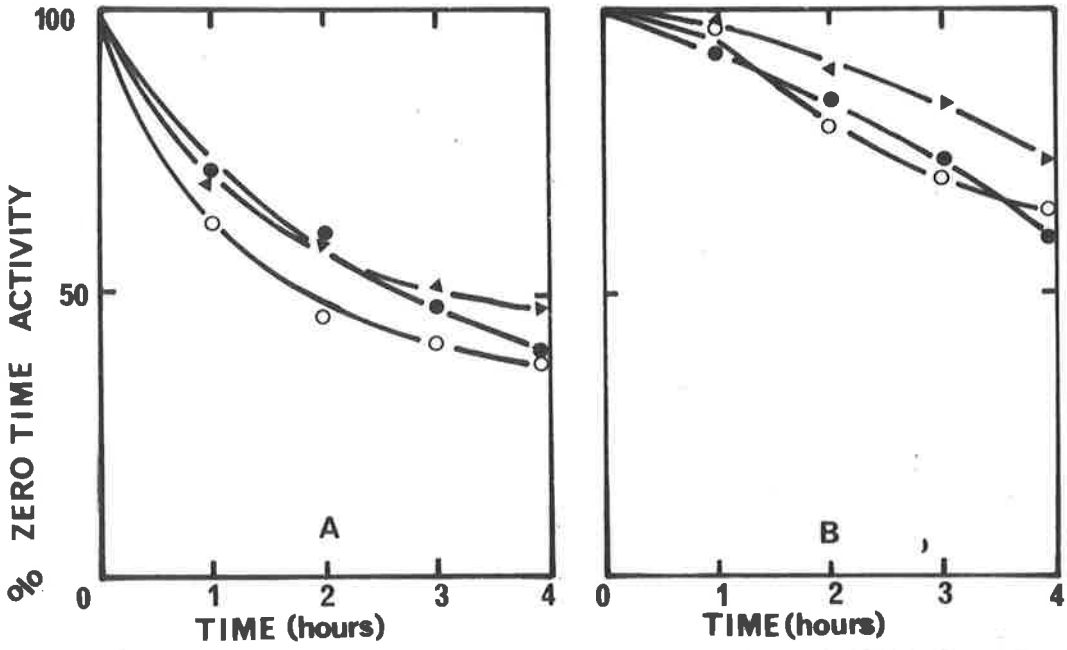


Fig. 3.6. The effect of metal ions on the stability of ALA synthetase in crude extracts of R. spheroides. (A) $MgCl_2$ and $ZnSO_4$ were added to separate portions of crude extract (13.5 mg of protein/ml) to give final concentrations of 1mM and 0.01mM respectively. 0.1 ml samples of each of these solutions, together with a control (original untreated extract), were immediately assayed for ALA synthetase activity as described in Fig. 3.1. The remaining portion of each of these solutions was maintained at 30°. At hourly intervals, 0.1 ml samples were withdrawn from each solution and ALA synthetase activity determined. Results are expressed as a percentage of the activity present in each solution when assayed at zero time. ALA synthetase activity of the original untreated extract was 78 nmole of ALA produced/mg of protein/60 min. ●—● , no additions to preincubation; ▲—▲ , $MgCl_2$ present; ○—○ , $ZnSO_4$ present.

(B) Experimental details were as described for Fig. 1.5A, except that all enzyme solutions contained 10mM pyridoxal 5-phosphate. Results are expressed as a percentage of the activity present in each solution when assayed at zero time. ●—● , no additions, ▲—▲ , $MgCl_2$ present; ○—○ , $ZnSO_4$ present.

encountered difficulties due to enzyme instability and to lack of reproducibility of DEAE-cellulose fractionation procedures.

In the present work preliminary experiments showed that for absorption of ALA synthetase onto DEAE-cellulose the enzyme solution must not exceed 0.005M in buffer concentration. Pyridoxal 5-phosphate was added to stabilise the enzyme.

The following purification procedure was developed and used successfully a number of times. The method is described since purified bacterial ALA synthetase obtained from it was used in subsequent work.

Fractionation Procedure

Unless otherwise stated, all steps were carried out at 4°.

Step 1. 10 g offreeze-dried R. spheroides (p. 55) were suspended in 100 ml of phosphate buffer (pH 6.9) (0.005M) containing 1mM pyridoxal 5-phosphate. The cells were disrupted by ultrasonication for 2 minutes (in 20 ml batches), the temperature being kept below 4°. The mixture was centrifuged at 100,000 x g for 30 minutes and the supernatant (70 ml) collected.

Step 2. 5 ml of streptomycin sulphate were added slowly while stirring, and the precipitated nucleic acid removed by centrifugation. The supernatant was dialysed against 10 volumes of the phosphate-pyridoxal 5-phosphate buffer for 3 hours with 3 buffer changes. Volume of dialysate, 63 ml.

Step 3. 12.5 ml of the dialysate were mixed with an equal volume of saturated ammonium sulphate adjusted to pH 6.9 immediately before use. After 15 minutes the precipitate was centrifuged down at 45,000 x g for 20 minutes, dissolved in a minimal volume of phosphate-pyridoxal 5-phosphate buffer and dialysed as in Step 2.

Step 4. The solution was applied to a DEAE-cellulose column (10 x 1.5 cm) previously equilibrated with 0.005M phosphate buffer (pH 6.9). The column was washed with 15 ml of this buffer, followed by 20 ml of 0.075 M phosphate buffer (pH 6.9). The column was then eluted with a linear phosphate buffer gradient (50 ml of 0.1M buffer and 50 ml of 0.3M buffer, both at pH 6.9, in the two vessels). 5.5 ml fractions were collected. ALA synthetase appeared as a broad peak with only a small purification achieved.

Step 5. The pooled fractions (Nos. 10-17) containing AlA synthetase were treated with an equal volume of saturated ammonium sulphate (pH 6.9). The precipitate was centrifuged down, redissolved and dialysed as in Step 3.

The purification achieved only a 4 fold purification with a 14% yield (Table 3.2).

The enzyme was stored in small batches at -15°. Approximately 50% of the original activity was present after 3 to 4 weeks.

TABLE 3.2

PURIFICATION OF ALA SYNTHETASE FROM R. SPHEROIDES

Experimental details were as described in the text.

Fraction	Volume (ml)	Concentration of protein (mg/ml)	Specific Activity (mμmole ALA/ hr/mg protein)	Recovery (%)
Step 1 - crude extract	70	13.5	105	100
Step 2 - supernatant after treatment with streptomycin sulphate and dialysis	68	11.0	147	110
Step 3 - redissolved precipitate from treatment with 50% $(NH_4)_2SO_4$ and dialysis	6.7	8.7	245	72
Step 4 - eluate from DEAE-cellulose (fractions 10-17)	44	0.32	383	27
Step 5 - redissolved precipitate from treatment with 50% $(NH_4)_2SO_4$ and dialysis	2.5	2.7	430	14

2. DISCUSSION

The main achievement of this preliminary work was the finding that ALA synthetase of R. *sphaeroides* is stabilised by pyridoxal 5-phosphate. A similar observation has since been reported by Neuberger (1968) who found that this compound, together with succinate and glycine, markedly increased the stability of the bacterial enzyme.

Fortunately, the liver enzyme proved to be similar to the bacterial enzyme in this respect and use of pyridoxal 5-phosphate permitted its storage and handling with predictable results. (This is described in Chapter 7, p. 134.)

The purification achieved here with the bacterial enzyme has no particular significance since it is not superior to that of Burnham and Lascelles (1963). It did however, provide a preparation of enzyme useful in subsequent work which is described in the next chapter.

CHAPTER 4

DEVELOPMENT OF A SENSITIVE RADIOCHEMICAL ASSAY FOR ALA SYNTHETASE

Although the incorporation of ^{14}C-succinate and ^{14}C-glycine into ALA has been demonstrated (Laver, Neuberger and Udenfriend, 1958; Kikuchi *et al.*, 1958), these observations have not been utilised as a measure of ALA synthesis. Recently however, the decarboxylation of ^{14}C-1-glycine, under appropriate restrictive conditions, has been shown to parallel ALA synthesis (Lewis *et al.*, 1967).

ALA synthetase activity is usually determined by converting the ALA produced to a pyrrole by condensation with acetyl acetone, the pyrrole then being measured colorimetrically after treatment with Ehrlich reagent. One of the difficulties attached to colorimetric assays of ALA synthetase in crude preparations of this enzyme is that liver and other cells form AA(Elliott, 1959, 1960a; Green and Elliott, 1964) as well as ALA (Granick and Urata, 1963) and AA is also converted to a pyrrole on condensation with acetyl acetone. For the measurement of ALA synthetase activity it is therefore necessary to separate ALA and AA. Methods have been described in which separation is achieved

on resin columns either before (Urata and Granick, 1963) or after conversion to the pyrroles (Marver et al., 1966a). The pyrroles may also be separated by extracting AA pyrrole into ether, the ALA pyrrole remaining in the aqueous layer (Granick, 1966; Bottomley and Smithee, 1968).

While these procedures are satisfactory for the measurement of ALA synthetase in preparations of porphyric liver, reticulocytes or R. spheroides in which activities are high, difficulties are encountered when they are applied to tissues of low activity, such as normal liver (Marver et al., 1966b), or when only small amounts of tissue are available (Granick, 1966).

The difficulties inherent in these methods is illustrated by the work of Waxman et al. (1966) who had to use drug induction to amplify the fluctuations in liver ALA synthetase activity in response to haem levels. If normal levels of the enzyme are to be measured with any degree of accuracy, a much more sensitive method is required. Such an assay is also needed for the measurement of ALA synthetase activity in small amounts of tissue for example in chick embryo tissue culture systems, for which semi-quantitative indirect assays have had to be used (Granick, 1966; Levere and Granick, 1967).

The potential advantages of using ^{14}C-succinate as a precursor in the synthesis of ALA were two-fold. (1) The sensitivity of the assay could be greatly increased and (2) AA synthesis could be excluded, provided of course that appropriate requirements could be met.

This chapter describes the development of such an assay in which the incorporation of ^{14}C-succinate into ALA can be accurately measured. The procedure finally adopted for crude preparations of the enzyme, such as mitochondria and homogenates of livers from porphyric guinea pigs, depends on (a) restriction of the metabolism of succinate during incubation, so far as possible, to ALA synthesis; (b) removal of excess ^{14}C-succinate by Dowex 50 (H^+) absorption of ALA; (c) the separation of labelled ALA from other radioactive Dowex absorbable compounds by the extraction of ALA pyrrole into ethyl acetate, and (d) identification of labelled ALA pyrrole by thin layer chromatography and autoradiography (the latter step not being part of the routine assay, however).

1. RESULTS

A. Preliminary Studies on Incorporation of ^{14}C-Succinate into ALA by ALA Synthetase in Crude and Purified Extracts of R. spheroides

Although the assay was intended for use in animal tissues, initial studies were made using the bacterial enzyme since active and reasonably stable preparations were readily available which was not the case with the animal enzyme. For crude bacterial extracts, the method finally adopted was to permit incorporation of ^{14}C-1,4-succinate into ALA. After removal of protein, ALA was absorbed onto Dowex 50 (H^+) and excess succinate washed away with water. The ALA was eluted with pyridine-acetate buffer and converted to the pyrrole which was extracted into ethyl acetate. In order to increase the sensitivity of the method, the ethyl acetate was removed by evaporation and the residue dissolved in a minimal volume of methanol; radioactivity was determined by scintillation counting. Samples of the final methanol solution were chromatographed on thin layer plates to verify that all the radioactivity was in fact present as ALA pyrrole.

The final method adopted is described in the legend to Fig. 4.3. A description of the investigations made at each step of the procedure will now be given since these were essential in establishing the validity of the method.

(a) Separation of ALA from ^{14}C-succinate by Dowex 50 (H^+)

(i) Absorption of ALA into Dowex 50 (H^+) and its elution

The conditions under which ALA in 0.15% trichloracetic acid is absorbed onto Dowex 50 (H^+) and 90% of this reproducibly eluted are shown in Table 4.1. The same recovery was achieved when 50 μmoles of ALA were added to the trichloracetic acid supernatant of an incubation mixture containing enzymically formed ALA.

(ii) Removal of ^{14}C-succinate from the ALA fraction by Dowex 50 (H^+)

Table 4.2 shows that a 10 ml water wash removed more than 99.7% of added succinate from the Dowex column. This procedure was slightly modified for use with trichloracetic acid supernatants of incubation mixtures. 100 μmoles of unlabelled potassium succinate and 1 μmole of carrier ALA were added to these solutions before the treatment with Dowex 50 (H^+) and the amount of water used to wash the columns was increased to 30 ml. Examination of trichloracetic acid supernatants of zero time incubations (experimental

TABLE 4.1

RECOVERY OF ALA BY ELUTION FROM DOWEX 50 (H^+)

Known amounts of ALA-HCl in 4.0 ml volumes of 0.15M trichloracetic acid were applied to Dowex 50 (H^+) columns (3 x 1 cm). The columns were washed with 30 ml of water and ALA eluted with 10 ml of 2M pyridine-acetate buffer, pH 6.0. The eluates were collected in 100 ml round bottomed flasks, containing 1 drop of concentrated HCl (added to maintain an acid pH during evaporation of the buffer) and twice evaporated to dryness under reduced pressure on a Buchi Rotovapor. Residues in each flask were dissolved in 2.5 ml of water. 2 ml Samples were taken for assay of ALA by the procedure described in Materials and Methods (p. 57).

ALA-HCl added (μmole)	ALA recovered (μmole)	% Recovery
50	45	90
100	90	90
150	137	91.5
200	185	92

TABLE 4.2

SEPARATION OF ^{14}C-1,4-SUCCINIC ACID FROM ALA BY DOWEX 50 (H^+)

0.11 µmole of ^{14}C-1,4-succinic acid* in a 4 ml volume of 0.15M trichloracetic acid was applied to a Dowex 50 (H^+) column (3 x 1 cm). The column was washed with 10 ml of water and the total eluate collected. This was followed by 10 ml of 2M pyridine-acetate buffer, pH 6.0 and the eluate collected separately. The volumes of both eluates were made up to 15 ml with water. A control, which was not subjected to the Dowex 50 (H^+) treatment, contained 0.11 µmole of ^{14}C-1,4-succinic acid,* 4 ml of 0.15M trichloracetic acid and water to a final volume of 15 ml. 0.05 ml samples of each solution were dried on filter paper discs for scintillation counting. Observed counts shown are corrected for background, but not for counting efficiency.

Treatment	Observed cpm	%
None	2,271	100
Dowex 50 (H^+) water wash	2,258	99.7
Dowex 50 (H^+) buffer eluate	5	0.3

* Specific activity 8.8 µC/µmole.

details given in Fig. 4.3) showed that under these conditions the radioactivity eluted in the ALA fraction was less than 0.3% of the total.

(b) Extraction of pyrroles into ethyl acetate

Fig. 4.1 shows that ethyl acetate extraction of ALA pyrrole, formed from known amounts of ALA-HCl, gave 85% recovery and that this was linear up to at least 200 mμmoles of ALA, AA pyrrole could also be extracted into ethyl acetate but no quantitative measurements of its recovery were made.

(c) Thin layer chromatography of pyrroles

Under the conditions described in Fig. 4.2, ALA pyrrole is readily identified on thin layer chromatograms and is well separated from AA pyrrole. In the system used, ALA and AA pyrroles moved, with R_F values of 0.5 and 1.0 respectively.

(d) Incorporation of ^{14}C-succinate into ALA by crude extracts of R. spheroides

Crude extracts of R. spheroides ALA synthetase were prepared as described in Materials and Methods (p. 55). These were used in preliminary experiments carried out to determine whether reproducible estimations of the incorporation of ^{14}C-succinate into ALA could be obtained by the use of all

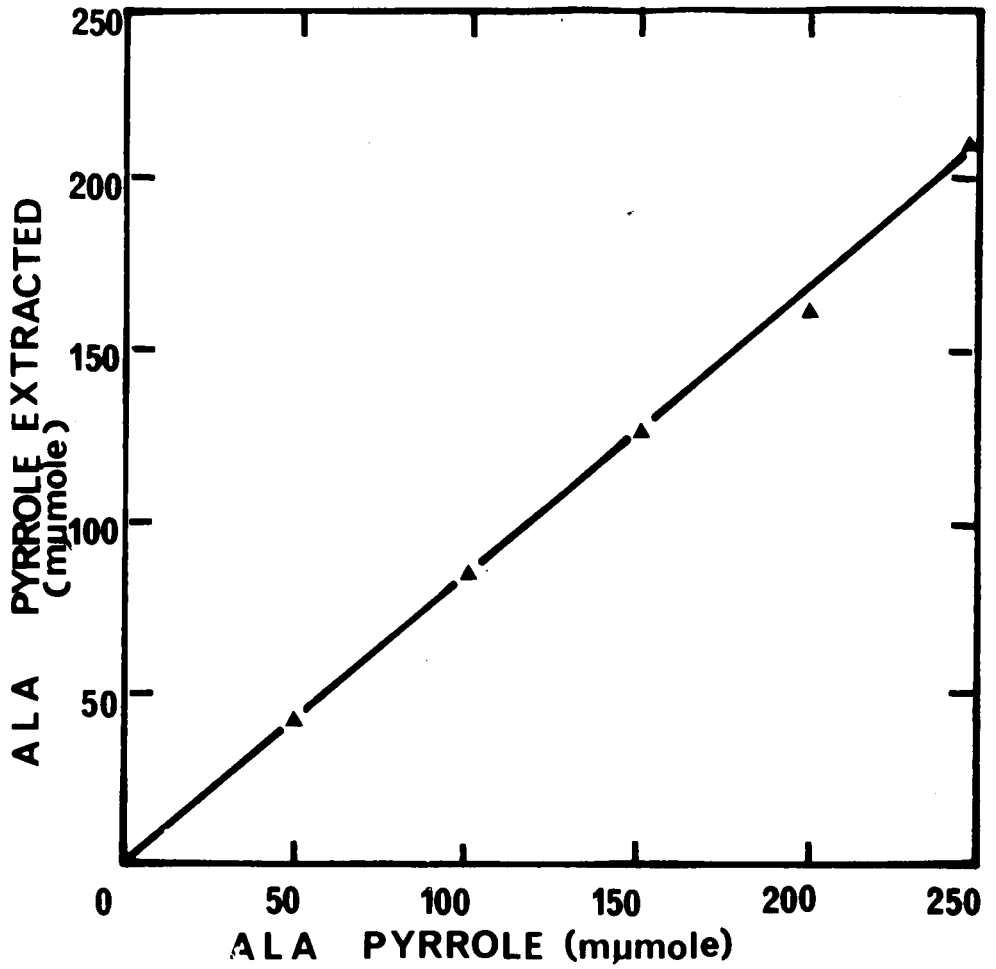


Fig. 4.1. Extraction of ALA pyrrole by ethyl acetate.

Known amounts of ALA-HCl in 2 ml volumes of 0.15M trichloracetic acid were converted to ALA pyrrole as described in Materials and Methods (p.46) and the solution extracted with 25 ml of ethyl acetate. The ethyl acetate layer was washed with 3 ml of water and was then evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml of water and 5 ml of Ehrlich reagent added. The figures on the abscissa represent the original amounts of ALA pyrrole before extraction.

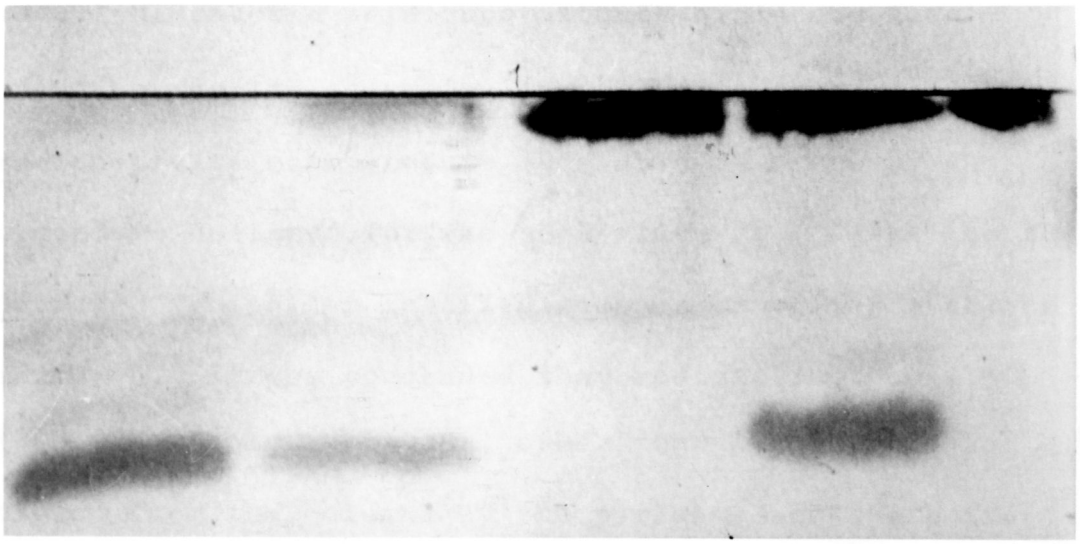


Fig. 4.2. Thin layer chromatography of ALA and AA pyrroles. 200 mμmoles each of ALA-HCl and the p-toluene sulphonic acid salt of AA were separately converted to their respective pyrroles as described in Materials and Methods (p. 46). The pyrroles were extracted into ethyl acetate as described in Fig. 4.1. The ethyl acetate was evaporated under reduced pressure and the residues were dissolved in 0.5 ml of methanol. Samples (0.01 ml) were spotted on glass plates coated with Kieselgel G and chromatographed in methyl acetate-isopropanol-25% NH_4OH (45-35-20). Both pyrroles were identified by spraying the plates with modified Ehrlich reagent (b) (Materials and Methods, p.47).

(1) ALA pyrrole
(2) ALA pyrrole + AA pyrrole
(3) AA pyrrole
(4) ALA pyrrole + AA pyrrole
(5) AA pyrrole

of the procedures described above when they were applied to trichloracetic acid supernatants of incubations.

The results shown in Fig. 4.3 were calculated from radioactivity measurements made at each stage of the procedure designed for the separation of ^{14}C-ALA (as the pyrrole) from other labelled components of the reaction mixtures. It was concluded that radioactive Dowex 50 (H^+) absorbable compounds, other than ALA, were also eluted from the columns by the pyridine-acetate buffer since the incorporation of ^{14}C-succinate into ALA as determined from measurements of the radioactivity present in the Dowex 50 eluate was approximately 70% higher than that determined from measurements of the radioactivity present in either the methanol solution of ALA pyrrole or in the silica gel containing ALA pyrrole isolated by thin layer chromatography. For the estimation of ALA synthetase activity in crude preparations of the enzyme it seemed therefore that formation and extraction of ALA pyrrole were both essential steps in the procedure.

Fig. 4.3 also shows that further separation of ALA pyrrole by thin layer chromatography was not essential and furthermore that the incorporation of ^{14}C-succinate into ALA, as determined from radioactivity measurements of the

Fig. 4.3. Incorporation of ^{14}C-1,4-succinate into ALA by crude extracts of R. sphaeroides.

Incubation mixtures were as described in Fig. 3.1, except that unlabelled potassium succinate was omitted and 0.57 μmole of ^{14}C-1,4-succinic acid (specific activity 8.8 μC/μmole) included instead. The reactions were stopped by the addition of 2 ml of 0.3M trichloracetic acid containing 100 μmoles of unlabelled potassium succinate and 1 μmole of carrier ALA. 3 ml of the protein free supernatants were applied to Dowex 50 (H^+) columns and the ^{14}C-1,4-succinic acid washed through with 30 ml of water. ALA was eluted with 10 ml of 2N pyridine-acetate buffer (pH 6.0) and the eluates collected in 100 ml round bottomed flasks containing 1 drop of concentrated HCl. The buffer was removed by evaporation twice to dryness under reduced pressure and the residues were dissolved in 1 ml of water. 0.05 ml samples of these solutions were dried onto filter paper discs for counting in a Packard scintillation spectrometer. ALA in 0.5 ml of these solutions was converted to ALA pyrrole (as described in Materials and Methods, p. 46) which was extracted into 25 ml of ethyl acetate. The ethyl acetate was removed by evaporation under reduced pressure and the residues were dissolved in 0.4 ml of methanol. 0.02 ml samples of these solutions were dried onto filter paper discs for scintillation counting. At the same time 0.02 ml samples of the methanol solution containing ALA pyrrole were chromatographed on thin layer plates as described in Fig. 4.2 and these were sprayed with Ehrlich reagent (b). The areas containing ALA pyrrole were scraped off the plates and the silica gel from each spot was suspended

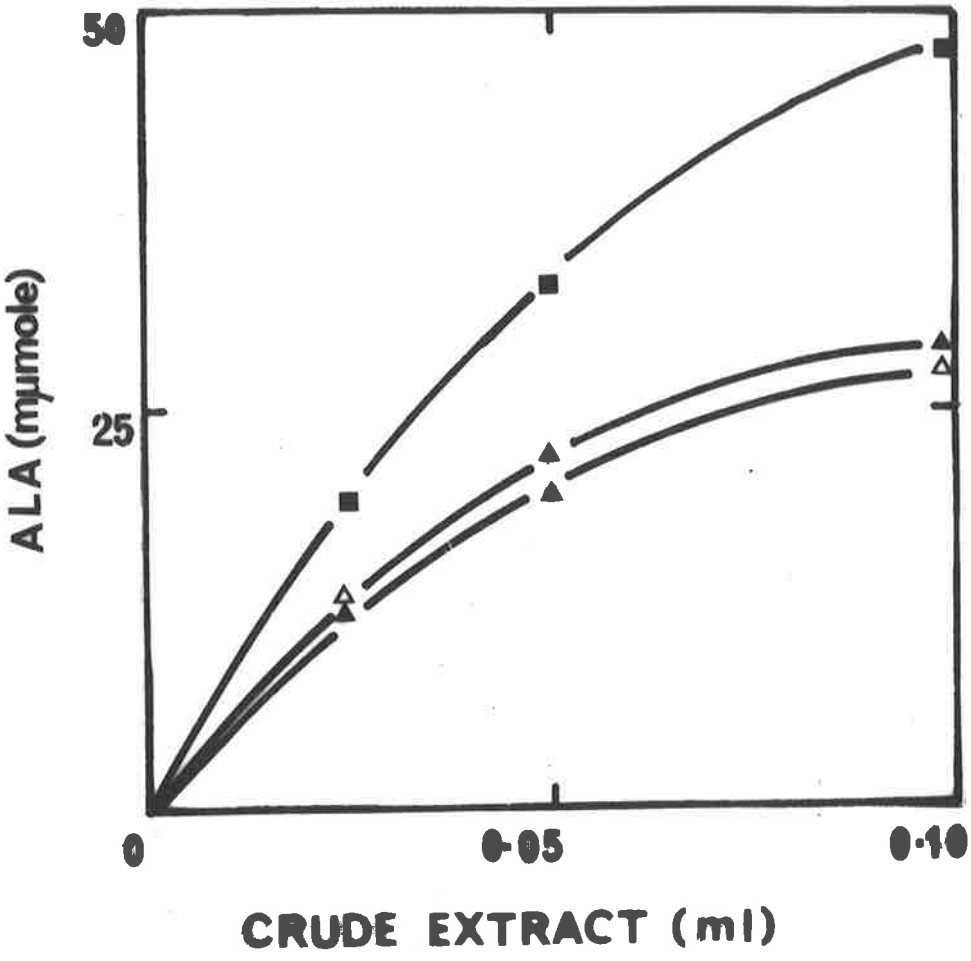


Fig. 4.3 (Con'td.)

in thixotropic gel for scintillation counting. All radioactivity measurements were corrected for counting efficiency. The figures on the ordinate represent the total amount of ALA formed during incubations and are corrected for values obtained from zero time incubations processed at the same time. Protein concentration of the R. spheroides extract was 15.7 mg/ml.

■—■ , ALA calculated from radioactivity measurements of the Dowex 50 eluate; ▲—▲ , ALA calculated from radioactivity measurements of the methanol solution of ALA pyrrole; △—△ , ALA calculated from radioactivity measurements of the pyrrole isolated from thin layer chromatograms.

methanol solution of ALA pyrrole, was approximately proportional to the amount of bacterial enzyme added.

(e) Comparison of colorimetric and radiochemical assays of ALA synthetase activity in purified preparations of the bacterial enzyme

ALA synthetase was partially purified from R. spheroides as described in Chapter 3 (p. 67) and the activity of this preparation was determined by the standard colorimetric assay procedure (as described in Fig. 3.1, p. 61). A linear response to enzyme concentration was obtained and this is shown in Fig. 4.4. Also shown are the results of assays in which ^{14}C-succinate replaced unlabelled potassium succinate as the substrate. Although these values were only 60% of those obtained by the colorimetric procedure, they also showed a linear response to enzyme concentration.

By contrast with the results obtained using crude bacterial extracts (Fig. 4.3), the total amount of radioactivity present in the Dowex 50 eluate was virtually the same as that present in the methanol solution of ALA pyrrole (Fig. 4.4). This indicated that for purified preparations of bacterial ALA synthetase, the

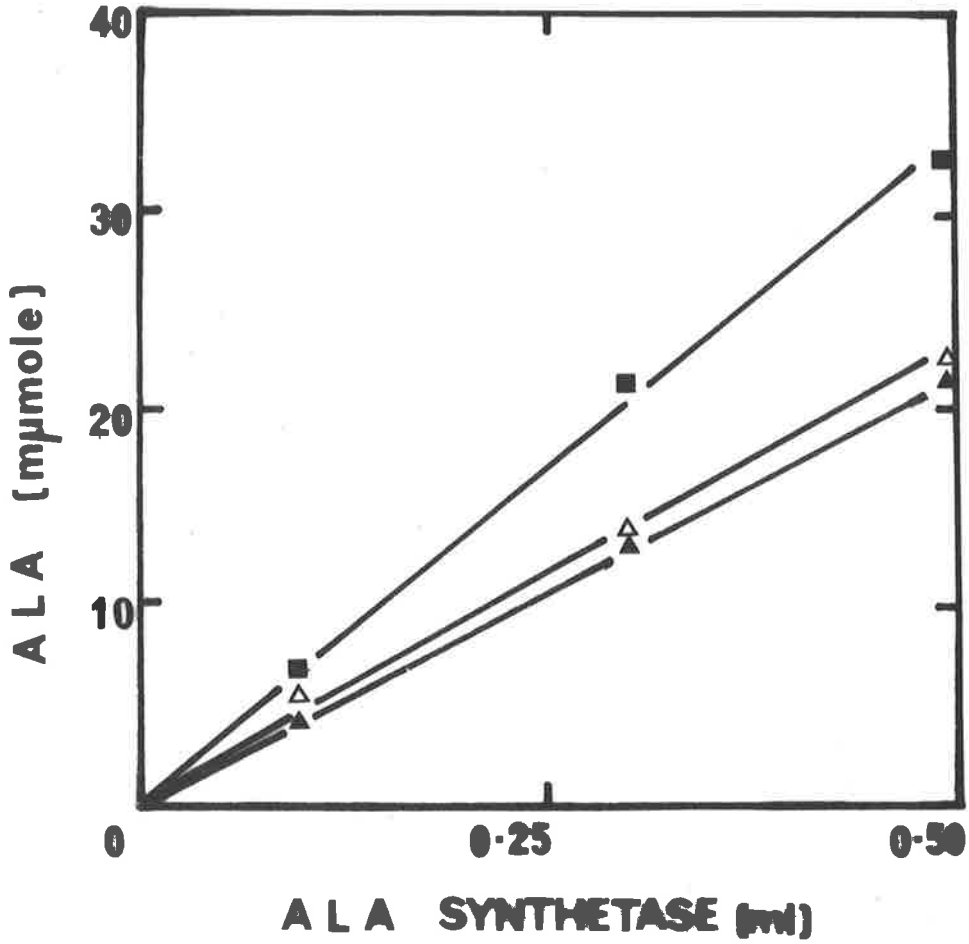


Fig. 4.4. Comparison of colorimetric and radiochemical assays of ALA synthetase activity in a purified preparation of R. spheroides. For the colorimetric assay, incubation conditions and experimental procedures were as described in Fig. 3.1 (p. 61). For the radiochemical assay, incubation conditions and experimental procedures were as described in Fig. 4.3. except that the thin layer chromatography step was omitted. The figures shown on the ordinate represent the total amount of ALA formed during incubations and are corrected for the values obtained from zero time incubations processed at the same time. Protein concentration of the enzyme solution was 0.41 mg/ml.

■—■ , colorimetric assay; △—△ , ALA calculated from radioactivity measurements of the Dowex 50 eluate; ▲—▲ , ALA calculated from radioactivity measurements of the methanol solution of ALA pyrrole.

procedure could be simplified in that it was unnecessary to convert ALA to the pyrrole since almost all of the radioactivity present in the Dowex eluate could be attributed to ALA.

The great potential value of a sensitive radiochemical assay when dealing with low levels of the enzyme is illustrated by the fact that an observed value of 1083 cpm (representing the synthesis of 5 mμmoles of ALA) was obtained, while the corresponding optical density reading for the colorimetric assay was 0.021.

Optimal incubation conditions were not further investigated for the radiochemical assay of the purified bacterial ALA synthetase since these experiments were only a preliminary towards establishing a technique which would be applicable to the liver enzyme.

(f) Verification of the validity of the radiochemical assay, as applied to the purified bacterial enzyme, by thin layer chromatography

The extracted pyrrole derived from ^{14}C-ALA synthesised by the purified bacterial enzyme was subjected to thin layer chromatography and the resulting chromatogram scanned for radioactivity. Fig. 4.5 shows that radioactivity was located entirely in the ALA pyrrole region. It was found however, that long exposure of chromatograms to X-ray film indicated additional radioactive spots; these could not have contributed significantly toALA values since they were not detectable in scans of the thin layer plates.

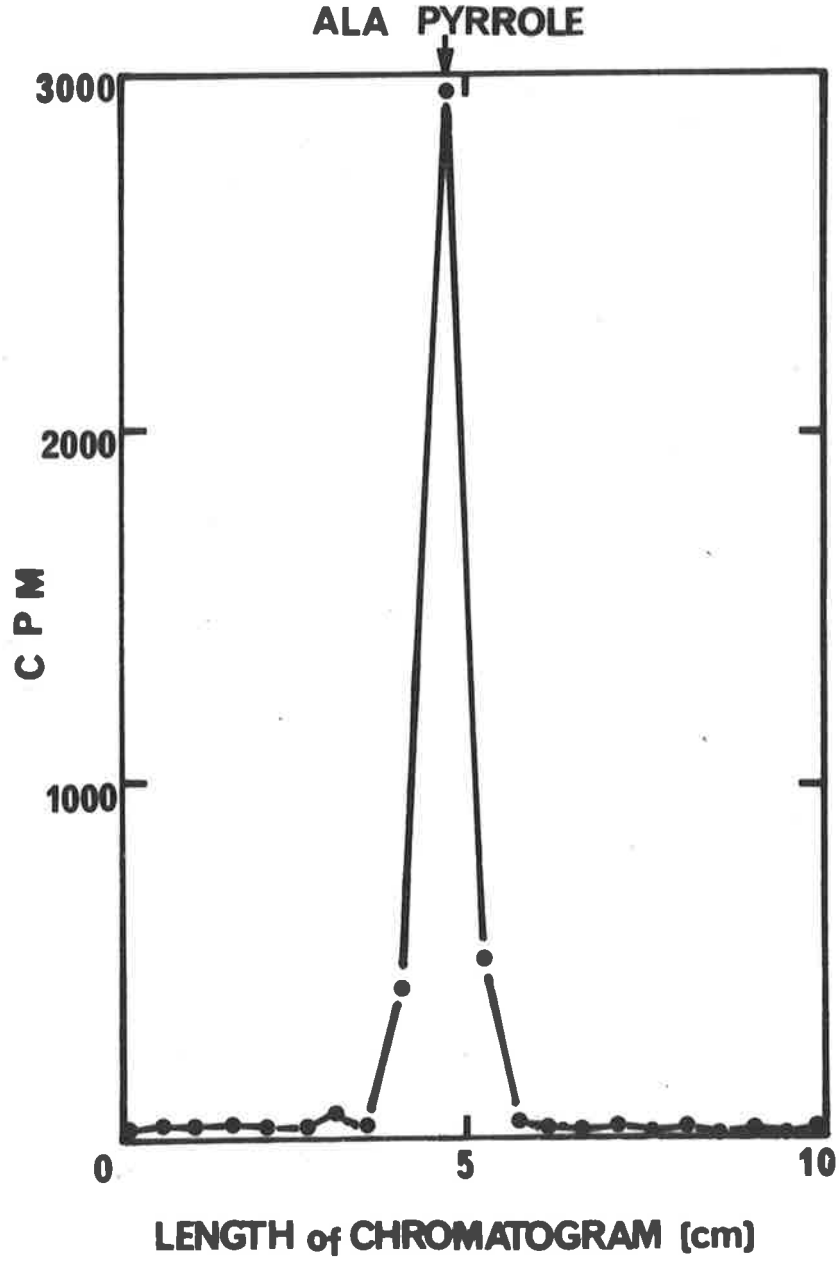
This work confirmed the results obtained using crude extracts of bacterial ALA synthetase (see Fig. 4.3) which had indicated that the assay was valid without the final chromatographic step.

B. Development of a Radiochemical Assay for ALA Synthetase in the Livers of Porphyric Guinea Pigs

Guinea pigs were made porphyric by treatment with DDC as described in Materials and Methods (p. 49). Twenty-four

Fig. 4.5. Thin layer chromatography of ALA pyrrole derived from ^{14}C-ALA formed during incubations of bacterial ALA synthetase.

A methanol solution containing labelled ALA pyrrole was prepared from the trichloracetic acid supernatant of an incubation of the purified bacterial enzyme as described in Fig. 4.3. A 0.2 ml sample of this solution was streaked on a thin layer plate over a distance of 3 cm and chromatographed as described in Fig. 4.2. After drying the plate, successive areas (1 x 0.5 cm) were scraped off and the silica gel suspended in thixotropic gel for scintillation counting. Values shown on the ordinate are corrected for background (20 cpm). The remainder of the chromatogram was sprayed with Ehrlich reagent (b) to identify the position of the ALA pyrrole. The arrow shows the position of the ALA pyrrole located colorimetrically.


ALA PYRROLE
3000
2000
1000
CPM
0
5
10
LENGTH of CHROMATOGRAM (cm)

hours after dosing the animals were killed and the livers were removed and placed on ice. Mitochondria were isolated as described in Materials and Methods (p. 50) and resuspended in 0.25% sucrose containing 10^{-3}M tris-HCl, 5×10^{-4}M EDTA and (unless otherwise stated) 2×10^{-3}M pyridoxal 5-phosphate (pH 7.4). (The effect of these compounds on the stability of liver AlA synthetase is described in Chapter 7, p. 134). Suspensions of mitochondria were stored frozen in small batches, and throughout the work described in this chapter, preparations used were once frozen and thawed. Mitochondria isolated from the livers of DDC treated guinea pigs are designated DDC liver mitochondria. Homogenates (prepared as described in Materials and Methods, p. 50) of the livers of DDC treated guinea pigs are designated DDC liver homogenates.

(a) ALA synthetase activity in DDC liver mitochondria

(1) Colorimetric assay

Fig. 4.6 shows that the standard procedure for the colorimetric assay, as described in Chapter 3 for the bacterial ALA synthetase (see Fig. 3.1, p. 61) gave the type of response to enzyme concentration which might have been expected from earlier studies of ALA synthetase in DDC

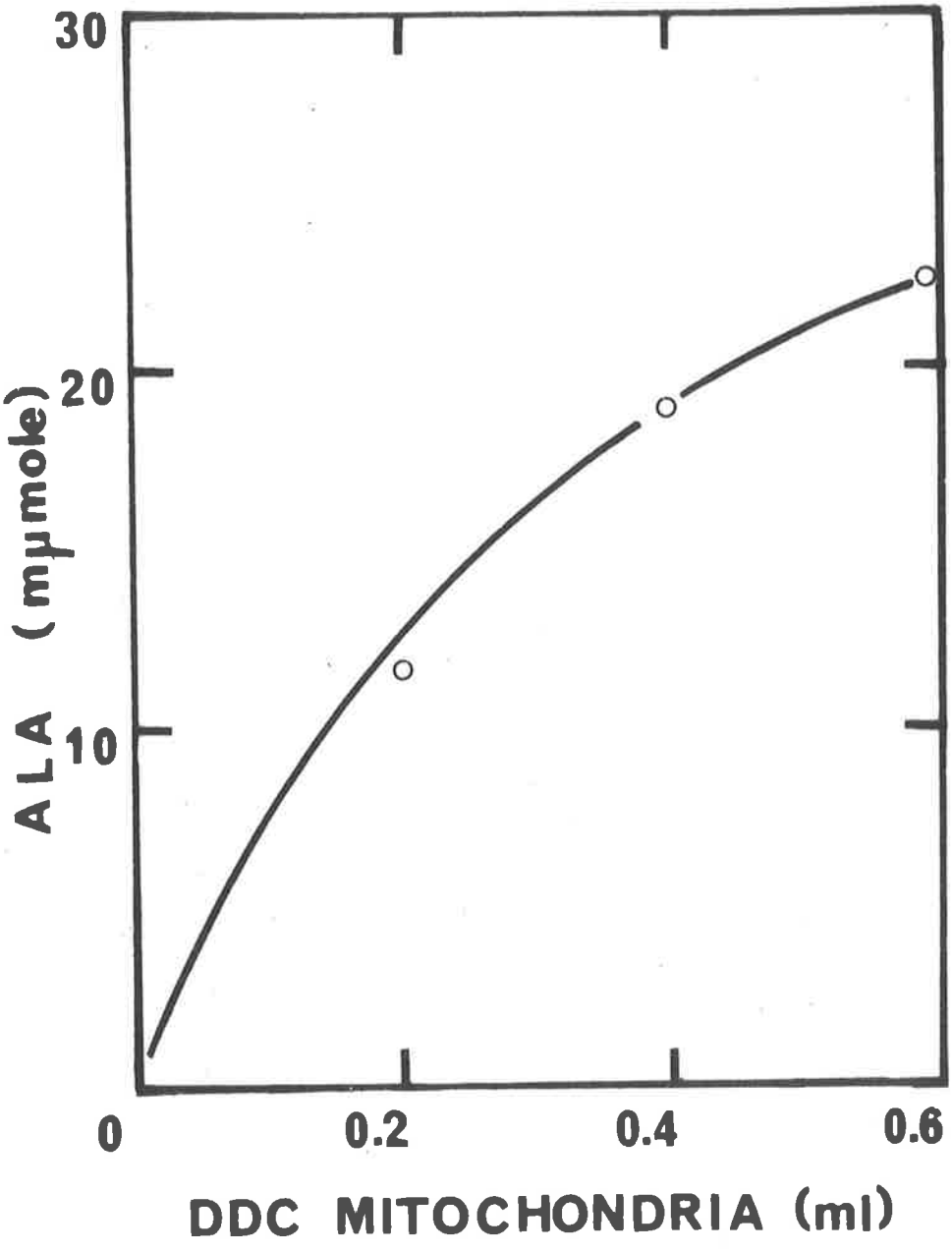


Fig. 4.6. Colorimetric assay of ALA synthetase in DDC liver mitochondria.

Incubation conditions and method of assay were as described in Fig. 3.1, except that the enzyme source was DDC liver mitochondria. (Protein concentration of the mitochondrial suspension was 20 mg/ml.) The figures on the ordinate represent the total amount of ALA formed during incubations.

liver mitochondria (Granick and Urata, 1963).

(ii) Application of the radiochemical assay

It was anticipated that the procedures described in Fig. 4.3 for radiochemical assays of bacterial ALA synthetase would, with perhaps a minor adjustment to the amount of substrate used, be directly applicable to the mitochondrial enzyme. The results in Fig. 4.7 show however, that while labelled compounds were eluted from Dowex 50 (H^+) in the ALA fraction, less than 1% of these were extracted into ethyl acetate after treatment of the Dowex 50 eluate with acetyl acetone. Moreover, there was no significant increase in the amount of labelled compounds extracted into ethyl acetate when increasing amounts of mitochondria were included in incubations. The method was therefore unsatisfactory in this form as a measure of ALA synthetase activity in DBC liver mitochondria.

It was therefore decided to attempt to minimise succinate metabolism using inhibitors of the Krebs cycle, i.e. to arrange that the labelled acid was converted to succinyl CoA but was not metabolised via the Krebs cycle.

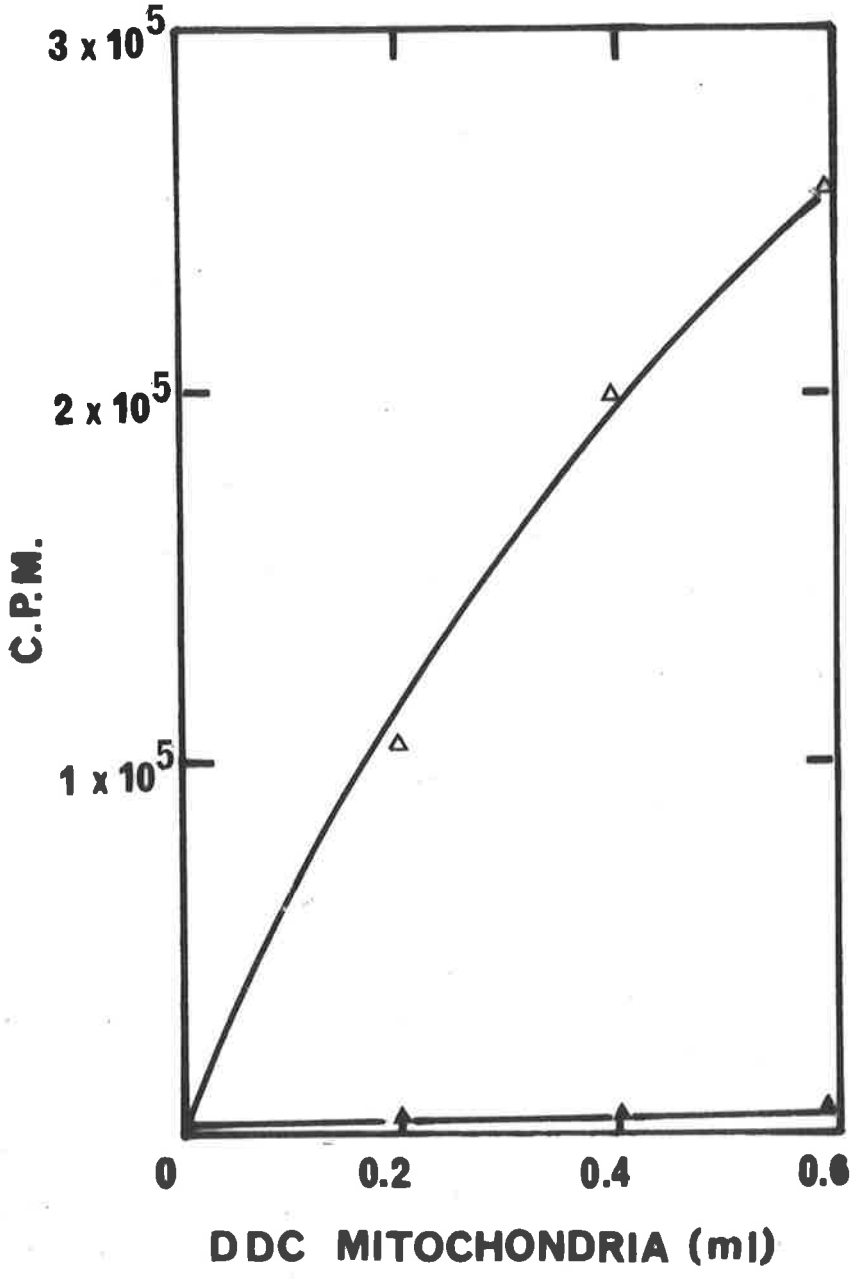


Fig. 4.7. Application of the radiochemical assay to ALA synthetase in DDC liver mitochondria.

Incubation conditions and experimental procedures were as described in Fig. 4.4, except that the source of the enzyme was DDC liver mitochondria. Values refer to complete incubations and are corrected for zero time levels.

△—△, Dowex eluate; ▲—▲, ALA pyrrole fraction.

(b) The effect of Krebs cycle inhibitors on the metabolism of ^{14}C-succinate in mitochondrial preparations

When mitochondria were incubated with ^{14}C-succinate (without components necessary for ALA synthesis), a measure was obtained of the incorporation of ^{14}C-succinate into metabolites which were absorbed onto Dowex 50 (H^+) and which could interfere with the ALA assay. Such incorporation was found to be large but this was substantially reduced by either antimycin A or sodium malonate which inhibit succinic dehydrogenase, or by ^{12}C-malate which traps any ^{14}C-malate which might be formed and which is then removed by the Dowex 50 treatment. A combination of all three of these compounds reduced by 96% the incorporation of label into Dowex 50 (H^+) absorbable compounds which were eluted in the ALA fraction (Table 4.3).

The effect of these agents on ALA synthetase of DDC liver mitochondria and of R. spheroides was examined with the colorimetric assay procedure. It can be seen from Table 4.4 that no inhibition occurred with any one of the inhibitors alone or with a mixture of all three.

(c) The effect of sodium arsenite on ALA synthetase activity

Since it was also desirable in this system to prevent

TABLE 4.3

EFFECT OF ANTIMYCIN A, MALONATE AND MALATE ON INCORPORATION OF ^{14}C-SUCCINATE INTO DOWEX 50 ABSORBABLE COMPOUNDS

Additions			Per cent incorporation of control
Antimycin A (µg)	Sodium malonate (µmole)	Sodium DL-malate (µmole)	
-	-	-	100.0
2.5	-	-	32.0
5.0	-	-	31.0
-	20	-	6.4
-	-	10	23.0
5.0	20	-	5.9
5.0	-	10	14.1
-	20	10	6.7
5.0	20	10	4.3

Incubations contained DDC liver mitochondrial suspension (20 mg of protein/ml), 0.4 ml; tris-HCl (pH 7.4), 100 µmoles; ^{14}C-1,4-succinic acid (specific activity 8.8 µC/µmole), 0.576 µmole; additions shown in the table and water to a final volume of 2 ml. Tubes were incubated at 37° in a shaking water bath for 1 hour and the reaction stopped by the addition of 2 ml of 0.3M trichloracetic acid containing 100 µmoles of unlabelled potassium succinate; protein free supernatants were treated on Dowex 50 (H^+) columns as in Fig. 4.3. The fraction eluted was evaporated to dryness and the residue dissolved in 1 ml of water. 0.05 ml samples were dried on glass fibre discs and radioactivity determined by scintillation counting. The observed value for the control (no additions) was 7,292 cpm.

TABLE 4.4

ACTIVITY OF BACTERIAL AND MITOCHONDRIAL ALA SYNTHETASE IN THE PRESENCE OF ANTIMYCIN A, MALONATE AND MALATE

Source of enzyme	Antimycin A (µg)	Sodium DL-malate (µmoles)	Sodium malonate (µmoles)	ALA formed (mµmoles)
R. spheroides	-	-	-	73.6
"	2.5	-	-	77.2
"	-	10	-	74.8
"	2.5	-	20	75.2
"	2.5	10	20	70.4
DDC mitochondria	-	-	-	33.6
" "	2.5	10	20	32.6
" "	5.0	10	20	39.0

Incubation conditions (except for additions shown) and colorimetric method of assay were as described in Fig. 4.6. The 2 ml incubation mixture contained 0.5 ml of R. spheroides extract (protein content, 0.4 mg/ml) or 0.5 ml of DDC liver mitochondrial suspension (protein content, 20 mg/ml).

succinyl CoA formation from α-oxoglutarate, the effect of sodium arsenite, which inhibits the α-oxoglutarate oxidase system, on ALA synthetase activity was examined.

In DDC liver mitochondria the synthesis of ALA, when α-oxoglutarate is the source of succinyl CoA, is inhibited when sodium arsenite is also present (Granick and Urata, 1963). The effect of arsenite on the synthesis of ALA from glycine, succinate and CoA by both the mitochondrial and bacterial enzymes is shown in Fig. 4.8. The activity of the purified bacterial enzyme was slightly stimulated while that of the mitochondrial enzyme was inhibited. When incubations contained 5 μmole of sodium arsenite/ml, a concentration of arsenite which had been shown to inhibit almost completely ALA synthesis from α-oxoglutarate, CoA and glycine (Granick and Urata, 1963), the inhibition of enzyme activity in the present system was only 2.5%. This concentration of arsenite was used in subsequent work.

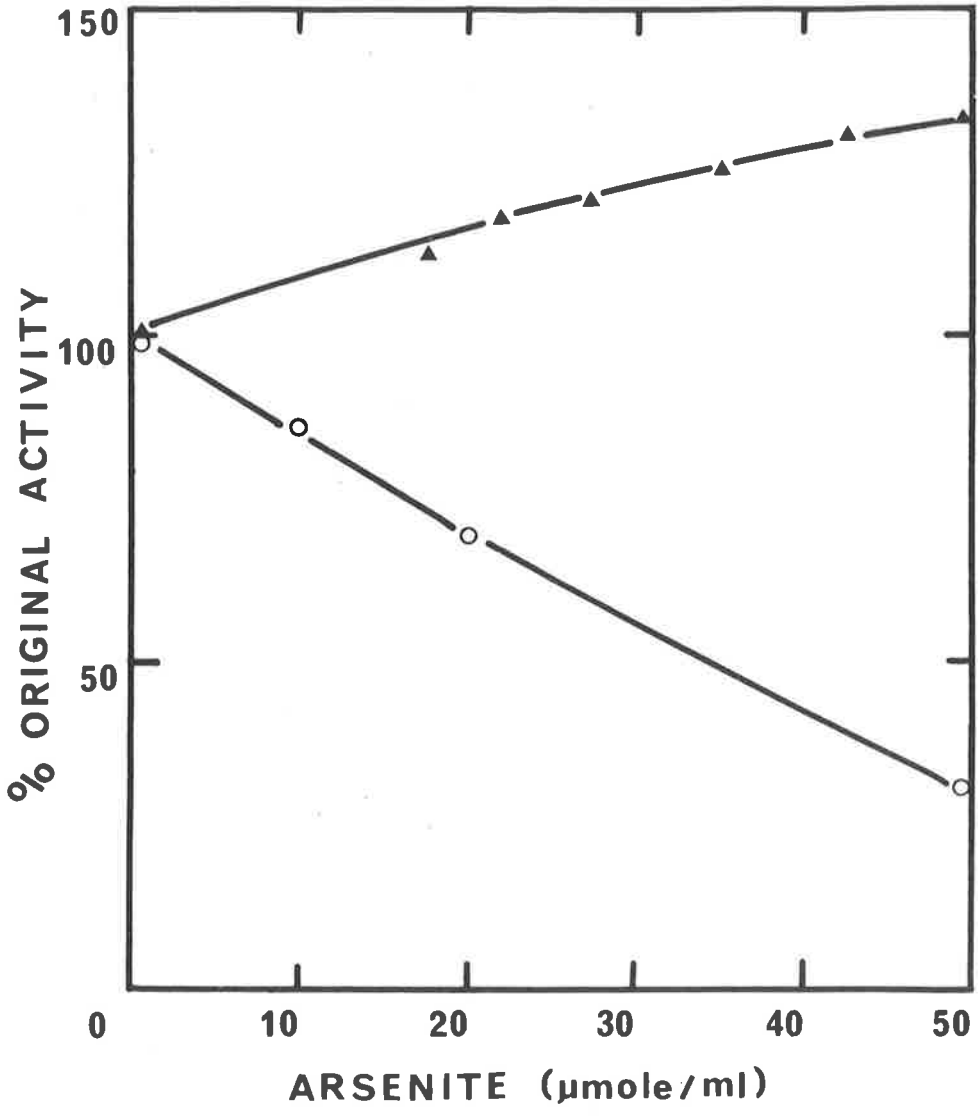


Fig. 4.8. The effect of arsenite concentration on ALA synthetase activity. Incubation conditions and colorimetric method of assay were as described in Fig. 4.6 except that incubation mixtures also contained 5 µg of antimycin A, 20 µmoles of sodium malonate and 10 µmoles of sodium DL-malate. In the absence of sodium arsenite, 40.2 mµmoles of ALA were produced with 0.5 ml of R. spheroides extract (0.4 mg of protein/ml) and 107.2 mµmoles of ALA with 0.2 ml of DDC liver mitochondria suspension (45 mg of protein/ml).

▲—▲ , R. spheroides enzyme,
○—○ , DDC liver mitochondria.

(d) Determination of optimum substrate concentrations for assay of ALA synthetase in DDC liver mitochondria

Restriction of the metabolism of ^{14}C-succinate to the synthesis of ALA by the use of malonate, antimycin A and arsenite, together with the extra precaution of excess ^{12}C-malate, resulted in a small measurable incorporation of label into ALA during incubations of DDC liver mitochondria (Fig. 4.9). The amount of ALA formed, which was detected by this method, was however, less than 10% of that determined by the standard colorimetric assay procedure. That the low concentration of ^{14}C-succinate in incubation mixtures was a limiting factor is apparent from the results shown in Fig. 4.10 which indicated the necessity for relatively high concentrations of succinate.

The requirement for all substrates and cofactors by the liver enzyme was examined in greater detail (Fig. 4.11); (colorimetric assays were used for purposes of economy). The results indicated that the concentrations of glycine, CoA and ATP (Fig. 3.1) used in all colorimetric assays so far described in this thesis were in fact limiting and that the full potential of the mitochondrial enzyme had not been measured. The almost complete dependence of the reaction on

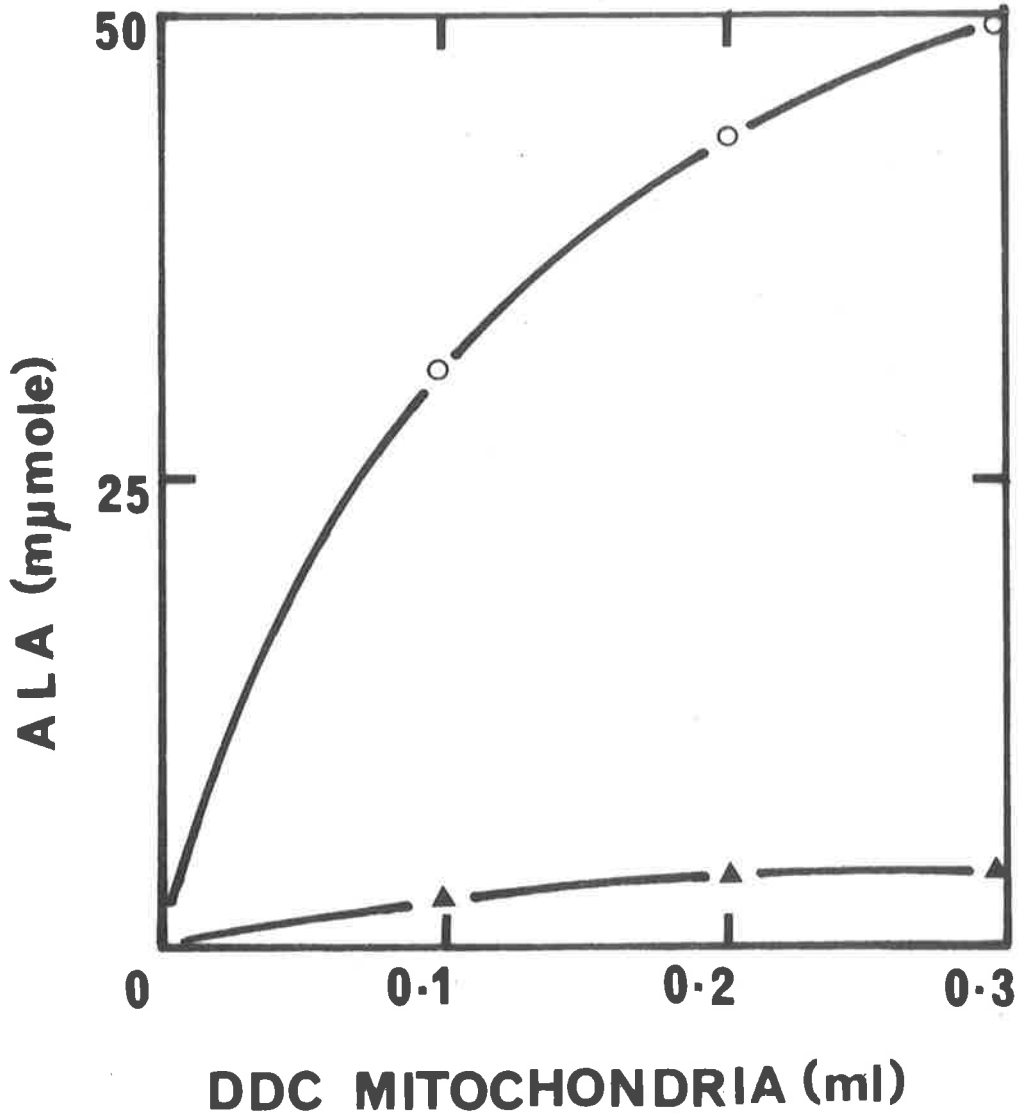


Fig. 4.2. Comparison of radiochemical and colorimetric assay of ALA synthetase activity in DDC liver mitochondria.

For the colorimetric assay, incubation conditions and experimental procedures were as described in Fig. 4.8, except that all incubations contained 10 μmole of sodium arsenite. For the radiochemical assay incubations were as described for the colorimetric assay except that 0.576 μmole of ^{14}C-1,4-succinic acid (specific activity 8.8 μC/μmole) were included instead of 100 μmole of unlabelled potassium succinate; experimental procedures were as described in Fig. 4.7. After evaporation of ethyl acetate, the residue was dissolved in 0.5 ml of methanol and 0.05 ml samples dried on glass fibre discs for scintillation counting. ○—○, colorimetric assay; ▲—▲, radiochemical assay.

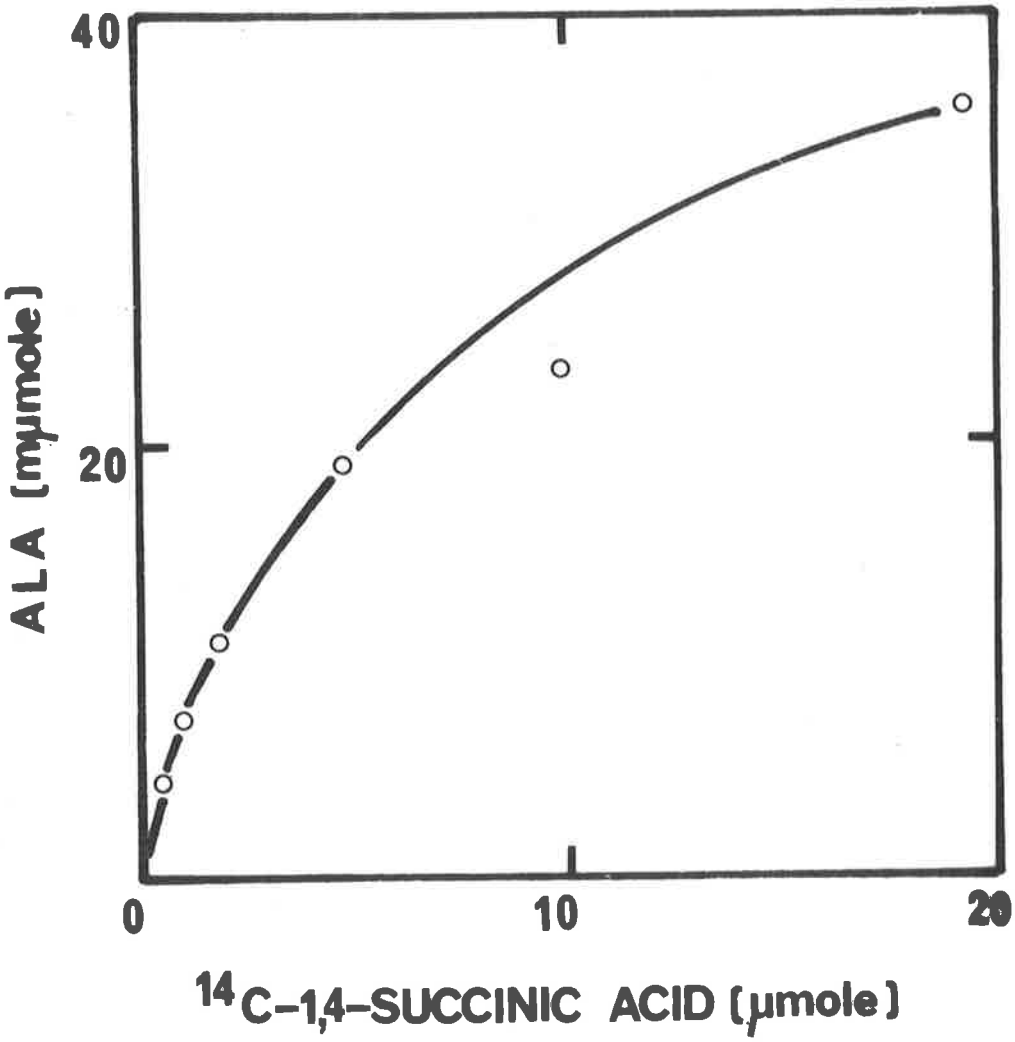


Fig. 4.10. The effect of succinate concentration on the synthesis of ^{14}C-ALA by DDC liver mitochondria. Incubation conditions and experimental procedures were as described in Fig. 4.9 for the radiochemical assay except that all incubations contained 0.05 ml of DDC liver mitochondrial suspension, the concentration of ^{14}C-1,4-succinate was varied and its specific activity was 0.475 μC/μmole. Figures on the abscissa represent the amount of ^{14}C-succinate added to the 2 ml incubations.

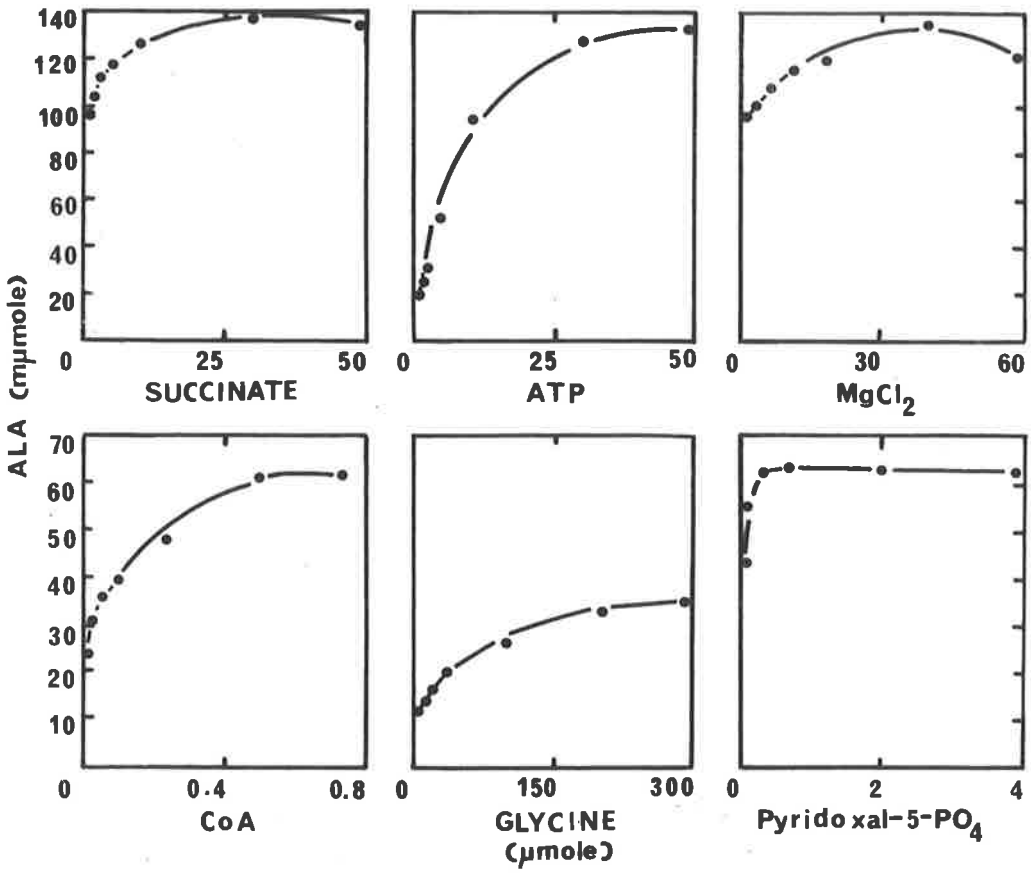


Fig. 4.11. Effect of substrate and cofactor concentrations on ALA synthetase activity of DDC liver mitochondria. Except for substrate varied, incubation mixtures contained (in μmoles unless otherwise stated): glycine, 100; potassium succinate, 100; tris-HCl buffer, 100; $MgCl_2$, 40; CoA, 0.85; pyridoxal 5-phosphate, 2; GSH, 4; ATP, 50; sodium malonate, 20; sodium DL-malate, 10; sodium arsenite, 10; antimycin A, 5 μg; succinyl CoA synthetase solution, 0.2 ml; DDC liver mitochondrial suspension (stored in the absence of added pyridoxal 5-phosphate) 0.2 ml; water to a final volume of 2 ml. Method of assay was as described in Fig. 4.8. Figures shown on abscissae represent the amounts added to the 2 ml incubations.

added ATP confirmed the absence of succinyl CoA synthesis by the α-oxoglutarate oxidase system.

The concentrations of succinate, CoA, glycine, ATP, pyridoxal 5-phosphate and Mg^{++} used in subsequent experiments are based on these results.

It was found however, that ALA synthesis by DDC liver mitochondria was not dependent on added E. coli succinyl CoA synthetase. This indicated that endogenous succinyl CoA synthetase was responsible for the production of succinyl CoA. The effect of GTP on ALA synthesis was therefore examined, but there was no significant increase in activity when this compound was also included in incubations; presumably adequate endogenous GTP was available.

As a precaution, the bacterial succinyl CoA synthetase was included in the final system adopted so that the assay would also be applicable to more purified preparations of ALA synthetase. In order that the sensitivity of the method might be increased, the volume of incubation mixtures was reduced from 2 ml to 0.5 ml and at the same time the relative concentration of ^{14}C-succinate was increased.

(e) Final radiochemical assay procedure

The method finally adopted is described in full below; for convenience of reference the entire procedure is described despite the fact that parts of it have already been given in legends to figures.

Incubation mixtures contained the following (in μmoles/ml unless otherwise stated): tris-HCl buffer, pH 7.4, 50; glycine, 100; unlabelled potassium succinate, 10; ^{14}C-1,4-succinic acid, 0.576 (5 μC); $MgCl_2$, 20; GSH, 2; pyridoxal 5-phosphate, 1; ATP, 25; CoA, 0.425; antimycin A, 2.5 μg; sodium malonate, 10; sodium DL-malate, 5; sodium arsenite, 5; purified bacterial succinyl CoA synthetase, 0.1 ml; enzyme and water to a final volume of 0.5 ml.

Tubes were incubated at 37° for 1 hour in a shaking water bath and the reaction stopped by the addition of 2 ml of 0.3M trichloracetic acid. 1.5 ml of water, containing 1 μmole of carrier ALA and 100 μmoles of unlabelled potassium succinate, were added and protein removed by centrifugation. 3 ml of the supernatant were added to Dowex

50 (H^+) columns (3 x 1 cm) and washed through the column with 30 ml of water. ALA was eluted with 10 ml of 2M pyridine-acetate buffer (pH 6.0) and the eluate collected in 100 ml round bottomed flasks containing 1 drop of concentrated HCl. The buffer was removed by evaporation under reduced pressure and the residue dissolved in 2 ml of water. 1.8 ml samples of this solution were mixed with 1 ml of 2M acetate buffer (pH 4.6) and 0.1 ml of acetyl acetone. The tubes were heated in a boiling water bath for 15 minutes and the ALA pyrrole was extracted into 25 ml of ethyl acetate. This was washed with 3 ml of water; the ethyl acetate layer was then dried and the residue dissolved in 1 ml of methanol. Samples (0.8 ml) were plated onto metal planchettes and counted in a Nuclear-Chicago gas flow counter. Alternatively, if scintillation counting was desired, 0.1 ml samples were dried on glass fibre discs.

When comparable colorimetric assays were required, ^{14}C-succinate was omitted from the incubation mixtures, all other conditions being the same. The reaction was stopped with 2 ml of 0.3M trichloracetic acid and 1.5 ml of water added. The determination of ALA produced was then as described in Fig. 3.1 (p. 61).

Fig. 4.12 compares the synthesis of ALA by DDC liver mitochondria, measured both by the colorimetric and radiochemical assay procedures. The amount of ALA synthesised, as measured by the ^{14}C incorporation agreed reasonably well with that determined in duplicate colorimetric assays; in both cases the synthesis of ALA was proportional to the amount of mitochondria added. The greater sensitivity of the radiochemical method was again noticeable; an observed value of 1675 cpm (representing the synthesis of 20 nmoles of ALA) was compared with an optical density reading of 0.096. In addition, providing the succinate requirement of the system for the synthesis of ALA was fulfilled, the incorporation of ^{14}C-succinate into ALA was doubled when the specific activity of the added succinate was raised from 0.47 to 0.95 $\mu C/\mu mole$. Thus the sensitivity of the assay can be readily adjusted to the requirements of any particular experiment.

Verification that the radioactivity measured in the methanol solution of ALA pyrrole was present as ALA pyrrole was obtained from scans of thin layer chromatograms. The single peak of radioactivity shown in Fig. 4.13 corresponded with the ALA pyrrole region, located colorimetrically. This

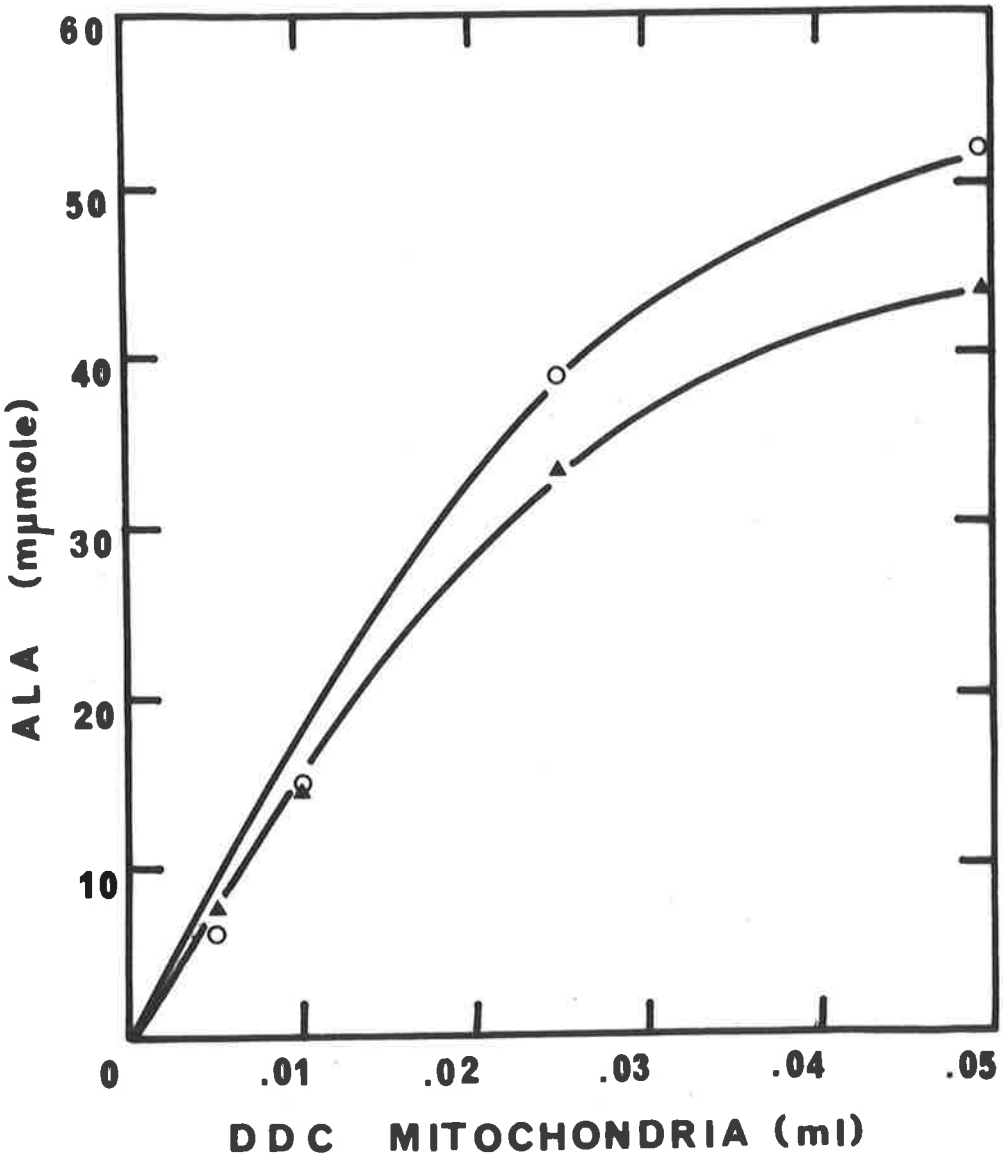


Fig. 4.12. Comparison of radiochemical and colorimetric assay methods for ALA synthesis by DDC liver mitochondria.

Incubation conditions and procedures for the radiochemical assay are fully described on p. 92 . Gas flow counting was used. (The radiochemical assays contained 10.6 μmoles of potassium succinate, specific activity 0.47 μC/μmole.)

○—○ , colorimetric assay; ▲—▲ , radiochemical assay.

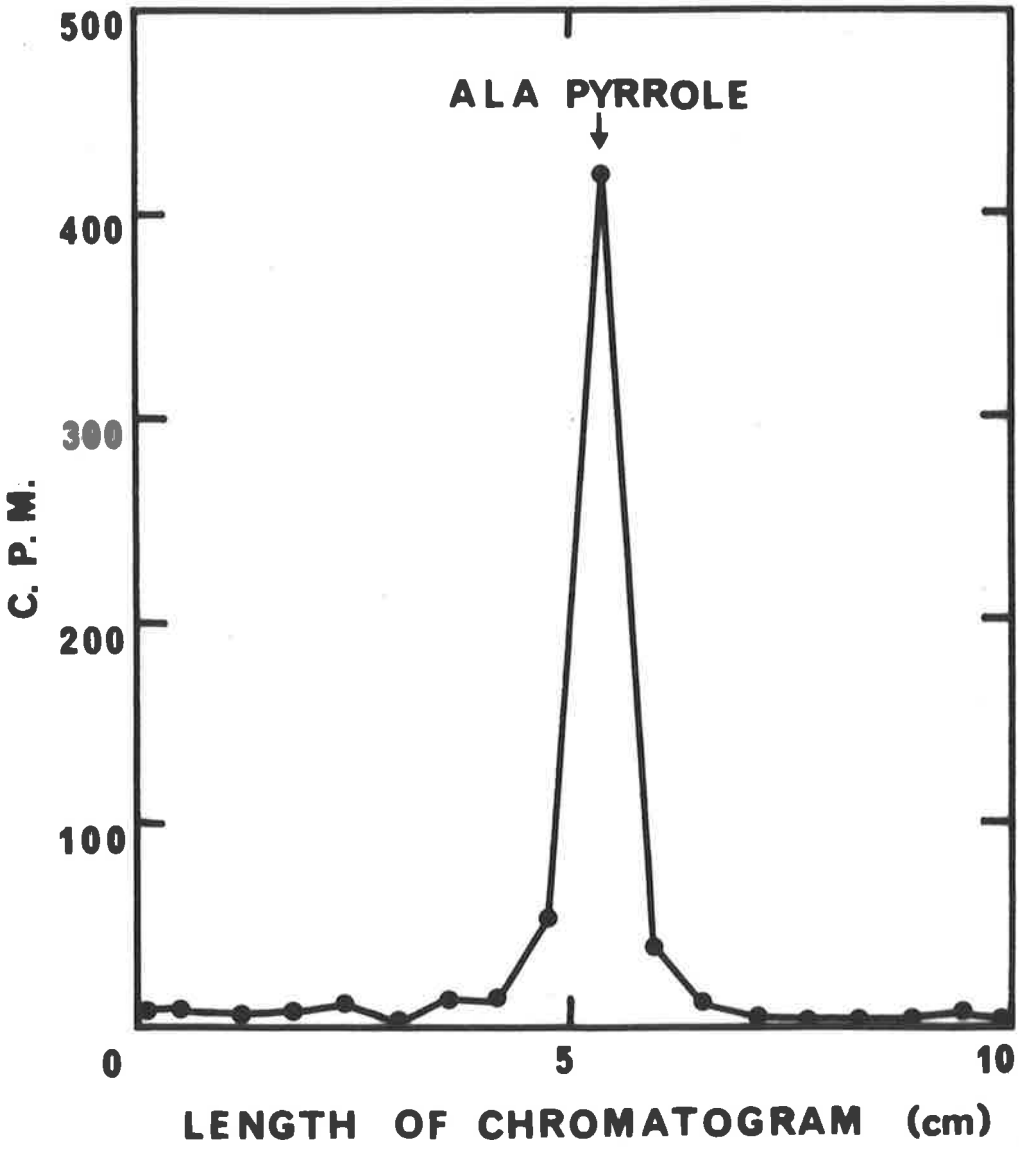


Fig. 4.13. Thin layer chromatography of ALA pyrrole derived from ^{14}C-ALA formed during incubations of DDC liver mitochondria. Experimental conditions were as described in Fig. 4.12. A sample, 0.2 ml, of the methanol solution of ALA pyrrole was chromatographed and the resulting chromatogram scanned for radioactivity as described in Fig. 4.5. Values shown on the ordinate are observed counts (corrected for background). The arrow shows the position of ALA pyrrole, located by spraying the remainder of the chromatogram with Ehrlich reagent (b).

result was confirmed by autoradiographs made by exposing X-ray film to the plates for three months. A single spot appeared on films after development, corresponding in position to ALA pyrrole.

One of the difficulties attached to the radiochemical method was the length of time involved in completing assays, especially if large numbers were required to be done simultaneously. Tests showed that trichloracetic acid supernatants of incubations could be stored frozen at -15° without affecting the results. If methanol solutions of ALA pyrrole were required for chromatography, the storage of these at -15° was also necessary.

(f) Application of the radiochemical assay to homogenates of livers of porphyric guinea pigs

Incubation mixtures contained, in addition to the constituents listed in Section (e) (p. 92), 1mM EDTA since the formation of porphobilinogen by liver homogenates was shown to be minimised when EDTA is included (Marver et al., 1966b). While no porphobilinogen was detected by direct Ehrlich reagent treatment of trichloracetic acid supernatants of incubation mixtures in the present work, the possible

effect of Mg^{++} (present to activate succinyl CoA synthetase) on the inhibition of porphobilinogen formation by EDTA has not been investigated.

The incorporation of ^{14}C-succinate into ALA by crude DDC liver homogenates is shown in Fig. 4.14. Values were proportional to the amount of homogenate added up to 5 mμmoles of ALA formed, but were approximately half those obtained from duplicate colorimetric assays. Optical density values were extremely low however, (ranging from 0.02 to 0.05 approximately) so that it is impossible to place significance upon them. In further tests with homogenates it was found that the incorporation of ^{14}C-succinate into ALA by frozen and thawed homogenates did not vary by more than 10% from values obtained with freshly prepared homogenates. It did not appear therefore that mitochondrial impermeability to succinate was a restricting factor in the assay. Autoradiographs of chromatograms of the pyrrole fraction showed one radioactive spot, located in the ALA pyrrole region.

In terms of sensitivity, an observed value of 410 cpm was obtained from the radiochemical assay for 0.02 ml of homogenate; this corresponded to an observed optical density of

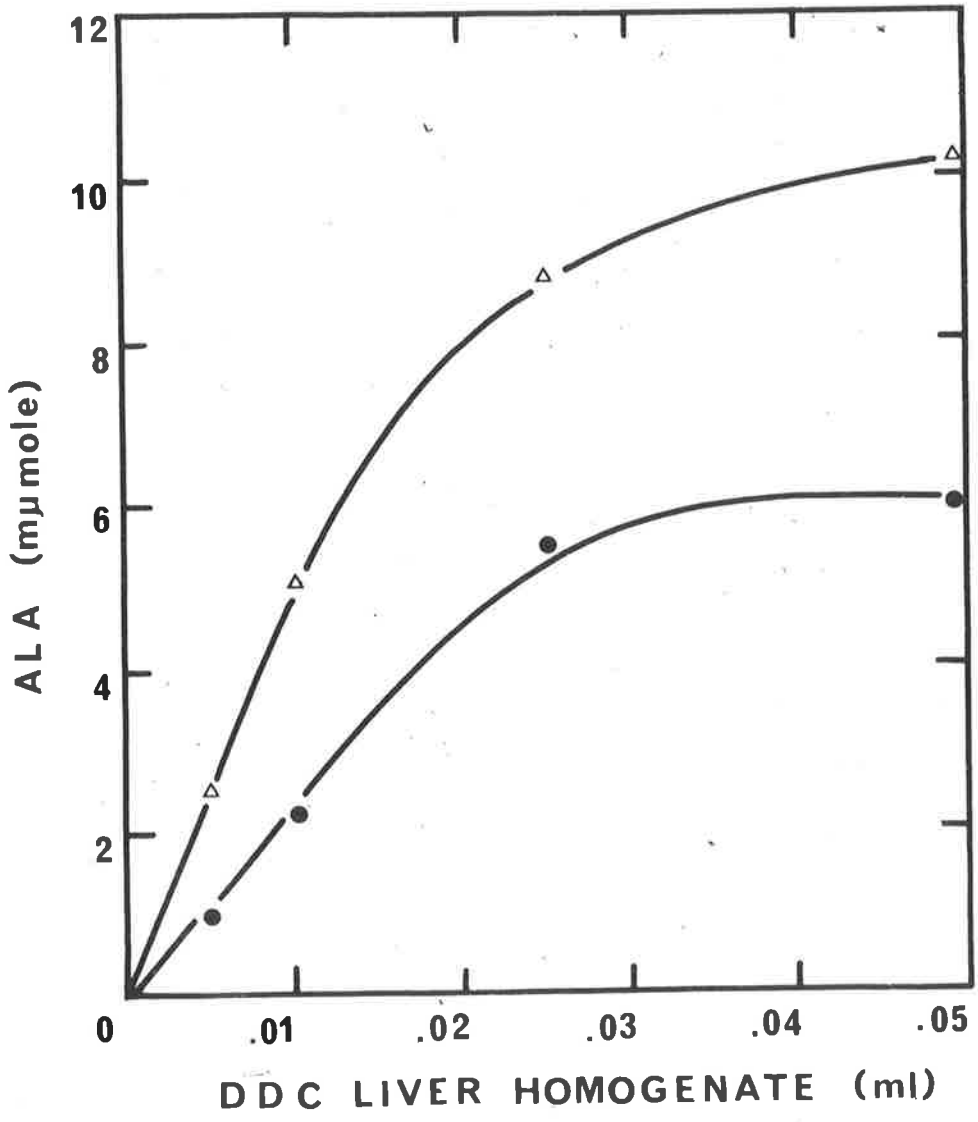


Fig. 4.14. Comparison of radiochemical and colorimetric assays of ALA synthetase activity in crude DDC liver homogenates. Experimental conditions for both colorimetric and radiochemical assays were as described in Fig. 4.12, except that all incubations contained 1 mM EDTA. Gas flow counting was used. △—△, colorimetric assay; ●—●, radiochemical assay.

0.035 for the same amount of homogenate.

2. DISCUSSION

The work of this chapter describes the development of a radiochemical assay for ALA synthetase. The method can be applied with simplicity to purified preparations of the bacterial enzyme (Fig. 4.4, p. 81) since all of the radioactivity present in the Dowex eluate is derived from ALA. Suitable modification of incubation conditions and isolation of ALA pyrrole make the method applicable to crude preparations of the liver enzyme. The assay can be adjusted to almost any required degree of sensitivity by increasing the specific activity of the succinate added to incubations. When low levels of the enzyme are expected, gas flow counting is the method of choice since larger samples of the pyrrole solution can be counted; but in general, scintillation counting was found to be preferable since each sample could be counted in duplicate. Using glass fibre discs, counting efficiency of the scintillation spectrometer was 65%; an added advantage was the greater absorbancy of these discs compared with that of filter paper.

That the method could be successfully applied to such small amounts of liver homogenates as 0.02 ml indicates the potential of the method to measure levels of ALA synthetase, hitherto undetectable. The actual amount of liver (wet weight) in 0.02 ml of homogenate is only 6 mg and Granick (1966) had found it possible, by increasing the number of cells in chick embryo liver cultures so that the protein content ranged between 1 and 2 mg, to make quantitative measurements of porphyrin production. The radiochemical assay, with the sensitivity suitably adjusted, should therefore enable direct measurements of ALA synthetase to be made in such <u>in vitro</u> systems. The elimination of AA synthetase as an interfering element in assays as mentioned above (p. 72) is particularly important since in normal tissues this activity greatly exceeds that of ALA synthetase activity.

CHAPTER 5

APPLICATION OF THE RADIOCHEMICAL METHOD OF ASSAY OF ALA SYNTHETASE TO A VARIETY OF BIOLOGICAL SYSTEMS

The new method of assay described in the previous chapter was developed, as already mentioned, for two main purposes. (1) The general need was felt for a method capable of application to tissues of low activity (or to very small amounts of material) and (2) it was specifically intended to use the method for enzyme measurement in normal tissues. On this latter point, the aims were again two-fold - firstly, an accurate knowledge of the level and variations in normal tissues seemed important and secondly, the use of tissue such as normal liver, obtainable in large amounts, for enzyme purification had obvious attractions compared with induction of animals with drugs.

The aims of the present author's work centred on the latter aspect, namely enzyme isolation, since studies of the control of synthesis is itself a long term project and outside the scope of the present thesis. (A separate study of this, using tissue culture systems and the new method of assay is the subject of a separate project in the laboratory.)

It was decided first to make a survey of ALA synthetase in a variety of normal tissues and cells using the new radio-

chemical assay method. The survey is described in this chapter. The reason for choosing the particular systems will more conveniently be dealt with in each section.

2. RESULTS

A. Estimation of ALA Synthetase in Normal Liver

It was confidently expected that guinea pig liver mitochondria would have an activity readily detectable by the radiochemical assay method. The reason for this expectation was that colorimetric assay had indicated a normal value which was 20% of the DDC induced level (see Fig. 5.1). Moreover, Marver et al. (1966b) had reported values in rat liver homogenates equivalent to 5% of those in liver homogenates from porphyric rats while Labbe, Kurumada and Onisawa (1965) obtained a value in mouse liver mitochondria which was 10% of that in liver mitochondria from porphyric mice. Finally, in liver from normal human subjects, the level was 10% of that found in the liver of a porphyric patient (Tschudy et al., 1965).

When applied to normal guinea pig liver, the radiochemical assay invariably gave inconclusive results in that observed counts were little if at all, above those obtained for zero

time incubations. A comparison of the activity of increasing amounts of normal and DDC liver mitochondria by the ^{14}C-incorporation method is shown in Fig. 5.1. The DDC liver mitochondria were at least 50 times more active than those from normal liver and this contrasted with a value of only 5 times when determined colorimetrically (Fig. 5.1). The radiochemical assay values were so low as to be insignificantly above zero, and in fact increasing levels of normal mitochondria gave no significant increase in ^{14}C-succinate incorporation into ALA. (This type of response was obtained whether or not normal liver mitochondria were stored in the presence of pyridoxal 5-phosphate.)

Using the radiochemical assay, no ALA synthetase activity could be detected in any of the following systems: liver homogenates from normal adult guinea pigs; liver mitochondria and liver homogenates from foetal and 10 day-old guinea pigs, liver mitochondria and liver homogenates of normal adult male rats. When whole liver homogenates were assayed, incubation mixtures contained 1 mM EDTA (see Fig. 4.14).

The assay in normal liver did not appear to be limited by the amounts of bacterial succinyl CoA synthetase included

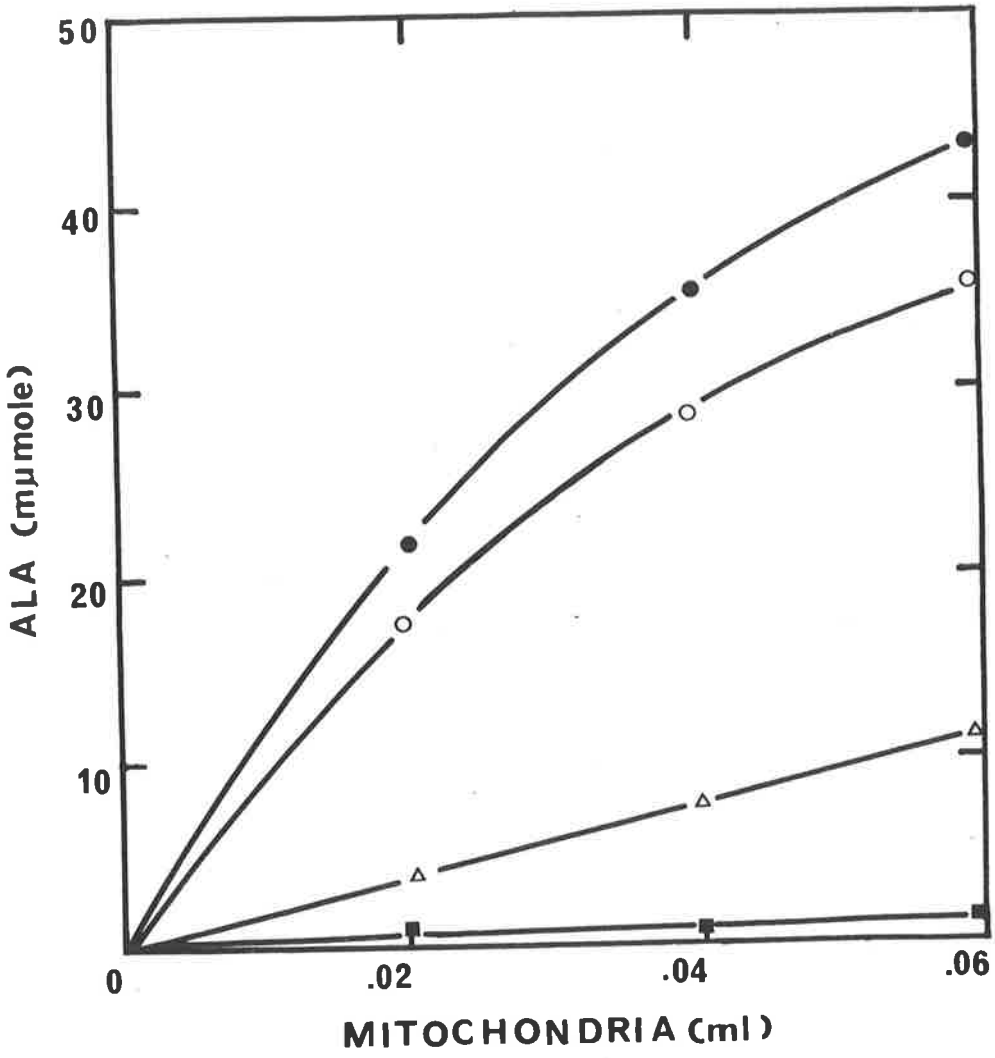


Fig. 5.1. Comparison of ALA synthetase activity in normal and DDC liver mitochondria as determined by both radiochemical and colorimetric assay procedures. Incubation conditions and procedures were as described in Chapter 4 (p. 92) for both colorimetric and radiochemical assays. Radioactivity in the methanol solution of ALA pyrrole (0.5 ml) was determined by scintillation counting of 0.15 ml aliquots dried on glass fibre discs.

●—● , colorimetric assay of DDC mitochondria;
○—○ , radiochemical assay of DDC mitochondria;
△—△ , colorimetric assay of normal mitochondria;
■—■ , radiochemical assay of normal mitochondria.

in incubation mixtures, since a three-fold increase did not significantly affect observed counts. When the sensitivity of the method was increased by raising the specific activity of the ^{14}C-succinate from 0.475 μC/μmole to 1.42 μC/μmole, there was still no detectable increase in the incorporation of ^{14}C-succinate into ALA with increasing amounts of mitochondria.

Scans of thin layer chromatograms of the pyrrole fraction obtained from radiochemical assays of normal guinea pig liver mitochondria showed no localisation of radioactivity in the ALA pyrrole region (Fig. 5.2) as was shown by DDC liver mitochondria (Fig. 4.13).

When the pyrrole fractions obtained from processing reaction mixtures of colorimetric assays of normal guinea pig liver mitochondria, were subjected to thin layer chromatography (see Fig. 4.2 for details) all of the detectable pyrrole present was derived from AA; in contrast, chromatograms obtained by similar processing of incubations of DDC liver mitochondria showed both AA and ALA pyrroles (Fig. 5.3).

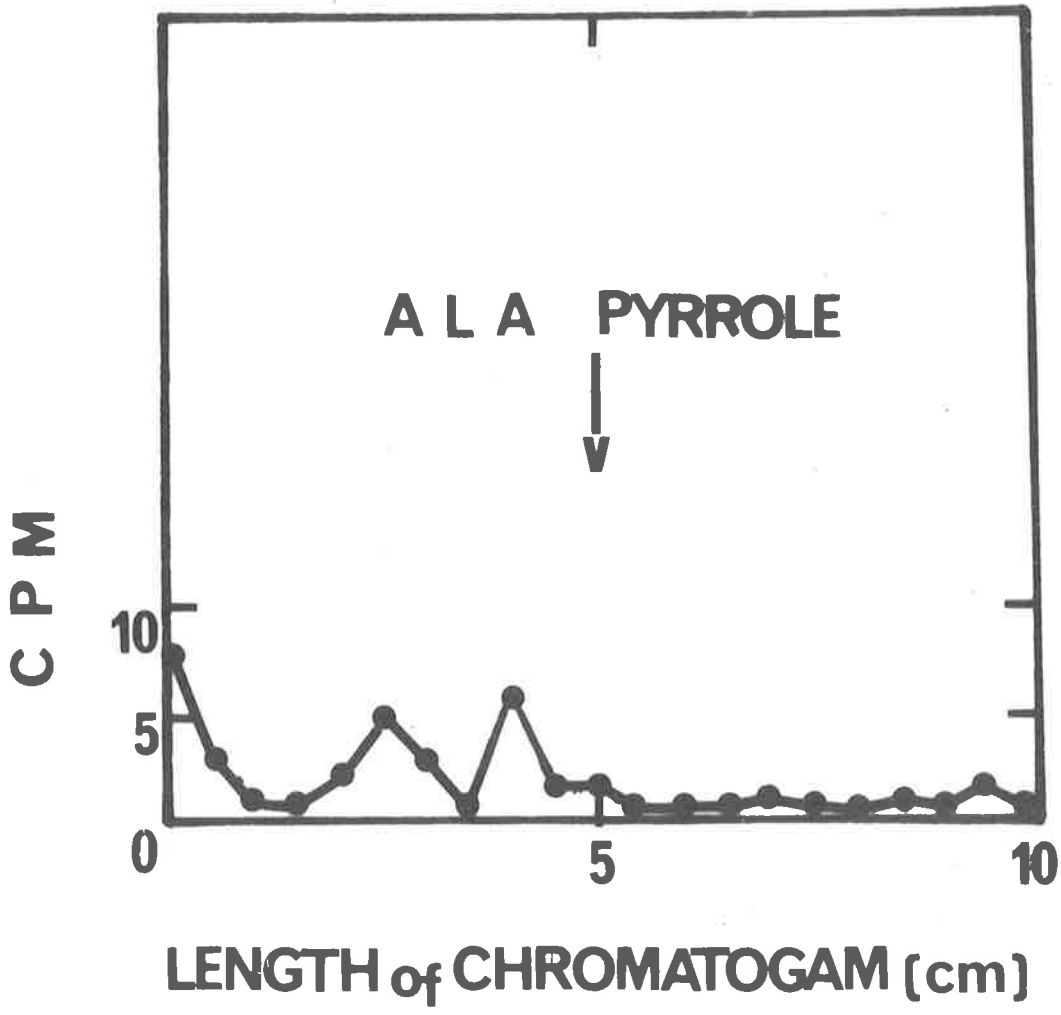


Fig. 5.2. Radioactivity scan of a chromatogram of the pyrrole fraction obtained from processing incubations of normal liver mitochondria. Incubation conditions and experimental procedures were as described in Fig. 5.1 for the radiochemical assay except that the specific activity of the ^{14}C-succinate was 0.95 μC/μmole and the volume of the final methanol solution was 0.4 ml. A sample, 0.2 ml, of this solution was chromatographed and the resulting chromatogram scanned for radioactivity as described in Fig. 4.13. The figures on the ordinate are corrected for background (20 cpm). The arrow shows the position of ALA pyrrole.

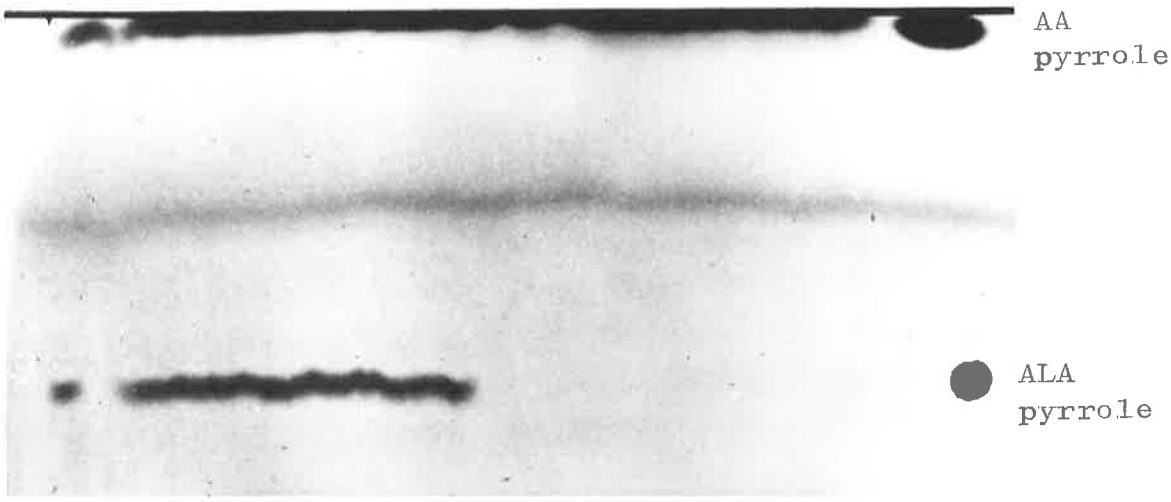


Fig. 5.3. Thin layer chromatography of the pyrroles derived from ALA and AA formed during incubations of liver mitochondria. Incubation conditions were as described in Fig. 5.1 for the colorimetric assay procedure. ALA and AA in the trichloracetic acid supernatants of incubations were converted to pyrroles and the ethyl acetate extract chromatographed and sprayed as described in Fig. 4.2. Marker pyrroles were prepared from synthetic ALA-HCl and AA-p-toluene sulphonic salt as described on p. 46 . (1), Incubations contained mitochondria isolated from the livers of normal guinea pigs, (2) incubations contained mitochondria isolated from the livers of DDC treated guinea pigs; (3) marker pyrroles. (The continuous streak is a solvent effect, not a pyrrole.)

B. Assay of ALA Synthetase of Reticulocytes

Immature red blood cells are the only 'normal' cells of animal tissues in which ALA synthetase activity is high enough to be readily detected (Laver, Neuberger and Udenfriend, 1958), a phenomenon presumably associated with haemoglobin synthesis. Considerable interest in ALA synthetase exists in view of its apparent role as the pacemaker in haem biosynthesis and in turn of haem biosynthesis regulating haemoglobin synthesis (Bruns and London, 1965; Grayzel, Horchner and London, 1966; Levere and Granick, 1967; Wainwright and Wainwright, 1967).

The successful application of the radiochemical assay method to such cells is shown in Fig. 5.4.

During maturation of the immature reticulocyte to the mature red blood cell, the capacity to synthesise haemoglobin diminishes, among many other capacities. It is possible to separate reticulocytes on the basis of stage of maturity using centrifugation in a density gradient of bovine serum albumin. With the collaboration of Mr. A.F. Williams, a study of the level of ALA synthetase in cells of different ages from such a gradient was made. It can be seen in Fig. 5.5. that ALA synthetase activity progressively disappears from cells as they mature. The cells

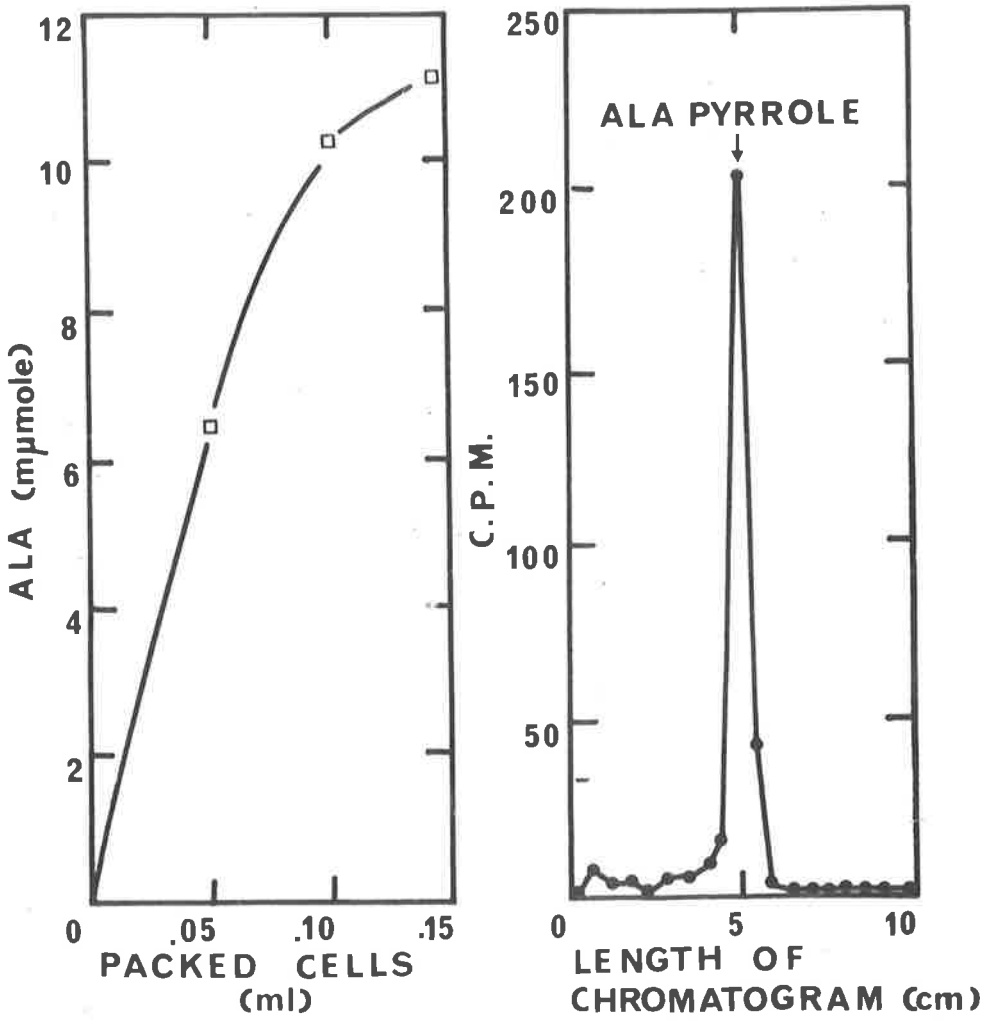


Fig. 5.4. Radiochemical assay of ALA synthetase in reticulocytes from the blood of anaemic chickens.

Incubation mixtures and procedures were as described in Fig. 5.1, except that the specific activity of the ^{14}C-succinate was raised to 1.1 μC/μmole. Values are calculated to represent the total ALA produced in the incubation mixtures. The methanol solution of ALA pyrrole was chromatographed and scanned for radioactivity as described in Fig. 5.2. Figures on the ordinate are corrected for background (20 cpm). The arrow shows the position of ALA pyrrole, located as described in Fig. 5.2.

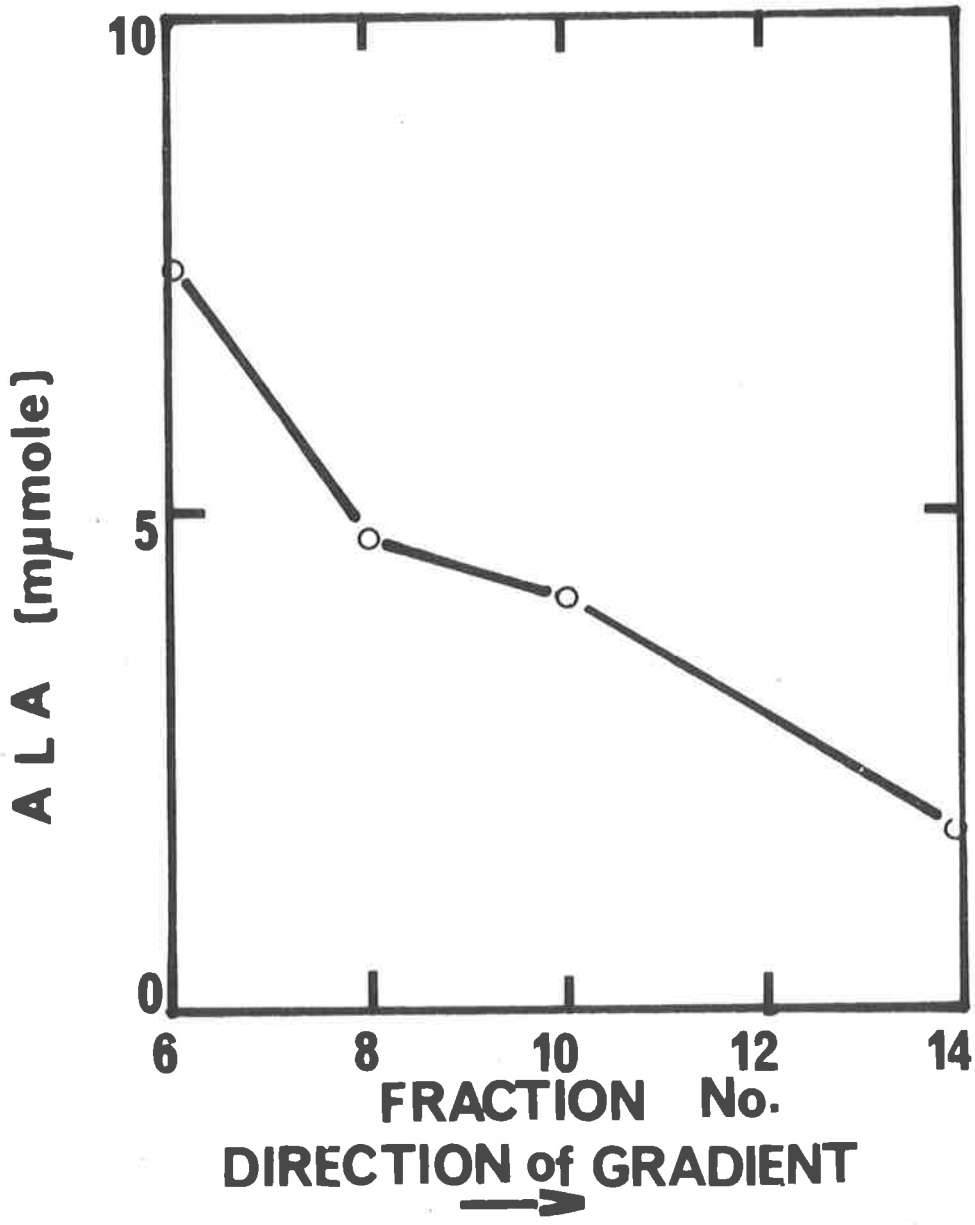


Fig. 5.5. Radiochemical assay of ALA synthetase in fractions of cells of different stages of maturity from the blood of anaemic chickens, separated by density gradient centrifugation.

Cells were fractionated as described in Materials and Methods (p. 51) and 0.05 ml samples of the fractions shown on the abscissa were assayed for ALA synthetase activity. (The volumes of each fraction were adjusted to contain 2 x 10^9 cells/ml.) Incubation mixtures and procedures were as described in Fig. 5.4. Values are calculated to represent the total amount of ALA produced in incubation mixtures.

from the gradient show good correlation between position on the gradient and cytological and biochemical maturation (A.F. Williams, personal communication). Since only small amounts of cells are available from such gradients, the usefulness of the sensitivity of the assay method is perhaps illustrated here.

The loss of ALA synthetase does parallel the loss of haemoglobin synthesising capacity, but there is of course no evidence that ALA synthetase decay is causally related to the cessation of haemoglobin synthesis. Mitochondria, ribosomes and other cellular components are also lost (Rubenstein, Ottolenghi and Denstedt, 1956; Glowacki and Millette, 1965, Rowley and Morris, 1967; Simpson and Kling, 1968).

C. Assay of ALA Synthetase of Micro-organisms

ALA synthetase has been reported in R. spheroides and Rhodospirillum rubrum (Kikuchi et al., 1958), but in no other micro-organism. With the collaboration of Dr. R.J. Porra, an investigation was made of ALA synthetase levels in a variety of micro-organisms - other than members of the Athiorhodaciae group - known to synthesise either haem and haem proteins or photosynthetic pigments and which must presumably use ALA.

Using the radiochemical assay, ALA synthetase activity was not detected in any of the following preparations: (1) crude extracts of Euglena gracilis grown either in light or darkness; (2) crude extracts of Saccharomyces cerevisiae grown aerobically on glucose or lactate, or anaerobically on glucose; (3) crude extracts of Torulopsis utilis (T. utilis) grown aerobically on glucose medium. ALA synthetase activity was not detected in either the pellets or supernatants of these crude extracts after centrifugation at 100,000 x g for one hour.

However, when T. utilis was grown anaerobically (as described in Materials and Methods, p. 54) ALA synthetase activity was readily detected in crude extracts of the organism by the radiochemical assay (Fig. 5.6). The assay was verified by thin layer chromatography of the pyrrole fraction, the peak of radioactivity corresponding with the ALA pyrrole band located colorimetrically (Fig. 5.6).

The haem content of this extract (determined as described in Materials and Methods, p. 57) was only 0.045 μmole of haem/mg of protein, while an identical extract from aerobically grown T. utilis contained 1.45 μmole of haem/mg of protein. Furthermore, adaptation of anaerobically-grown T. utilis

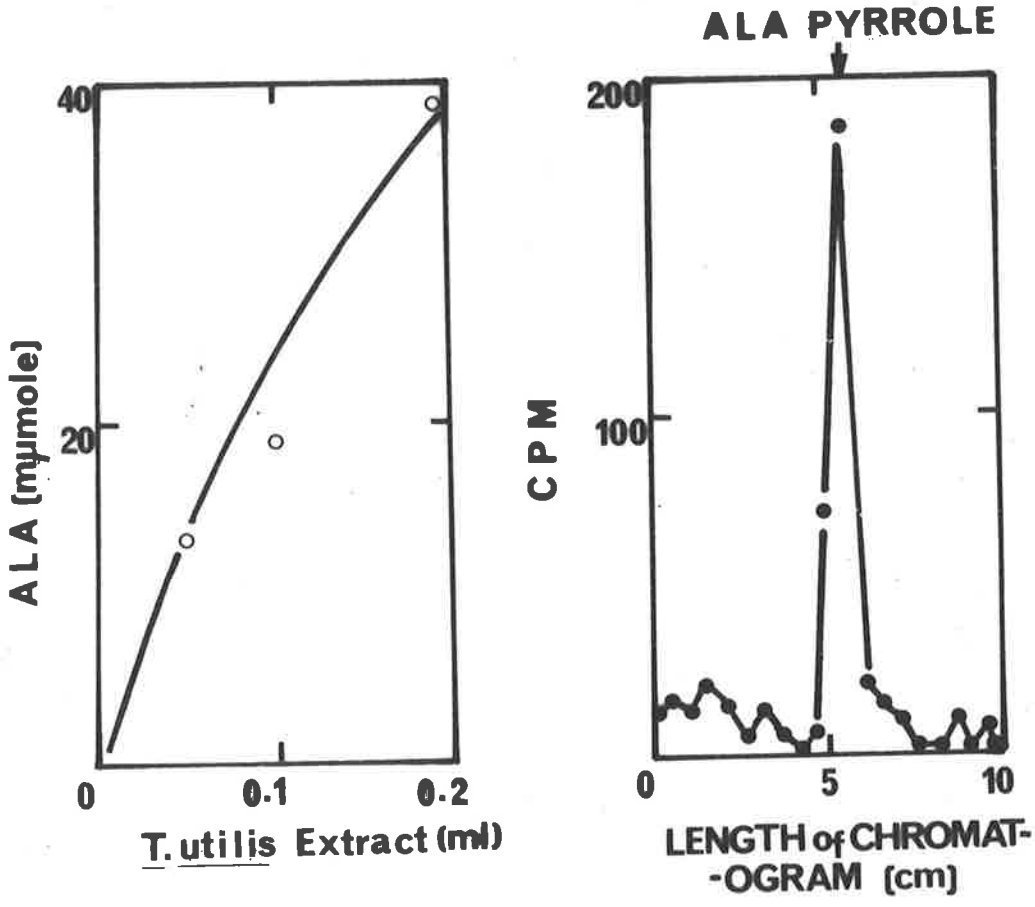


Fig. 5.6. Radiochemical assay of ALA synthetase in a cell free extract of T. utilis.

The organism was grown anaerobically as described in Materials and Methods. Protein concentration of the extract was 74 mg/ml. Incubation conditions and procedures were as described in Fig. 5.1. Values are calculated to represent the total amount of ALA produced in incubation mixtures. A sample of the methanol solution of ALA pyrrole was chromatographed and scanned for radioactivity as described in Fig. 5.4. The arrow shows the position of ALA pyrrole located colorimetrically.

to aerobic conditions resulted in complete loss of detectable ALA synthetase activity within one hour.

D. Estimation of ALA Synthetase in Plant Material

The presence of all the enzymes required for the conversion of ALA to haem (Porra and Lascelles, 1968) and protochlorophyll (Granick, 1961) has been demonstrated in plant proplastids. Granick and Gibor (1967) have discussed the possible autonomy of plant chloroplasts and proplastids for haem biosynthesis, but as yet the presence of ALA synthetase in these plant organelles has not been demonstrated.

In collaboration with Dr. R.J. Porra, the question of the ability of such plant organelles to incorporate ^{14}C-succinate into ALA was examined. However, no ALA synthetase activity was detected in any of the plant fractions tested. These included beam chloroplasts, proplastids and mitochondria; and spinach chloroplasts. Incubations were carried out at 26^{o} for varying lengths of time up to 120 minutes; some preparations were frozen and thawed before assay and in others 1 mM EDTA was included in incubations to inhibit the further metabolism of any ALA which might be

formed to PBG.

Furthermore, the enzyme could not be detected in crude extracts, or in isolated chloroplast, mitochondrial, or soluble fractions of primary leaves of etiolated pea (Pisum sativum) plants grown as described for etiolated bean plants (see Materials and Methods, p. 51), but in which chloroplast formation was induced by exposure to light (800 foot candles) for four hours prior to harvesting the leaves. (Chloroplast formation is complete after twenty-four hours, but after four hours the chlorophyll content is rising steeply (N.K. Boardman, personal communication). (The protein and chlorophyll content of the plant and algal preparations tested is shown in Table 5.1 for reference.)

5. DISCUSSION

The work of this chapter illustrates that the radiochemical assay for ALA synthetase can be applied to different types of tissue satisfactorily.

It was surprising to find that, despite its greater sensitivity, the method failed to detect any enzyme even in preparations of plant cells in which chlorophyll is produced

TABLE 5.1.

The various fractions listed were prepared as described in Materials and Methods (p. 51). Protein and chlorophyll determinations were carried out as described in Materials and Methods (p. 58). (No ALA synthetase activity was detected in any of the fractions listed.)

TABLE 5.1

PROTEIN AND CHLOROPHYLL CONTENT OF PLANT AND ALGAL PREPARATIONS USED FOR ALA SYNTHETASE ASSAYS DESCRIBED IN THE TEXT

Plant or algae	Fraction	Fresh wt. of leaves (g)	Volume of fraction (ml)	Protein (mg/ml)	Chloro-phyll (µg/ml)
Spinach	Washed chloroplast	20.0	10.0	3.3	67
Bean	" "	20.0	3.0	9.5	604
"	Mitochondria	20.0	3.0	4.8	331*
Etiolated bean	Washed proplastids	15.0	3.0	3.0	0
"	Mitochondria	20	3.0	2.7	0
Euglena gracilis (light-grown)	Crude extract	-	-	18.0	600
Euglena gracilis (dark-grown)	Crude extract	-	-	6.6	0
Pea	Crude extract	5.5	13.5	6.3	6.4
"	Washed chloroplasts	10.0	3.0	10.5	19.0
"	Mitochondria	10.0	3.0	11.0	12.4*
"	Supernatant	10.0	26.0	10.3	8.2*

* These fractions were contaminated with chloroplast fragments.

(see Table 5.1). It is possible, in plant tissues that ALA is synthesised by an alternative pathway; Gassman, Pluscec and Bogorad (1968) have isolated a transaminase in Chlorella vulgaris which converts γ-δ-dioxovaleric acid to ALA using L-α-alanine, L-glutamic acid or L-phenylalanine as an amino group donor. However, the significance of this enzyme is difficult to determine since the equilibrium constant is so small; in addition, the intracellular concentration of the enzyme is not increased by treatments that induce chlorophyll formation.

The discovery of ALA synthetase in anaerobically but not aerobically grown T. utilis is consistent, perhaps, with the finding of Harriott, Neuberger and Tait (1969) that a low molecular weight, non-protein activator of ALA synthetase is formed in R. spheroides grown under semi-anaerobic conditions, while a low molecular weight, non-protein factor which abolishes this activation is formed in aerobically-grown cells. While the presence of such factors might account for the rapid loss of enzyme activity when anaerobically grown cells are adapted to aerobic conditions, it would not explain either the complete loss of activity under these conditions or the complete absence of detectable activity when T. utilis is grown aerobically.

Furthermore, the presence of such activators and inhibitors in R. spheroides is consistent with the metabolic changes that occur in aerobic and anaerobic conditions in R. spheroides but not in T. utilis; the adaptation of R. spheroides from non-photosynthetic (aerobic-dark) to photosynthetic (anaerobic-light) conditions is accompanied by increased intracellular ALA synthetase activity (Lascelles, 1959) and increased intracellular concentrations of tetrapyrroles, i.e. bacteriochlorophyll and haems (Porra and Lascelles, 1965) whereas cells of anaerobically grown T. utilis contain less haem than those grown aerobically (Linnane, Vitols and Newland, 1962).

The possibility was also considered that, as a result of the higher haem content of crude extracts of the latter cells, ALA synthetase activity was inhibited. Again, this would not explain the complete absence of detectable activity in these extracts since even at high concentrations haem does not inhibit completely the ALA synthetase of R. spheroides (Burnham and Lascelles, 1963). Thus it is not possible, on the basis of existing knowledge of ALA synthetase, to explain the detection of this enzyme in anaerobically - but not aerobically - grown T. utilis.

It should perhaps be emphasised that all of the applications of the method described in this chapter constituted a general survey of a preliminary kind and no attempt has been made to investigate any of the areas in depth, due to limitation of the author's time.

In view of this it would be premature to conclude that no enzyme exists in some of the tissues studied. Thus it may be that the level of ALA synthetase in normal liver varies according to species, age, sex and physiological state. That this may be so is evidenced by the detection of small but significant activity in the livers of female rats by Marver et al. (1966b) who used a method which carefully excluded AA interference.

The failure to readily detect ALA synthetase in normal liver made the idea of using such tissues as a source of enzyme for purification impracticable. No attempt has therefore been made to follow up this work with an investigation of variations in normal levels.

CHAPTER 6

THE INHIBITION OF ALA SYNTHETASE ACTIVITY BY A FACTOR IN NORMAL LIVER MITOCHONDRIA

ALA synthetase activity in normal liver mitochondria and liver homogenates was not detectable by the radiochemical method of assay. While this apparent lack of activity could be due to absence of the enzyme, there is the alternative possibility that it is due to some factor in normal mitochondria affecting the assay.

To test this possibility mixing experiments between normal and DDC liver mitochondria and mitochondrial extracts were carried out. As a first approach to the problem, the effect of normal liver mitochondria on the incorporation of ^{14}C-succinate into ALA by DDC liver mitochondria was investigated. Preliminary experiments indicated that ALA synthetase of DDC liver mitochondria was inhibited by normal liver mitochondria and this chapter describes an investigation of this effect.

1. RESULTS

A. ALA Synthesis in Mixtures of Normal and DDC Liver Mitochondria

Unless otherwise stated mitochondrial preparations

used were once frozen and thawed, and were stored in the presence of pyridoxal 5-phosphate, added to stabilise the enzyme (see Chapter 4, p.84).

Fig. 6.1 shows the effect of increasing amounts of normal liver mitochondria on the incorporation of ^{14}C-succinate into ALA by a fixed amount of DDC liver mitochondria. The activity in 0.05 ml of the DDC mitochondrial suspension was reduced by 10% when 0.01 ml of the normal liver preparation was added and by more than 70% when this was increased to 0.2 ml.

Since these results were obtained using the radiochemical assay method, it was possible to check their validity by thin layer chromatography of the methanol solutions of ALA pyrrole (Fig. 6.2). The decreasing height of the radioactive peak, corresponding with the ALA pyrrole region of the chromatogram, clearly showed that as the amount of normal liver mitochondria included in incubations was increased, so the incorporation of ^{14}C-succinate into ALA was decreased. For comparison, the scan of the chromatogram obtained by similar processing of an incubation of normal liver mitochondria alone is also shown (bottom curve); there was no localisation of radioactivity in the ALA pyrrole region.

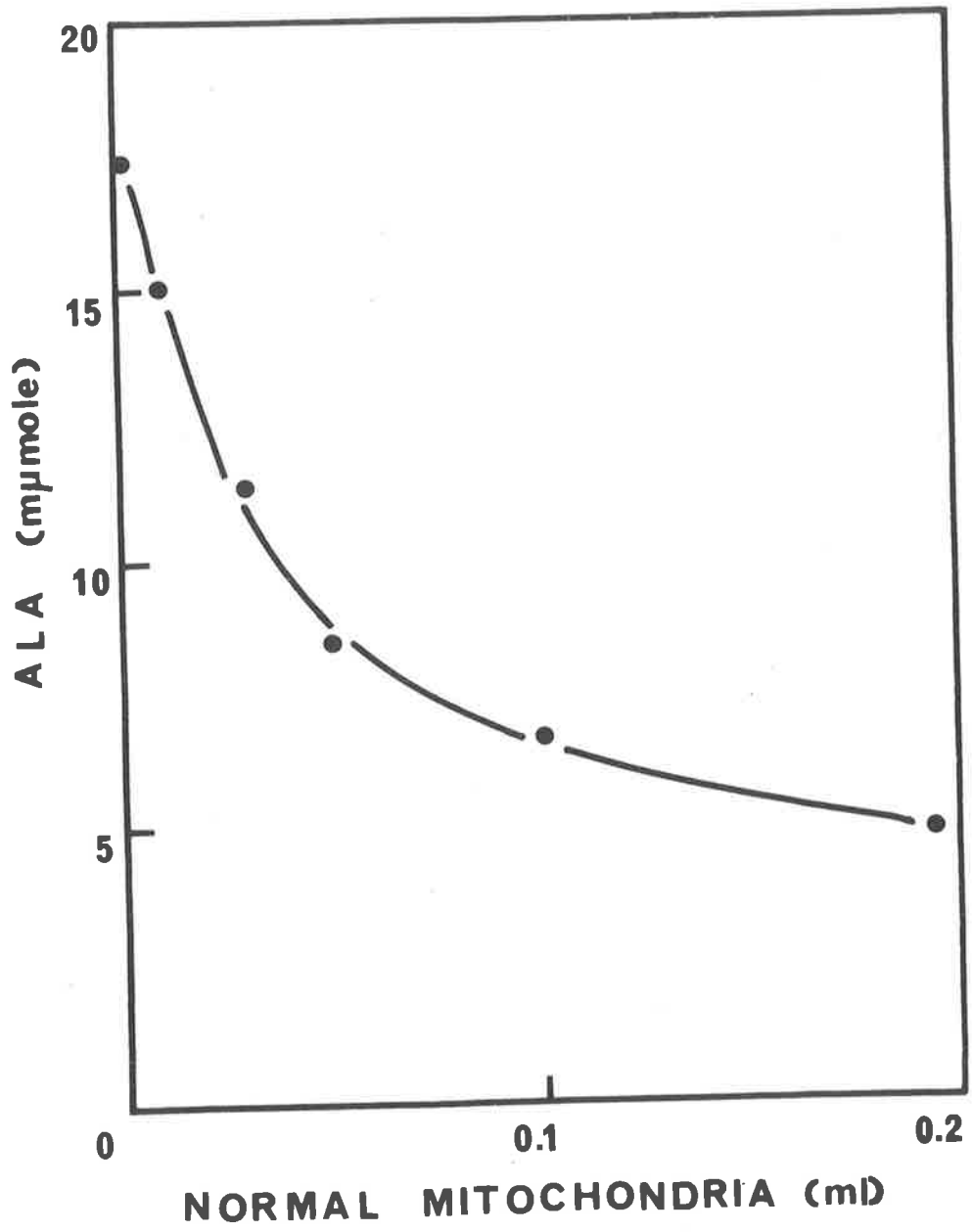


Fig. 6.1. The effect of normal liver mitochondria on ALA synthesis by DDC liver mitochondria. Incubation conditions and experimental procedures were as described in the text (Chapter 4, p. 91) for the radiochemical assay method. All incubations contained 0.05 ml of DDC liver mitochondria and the figures on the ordinate represent total ALA produced in each incubation.

Fig. 6.2. Thin layer chromatography of ALA pyrrole from experiments in Fig. 6.1.

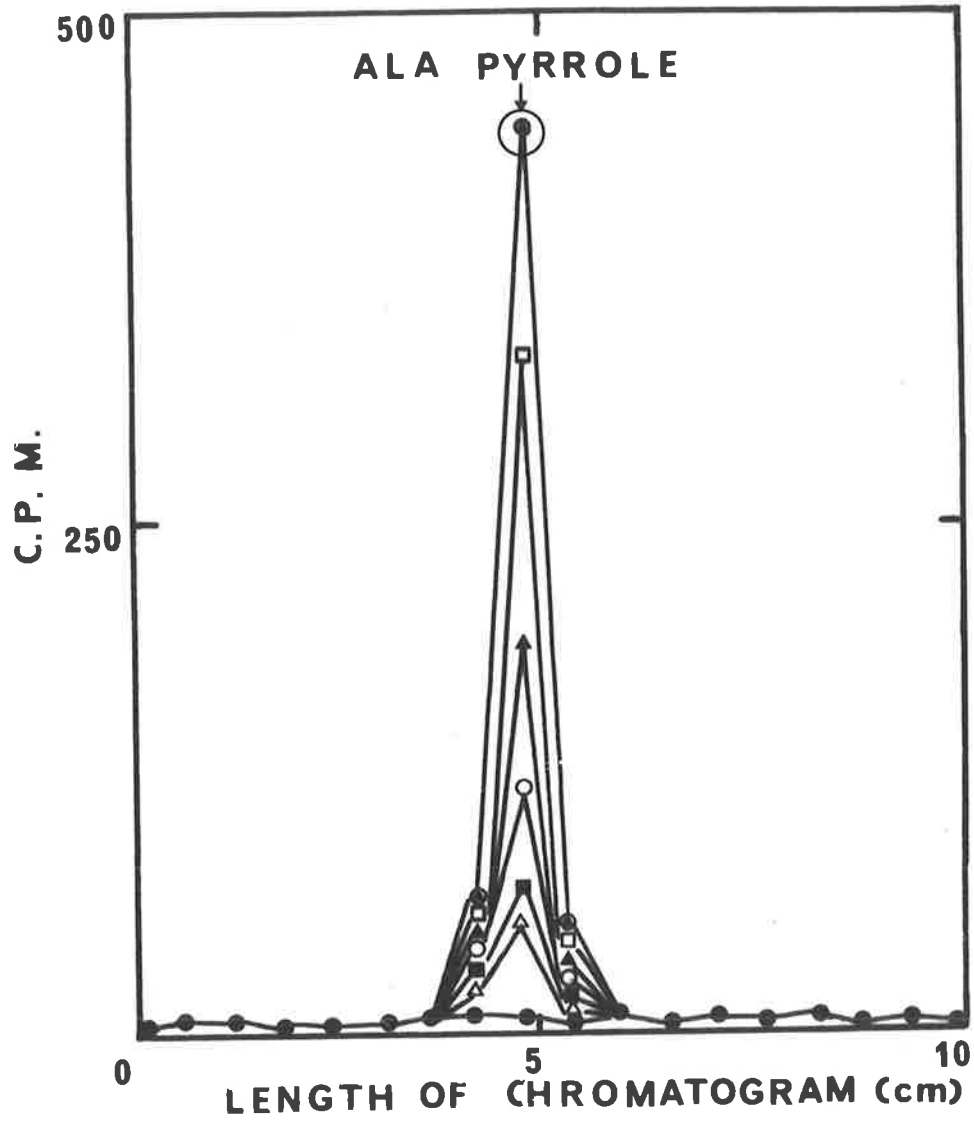
The methanol solutions of ALA pyrrole from which the points in Fig. 6.1 were obtained were chromatographed and the chromatograms scanned for radioactivity as described in Fig. 5.2. In addition an incubation containing only normal mitochondria was similarly treated. The arrows shows the centre of the ALA pyrrole spot located colorimetrically. Volumes of normal mitochondria were added to 0.05 ml of DDC mitochondria:

⊙ , none; □ , 0.01 ml;

▲ , 0.03 ml; ○ , 0.05 ml;

■ , 0.10 ml; △ , 0.20 ml;

● , control (no DDC mitochondria; 0.10 ml of normal mitochondria).


500
ALA PYRROLE
C.P.M.
250
0
5
10
LENGTH OF CHROMATOGRAM (cm)

A similar investigation of ALA synthesis in mixtures of normal and DDC liver mitochondria was carried out using the colorimetric assay method and the results (Fig. 6.3) confirmed those obtained by the radiochemical assay procedure. The effect is less here; some variability in inhibition obtained occurs. ALA synthesis by increasing amounts of DDC liver mitochondria in the presence of a fixed amount of normal liver mitochondria is also shown; the parabolic shape of the curve indicates that the inhibitory effect is overcome as the amount of DDC liver mitochondria is increased.

Since the results of colorimetric assays confirmed the inhibitory effect of normal liver mitochondria on ALA synthesis by DDC liver mitochondria, and in view of the cost of the ^{14}C-succinic acid, the results in the further work described in this chapter were obtained (unless otherwise stated) using the colorimetric assay method.

The effect of mitochondria freshly isolated from the livers of normal guinea pigs (i.e. not frozen and thawed) on ALA synthesis by mitochondria freshly isolated from the livers of DDC treated guinea pigs was also examined. Fig. 6.4 shows that the inhibition of ALA synthetase activity was much lower under these circumstances, being only 17% when incubations included 0.2 ml of the normal

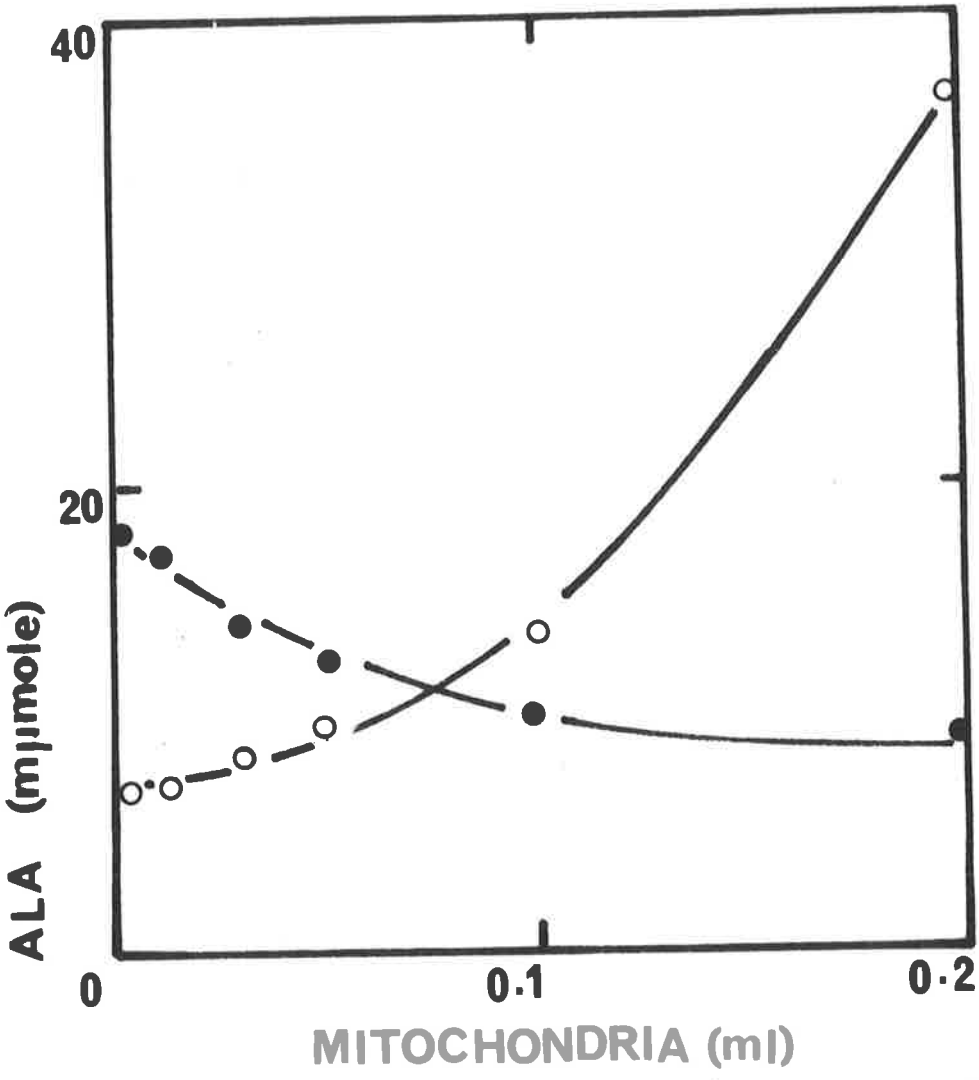


Fig. 6.3. ALA synthesis in mixtures of normal and DDC liver mitochondria, measured by the colorimetric assay method.

Incubation conditions and experimental procedures were as described in the text (Chapter 4, p. 93) for the colorimetric assay method. Figures on the ordinate represent total ALA produced in each incubation.
●—● , 0.05 ml of DDC liver mitochondria and variable normal liver mitochondria; ○—○ , 0.1 ml of normal liver mitochondria and variable DDC liver mitochondria.

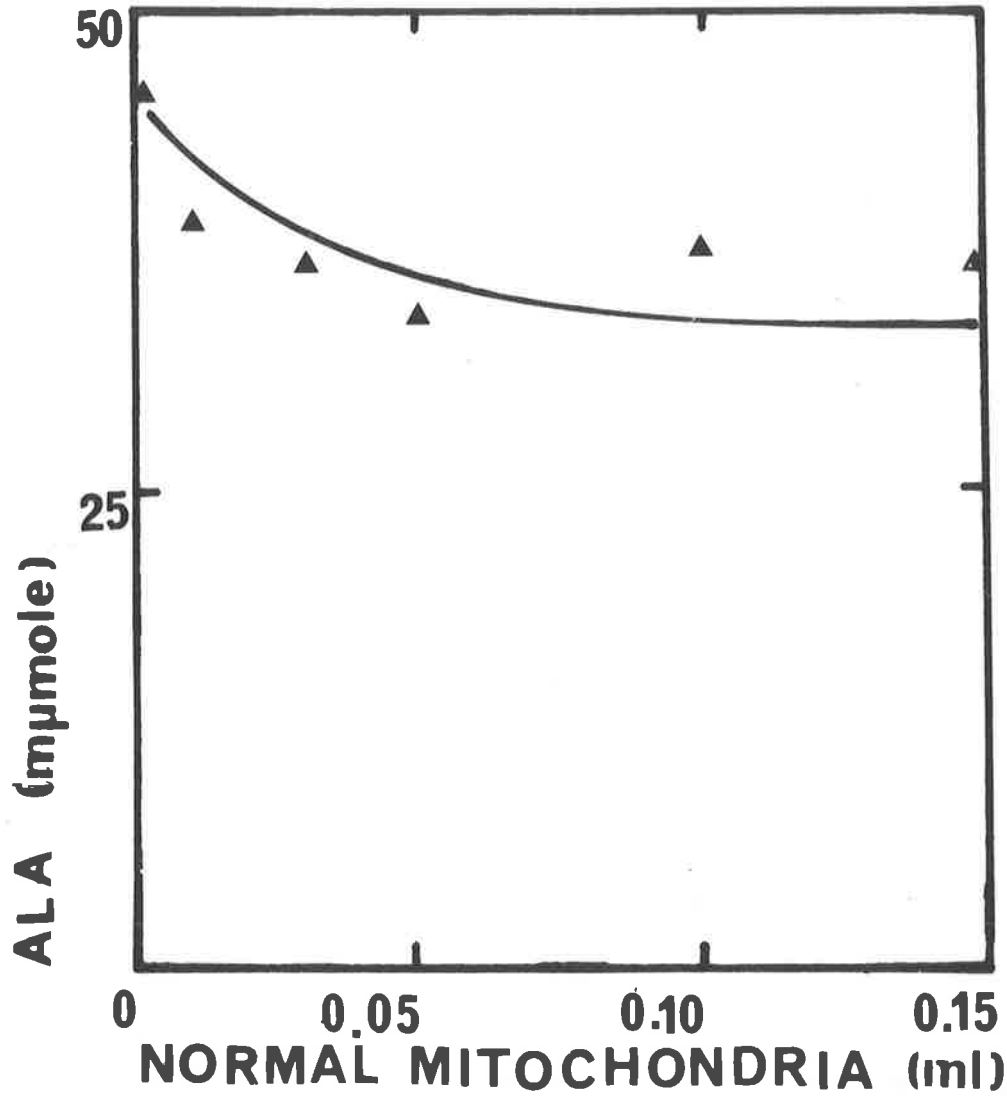


Fig. 6.4. The effect of mitochondria, freshly isolated from the livers of normal guinea pigs, on the synthesis of ALA by freshly isolated DDC liver mitochondria.

Incubation conditions and experimental procedures were as described in Fig. 6.3 except that all incubations contained 0.05 ml of DDC liver mitochondria. Figures on the ordinate represent total ALA produced in each incubation.

preparation.

B. Location of the Inhibitory Factor in Liver Mitochondria

The obvious decrease in the inhibitory effect of normal liver mitochondria when they were not frozen and thawed indicated that the degree of inhibition might be related to the structural integrity of the mitochondria. That this may be so is also suggested by the work of Harver *et al.* (1966d) who found no effect on AIA synthesis when normal liver homogenates were mixed with liver homogenates from AIA treated rats, in which presumably, mitochondria were intact.

Accordingly, the effect of sonic disruption on the inhibitory properties of normal liver mitochondria was examined and an investigation was made to see whether they were confined to the soluble or insoluble fraction of ultrasonicated mitochondrial suspensions.

A suspension of normal liver mitochondria was subjected to ultrasonication for 15 seconds and was then centrifuged at 100,000 x g for 60 minutes. The supernatant is designated the soluble fraction.

The insoluble residue remaining after removal of the soluble fraction was resuspended in 0.1M tris-HCl (pH 7.4) using a Potter-Elvejhem homogeniser and the volume of the suspension was adjusted to that of the original mitochondrial suspension prior to centrifugation. Such preparations are designated normal mitochondrial debris in this thesis.

(a) The effect of the soluble fraction of normal mitochondria on ALA synthetase activity in DDC liver mitochondria

Increasing amounts (0.01 to 0.2 ml) of the soluble fraction were added to incubations containing a fixed amount of DDC liver mitochondria; virtually no inhibitions of ALA synthetase activity was caused by the soluble fraction. The soluble fraction itself was also assayed for ALA synthetase activity using the radiochemical method; no activity was detected however.

(b) The effect of normal mitochondrial debris on ALA synthetase activity in DDC liver mitochondria

In contrast to the soluble fraction, normal mitochondrial debris had a marked inhibitory effect on ALA synthetase activity in DDC liver mitochondria. Fig. 6.5 shows that ALA synthesis by a fixed amount of DDC liver mitochondria

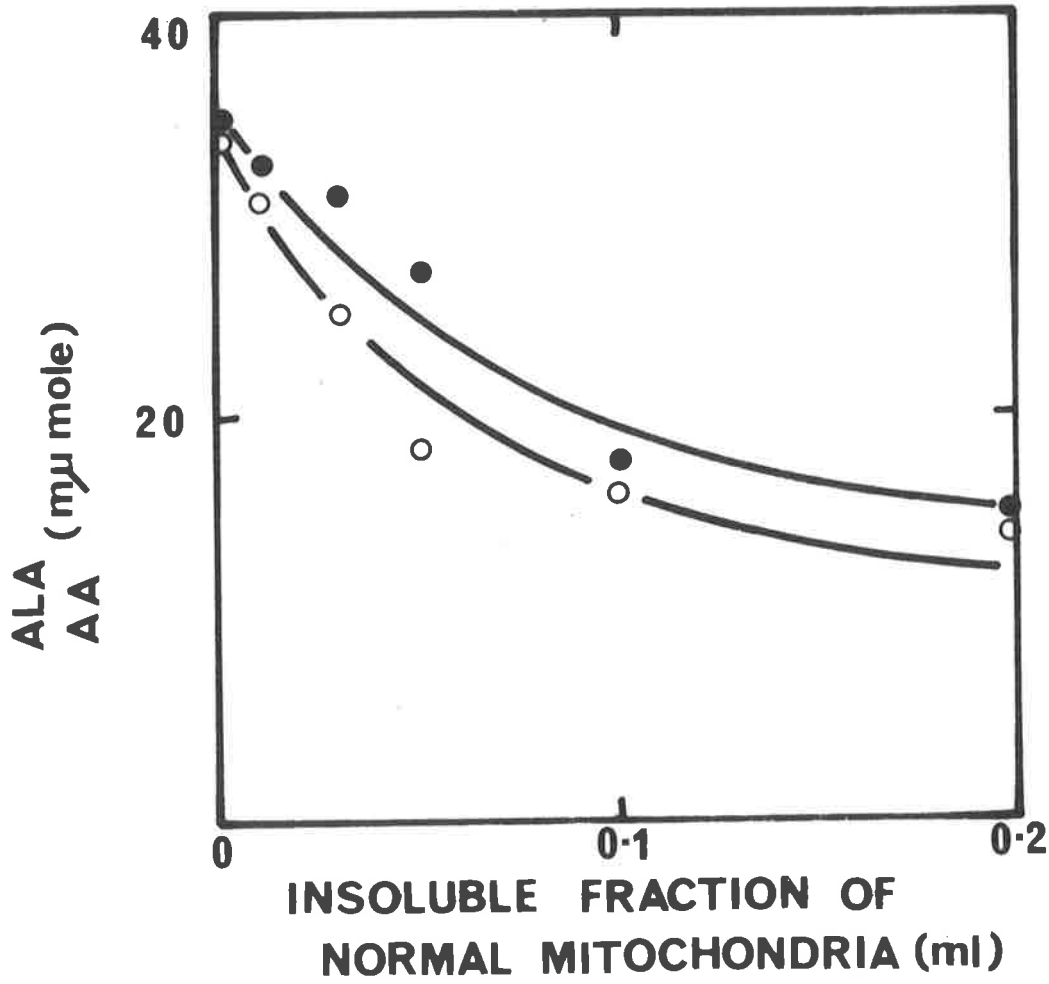


Fig. 6.5. The effect of normal mitochondrial debris on ALA and AA synthesis by DDC liver mitochondria.

Incubation conditions and experimental procedures were as described in Fig. 6.4. The concentration of protein in the suspension of mitochondrial debris was 27 mg/ml. Figures on the ordinate represent total amounts of ALA or AA produced in each incubation. ○—○ , ALA synthesis; ●—● , AA synthesis.

was progressively inhibited as the amount of normal mitochondrial debris included in incubations was increased; and furthermore the degree of inhibition was comparable with that caused by the same volumes of the original mitochondrial suspension (see Fig. 6.3). (It was noted that the synthesis of AA by DDC liver mitochondria was decreased in the presence of normal mitochondrial debris and this effect is also shown in Fig. 6.5.)

(c) The effect of the insoluble fraction of ultrasonicated DDC liver mitochondria on ALA synthetase activity

In view of the above results, it seemed desirable to determine whether or not a similar preparation of DDC liver mitochondria also inhibited ALA synthetase activity. A suspension of the insoluble fraction of ultrasonicated DDC liver mitochondria was prepared as described above for the preparation of normal mitochondrial debris; this preparation is designated DDC mitochondrial debris.

It was found that DDC mitochondrial debris also inhibited ALA synthetase activity (Fig. 6.6) but that this inhibition was much less than was seen with a comparable preparation of normal mitochondrial debris. (This is also shown in Fig. 6.6.) While this could have been due to the

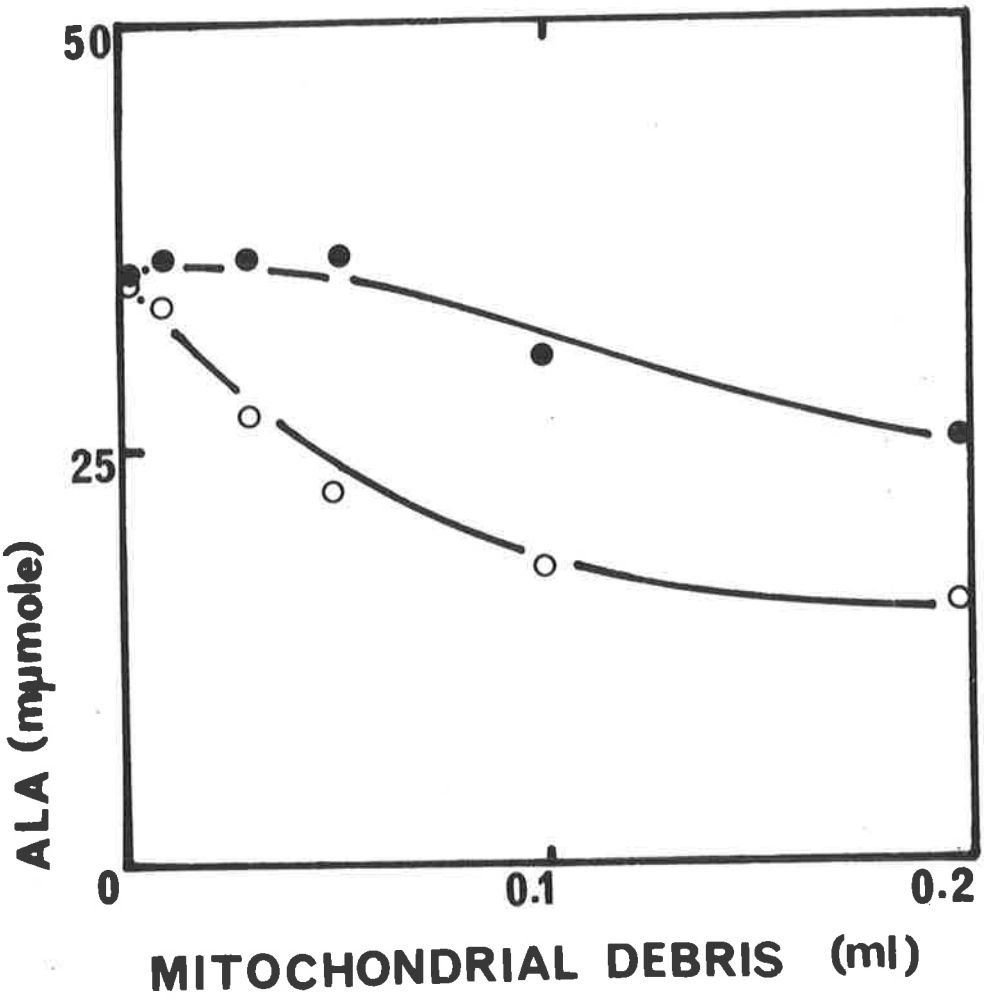


Fig. 6.6. The effect of normal and DDC mitochondrial debris on ALA synthesis by DDC liver mitochondria. Incubation conditions and experimental procedures were as described in Fig. 6.5. All incubations contained 0.05 ml of DDC liver mitochondria. The concentration of protein in the suspension of DDC mitochondrial debris was 21 mg/ml and that of the normal preparation was 27 mg/ml. Figures on the ordinate represent total ALA produced in each incubation. ●—●, DDC mitochondrial debris; ○—○, normal mitochondrial debris.

fact that the protein concentration of the latter (27 mg/ml) was greater than that of the former (21 mg/ml), the two preparations were comparable in that the protein concentrations of the original mitochondrial suspensions was the same. (It was noted throughout this work however, that DDC liver mitochondrial suspensions invariably contained less debris than comparable preparations from normal liver.)

The possibility that DDC mitochondrial debris contained ALA synthetase was also investigated since its inhibitory properties could have been influenced by the presence of the enzyme; however, no activity was detected.

(d) The effect of normal mitochondrial debris on ALA synthetase activity in crude extracts of R. spheroides

In case the inhibition of ALA synthesis in DDC liver mitochondria by normal mitochondrial debris was due to some peculiarity of the mitochondrial system, the effect of this material on ALA synthetase of R. spheroides was also examined. Fig. 6.7 shows that the activity of the bacterial enzyme was also inhibited.

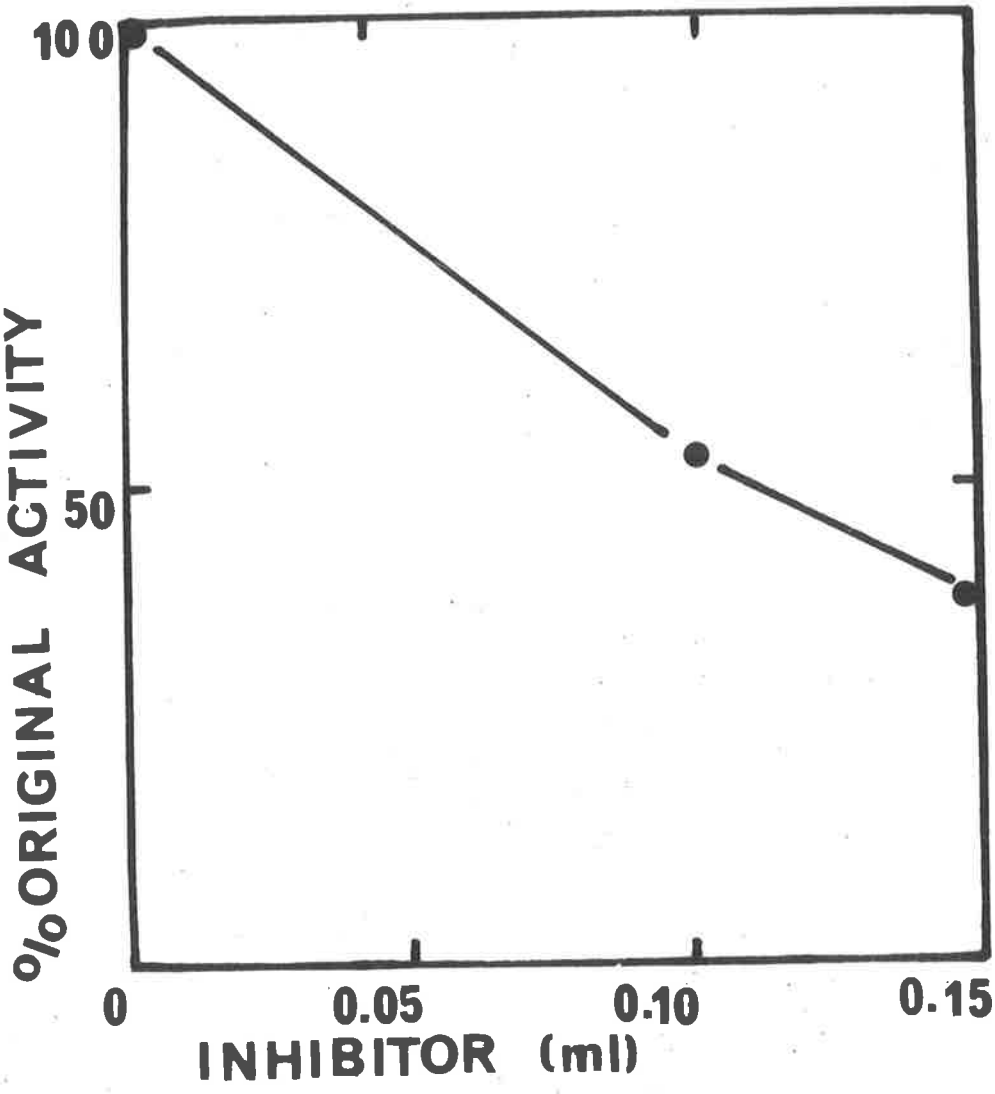


Fig. 6.7. The effect of normal mitochondrial debris on theactivity of ALA synthetase in crude extracts of R. spheroides. Crude extracts of the bacterial enzyme were prepared as described in Materials and Methods (p. 55). Incubation conditions and method of assay were as described in Fig. 6.6. ALA synthesis is expressed as a percentage of the activity in 0.05 ml of crude extract (53 mμmoles of ALA produced).

C. A Study of the Properties of Normal Mitochondrial Debris Relating to its Inhibitory Effect on ALA Synthetase Activity

In an attempt to establish the point at which the inhibition by normal mitochondrial debris was acting, the effect of incubation conditions on this inhibition was examined.

(a) The effect of normal mitochondrial debris on succinyl CoA synthetase activity

It was conceivable that the inhibitory effect of normal mitochondrial debris on ALA synthetase activity was mediated through an inhibitory action on the activity of E. coli succinyl CoA synthetase (included in incubations as a precaution to ensure adequate succinyl CoA formation). Preliminary experiments showed that the activity of this enzyme was inhibited, a 25% inhibition occurred in the presence of 0.2 ml of normal mitochondrial debris. (Succinyl CoA synthetase activity was determined by the method of Kaufman, 1955) (see Materials and Methods, p. 56). However, as shown in Table 6.1, the inhibition of ALA synthetase activity both in DDC liver mitochondria and crude extracts of R. sphaeroides was not overcome when incubations contained

TABLE 6.1

THE EFFECT OF E. COLI SUCCINYL CoA SYNTHETASE ON THE INHIBITION OF ALA SYNTHETASE ACTIVITY BY MITOCHONDRIAL DEBRIS

Source of enzyme	Additions: Succinyl CoA synthetase (ml)	Additions: Normal Mitochondrial debris (ml)	ALA synthetase (% original activity)
DDC liver mitochondria	-	-	100
" " "	-	0.15	59
" " "	0.05	-	100
" " "	0.05	0.15	51
R. spheroides	-	-	100
" "	-	0.15	43
" "	0.05	-	103
" "	0.05	0.15	44

Incubation mixtures were as described in Fig. 6.5. (The succinyl CoA synthetase referred to in the table is in addition to the 0.05 ml present in all.) All incubations contained 0.1 ml of the enzyme preparation and the results are expressed as a percentage of the activity determined using the standard incubation conditions (DDC liver mitochondria, 21 mμmole of ALA produced/0.05 ml; R. spheroides extract, 35 mμmole of ALA produced/0.05 ml).

twice the amount of succinyl CoA synthetase normally used.

(b) The effect of incubation time on the inhibition of ALA synthetase activity by normal mitochondrial debris

Since it was possible that the time of incubation could be affecting the degree of inhibition observed, this was also examined. However, the results in Fig. 6.8 show that this was not the case; furthermore the addition of normal mitochondrial debris after incubation is without effect on the ALA synthetase activity measured.

(c) The effect of succinate on the inhibition of ALA synthetase activity by normal mitochondrial debris

The results in Fig. 6.9 show that the effect of the inhibitor is not reduced by increasing the concentration of succinate ten-fold. This was done to eliminate the (unlikely) possibility that the inhibitor might bind succinate and thus cause inhibition of ALA synthetase activity.

(d) The effect of substrate and cofactor concentration on the inhibition of ALA synthetase activity by normal mitochondrial debris

Although the activity of the enzyme in the presence of

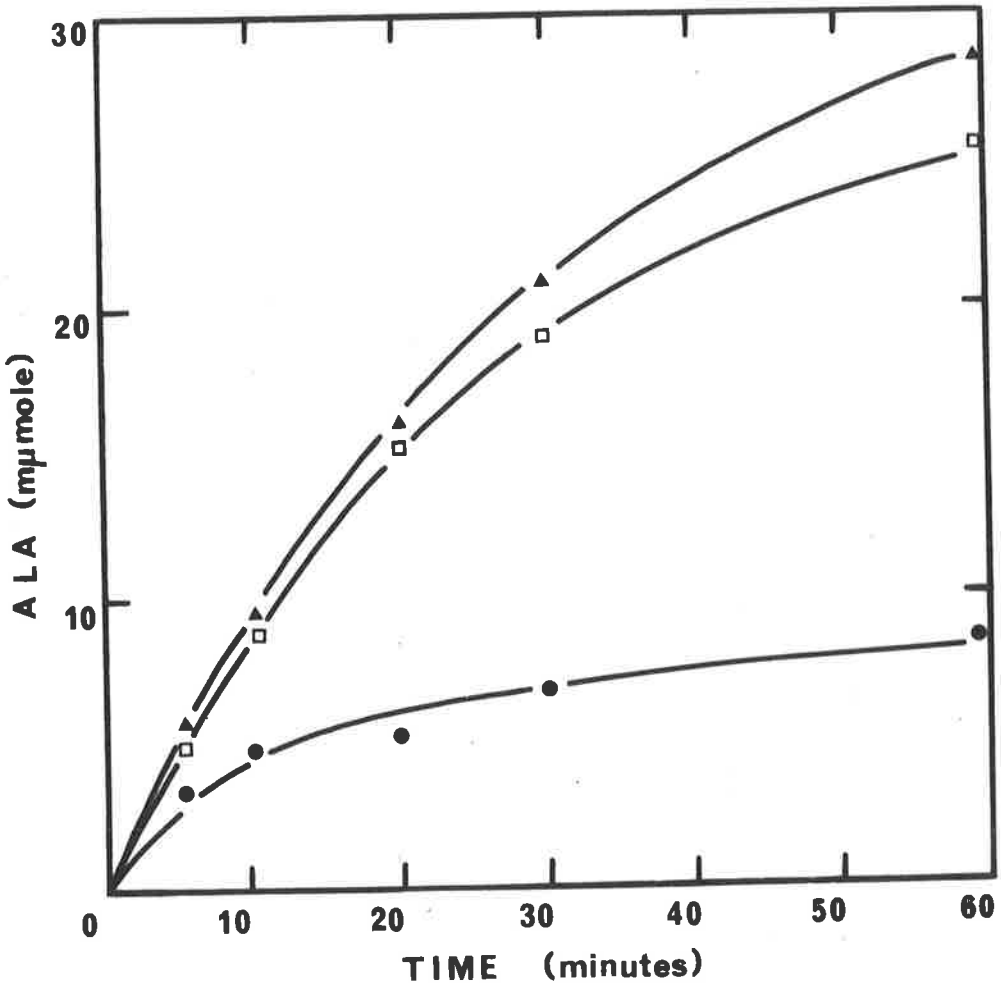


Fig. 6.8. The effect of incubation time on the inhibition of ALA synthetase activity in DDC liver mitochondria by normal mitochondrial debris.

Incubation conditions and method of assay were as described in Fig. 6.6, except that the time of incubation was varied. All incubations contained 0.05 ml of DDC liver mitochondria. Figures on the ordinate represent total ALA produced.

□—□ , no inhibitor; ▲—▲ , 0.15 ml of inhibitor added after incubation; ●—● , 0.15 ml of inhibitor added before incubation.

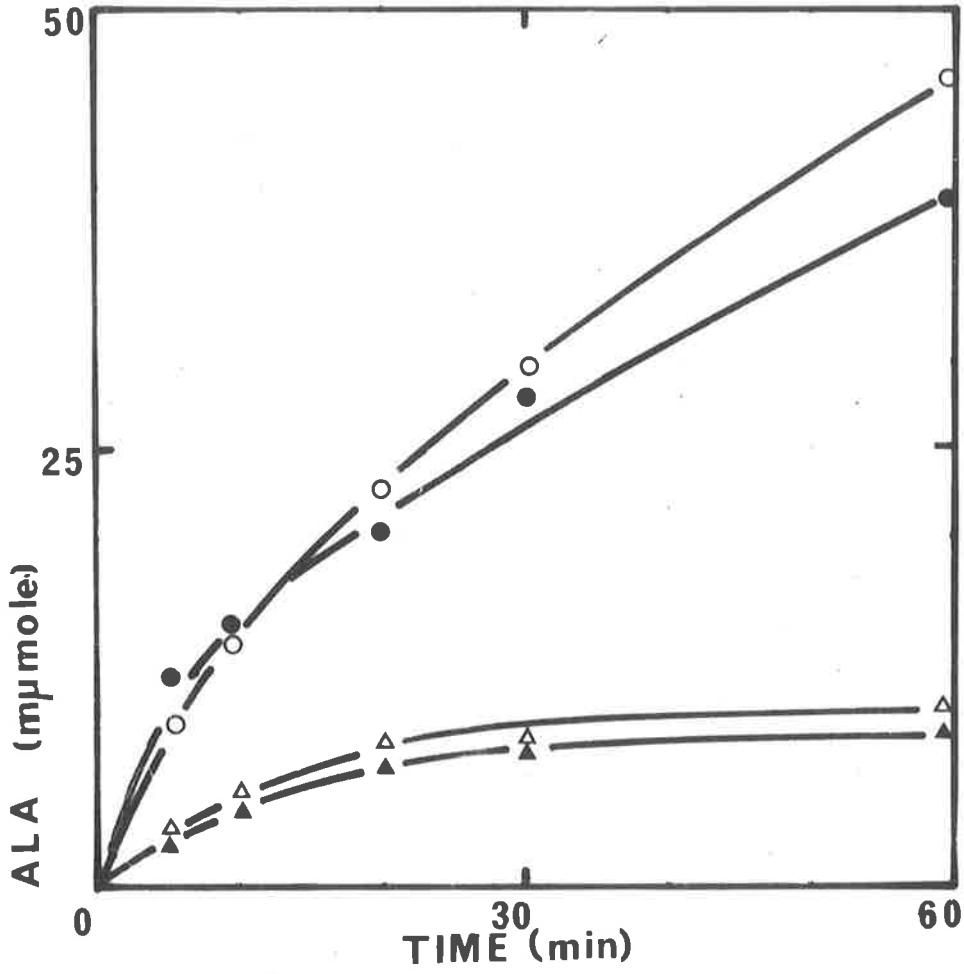


Fig. 6.9. The effect of succinate concentration on the inhibition of ALA synthetase activity by normal mitochondrial debris. Incubation conditions and method of assay were as described in Fig. 6.6, except that the time of incubation was varied. All incubations contained 0.05 ml of DDC liver mitochondria. Figures on the ordinate represent total ALA produced. ○— ○ , no inhibitor, 10mM succinate; ●— ● , no inhibitor, 100 mM succinate; △ — △ , 0.15 ml of inhibitor, 10mM succinate; ▲— ▲ , 0.15 ml of inhibitor, 100mM succinate.

normal mitochondrial debris was not limited by the succinate concentration, the possibility that it was limited by the concentrations of other components of the reaction mixture could not be overlooked. Fig. 6.10 shows that inhibition occurred at all substrate concentrations, even up to twice that normally used. (The mitochondrial preparations used in these experiments were stored in the absence of pyridoxal 5-phosphate.)

D. The Nature of the Inhibitor

(a) The stability of the inhibitor

The capacity of the inhibitor to prevent ALA synthesis was not affected by such drastic treatments as heat (5 minutes at 100°) or acid (1 hour in 1N HCl at 100°). After the latter treatment, the insoluble residue was centrifuged down, resuspended in water and recentrifuged; this procedure was continued until the supernatant was free from acid. The precipitate was then resuspended (using a Potter-Elvejhem homogeniser) in water up to the original volume before the treatment. ALA synthesis was reduced by 56% when 0.15 ml of the heat treated material was included in

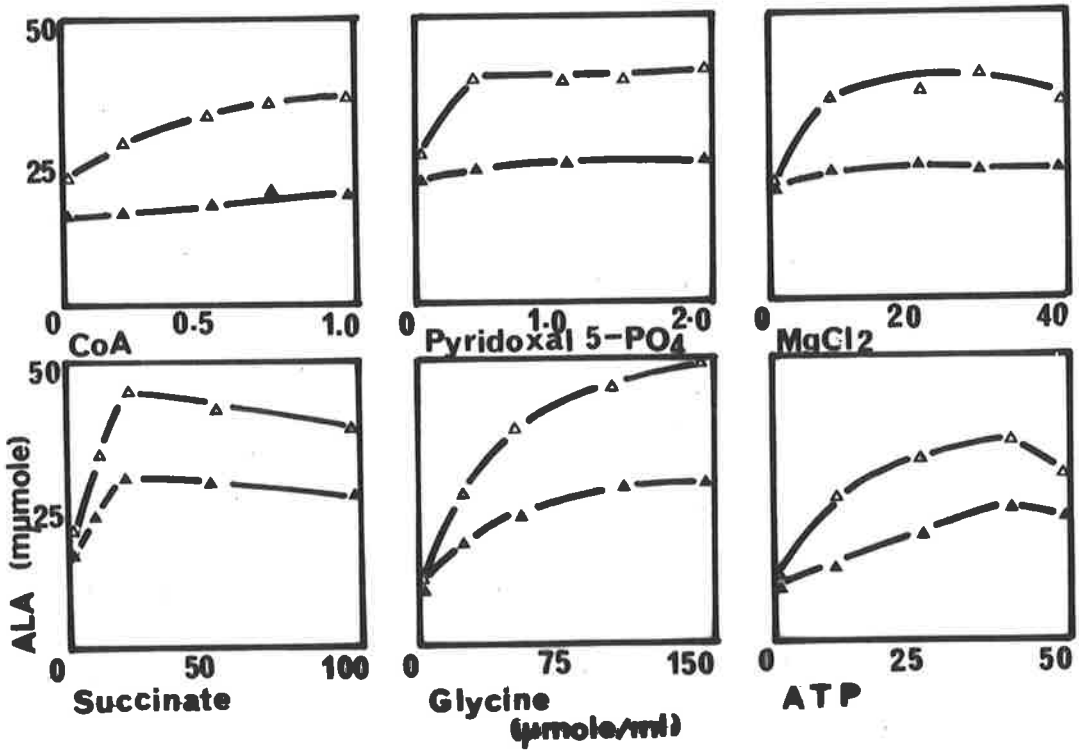


Fig. 6.10. The effect of substrate and cofactor concentration on the inhibition of ALA synthetase activity by normal mitochondrial debris. Except for substrate varied, incubation conditions and experimental procedures were as described in Fig. 6.6. All incubations contained 0.05 ml of EDC liver mitochondria (stored without pyridoxal 5-phosphate). Figures on the ordinate represent total ALA produced. △— △ , no inhibitor; ▲— ▲ , 0.15 ml of inhibitor.

the incubation mixture and by 41% when the same volume of the acid treated material was included. All capacity to inhibit was however, lost after mitochondrial debris had been ashed in a muffle furnace at 1,000°F. (The ash was dissolved in an amount of water equal to the original volume of the suspension before ashing; the effect of 0.2 ml of this solution on ALA synthesis by DDC liver mitochondria was tested.)

(b) Attempts to imitate the inhibitory effect of normal mitochondrial debris

(i) The effect of heat denatured albumin on ALA synthetase activity in DDC liver mitochondria

As both normal and DDC mitochondrial debris inhibited ALA synthetase activity, it was possible that this inhibition was merely the reflection of some non-specific effect due to an excess of insoluble protein material present in reaction mixtures. Accordingly, the effect of purified bovine serum albumin, rendered insoluble by heat treatment, was examined. A solution of bovine serum albumin (40 mg/ml) in 0.1M tris-HCl (pH 7.4) was heated at 100° for 5 minutes and the precipitate resuspended using a Potter-Elvejhem homogeniser. Increasing amounts of this

material (up to 0.2 ml) had no effect whatsoever on ALA synthesis by 0.05 ml of DDC liver mitochondria.

(ii) The effect of haem and haem proteins on ALA synthetase activity in DDC liver mitochondria

Since it was known that haem and haem proteins inhibit the activity of R. spheroides ALA synthetase (Burnham and Lascelles, 1963) and since mitochondrial debris constitutes a source of haem proteins, the effect of haem, haemoglobin and myoglobin on ALA synthetase activity in DDC liver mitochondria was examined. The results (Table 6.2) show that the activity of the crude liver enzyme was inhibited by each of these compounds. It was therefore surprising to find in later experiments using the purified liver enzyme (see Table 8.1, p. 157) that, although haem itself was inhibitory, neither haemoglobin or myoglobin had any inhibitory action on ALA synthesis. The reason for this difference between the crude and the purified enzyme has not yet been determined.

It was noted that AA synthesis was also inhibited by haem, 32% inhibition occurring in the presence of 0.2mM haem.

TABLE 6.2.

THE EFFECT OF HAEM AND HAEM PROTEINS ON ALA SYNTHETASE ACTIVITY IN DDC LIVER MITOCHONDRIA

Incubation conditions and experimental procedures were as described in Fig. 6.6. All incubations contained 0.05 ml of DDC liver mitochondria (stored without pyridoxal 5-phosphate) and additions shown. The results are expressed as a percentage of the original activity (77.5μmoles of ALA produced).

Addition	Final Concentration (mM)	ALA Synthetase (% original activity)
None	-	100
Haem	0.02	102
	0.20	74
Haemoglobin	0.005	102
	0.05	76
Myoglobin	0.02	95
	0.20	65

2. DISCUSSION

As already mentioned, it was anticipated that the radiochemical assaymethod would readily detect ALA synthetase activity, not only in normal liver but in other biological systems which must also use ALA for the synthesis of protoporphyrin, the precursor common to both the haem and chlorophyll biosynthetic pathways.

The discovery that normal liver mitochondria contained a factor which inhibited ALA synthesis *in vitro* could account for the failure of the assay to detect ALA synthetase activity in normal liver.

Since ALA synthesis was only slightly inhibited when freshly isolated mitochondria were used, it seemed that the effect *in vitro* was dependent on disruption of the mitochondrial membranes. This is supported by the finding that the inhibitory properties of normal mitochondria were confined to the insoluble fraction of ultrasonicated preparations. Consequently, the question of how such an inhibitory effect might be mediated became more difficult.

It was also found that all mitochondrial debris, whether prepared from normal or DDC liver mitochondria, inhibited ALA synthetase activity. Although normal mito-

chondrial debris exerted a greater inhibitory effect than did that from DDC liver mitochondria, the significance of this is uncertain since the protein content of the former was also greater. Another difference was that ultrasonicated suspensions of normal liver mitochondria invariably contained a greater proportion of debris than comparable preparations of DDC liver mitochondria and this could be a reflection of some effect of inducing drugs on the structural components of liver mitochondria, a possibility indicated by the report (Granick and Urata, 1963) that mitochondria in the livers of DDC treated guinea pigs were enlarged.

The inhibitor did not appear to be a specific protein since such drastic treatments as heat and acid failed to inactivate it*. However, the inhibitory properties were lost after the material was ashed; this indicated that the effect was not associated with metals in the oxidised form. (For example, bacterial ALA synthetase activity is inhibited by Fe^{++}, but not by Fe^{+++} (Burnham and Lascelles, 1963).)

Since heat denatured albumin failed to reproduce the inhibition, it did not appear that the inhibitory effect of mitochondrial debris was entirely non-specific and it

*See Appendix IV.

could be related to the haem content of this material.

No evidence was found to indicate that the inhibitory effect was related to any of the substrates or cofactors used in reaction mixtures since it was not overcome when the concentration of these was increased.

In considering the apparent lack of ALA synthetase activity in normal mitochondria, another possibility was that removal of inhibitory substances in the debris would leave an active supernatant, but this was not so. On the other hand, if the enzyme became accessible to the inhibitor only after disruption of mitochondria by freezing and thawing then the radiochemical assay method should have detected ALA synthetase activity in freshly prepared homogenates of liver obtained from normal animals; again this was not found (see p. 101).

While it seems doubtful that the inhibitory effect of mitochondrial debris on ALA synthetase activity has physiological significance, it is important to the assessment of the results of assays since it probably prevents a true estimate of ALA synthetase activity in normal liver mitochondria.

CHAPTER 7

PURIFICATION OF ALA SYNTHETASE FROM LIVER MITOCHONDRIA OF DDC TREATED GUINEA PIGS

Although, as already described, ALA synthetase of bacterial origin has been studied in partially purified preparations (Kikuchi, Kumar and Shemin, 1959; Burnham and Lascelles, 1963), there have been no classical enzymological studies of animal ALA synthetase carried out. The work done has been of two main types. Firstly, the work of Laver, Neuberger and Udenfriend (1958) was concerned with ALA synthetase of chicken red blood cells and in this washed (Laver, Neuberger and Udenfriend, 1958) or freeze-dried particles (Gibson, Laver and Neuberger, 1958b) from haemolysates were studied; the nature of the reaction and the product was established and the inhibitory effect of agents such as cyanide and penicillamine demonstrated. It was also shown that haemolysates synthesised AA as well as ALA and it was concluded that ALA synthetase had a low degree of specificity and was capable of using acetyl, propionyl or succinyl CoA. Secondly, the work of Granick's group and of Tschudy's group has dealt with the characteristics and measurement of ALA synthetase activity either in liver mitochondria (Granick and Urata, 1963; Marver et al., 1966b) or liver homogenates (Marver et al., 1966b). Since the main

aims of these workers was to study the control of ALA synthetase levels in liver, little work on the nature of ALA synthetase itself has been done. Apart from reports that ALA synthetase activity was detected in high speed supernatant solutions of liver homogenates prepared in 0.25M sucrose (Granick and Urata, 1963) and that the enzyme, solubilised from liver mitochondria, was not inhibited by haem (Marver et al., 1966d), no references to detailed studies of the solubilised liver enzyme are available in the literature.

The aims of carrying out the work in this chapter were several. Firstly, since ALA synthetase appears to be the controlling factor in haem and porphyrin production, it seemed desirable for a proper understanding of the control of ALA synthetase, both activity and production, that the nature and properties of the enzyme should be known. In particular, the relationship to other mitochondrial enzymes seemed to be of interest in connection with its synthesis.

Secondly, a study of the enzyme itself could offer the possibility of designing specific inhibitors for ALA synthetase - on some of the current hypotheses it might be thought that such inhibitors would constitute rational

therapeutic agents for the treatment of acute intermittent porphyria. In this connection it might be mentioned that this situation offers an almost unique challenge and opportunity for the application of "pure" biochemistry in combatting a genetic disease - unique because it is the only known situation in which a disease is apparently due to an overproduction of an enzyme (with the important qualification however, that at present no direct causal connection between ALA production and the clinical symptoms of acute intermittent porphyria has been established).

Finally, purification of the enzyme automatically carried with it the opportunity of investigating its relationship to AA synthetase.

Inherent disadvantages were the necessity to utilise drug treated animals for enzyme preparations, coupled with the knowledge that the enzyme was unstable. However, the desirability of enzyme studies seemed to outweigh the difficulties involved. This chapter describes the solubilisation and partial purification of the enzyme.

1. RESULTS

A. Preliminary Studies on Purification Procedures

(a) Source of the enzyme

The enzyme was obtained from the livers of guinea pigs dosed with DDC (Granick and Urata, 1963). In order to determine the optimum time for killing guinea pigs, a series of tests were made on the time course of ALA synthetase induction after administration of DDC. These revealed that the level of ALA synthetase in liver mitochondria was highest 24 hours after the administration of 2 g of DDC orally as a single dose. When killed 48 hours after dosing, enzyme levels were less than half those at 24 hours, and approximately two-thirds of those obtained when the administration of the drug was spread out over a period of 24 to 48 hours as described by Granick and Urata (1963).

The effect of repeated injections of sodium malonate (subcutaneously, as described by Gajdos et al. (1966)) on levels of ALA synthetase in the livers of guinea pigs was also investigated. Although high levels of PBG were present in the urine (demonstrated by the qualitative Watson-Schwartz test), the level of ALA synthetase in liver homogenates and liver mitochondria was not affected.

In an endeavour to obtain greater amounts of liver, attempts were made to induce experimental porphyria in sheep, either by oral administration of DDC or a subcutaneous injection of AIA. Initial attempts to do this did not meet with success (possibly due to having used young sheep), and because of practical difficulties involved, it was decided to continue with guinea pigs.

(b) Solubilisation of the enzyme

In the earlier studies, liver mitochondria were prepared as described in Materials and Methods (p. 50) and the activity of these was measured using the standard colorimetric assay procedure described in Chapter 3 (see Fig. 3.1, p. 61). It was found that disruption of mitochondria with Tween 80, sodium dodecyl sulphate or sodium deoxycholate caused almost total loss of the enzyme.

Attempts were also made to solubilise the enzyme by ultrasonication of mitochondrial suspensions. It was found that a 15 second treatment resulted in approximately 50% of the original activity being rendered non-sedimentable in 1 hour at 100,000 x g. Further work showed that, provided pyridoxal 5-phosphate was present (final concentration 10^{-2}M), 100% of the activity originally present was recovered in the soluble fraction of ultrasonicated DDC liver mitochondria.

The value of pyridoxal 5-phosphate in stabilising the solubilised liver enzyme is clearly illustrated by the results shown in Fig. 7.1. In the presence of pyridoxal 5-phosphate, enzyme activity remaining after four hours at 30° was approximately 70% of that present at the beginning of the experiment, whereas in the absence of this cofactor, only 40% of the original activity remained.

Fig. 7.1 also shows that no additional advantage was to be gained by the addition of $MgCl_2$ as well as pyridoxal 5-phosphate, as might have been expected from the results obtained in the studies on the stability of the bacterial enzyme (see Fig. 3.6). (The inclusion of 1mM EDTA did not affect the ability of pyridoxal 5-phosphate to stabilise the mitochondrial enzyme; this is also shown in Fig. 7.1.)

(c) Ammonium sulphate fractionation

It was found essential to adjust the pH of the ammonium sulphate solutions to pH 7.0; at pH's below 6.8 considerable

Fig. 7.1. (Cont'd.)

▲—▲ , no additions;

□—□ , pyridoxal 5-phosphate present;

■—■ , pyridoxal 5-phosphate and EDTA present;

△—△ , pyridoxal 5-phosphate and $MgCl_2$ present.

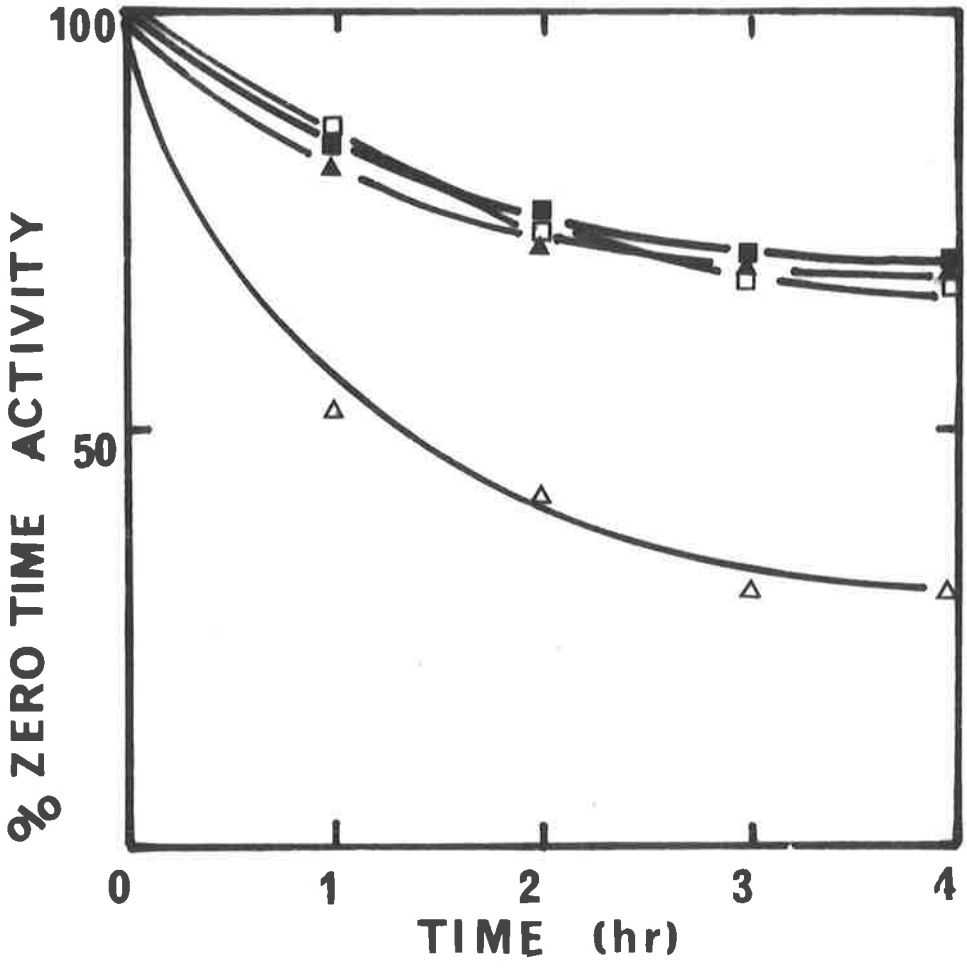


Fig. 7.1. The effect of pyridoxal 5-phosphate on the stability of ALA synthetase in the soluble fraction of ultrasonicated DDC liver mitochondria.
The soluble fraction of ultrasonicated DDC liver mitochondria was prepared as described in the text, except that pyridoxal 5-phosphate was absent. Separate samples were maintained at 30°. One sample contained no additions, while pyridoxal 5-phosphate (pH 7.4) was added to the remaining three samples to a final concentration of 10^{-2} M. Of the latter, the first had no further additions, the second contained 1mM EDTA and the third, 1 mM $MgCl_2$. ALA synthetase activity in 0.1 ml samples, withdrawn from each solution at various intervals of time (0 to 4 hours), was determined by the colorimetric assay procedure as described in Chapter 4 (p. 93). Results are expressed as a percentage of the activity present in each solution at zero time.

losses occurred. Having shown the value of pyridoxal 5-phosphate in stabilising the enzyme, no attempt was made to determine the extent of losses during ammonium sulphate fractionation in the absence of this compound.

(d) Sephadex gel filtration

It was originally intended to use DEAE-cellulose columns for further fractionation as were used for the bacterial enzyme preparation. However, two difficulties arose. Firstly, it was necessary to dialyse the enzyme after ammonium sulphate fractionation but dialysis for two hours against tris-pyridoxal 5-phosphate buffer resulted in a 30% loss of enzymic activity. (Marriott, Neuberger and Tait (1969) have recently found that bacterial ALA synthetase is also unstable to dialysis.) Secondly, it was impossible, on a large scale, to equilibrate columns with buffer containing pyridoxal 5-phosphate since the latter was retained at the top of the column. For these reasons Sephadex gel filtration was used instead of DEAE-cellulose column chromatography.

It is perhaps worth noting at this point that it was surprising to find that while chromatography on Sephadex G-100 gave good recoveries of the enzyme, the same procedure

applied to Sephadex G-200 resulted in total loss of activity. This is illustrated in Fig. 7.2 which shows that there was a wide distribution of protein amongst the fractions but enzyme activity was zero. Recombination of fractions failed to restore any activity. This phenomenon has not yet been investigated due to lack of time but work is in progress to examine it since it has potential interest.

Of the various techniques used to purify the enzyme only those finally adopted as most useful will be described here.

B. Procedure for Purification of ALA Synthetase of Liver

Starting material. Guinea pigs were treated with DDC and suspensions of liver mitochondria prepared as described earlier in Materials and Methods (p. 50). Pyridoxal 5-phosphate (pH 7.4) was added to give a final concentration of 10^{-2}M and the mitochondrial suspensions were rapidly frozen in small batches and stored at -15°. Under these conditions the enzyme was stable for at least three months and the frozen material provided a uniform starting material for purification. (This contrasts markedly with storage in

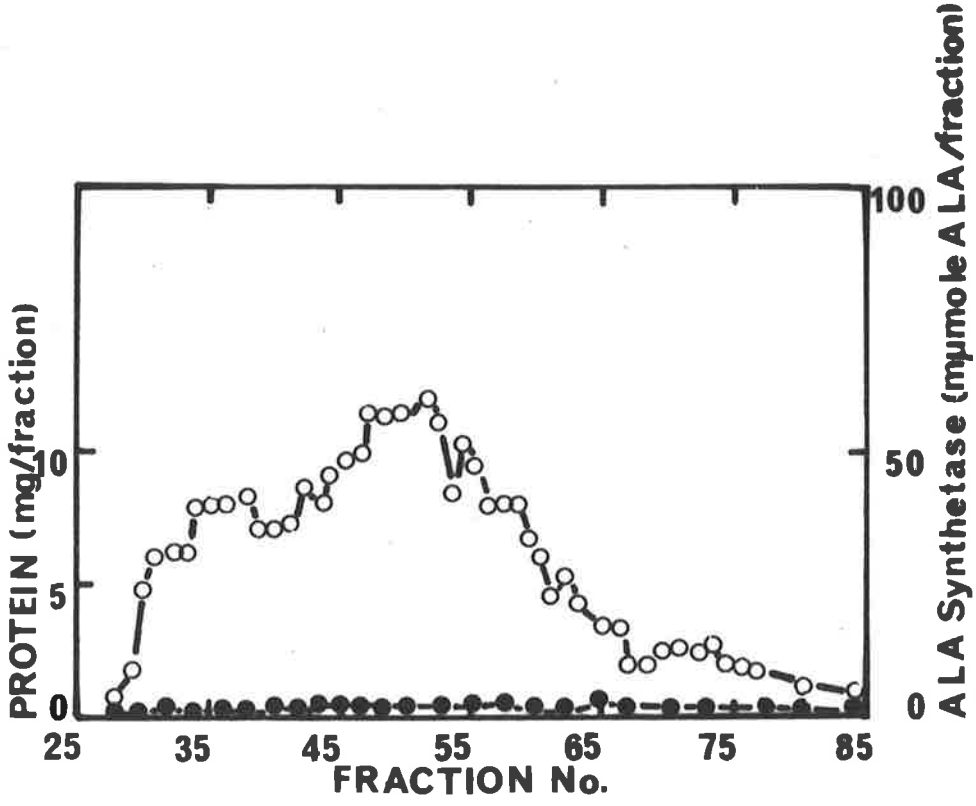


Fig. 7.2. Elution of protein from Sephadex G-200.

Steps 1 and 2 of the purification procedure were as described in the text (p.137). The redissolved fraction from the ammonium sulphate fractionation was subjected to gel filtration on Sephadex G-200 as described in the text for Sephadex G-100. Total ALA synthetase activity in the ammonium sulphate fraction was 10,520 mμmoles of ALA and the specific activity was 45 mμmole of ALA/mg of protein. Sephadex fractions were assayed for protein as described in Materials and Methods and for ALA synthetase activity as described in Fig. 7.1. o—o , protein; ●— ● , ALA synthetase activity.

the absence of the coenzyme when unpredictable and often complete losses of activity occurred.)

All subsequent purification steps were carried out at 4°.

Step 1. Preparation of the soluble enzyme. 15 ml samples of once frozen and thawed mitochondrial suspensions were subjected to ultrasonication for 15 seconds. Suspensions were placed in heavy glass containers (diameter, 3 cm and height 8 cm) held in ice and ultrasonication was carried out using a Soniprobe (Type 1130 A (20 Kc/sec), Dawe Instruments) on the maximum setting (8). Under these conditions the current registered was between 2 and 4 m Amps. The suspensions were centrifuged at 100,000 x g for one hour and the supernatant containing the enzyme collected. Provided pyridoxal 5-phosphate is present, the enzyme is stable at this stage and can be frozen and stored at -15° without loss of activity.

Step 2. Ammonium sulphate precipitation. The enzyme solution (total protein between 700 and 800 mg) was diluted with 0.01M tris-HCl containing 10^{-4}M pyridoxal 5-phosphate (pH 7.4) to give a protein concentration of 24 mg/ml. Saturated ammonium sulphate solutions adjusted to pH 7.0,

was added slowly with stirring to give 33% saturation; after 15 minutes the precipitate was removed by centrifugation at 45,000 x g for 20 minutes. The supernatant was made 50% saturated by further addition of the saturated ammonium sulphate solution. The precipitate was collected and dissolved in a minimal volume of the tris pyridoxal 5-phosphate buffer. This step removed approximately half the protein present in the supernatant from Step 1, and usually gave a three-fold purification of the enzyme present in the original mitochondrial suspension.

Step 3. Sephadex gel filtration. A column (80 x 2.5 cm) of Sephadex G-100 was prepared according to manufacturer's instructions and was equilibrated with the tris-pyridoxal 5-phosphate buffer. The enzyme solution from Step 2 (containing 300 to 400 mg of protein) was applied to the column which was developed using the tris-pyridoxal 5-phosphate buffer. Fractions 5.5 ml in volume were collected. The elution profiles of ALA synthetase and protein are shown in Fig. 7.3; (ALA synthetase was not retarded and was eluted with the void volume).

A summary of a typical purification is given in Table 7.1. Sephadex eluate fractions were not pooled, but

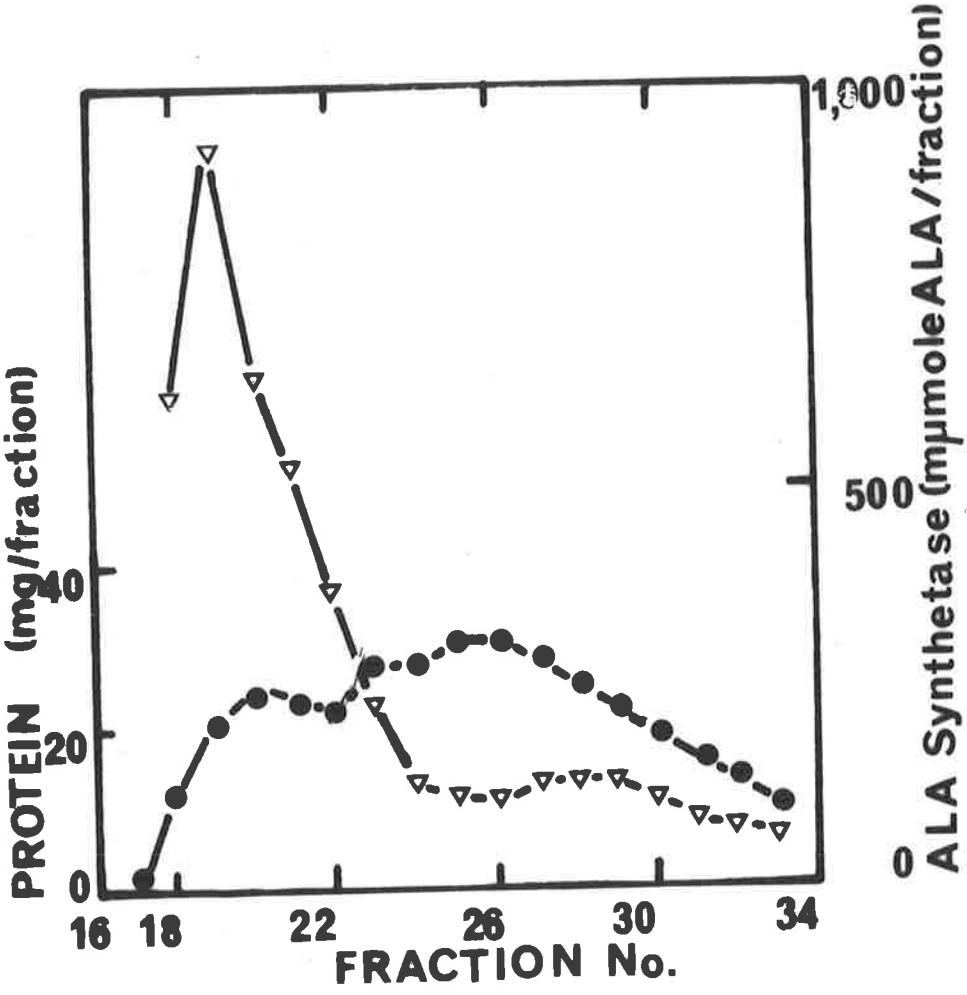


Fig. 7.3. The elution of ALA synthetase from Sephadex G-100. The purification procedure was as described in the text (p. 137). Sephadex fractions (0.25 ml samples) were assayed for protein as described in Materials and Methods (p. 58) and for ALA synthetase activity as described in Fig. 7.1.
●—●, protein; △—△, ALA synthetase activity.

TABLE 7.1

PURIFICATION OF ALA SYNTHETASE FROM DDC LIVER MITOCHONDRIA

Fraction	Volume (ml)	Protein Concentration (mg/ml)	ALA synthetase	
			Total Activity (mμmole ALA)	Specific Activity (mμmole/ALA/mg protein)
Mitochondrial suspension	19.0	61.0	18,800	16.2
Step 1 - high speed supernatant	14.8	51.5	19,900	26.2
Step 2 - ammonium sulphate fraction	4.5	73.5	16,200	49.0
Step 3 - Sephadex eluate fractions				
Σ 18 - 25	44.0	3.9	10,360	60.0
20	5.5	4.2	2,250	97.5

Experimental details of the purification procedure were as described in the text. Protein determinations were as described in Materials and Methods (p. 58). For colorimetric assays of ALA synthetase activity in the original mitochondrial suspension and the fractions from the first two steps of the purification procedure, incubation conditions and method of assay were as described in Fig. 7.1; and for the fractions of the Sephadex eluate as described in Fig. 7.4.

summation of the activities present in the fractions containing ALA synthetase indicated that 64% of the enzyme present in the ammonium sulphate fraction (Step 2) was eluted in these fractions. An overall yield of 55% was achieved with a four-fold purification, or six-fold in the fraction at the centre of the ALA synthetase peak. Radiochemical assays carried out at the same time gave similar results.

C. Stability of the Purified Enzyme

The enzyme was unfortunately extremely unstable after Sephadex gel purification and little activity remained after storage at -15° for one week. Further addition of pyridoxal 5-phosphate had little stabilising effect at this stage. Because of the rapid deterioration of enzyme activity, it was impossible to fully utilise pooled fractions from any single experiment. For this reason only the two or, at the most, three fractions at the centre of the enzyme peak eluted were used in subsequent studies.

The purified enzyme exhibited a consistent troublesome

physical phenomenon. The first few protein containing fractions eluted from Sephadex showed an extremely pronounced flow birefringence after standing at 4° for sometime with ALA synthetase activity being equally distributed between the precipitate and the supernatant (the specific activity of the latter being unchanged). After storage of such supernatants at -15°, on thawing, further flow birefringence appeared.

Electron micrographs of the precipitates revealed clumps of fibrous material devoid of any organised structure. In comparison, it is perhaps noteworthy that crude mitochondrial supernatants could be stored indefinitely and no insoluble material ever appeared.

D. Application of the Purification Procedure to Normal Liver Mitochondria

Despite the fact that no ALA synthetase activity was detected in high speed supernatant fractions of normal liver mitochondria by the radiochemical assay method (see p. 100), the possibility was considered that some ALA synthetase exists but cannot be assayed, due to inhibition by some other mitochondrial component specific to normal mitochondria.

For this reason, normal liver mitochondria were processed

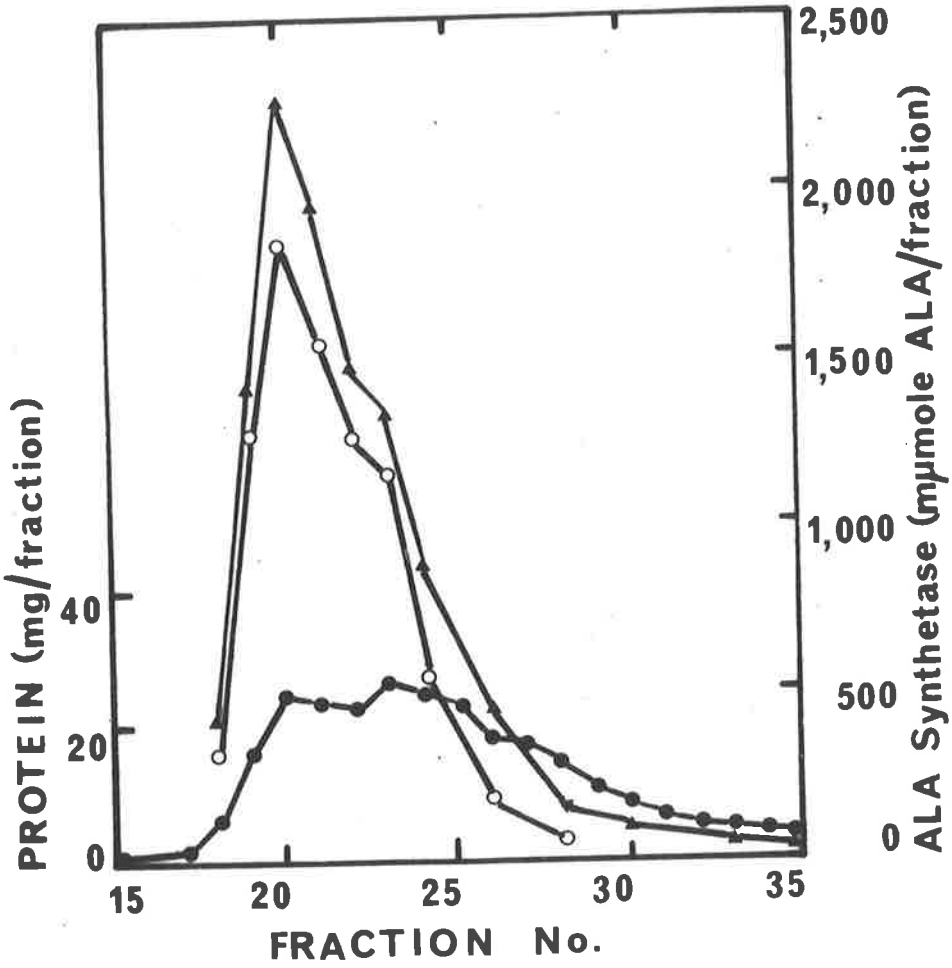
as were DDC liver mitochondria. Although the elution profile of proteins from Sephadex G-100 was the same as that obtained from processing DDC liver mitochondria, no ALA synthetase activity was detected in any fraction. Fractions were tested for ALA synthetase activity using the radiochemical assay procedure which gave maximum sensitivity and also agreed precisely with colorimetric assays when applied to column fractions of the enzyme from DDC liver mitochondria (see Fig. 7.4). (The validity of the radiochemical assays under the altered conditions - lower succinate levels and no Krebs cycle inhibitors - described in Fig. 7.4 is established in Chapter 8, p.151.)

E. Location of AA Synthetase Activity During the Purification Procedure

As already mentioned (p. 72), crude preparations such as DDC liver mitochondria synthesise both ALA and AA. Colorimetric assays carried out at each stage of the procedure developed for the purification of liver ALA synthetase showed that while considerable amounts of AA were also synthesised by both the high speed supernatant of ultrasonicated DDC liver mitochondria (Step 1 of the purification procedure) and the 33 to 50% ammonium sulphate fraction

Fig. 7.4. Comparison of radiochemical and colorimetric assays of ALA synthetase activity in Sephadex fractions.

Purification procedures were as described in the text (p.137). Proteins in Sephadex fractions were determined as described in Materials and Methods. Incubation conditions for the colorimetric assay of ALA synthetase activity in 0.25 ml samples of each fraction were as described in Fig. 7.3, except that malate, malonate, antimycin A and arsenite were omitted from the reaction mixture. The reaction was stopped by the addition of 1.5 ml of 0.2M trichloracetic acid and ALA in 1 ml of the protein free supernatant determined as described in Materials and Methods. Incubation conditions for the radiochemical assay of ALA synthetase activity in 0.05 ml samples of each fraction were as described above for the colorimetric assay, except that 5μmole of unlabelled potassium succinate was omitted and was replaced by 0.28 μmole of ^{14}C-1,4-succinic acid (specific activity 8.8 μC/μmole). The reaction was stopped by the addition of 2 ml of 0.3M trichloracetic acid and 1.5 ml of water containing 100 μmoles of unlabelled succinate and 1 μmole of carrier ALA were added. ALA in 3ml of the protein free supernatant was absorbed onto Dowex 50 (H^+) and eluted as described in Chapter 4 (p.110). The buffer was evaporated and the residue dissolved in 2 ml of water and radioactivity determined in 0.1 ml samples dried on glass fibre discs.



, ●—● , protein; ▲—▲ , ALA synthetase, colorimetric assay; ○—○ , ALA synthetase, radiochemical assay.

of such extracts (Step 2 of the purification procedure) there was no evidence of AA synthesis by Sephadex eluate fractions (numbers 18 to 24, Fig. 7.3) which exhibited ALA synthetase activity. Similar assays showed however, that small amounts of AA were synthesised by retarded fractions (numbers 24 to 34) and it seemed that the two enzymes might have been separated by the final step (gel filtration on Sephadex G-100) of the purification procedure.

It was of interest therefore to examine the AA synthetase activity of Sephadex eluate fractions using incubation conditions which were optimal for AA synthesis. The system used in these studies was devised by Walsh and Elliott (1969) and is dependent on the synthesis of acetyl CoA from acetyl phosphate and CoA by E. coli phosphotransacetylase. (The description of the complete system is given in the legend to Fig. 7.5.)

DDC liver mitochondria were processed as described in the text for the purification of ALA synthetase, and AA synthetase activity was determined by the method of Walsh and Elliott (1969) at each stage of the procedure. It was found that the two enzymes were not completely separated by the gel filtration procedure. Elution profiles consistently showed two peaks of AA synthetase activity; a small peak,

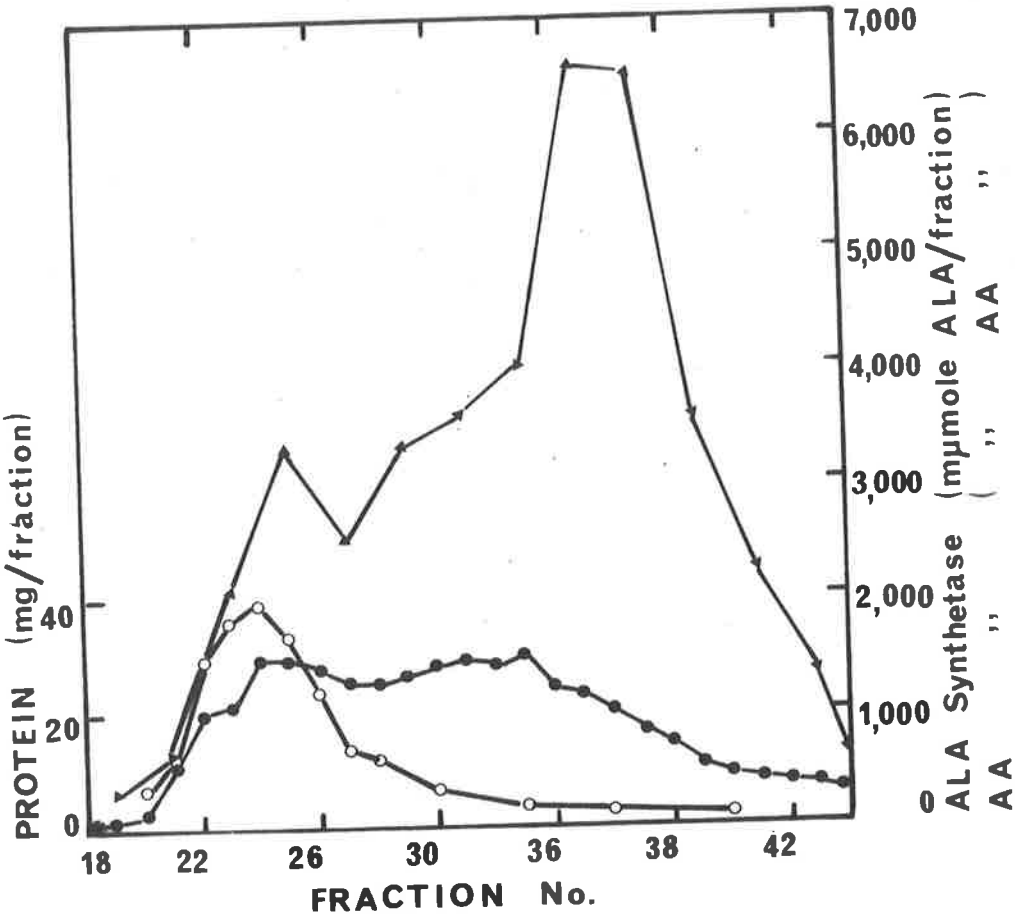


Fig. 7.5. Elution of ALA synthetase and AA synthetase from Sephadex G-100. DDC liver mitochondria were processed as described in the text for the purification of ALA synthetase, except that 4.5 ml fractions of the Sephadex eluate were collected. The protein content of Sephadex fractions was determined as described in Materials and Methods (p. 58) and ALA synthetase activity was determined as described in Fig. 7.4 for the colorimetric assay procedure. AA synthetase activity was determined by the method of Walsh and Elliott (1969). Incubations contained the following (in μmoles/ml, unless otherwise stated); glycine, 150; tris-HCl buffer (pH 7.4) 50; CoA, 0.425; GSH, 2; acetyl phosphate, 5; *E. coli* phosphotransacetylase, 0.05 ml; enzyme and water to a final volume of 0.5 ml. Tubes were incubated at 37° for 30 minutes in a shaking water bath; the reaction was stopped by the addition of 1.5 ml of 0.2N trichloracetic acid and AA in 1 ml of the protein free supernatant was determined as described in Materials and Methods (p. 57). ●—● , protein; ○—○ , ALA synthetase activity; ▲—▲ , AA synthetase activity.

invariably associated with the ALA synthetase peak, was eluted first and this was followed by a second much larger peak. The possibility, although remote, was considered that ALA might have been synthesised during these incubations, thus affecting the results obtained for AA synthesis by Sephadex eluate fractions of the first peak. Accordingly, trichloracetic acid supernatants of incubations were processed utilising the ether extraction procedure (see Fig. 3.1, p.61); there was however, no evidence that ALA pyrrole was present.

The elution profile of AA synthetase together with that of proteins and, for comparison, that of ALA synthetase is shown in Fig. 7.5. (In this particular preparation smaller fractions of the Sephadex eluate were collected (4.5 ml instead of 5.5 ml) and the two AA synthetase peaks were more defined.)

A summary of the purification is shown in Table 7.2; an overall recovery of 26% was achieved, with virtually no increase in specific activity of the enzyme, except in Fraction 36 which showed a three-fold purification. The major loss of activity appeared to occur during the ammonium sulphate fractionation, but this did not seem to be due to inadequate fractionation since no AA synthetase activity was

RECOVERY OF AA SYNTHETASE DURING THE PURIFICATION OF ALA SYNTHETASE FROM DDC LIVER MITOCHONDRIA

Purification procedures were as described in the text, except that 4.5 ml fractions of the Sephadex eluate were collected. Protein concentrations and AA synthetase activities were determined as described in Fig. 7.5.

Fraction	Volume (ml)	Protein Concentration (mg/ml)	AA Synthetase	
			Total Activity (mμmole AA)	Specific Activity (mμmole AA/mg protein)
Mitochondrial suspension	23	95	308,000	141
Step 1 - high speed supernatant	17	81	294,000	211
Step 2 - ammonium sulphate fraction	6.4	109	107,000	139
Step 3 - Sephadex eluate fractions				
Σ 19 - 44	117	4.2	80,200	163
Σ 19 - 27	40.5	4.8	17,270	79
Σ 28 - 44	76.5	3.6	62,930	231
25	4.5	6.6	3,780	100
36	4.5	4.6	6,580	380

detected in either the 33% fraction or in the dialysed supernatant of the 33 to 50% fraction. 75% of the enzyme applied to the Sephadex column was eluted (fractions 19 to 44). (In preparations in which 5.5 ml fractions were collected, the peak fraction for ALA synthetase was usually fraction 20, while the two peak fractions for AA synthetase were usually numbers 22 and 29.) The two peaks of AA synthetase activity will be further discussed in Chapter 9.

2. DISCUSSION

The results presented in this chapter show that ALA synthetase present in DDC liver mitochondria can be solubilised and partially purified. The purification achieved was not very great and problems of instability were encountered as was the case with the bacterial enzyme (Burnham and Lascelles, 1963). Another difficulty associated with purification of the enzyme was the presence of fibrous material which precipitated in Sephadex eluate fractions. In the light of experience obtained here, it might be better to commence the purification not by sonication of the mitochondria, but by more selective dissociation of mitochondria into

separate membrane fractions by well established methods, such as were used by Zuyderhoudt, Borst and Huijing (1969). These workers recently reported that ALA synthetase induced by ALA treatment of rats is either loosely bound to the mitochondrial inner membrane or present free in the matrix space.

Such techniques might also prove to be of value in studies of ALA synthetase in normal mitochondria, despite the fact that the present work showed that ALA synthetase activit could not be detected at any stage of the purification procedure when applied to normal mitochondria. In addition, more definite evidence might be obtained as the exact location of the inhibitory factor present in the insoluble fraction of sonicated mitochondria (Chapter 6).

Also arising from studies in this chapter is the question of a relationship between AA synthetase and ALA synthetase, and the significance of the two peaks of AA synthetase eluted from Sephadex G-100. Further investigations are described in Chapter 9.

Another feature of this purification work was the behaviour of ALA synthetase on Sephadex G-200. It might be speculated that this treatment separates out a subcomponent of the enzyme; on the other hand, since recombination of fractions

failed to restore activity, the known instability of the enzyme must also be taken into consideration.

At this stage of the work it was clear that any major further purification would be a lengthy research project and time did not permit this. Accordingly, it was decided to discontinue purification studies and examine the properties of the enzyme since a knowledge of the behaviour of the enzyme with respect to various substrate and cofactor concentrations could prove beneficial if further purification procedures were to be attempted. The properties of theenzyme are thus described in the next chapter.

CHAPTER 8

THE PROPERTIES OF ALA SYNTHETASE PURIFIED FROM MITOCHONDRIA ISOLATED FROM THE LIVERS OF PORPHYRIC GUINEA PIGS

This chapter describes the use of the radiochemical assay method in a study of some of the properties of ALA synthetase partially purified from liver. Since the assay system depends on two enzyme activities (succinyl CoA and ALA synthetases) it is not suitable for elaborate kinetic studies. The kinetic studies reported in this chapter are of a preliminary nature, carried out to determine the basic properties of the enzyme rather than as a complex kinetic study *per se*.

For all of the work described in this chapter, unless otherwise stated, the enzyme used was that present in the two or three fractions at the centre of the ALA synthetase peak eluted from Sephadex G-100. These fractions were pooled and were stored in small vials at -15°. Since the specific activity was not increased by removal of the birefringent precipitate which formed on standing (see p. 141) and since, in some cases, removal of the precipitate by centrifugation resulted in total and unpredictable loss of activity, the Sephadex eluate fractions used in this work had no further treatment.

1. RESULTS

A. Application of the Radiochemical Method of Assay to Purified ALA Synthetase

Despite the fact that the colorimetric assay was satisfactory for studies on the purified enzyme, the radiochemical method offered advantages, not the least of which was that the sensitivity was so great that only small amounts of enzyme need be used. This is important in view of the relative difficulty of obtaining the enzyme.

A further relevant point was that for all practical purposes with purified preparations of the bacterial ALA synthetase, ^{14}C-succinate was converted solely to ALA. Thus all the ^{14}C-isotope in the Dowex 50 eluate was ALA (see p.81, Fig. 4.4) and hence radioactivity measurement of this was all that was needed, eliminating pyrrole formation and subsequent extraction. The assay method was not only sensitive but also simple and rapid.

The application of the method to purified liver ALA synthetase was therefore investigated. Since it might be expected that the metabolism of succinate would now be severely curtailed (in comparison with the original mitochondria) the necessity for tricarboxylic acid cycle inhibitors was doubtful. Such proved to be the case -

good activity was obtained without the inhibitors (and as shown later, good quantitative agreement with the colorimetric assay method was obtained).

An interesting feature of the purified enzyme is that the assay system was saturated with a concentration of succinate approximately one twentieth of that required when crude mitochondrial preparations were used (see Fig.4.10). This has the fortunate effect that the cost of the assay becomes much less (less ^{14}C-succinate is needed) or from the alternative point of view, little ^{12}C-succinate need now be added. The resultant increase in specific activity permits great sensitivity.

The question was also examined of whether the method could be simplified by omitting the steps of pyrrole formation and extraction and simply determining radioactivity present in the Dowex 50 eluate. Using low concentrations of succinate (see above) it was found that all of the radioactivity in the Dowex 50 (H^+) elute was recovered in the ALA pyrrole fraction (Fig. 8.1). Unfortunately however, not all of the purified enzyme preparations behaved in this way. For routine assays therefore the full extraction procedure was essential; presumably further purification would eliminate this need.

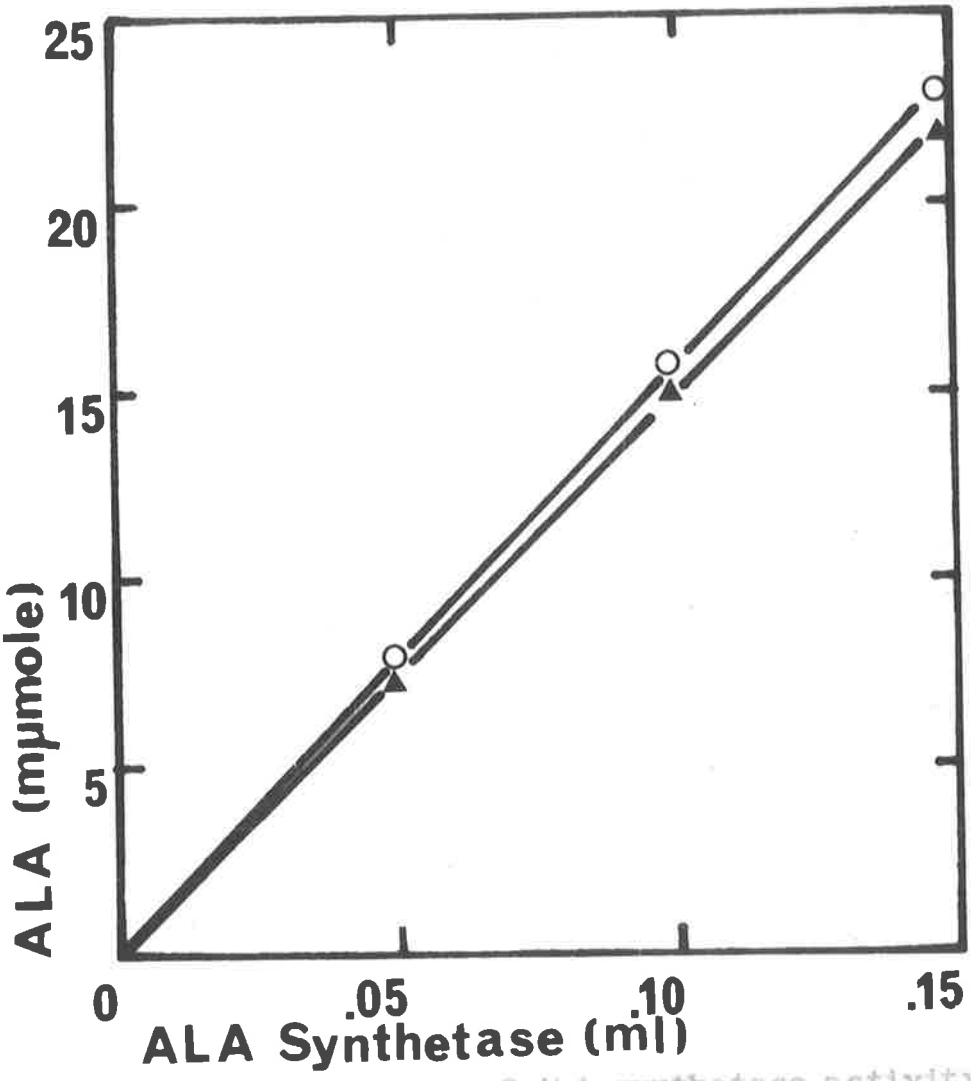


Fig.8.1. Radiochemical assay of ALA synthetase activity in purified preparations of the liver enzyme. Incubations contained the following (in μmoles/ml, unless otherwise stated): tris-HCl (pH 7.4), 50; glycine, 100; ^{14}C-1,4-succinic acid (specific activity 8.8 μC/μmole), 0.57; $MgCl_2$, 20; ATP, 25; CoA, 0.425; GSH, 2; pyridoxal 5-phosphate, 1; purified *E. coli* succinyl CoA synthetase, 0.1 ml; enzyme and water to a final volume of 0.5 ml. Experimental procedures were as described in the text (Chapter 4, p. 91). Figures on the ordinate represent the total amount of ALA formed during incubations. ○—○, mμmole of ALA calculated from radioactivity in the Dowex 50 eluate; ▲—▲, mμmole of ALA calculated from radioactivity in the methanol solution of ALA pyrrole.

Within the range tested, a precisely linear response to enzyme concentration was obtained with purified preparations of ALA synthetase (Fig. 8.1).

Unless otherwise stated, all assays were done with the radiochemical method employing ethyl acetate extraction of ALA pyrrole. Incubations contained ^{14}C-1,4-succinate at a concentration of 0.57 mM (specific activity ranging from 2.2 to 8.8 μC/μmole, depending on the activity of the enzyme preparation).

(a) Time course of the reaction

It can be seen from Fig. 8.2 that the synthesis of ALA by the purified liver enzyme was linear, under the standard conditions, for approximately 40 minutes. For comparison the experiment was carried out using both the colorimetric and radiochemical assay procedures. The two methods agreed reasonably well, in this case the value of ALA synthesis in the linear portion of the graph obtained by the latter method was 10% higher than by the former. In the colorimetric assays, ALA synthesis was calculated from optical density values obtained both before and after ether extraction (Granick, 1966) and the graph clearly shows

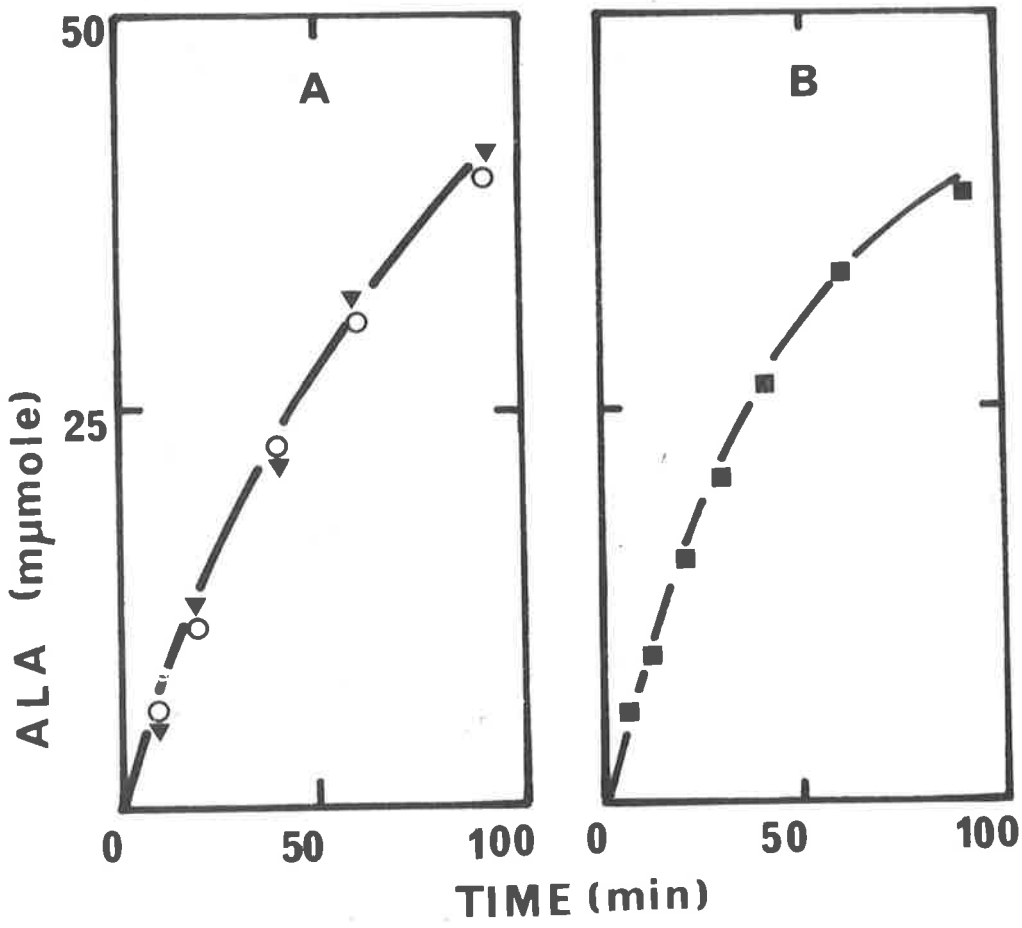


Fig. 8.2. Effect of time of incubation on ALA synthesis. A. Colorimetric assay. Incubation mixtures were as described in Fig. 8.1 except that ^{14}C-succinate was replaced by unlabelled potassium succinate (final concentration, 10 μmole/ml). Experimental procedures were as described in the text (Chapter 4, p.91), except that time of incubation was varied, and all incubations contained 0.2 ml of enzyme solution (5.2 mg of protein/ml).

▲—▲, ALA calculated from optical density before ether extraction; ○—○, ALA calculated from optical density after ether extraction. B. Radiochemical assay. Incubation conditions were as described in Fig. 8.1, except that time of incubation varied and all incubations contained 0.1 ml of enzyme solution. ALA production was calculated from radioactivity in the Dowex 50 eluate.

that in purified preparations of the enzyme, under conditions described in Fig. 8.2, no AA synthesis occurred during incubations.

(b) The effect of E. coli succinyl CoA synthetase on ALA synthesis

It was anticipated that, in the system used, ALA synthesis by purified preparations of the enzyme would be dependent on added succinyl CoA synthetase. Fig. 8.3 shows that this was the case and furthermore that the amount (0.05 ml) of the bacterial enzyme solution normally included in incubations was adequate for maximal ALA synthesis.

(c) The effect of glycine concentration on ALA synthetase activity

The results in Fig. 8.4A show the effect of glycine concentration on the synthesis of ALA by purified preparations of the liver enzyme. An apparent K_m value of 33.3 mM was calculated from the Lineweaver-Burk plot (Fig. 8.4B) of this data, a figure which closely agrees with that (34.5 mM) obtained from the results of colorimetric assays showing the effect of glycine concentration on ALA synthetase activity in DDC liver mitochondria (see Fig. 4.11).

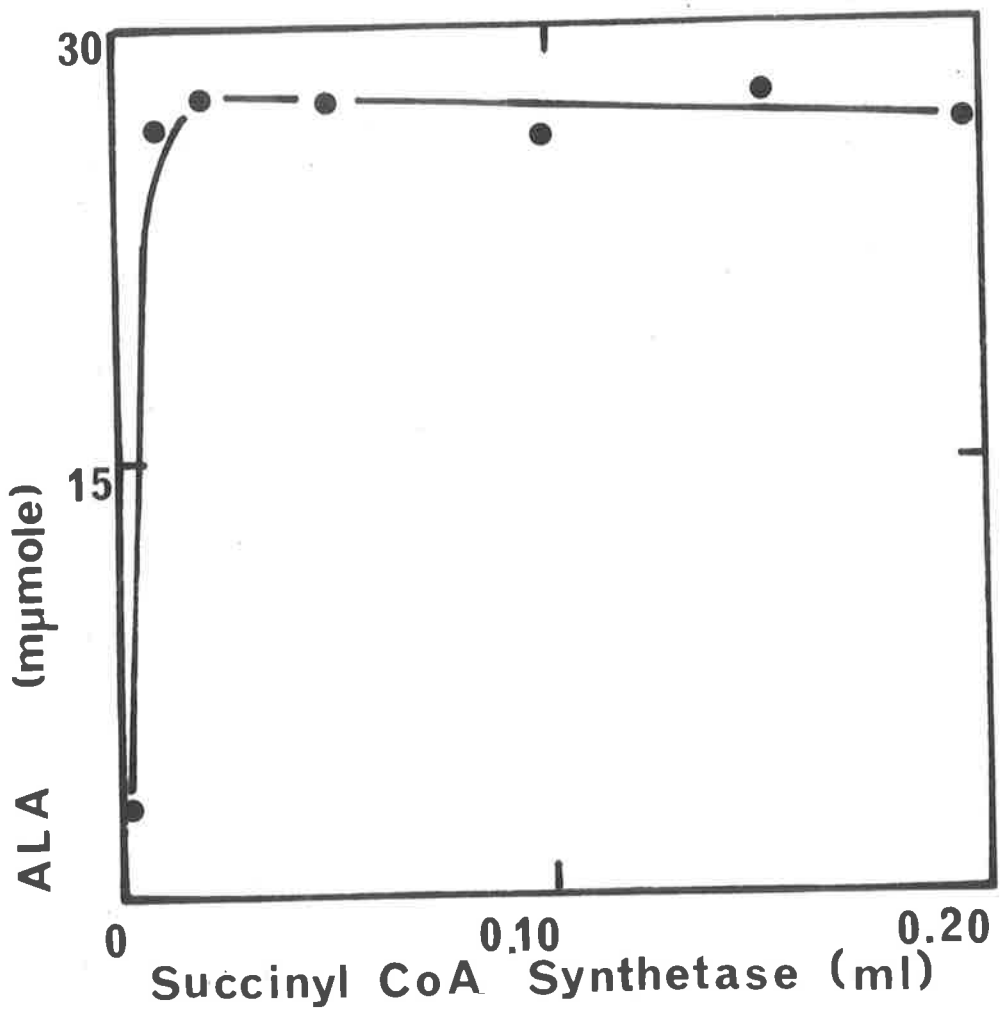


Fig. 8.3. The effect of E. coli succinyl CoA synthetase on the synthesis of ALA. Incubation conditions were as described in Fig. 8.2B, except the amount of succinyl CoA synthetase was varied and the incubation time was 30 minutes. All incubations contained 0.05 ml of ALA synthetase solution (5.2 mg of protein/ml). The activity of the succinyl CoA synthetase (determined as described in Materials and Methods (p. 56) by the method of Kaufman (1955)) was 9.0 μmoles of succinhydroxamic acid produced/30 minutes/ml of enzyme solution.

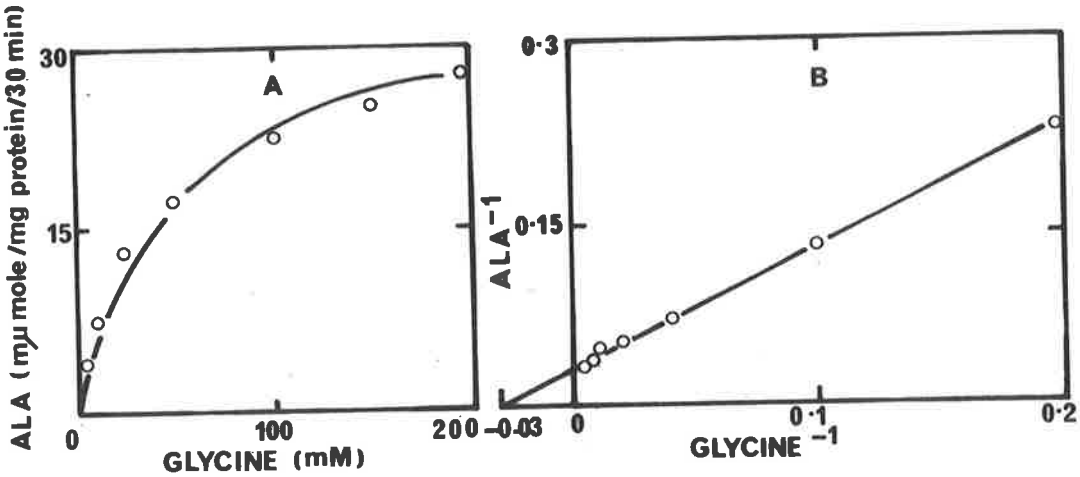


Fig. 8.4. The effect of glycine concentration on ALA synthetase activity. Incubation conditions were as described in Fig. 8.3 except that the glycine concentration was varied and the amount of succinyl CoA synthetase was held constant. The specific activity of the ^{14}C-1,4-succinic acid was 2.2 μC/μmole and all incubations contained 0.05 ml of enzyme solution (4.6 mg of protein/ml).
A. Velocity versus glycine concentration.
B. Lineweaver-Burk plot of the data in A.

(d) The effect of CoA, succinate and ATP concentration on ALA synthesis

For practical purposes, it was desirable to determine the optimum requirements of the system for CoA, succinate and ATP. Although the apparent K_m values so determined would not be functions of ALA synthetase itself, they would be relevant to the succinyl CoA synthetase also included in the reaction mixture.

The CoA concentration curve is shown in Fig. 8.5A and the Lineweaver-Burk plot of this data in Fig. 8.5B; the apparent K_m value for CoA in this system was 0.1mM. The requirement of the system was therefore unchanged when ALA synthetase was partially purified since the same value was calculated from the data shown in Fig. 4.11 which shows the effect of CoA concentration on ALA synthesis by DDC liver mitochondria.

The effect of succinate concentration on ALA synthesis is shown in Fig. 8.6A. An apparent K_m value of 0.1mM was calculated from the Lineweaver-Burk plot of this data, shown in Fig. 8.6B. For comparison, reciprocal plots of the data obtained for ALA synthesis by DDC liver mitochondria and soluble extracts of ultrasonicated preparations

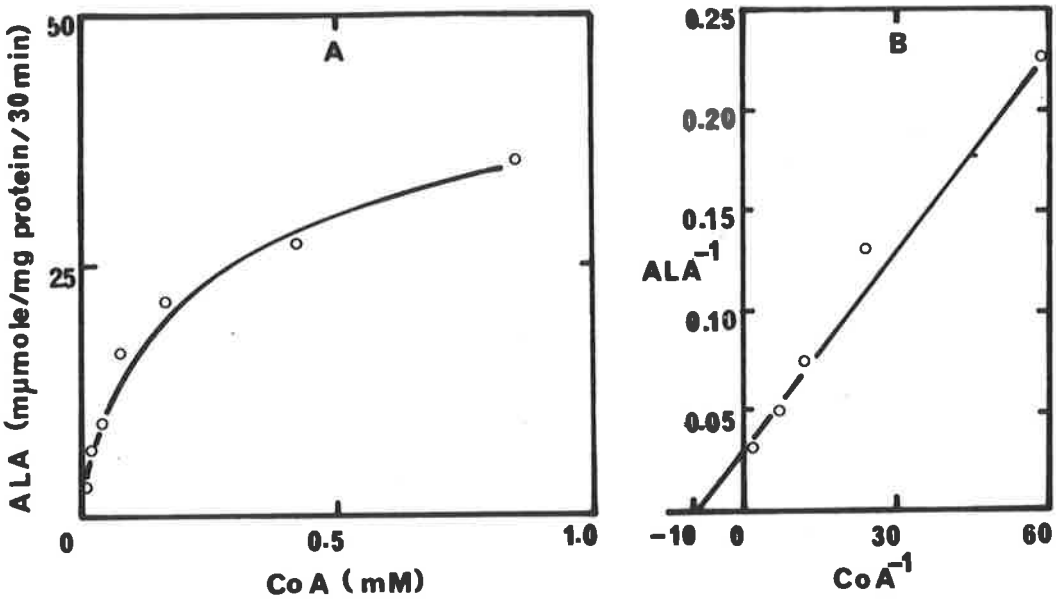


Fig. 8.5. The effect of CoA concentration on ALA synthesis. Incubation conditions and method of assay were as described in Fig. 8.4, except that the CoA concentration was varied and the glycine concentration was held constant. All incubations contained 0.05 ml of enzyme solution (7.0 mg of protein/ml).
A. Velocity versus CoA concentration.
B. Lineweaver-Burk plot of the data in A.

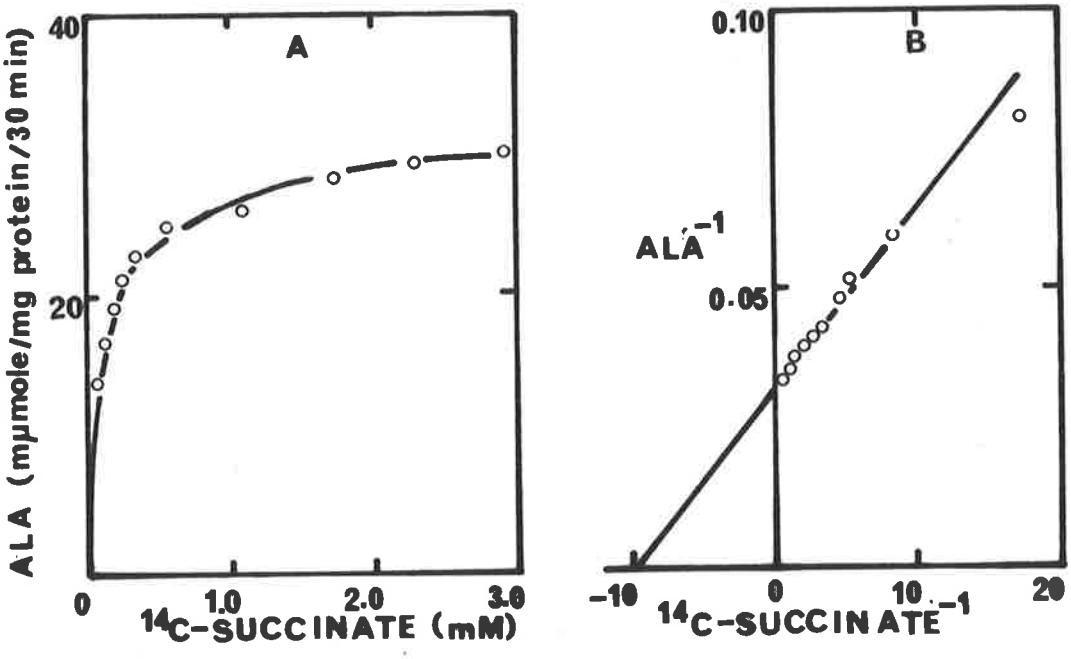


Fig. 8.6. The effect of succinate concentration on ALA synthesis. Incubation conditions and method of assay were as described in Fig. 8.5, except that the CoA concentration was constant and the succinate concentration was varied. All incubations contained 0.1 ml of enzyme solution (3.1 mg of protein/ml).
A. Velocity versus succinate concentration.
B. Lineweaver-Burk plot of the data in A.

are shown (Fig. 8.7). The apparent K_m value for succinate in the former system was 2.0mM while in the latter this was 0.65mM. Thus there was a twenty-fold drop in the apparent K_m for succinate when the enzyme was partially purified; this correlates with the lowered succinate requirement already mentioned.

The effect of ATP concentration on ALA synthesis is shown in Fig. 8.8 and as with crude preparations of the enzyme (see Fig. 4.11), the results illustrate the complete dependence of the assay system on added ATP.

(e) The effect of sodium malonate on ALA synthesis

Despite the high levels of PBG excreted in the urine of guinea pigs injected with sodium malonate (see p. 132), the level of ALA synthetase in the livers of such animals was not affected. It therefore seemed desirable to determine whether or not this compound affected the activity of the enzyme. No evidence was found however, to indicate that sodium malonate had a direct effect on ALA synthesis by purified preparations of the liver enzyme. Furthermore, the apparent K_m for succinate was not affected by sodium malonate either in the presence or in the absence of sodium DL-malate.

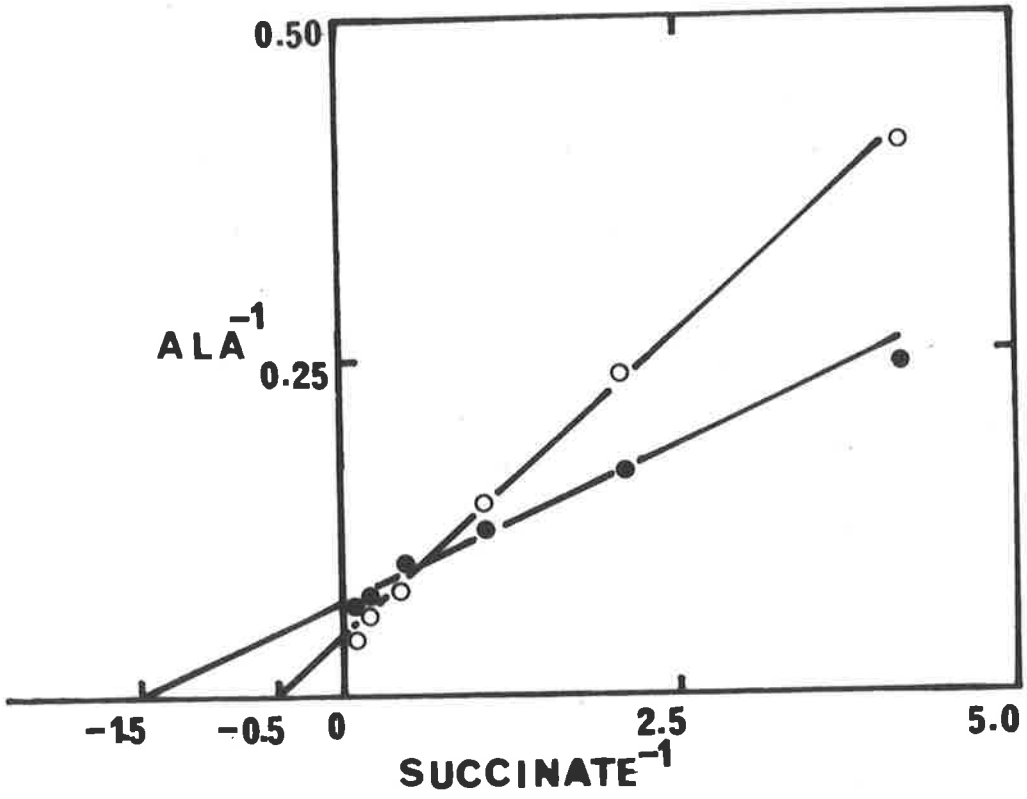


Fig. 8.7. Lineweaver-Burk plot of velocity versus succinate concentration for ALA synthesis by crude preparations of the enzyme. Incubation conditions and method of assay were as described in Fig. 4.10. Figures on the ordinate are the reciprocals of the amount of ALA formed (mμmole/mg of protein/30 minutes). ○—○, incubations contained 0.05 ml of DDC liver mitochondria; ▲—▲, incubations contained 0.05 ml of the high speed supernatant of ultrasonicated DDC liver mitochondria.

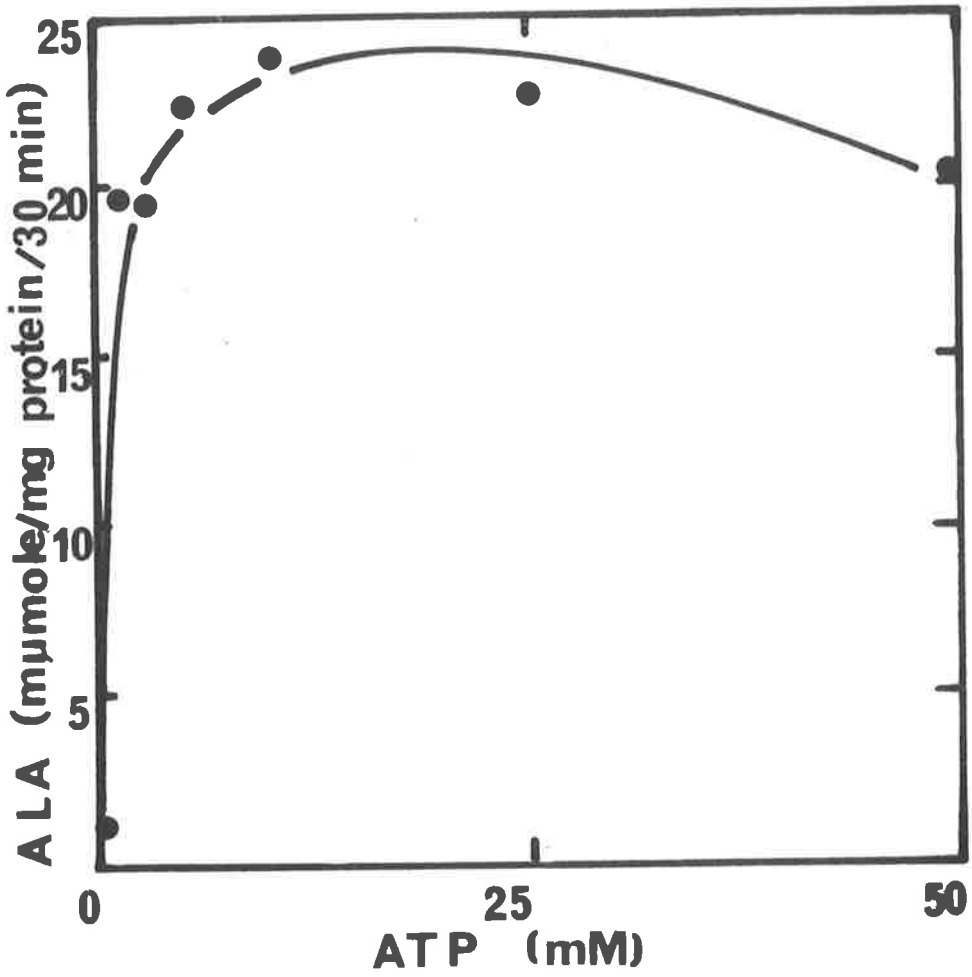


Fig. 8.8. The effect of ATP concentration on ALA synthesis. Incubation conditions and method of assay were as described in Fig. 8.6, except that ATP concentration was varied and succinate concentration was held constant. All incubations contained 0.05 ml of enzyme solution (5.4 mg of protein/ml).

B. Studies of the Effect of Haem on ALA Synthesis by Purified Preparations of the Liver Enzyme

As reported earlier (see p.124), ALA synthesis by DDC liver mitochondria was inhibited by haem. However, this inhibition was evident only at much higher concentrations of haem than have been used by other workers (Granick, 1966; Marver et al., 1966d) in attempts to demonstrate an inhibitory effect of haem on the activity of the liver enzyme.

Evidence has been obtained to indicate that feedback inhibition of ALA synthetase activity by haem may be one of the factors controlling porphyrin synthesis in R. spheroides (Burnham and Lascelles, 1963)*. It therefore seemed important that the inhibitory effect of haem on ALA synthesis by the liver enzyme should be examined in greater detail using purified preparations.

(a) The effect of haem and haem proteins on ALA synthesis

At a concentration of 0.4mM, haem inhibited ALA synthesis by approximately 70%. By contrast, none of the haem proteins tested had any inhibitory action (Table 8.1). Cytochrome C was included among these as a comparison since

---

*Lascelles and Hatch (1969).

TABLE 8.1.

THE EFFECT OF HAEM AND HAEM PROTEINS ON ALA SYNTHESIS

Incubation conditions and method of assay were as described in Fig. 8.8, except that all incubations contained 25 µmole of ATP/ml, additions shown, and 0.1 ml of enzyme solution (5.9 mg of protein/ml). Results are expressed as a percentage of the original activity in 0.1 ml of enzyme solution (15.3 mµmoles of ALA produced/ 30 minutes).

Addition	Final concentration (mM)	ALA synthetase (% Original activity)
None	-	100
Haem	0.4	29
Haemoglobin	0.1	133
Myoglobin	0.4	103
Cytochrome C	0.4	111

it does not inhibit the bacterial enzyme (Burnham and Lascelles, 1963). As already mentioned (see p. 124) it was surprising to find that haemoglobin and myoglobin, which inhibited ALA synthesis both by the bacterial enzyme (Burnham and Lascelles, 1963) and by DDC liver mitochondria (Table 6.2, p. 125), had no inhibitory effect on ALA synthesis by the purified liver enzyme*.

(b) The effect of haem concentration on succinyl CoA synthetase activity

Since assays of ALA synthetase activity of purified preparations were dependent on added succinyl CoA synthetase (see Fig. 8.3), it was essential to know whether or not the activity of this enzyme was also inhibited by haem. The effect of haem concentration on succinyl CoA synthetase activity is shown in Fig. 8.9. Enzyme activity was inhibited by approximately 50% when incubations contained 0.4mM haem. However, the results in Table 8.2 show that haem inhibition of ALA synthetase activity was not overcome when the amount of succinyl CoA synthetase included in incubation mixtures was doubled. These results indicated that the inhibition of ALA synthesis by haem was the result of a direct effect on ALA synthetase activity. Confirmation

---

*See Appendix IV.

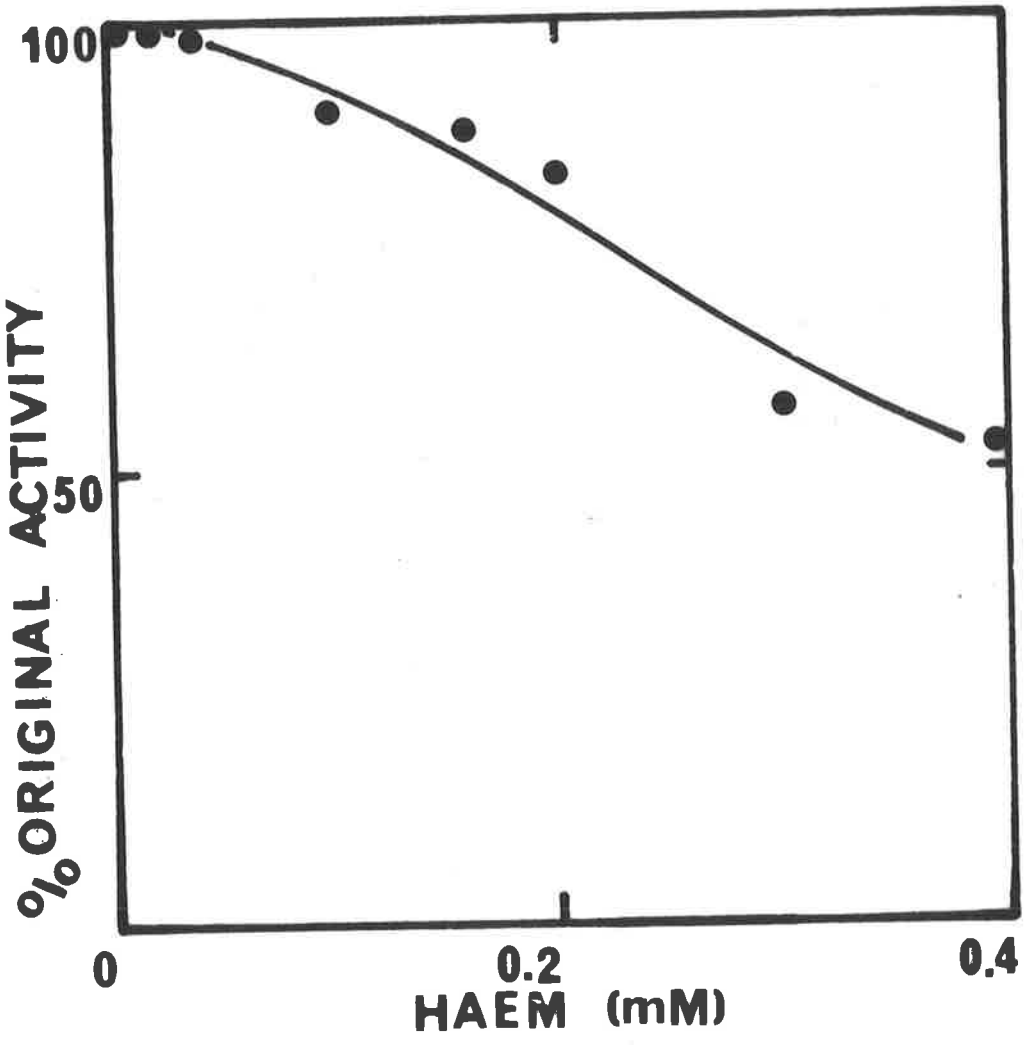


Fig. 8.9. The effect of haem concentration on E. coli succinyl CoA synthetase activity.
Incubation conditions and method of assay were as described in Materials and Methods (p. 56). All incubations contained 0.05 ml of enzyme solution. Results are expressed as a percentage of the original activity in 0.05 ml of enzyme solution (0.7 μmole of succinhydroxamic acid produced/30 minutes).

TABLE 8.2

EXPERIMENT TO TEST WHETHER INCREASED SUCCINYL CoA SYNTHETASE AFFECTS HAEM INHIBITION OF ALA SYNTHESIS.

Incubations contained the following (in μmoles/ml unless otherwise stated): tris-HCl (pH 7.4), 50; glycine, 100; potassium succinate, 10; CoA, 0.425; $MgCl_2$, 20; GSH, 2; ATP, 25; pyridoxal 5-phosphate, 1; additions shown, 0.1 ml of ALA synthetase (15 mg of protein/ml) and water to a final volume of 0.5 ml. Tubes were incubated for 30 minutes in a shaking water bath at 37° and the reaction stopped by the addition of 1.5 ml of 0.2M trichloracetic acid. ALA in the protein free supernatant was determined as described in Fig. 8.2 for the colorimetric assay. Results are expressed as a percentage of the original activity in 0.1 ml of enzyme solution (26 mμmole of ALA produced/30 minutes).

Additions		ALA synthetase (% original activity)
Succinyl CoA synthetase (ml)	Haem (mM)	
0.1	-	100
0.2	-	105
0.1	0.4	60
0.2	0.4	62

that this was so was obtained in a separate experiment in which reaction mixtures contained synthetic succinyl CoA (prepared by the method of Simon and Shemin, 1953) instead of the components used to generate this compound enzymically; this is shown in Table 8.3.

(c) The effect of haem concentration on ALA synthetase activity

Fig. 8.10 shows that while little inhibition occurred with concentrations of haem less than 0.05mM, ALA synthetase activity was inhibited to the extent of 75% when incubations contained 0.4mM haem. However, since the two stage enzyme system used in this work was unsuitable for complex kinetic studies, the inhibitory effect of haem was not examined in greater detail.

(d) The effect of glycine concentration on haem inhibition of ALA synthetase activity

As was the case with bacterial enzyme (Burnham and Lascelles (1963)), haem inhibition of ALA synthetase activity was not overcome by increasing concentrations of glycine (Fig. 8.11A). The Lineweaver-Burk plot (Fig. 8.11B) of this data indicated pure non-competitive inhibition at all levels of the inhibitor.

TABLE 8.3.

THE EFFECT OF HAEM ON ALA SYNTHETASE ACTIVITY

Incubation mixtures contained (in μmoles/ml, unless otherwise stated): tris-HCl, (pH 7.4), 50; glycine, 100; succinyl CoA, 0.5; pyridoxal 5-phosphate, 1; addition shown; 0.1 ml of enzyme solution (15 mg of protein/ml) and water to a final volume of 0.5 ml. Incubation conditions and method of assay were as described in Table 8.2. The result is expressed as a percentage of the original activity present in 0.1 ml of the enzyme solution (26 mμmole of ALA produced/30 minutes).

Addition	Final concentration (mM)	ALA synthetase (% Original activity)
None	-	100
Haem	0.4	38

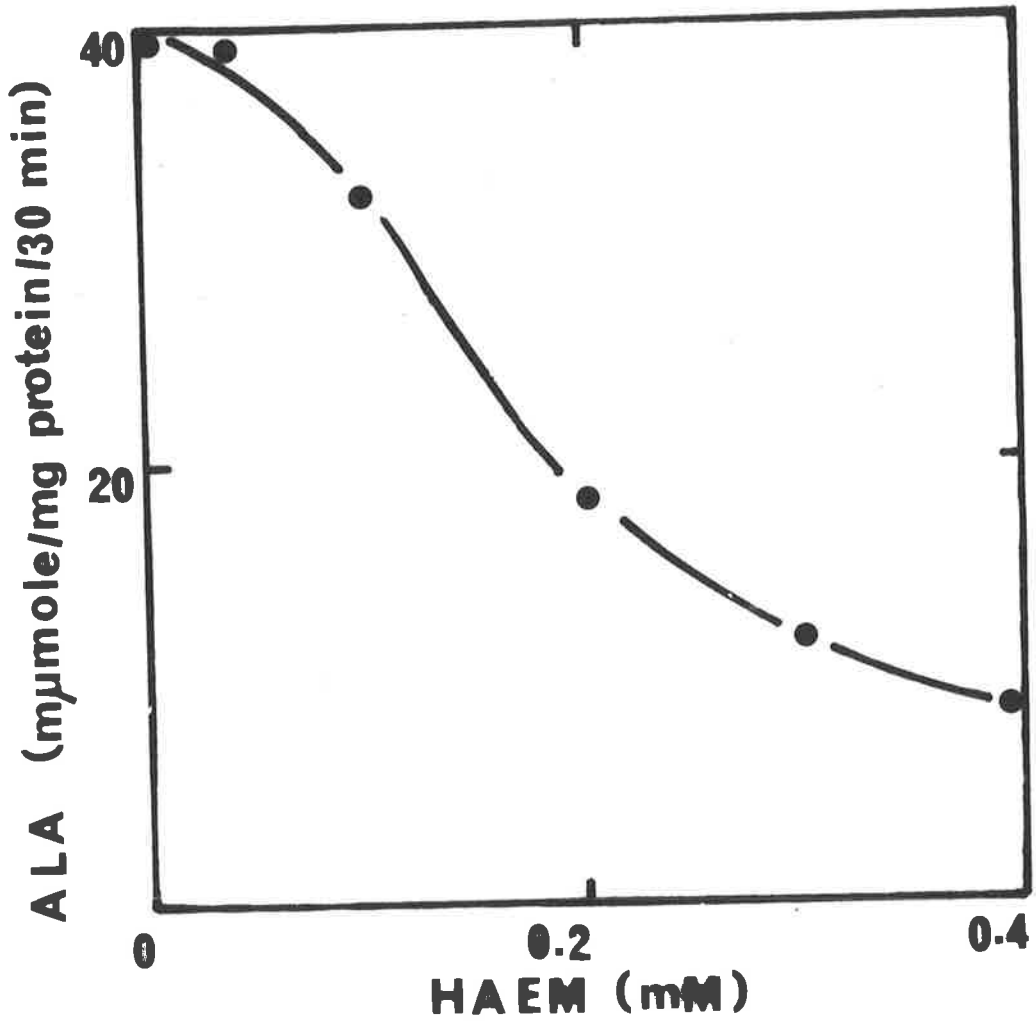


Fig. 8.10. The effect of haem concentration on ALA synthetase activity. Incubation conditions and method of assay were as described in Table 8.1, except that all incubations contained 0.05 ml of enzyme solution (4.3 mg of protein/ml).

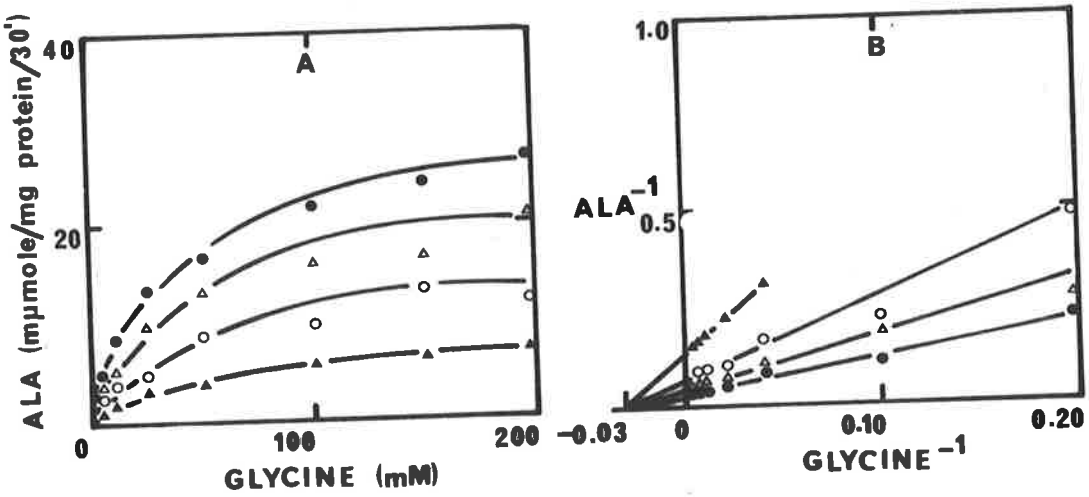


Fig. 3.11. The effect of glycine concentration on the haem inhibition of ALA synthetase activity.

Incubation conditions and method of assay were as described in Fig. 8.4, except that the specific activity of the ^{14}C-1,4-succinic acid was 4.4 μC/μmole. All incubations contained 0.05 ml of enzyme solution (4.5 mg of protein/ml).

A. Velocity versus glycine concentration.
● — ● , no haem; △ — △ , 0.08mM haem;
○ — ○ , 0.16mM haem; ▲ — ▲ , 0.24mM haem.

B. Lineweaver-Burk plot of the data in A.
● — ● , no haem; △ — △ , 0.08mM haem;
○ — ○ , 0.16mM haem; ▲ — ▲ , 0.24mM haem.

(c) The effect of succinate concentration on haem inhibition of ALA synthetase activity

The effect of succinate concentration on haem inhibition of ALA synthetase activity was also examined. The data of Fig. 8.12 shows that haem inhibition was not dependent on succinate concentration when low inhibitor concentration was used (less than 0.08mM). By contrast, the inhibition was apparently competitive at higher levels of haem. The significance of this has not been examined since a full kinetic study has not been possible.

C. The Effect of EDTA on ALA Synthesis by Purified Preparations of the Liver Enzyme

The fact that the administration of CaEDTA to patients suffering from various forms of hepatic porphyria is an apparently successful form of therapy (Peters, 1961; Woods et al., 1961; Peters et al., 1966; Donald et al., 1969) makes a study of the effect of EDTA on ALA synthetase activity of obvious interest.

(a) The effect of EDTA concentration on ALA synthesis

As shown in Fig. 8.13, at high concentrations (25 to 100 mM), EDTA markedly inhibited the synthesis of ALA.

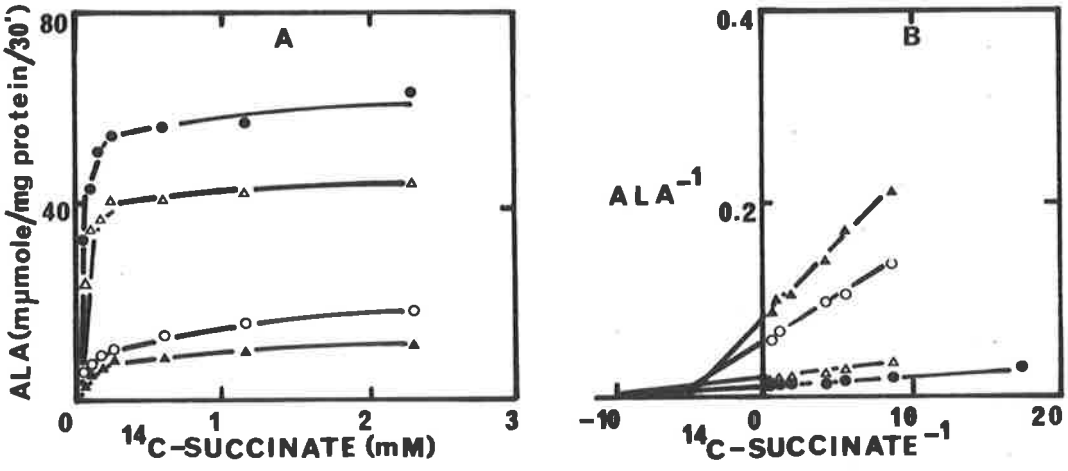


Fig. 8.12. The effect of succinate concentration on the haem inhibition of ALA synthetase activity. Incubation conditions and method of assay were as described in Fig. 8.6, except that the specific activity of the ^{14}C-1,4-succinic acid was 4.4 μC/μmole. All incubations contained 0.05 ml of enzyme solution (4.8 mg of protein/ml).

A. Velocity versus succinate concentration.
●—●, no haem; △—△, 0.08mM haem;
○—○, 0.16mM haem; ▲—▲, 0.24mM haem.

B. Lineweaver-Burk plot of the data in A.
●—●, no haem; △—△, 0.08mM haem;
○—○, 0.16mM haem; ▲—▲, 0.24mM haem;

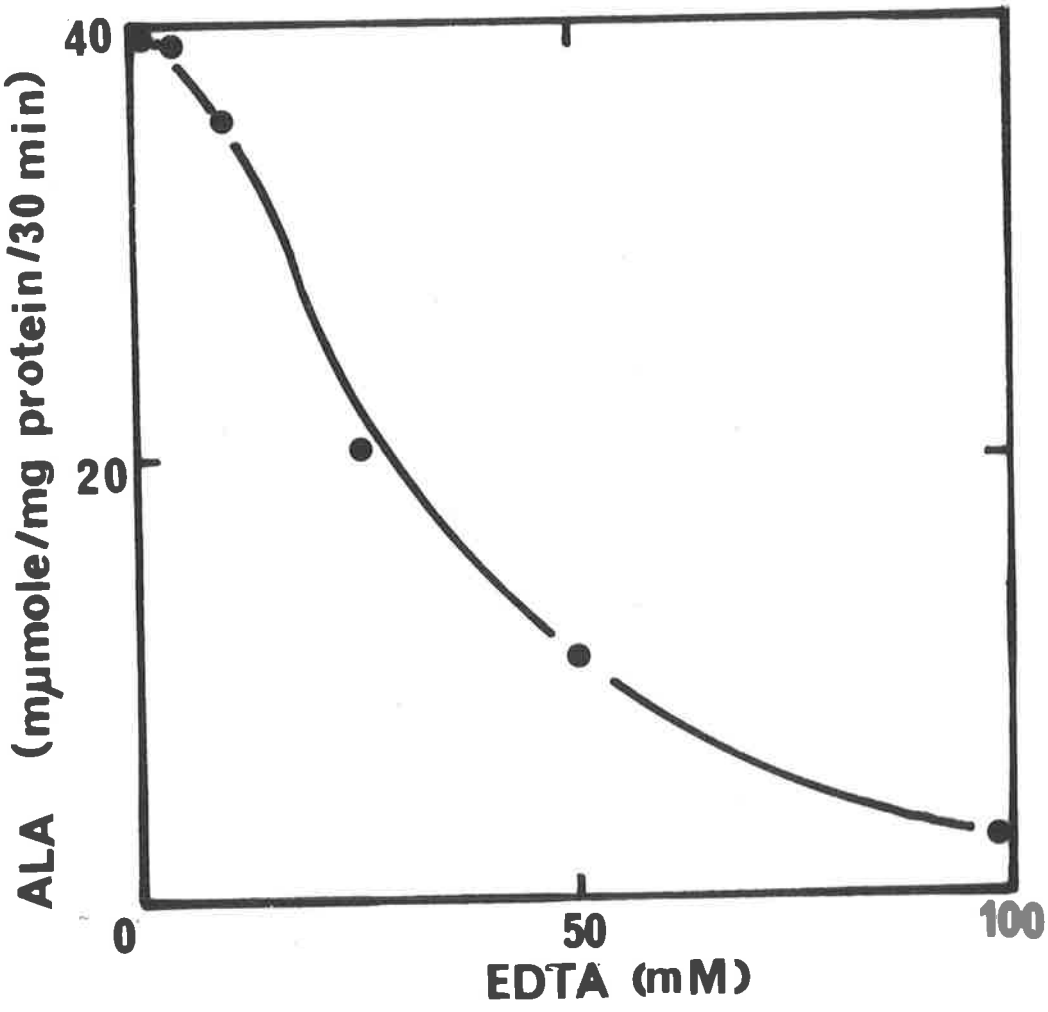


Fig. 8.13. The effect of EDTA concentration on ALA synthesis. Incubation conditions and method of assay were as described in Fig. 8.10 and all incubations contained 0.05 ml of enzyme solution (4.3 mg of protein/ml).

Since this effect could have been due to the removal of Mg^{++} (required by succinyl CoA synthetase) by EDTA, the concentration of Mg^{++} was increased four-fold. However, this procedure did not affect the inhibitory action of EDTA.

(b) The effect of EDTA concentration on succinyl CoA synthetase activity

Since the inhibitory effect of EDTA on ALA synthesis could have been the result of its inhibition of succinyl CoA synthetase activity, the effect of EDTA on the activity of this enzyme was also examined. As shown in Fig. 8.14, succinyl CoA synthetase activity was strongly inhibited by EDTA; approximately 80% inhibition occurred at EDTA concentrations of 25mM. Again the effect of Mg^{++} was studied, but even a four-fold increase in Mg^{++} concentration did not overcome the inhibitory effect of EDTA when the concentration of the latter was greater than 25mM.

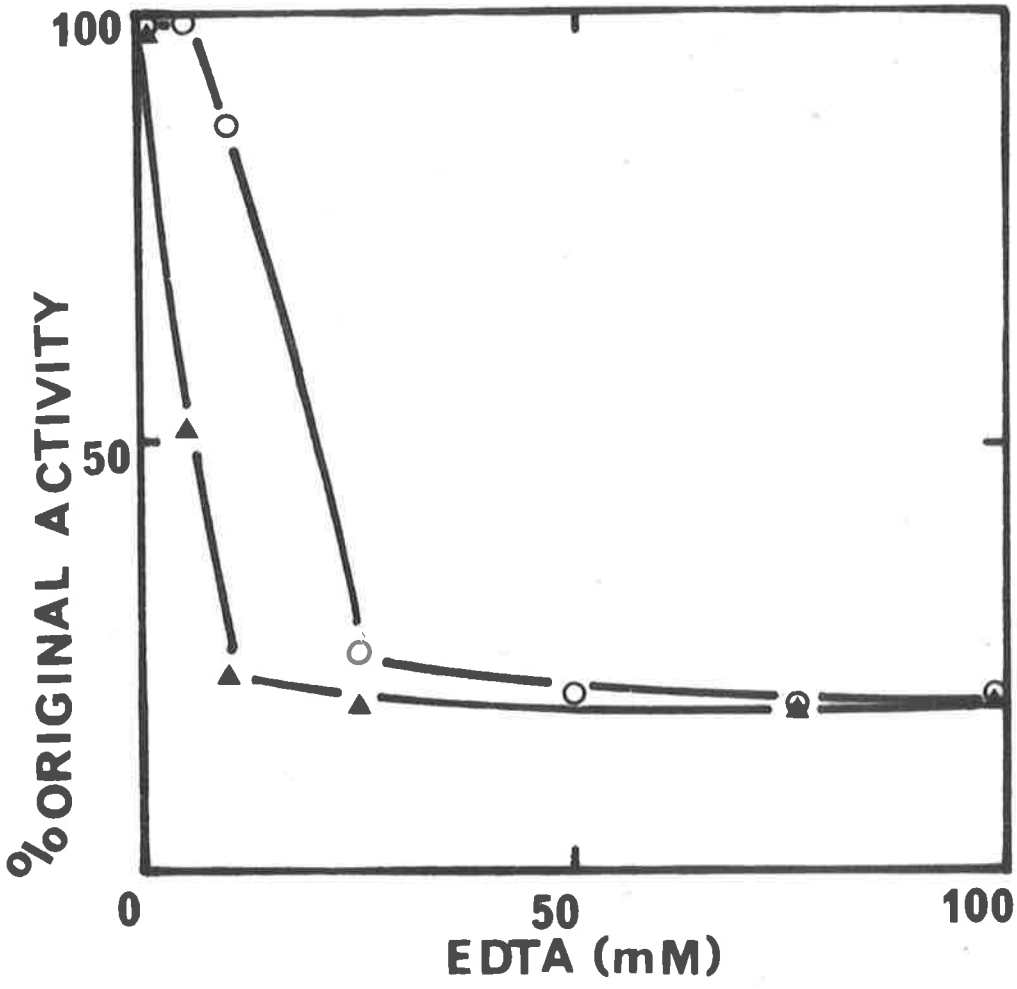


Fig. 8.14. The effect of EDTA concentration on *E. coli* succinyl CoA synthetase activity.
Incubation conditions and method of assay were as described in Materials and Methods (p. 56) except for the $MgCl_2$ concentration (shown below). All incubations contained 0.1 ml of enzyme solution. Results are expressed as a percentage of the original activity in 0.1 ml of enzyme solution (1.3 μmole of succinhydroxamic acid produced/30 minutes). ▲ — ▲ , 5mM $MgCl_2$ present; ○ — ○ , 20mM $MgCl_2$ present.

2. DISCUSSION

The value of the radiochemical method for assays of preparations of low ALA synthetase activity is further illustrated by the work of this chapter. While good quantitative agreement with the colorimetric method was obtained, the radiochemical assay was the method of choice because of its greater sensitivity. This was essential because the purified liver enzyme was extremely unstable and activities of the various preparations used were low. Moreover, only small amounts of the enzyme needed to be used, an added advantage when large numbers of comparable assays were required at any particular time (e.g. the experiments described in Figs. 8.11 and 8.12).

In comparing the optimum requirements of the mitochondrial bound enzyme and the purified enzyme for various components of reaction mixtures, the most striking feature was the dramatic decrease in the succinate requirement for obtaining maximal ALA synthesis. The two systems were not really comparable however, since, with purification of ALA synthetase, a number of factors which could have influenced the requirement of the crude mitochondrial assay system for succinate were presumably eliminated.

These include firstly, any effect due to the relative impermeability of frozen and thawed mitochondria (Granick and Urata, 1963). The fact that ALA synthesis by liver mitochondria was not dependent on E. coli succinyl CoA synthetase (see p. 91) as was the case with the purified enzyme would indicate that endogenous succinyl CoA synthetase may be preferentially involved in generating the substrate in mitochondria. It is possible that the amount of succinate required to fully saturate the endogenous enzyme may be high.

Alternatively, the destruction of succinyl CoA by a hydrolase in the mitochondria may have been eliminated during subsequent purification of ALA synthetase. There is also the possibility that, despite the presence of inhibitors of the tricarboxylic acid cycle, some of the added succinate is metabolised by other enzymes present in mitochondria. Furthermore, it was considered conceivable that the high apparent K_m value for succinate in the crude assay system might be due to a spurious effect of malate or malonate since these were omitted from assays when the purified enzyme was used. However, that this was not the case was shown by experiments using the purified enzyme to

which these compounds were added.

Nevertheless, th finding that the requirement of the system for succinate was markedly reduced when ALA synthetase was purified eliminated the need for diluting the ^{14}C-succinate with unlabelled succinate in order to saturate the system. The sensitivity of the assay was thereby increased to the extent that observed values of approximately 1,000 cpm (representing the synthesis of 1 mμmole of ALA) could be obtained if so desired.

If greater purification of ALA synthetase can be achieved, the present work illustrates the potential of the radiochemical assay method for complex kinetic studies and for studies of the chemistry of the active sites. In its present form, the method was not suitable for this type of work because of the dual enzyme system used to achieve the incorporation of labelled carbon atoms into ALA. Although this difficulty presumably could be overcome by the use of labelled succinyl CoA the true substrate of ALA synthetase, the instability of this compound might give rise to other problems.

The finding that haem inhibited the activity of liver ALA synthetase *in vitro* would indicate that feedback inhibition of enzyme activity could be a factor controlling the synthesis

of haem and porphyrins in liver as well as in other systems (Burnham and Lascelles, 1963; Kariban and London, 1965). The liver enzyme, even when purified, was less sensitive to haem inhibition than is the bacterial enzyme*. However, the recent demonstration (Marriott, Neuberger and Tait, 1969) that only the activated form of the bacterial enzyme is significantly inhibited *in vitro* by extremely small amounts of haem could be of relevance in assessing the possible physiological significance of the present results. It is conceivable that the isolated liver enzyme is analogous to the non-activated form of the bacterial enzyme.

The results with EDTA do not provide any information as to whether this compound directly inhibits ALA synthetase activity. Clearly this can be established by the use of succinyl CoA as substrate; it was not done due to insufficient time to prepare new enzyme but the work is being continued in this laboratory. Since it appears to be well established that 1mM EDTA does not inhibit in liver homogenates (Marver *et al.*, 1966b) there is as yet no biochemical rationale for the claimed effectiveness of EDTA therapy (Peters, 1961). The difficulty, pointed out by Lang *et al.* (1968) of thoroughly establishing the effectiveness of therapy for an intermittent disease, in which spontaneous remission of symptoms can occur, perhaps should be borne in mind.

---

*See Appendix IV.

CHAPTER 9

PURIFICATION OF AA SYNTHETASE FROM THE LIVERS OF NORMAL AND PORPHYRIC GUINEA PIGS

Like ALA synthetase, AA synthetase of liver is a mitochondrial enzyme and also catalyses a reaction between glycine and an acyl CoA compound to form an aminoketone (Urata and Granick, 1963). At one time it was thought that a series of aminoketones could be synthesised by one pyridoxal 5-phosphate requiring enzyme which was non-specific for acyl CoA compounds (Laver, Neuberger and Udenfriend, 1958; Neuberger, 1961), but it is now known that a separate enzyme specific for acetyl CoA exists for AA synthesis (Urata and Granick, 1963). These workers also found that in liver mitochondria the aminoketone synthesised from glycine and malonyl CoA was indistinguishable from AA; in order to account for this they proposed that malonyl CoA must first be decarboxylated to give acetyl CoA.

In liver and a number of other biological systems, AA is also synthesised by an alternative pathway and is an intermediate in the metabolism of L-threonine (Elliott, 1959; 1960b).

Because of the similarity of the reactions catalysed by AA synthetase and ALA synthetase, the question of a relation-

ship between the two enzymes has been considered by a number of workers. On the basis of their observation that AA was not excreted in the urine of animals treated with porphyrinogenic drugs, de Matteis and Rimington (1962) proposed that a balance between the amount of glycine available for metabolism either to AA or ALA might be the factor controlling the synthesis of porphyrins and haem; furthermore, they suggested that in the hepatic porphyrias, either hereditary or drug induced, glycine might be preferentially metabolised to ALA. However, Tschudy et al. (1963) reported that both non-porphyric and porphyric human subjects excreted AA in the urine; nevertheless, these workers found that the administration of glycine to a patient suffering from acute intermittent porphyria resulted in a four-fold increase in the amount of AA excreted.

The question has also been examined as to whether AA synthetase activity in liver is affected either in acute intermittent porphyria (Tschudy et al., 1965) or by porphyrinogenic drugs (Granick, 1966; Marver, Schmid and Schaltzel, 1968), but no evidence has been obtained to indicate that this might be so.

As already mentioned (p. 72), during incubation for ALA synthetase assay of porphyric liver preparations some AA

is also formed and while methods have been developed whereby AA and ALA formed during such incubations may be separately determined, little work has been done on AA synthetase itself. The opportunity to study AA synthetase in partially purified preparations obtained from the livers of normal and porphyric animals presented itself and this chapter reports the results of these investigations.

1. RESULTS

A. AA Synthetase in Normal and DDC Liver Mitochondria

Although no evidence has been found to show that there is induction of AA synthetase activity by drugs which cause increased levels of ALA synthetase in liver (Granick, 1966), it seemed worthwhile to re-investigate this question since the level of AA synthetase activity in DDC liver mitochondria is particularly high when measured using the acetyl phosphate-phosphotransacetylase system of Walsh and Elliott (1969).

Guinea pigs were therefore treated with the drug, killed at various intervals of time after dosing and liver mitochondria isolated. (All mitochondrial preparations used in this work were once frozen and thawed.) AA synthetase

activity of these preparations was determined, together with that of a preparation of mitochondria isolated from the livers of normal guinea pigs. In addition, samples of each of these preparations were ultrasonicated and the AA synthetase activity in the high speed supernatant fractions measured. Table 9.1 shows that on treatment with DDC there is an apparent fall in AA synthetase activity (with the exception of the single value at 18 hours). As with ALA synthetase (p. 133), AA synthetase was completely solubilised by the ultrasonication treatment. While AA synthetase levels decreased, ALA synthetase activity in each of the above preparations increased directly according to the time between dosing and killing (up to 24 hours). These results are also shown in Table 9.1; as expected negligible ALA synthetase activity was detected in comparable preparations of normal liver. The results presented in Table 9.1 have since been confirmed (M.J. Whiting, R.L. Walsh and W.H. Elliott, personal communication). The significance, if any, of this reduction in AA synthetase activity on DDC treatment is obscure.

TABLE 9.1

LEVELS OF AA AND ALA SYNTHETASES IN THE LIVERS OF GUINEA PIGS KILLED AT VARIOUS TIME INTERVALS AFTER DOSING WITH DDC

Guinea pigs, dosed with DDC, were killed at various intervals of time (6 to 24 hours) after dosing. Liver mitochondria were isolated and resuspended in 0.25M sucrose containing 10^{-2}M pyridoxal 5-phosphate (pH 7.4) (added to stabilise ALA synthetase). Mitochondria isolated from the livers of untreated guinea pigs served as a control. Each suspension contained mitochondria isolated from pooled livers of 10 guinea pigs. In addition, samples of each suspension were subjected to ultrasonification for 15 seconds and centrifuged at 100,000 x g for 60 minutes (see Chapter 7, p 137). AA synthetase activity was determined as described in Fig. 7.5 and ALA synthetase activity by the colorimetric assay method described in Chapter 4 (p. 93). Values are given as mμmoles of AA or ALA produced per mg of protein.

Time of killing after dosing with DDC (hours)	Mitochondria		Supernatant fraction	
	AA	ALA	AA	ALA
Control (not dosed)	255	0.1	321	0.2
6	213	4.0	250	8.8
14	183	9.1	255	16.5
18	212	12.0	297	22.0
24	156	16.2	230	27.5

It was previously shown (Fig. 7.5) that two peaks of AA synthetase activity are recovered from Sephadex gel filtration, one of which occupies the ALA synthetase position. (Since the latter is present in normal liver, it cannot represent an activity of ALA synthetase on acetyl CoA.) It was of interest to see whether any significant change occurred in these peaks on DDC treatment. Soluble extracts of normal and DDC liver mitochondria were fractionated with ammonium sulphate (see Chapter 7, p. 137) and samples fractionated on Sephadex columns by the procedure described in Fig. 7.3. It can be seen in Fig. 9.1 that irrespective of the time of DDC treatment, both peaks of AA synthetase were present. The large variations in amounts of activity are not necessarily of significance here since loss of activity due to instability or variable efficiency of ammonium sulphate precipitation has not been excluded. For comparison the changes in ALA synthetase are shown in Fig. 9.2.

B. Properties of Purified AA Synthetase

(a) The effect of glycine concentration on AA synthetase activity

The opportunity was taken to examine the glycine requirement of AA synthetase purified from normal and DDC liver mitochondria (guinea pigs killed 24 hours after dosing) since it was conceivable that the properties of the enzyme

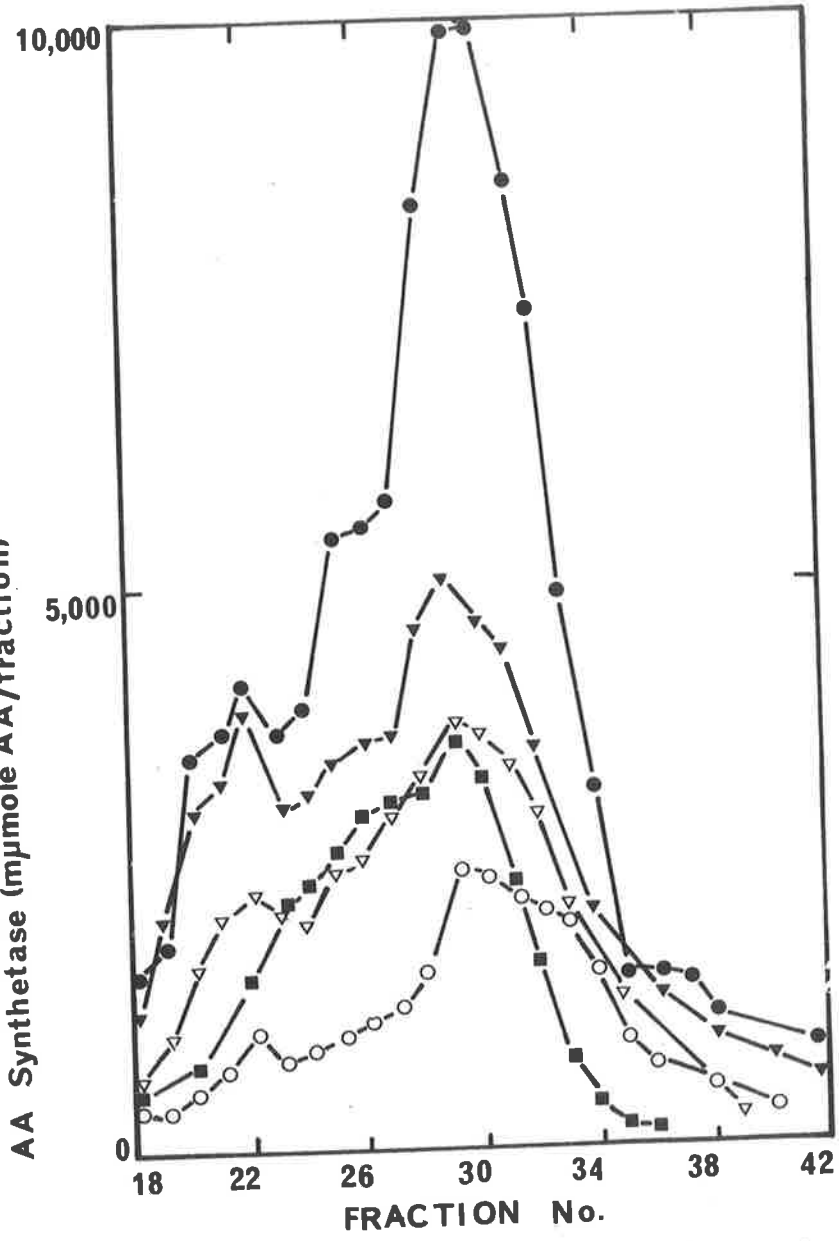


Fig. 9.1. The effect of DOC treatment of guinea pigs on the levels of AA synthetase activity in Sephadex G-100 eluate fractions. DOC treatment of guinea pigs, isolation of liver mitochondria and preparation of soluble extracts were as in Table 9.1. Fractionation procedures were as described in the text and AA synthetase activity of Sephadex eluate fractions was determined by the method given in Table 9.1. ●—● , Guinea pigs not treated; ■—■ , guinea pigs killed 6 hours after dosing; ○—○ , guinea pigs killed 14 hours after dosing; △—△ , guinea pigs killed 18 hours after dosing; ▲—▲ , guinea pigs killed 24 hours after dosing.

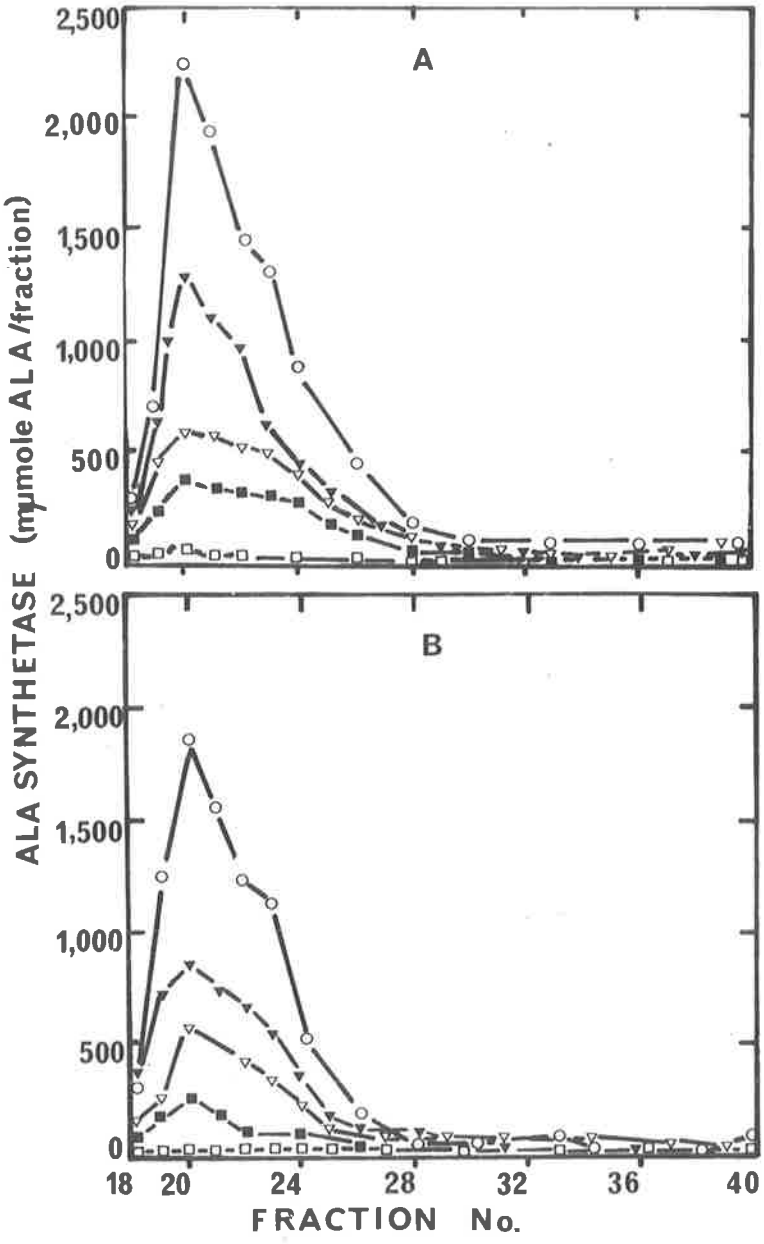


Fig. 9.2. The effect of the duration of DDC treatment of guinea pigs on the levels of ALA synthetase activity in Sephadex G-100 eluate fractions. Procedures were as described in Fig. 9.1. ALA synthetase activity of the Sephadex eluate fractions was determined by the colorimetric and radiochemical assay methods as described in Fig. 7.4. A. Colorimetric assay. B. Radiochemical assay. □—□, guinea pigs not dosed; ■—■, guinea pigs killed 6 hours after dosing; △—△, guinea pigs killed 12 hours after dosing; ▲—▲, guinea pigs killed 18 hours after dosing; ○—○, guinea pigs killed 24 hours after dosing.

might have been affected by the drug treatment. The Sephadex eluate fractions used were those at the centre of both peaks of AA synthetase activity (numbers 22 and 29, see Fig. 9.1). The effect of glycine concentration on AA synthetase activity is shown in Fig. 9.3, the only feature of note being the different shape of the curve for AA synthetase activity in fraction 22 of the preparation from DDC liver mitochondria.

(b) The effect of glycine concentration on haem inhibition of AA synthesis

During the investigation of the effect of haem on ALA synthesis by DDC liver mitochondria it was found that in this system AA synthesis was also inhibited (see p.124). The effect of haem on AA synthesis by the enzyme purified from normal and DDC liver mitochondria was therefore examined. Preliminary experiments indicated that AA synthesis was inhibited by haem. However, since AA synthesis in this system was dependent on the activity of the E. coli phosphotransacetylase also included, it was possible that the inhibitory effect of haem on AA synthesis could have been the result of an inhibition of phosphotransacetylase activity. However, up to a concentration of 0.2 mM haem did not affect the activity

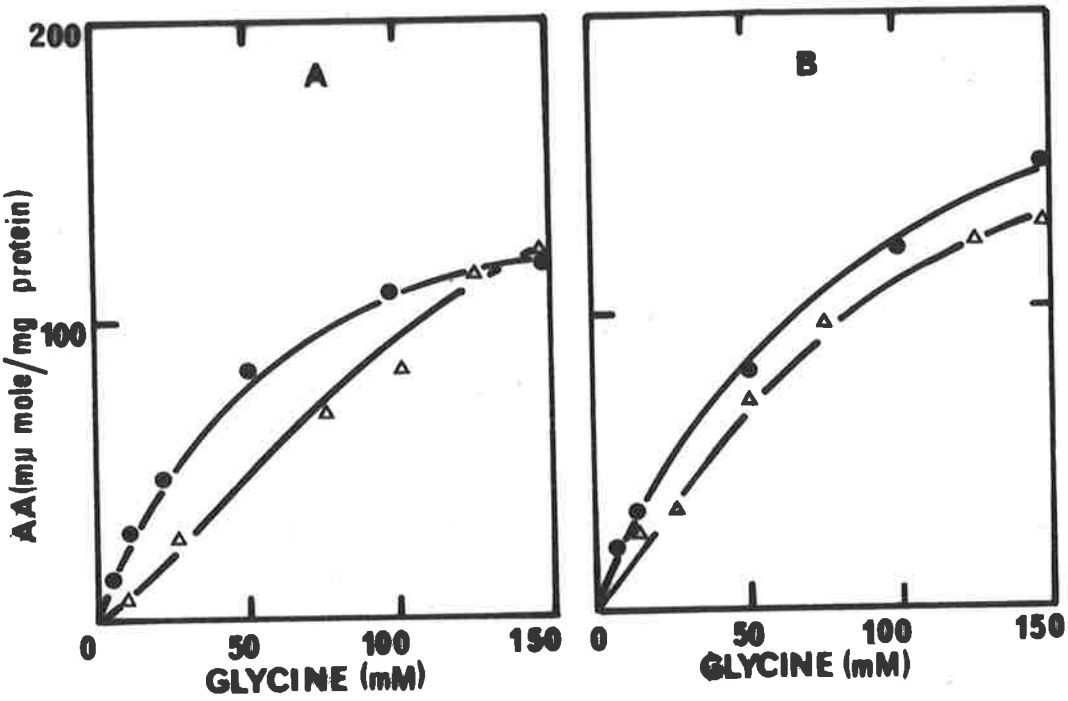


Fig. 9.3. The effect of glycine concentration on AA synthetase activity in purified preparations of the liver enzyme. The enzyme preparations used were Sephadex eluate fractions (numbers 22 and 29); the positions of these fractions in the elution profiles are shown in Fig. 9.2. Incubation conditions and method of assay were as described in Fig. 9.1 except that the glycine concentration was varied. A. Fraction 22. B. Fraction 29. ●—● , AA synthetase purified from normal liver mitochondria; △—△ , AA synthetase purified from DDC liver mitochondria (guinea pigs killed 24 hours after dosing).

of preparations of the enzyme used in this work; at higher concentrations of haem (0.4mM), activity was inhibited by approximately 10%.

The effect of glycine concentration on the haem inhibition of AA synthesis was investigated using the purified AA synthetase preparations described in Fig. 9.3. The results obtained with the enzyme purified from normal liver mitochondria are shown in Fig. 9.4 and as seen AA synthesis is inhibited by approximately 50% by 0.1mM haem. The Lineweaver-Burk plot (Fig. 9.5) of this data shows that the inhibition is non-competitive with glycine; similar results were obtained when the enzyme was purified from DDC liver mitochondria.

2. DISCUSSION

The results in this chapter clearly confirm AA synthetase as an enzyme distinct from ALA synthetase, a fact established by Urata and Granick (1963). The most striking feature of the work in this chapter was the presence of a small second peak in the same position as ALA synthetase in eluates from Sephadex G-100. However, this second peak occurred in gel filtrates of preparations from normal liver

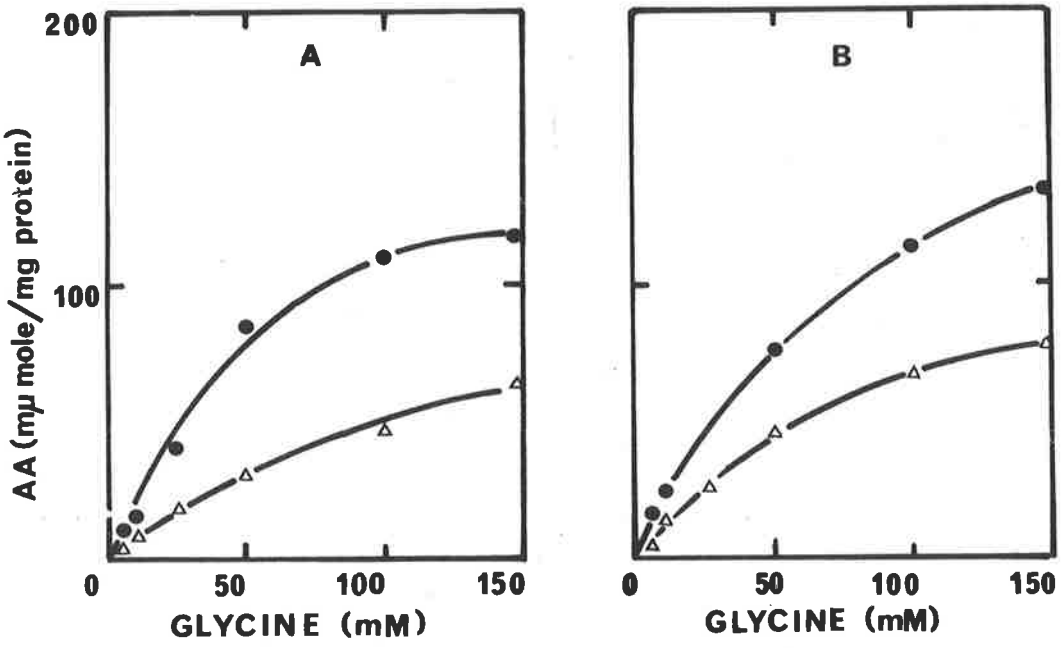


Fig. 9.4. The effect of glycine concentration on haem inhibition of AA synthetase activity.
The enzyme preparations used were Sephadex eluate fractions (numbers 22 and 29) obtained from processing normal liver mitochondria as described in the text. Incubation conditions and method of assay were as described in Fig. 9.3. A. Fraction 22. B. Fraction 29. ●—● , no haem present; △—△ , 0.1mM haem present.

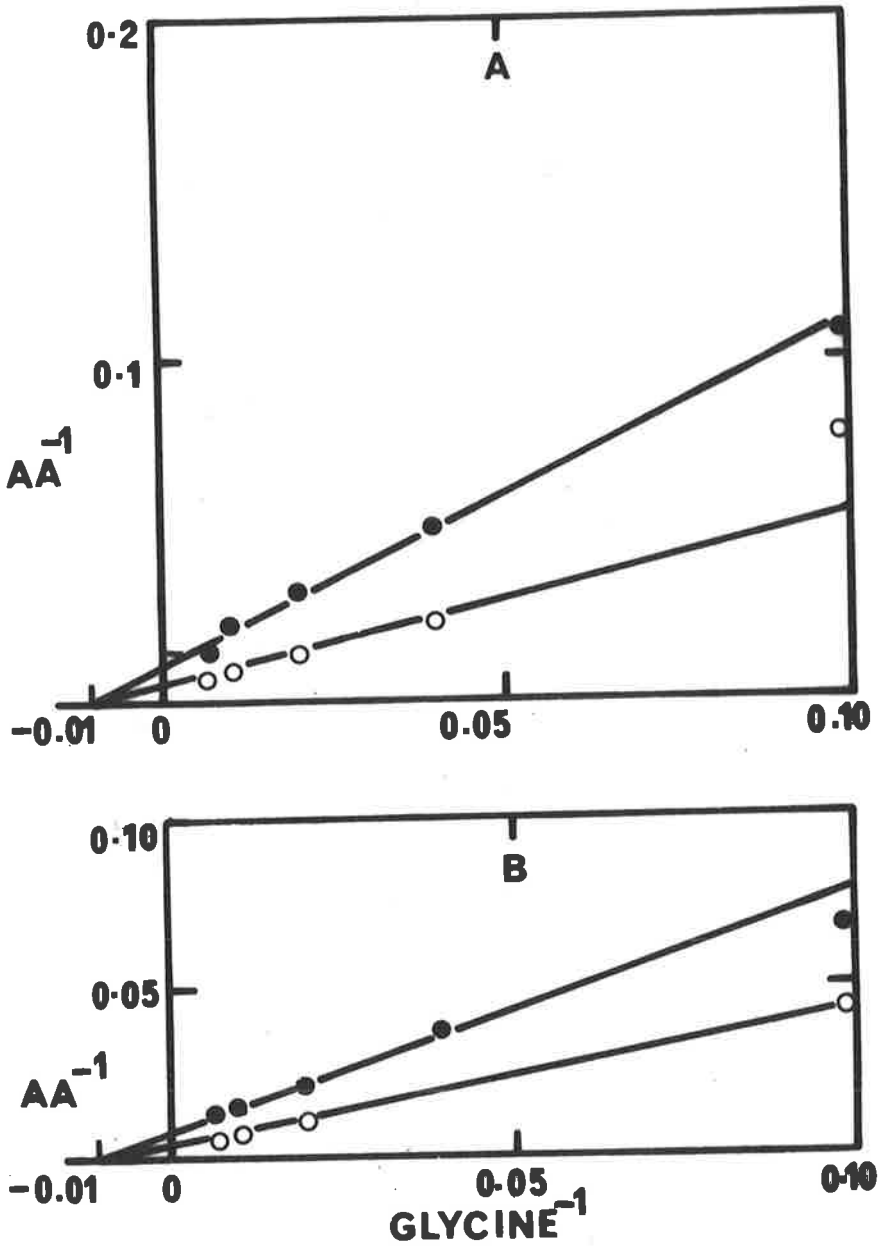


Fig. 2.5. Lineweaver-Burk plot of data in Fig. 2.4. A. Fraction 22. B. Fraction 29. ○—○ , no haem; ●—● , 0.1mM haem.

mitochondria in which ALA synthetase activity could not be detected.

AA synthetase was inhibited by haem, much as was ALA synthetase (see Chapter 6, p.160). There is no obvious reason why this should be unless haem happens to act at some common structural feature of both enzymes, but does not have physiological significance.

The similarity of the AA and ALA synthetase reactions, together with the fact that both systems are inhibited by haem, tends to make the possibility of some physiological relationship between the two present itself. (The requirement for protein synthesis in ALA synthetase induction obviously eliminates any simple interconversion.) While modification of one activity into another is an interesting and attractive idea for a system of such widely fluctuating activity as the ALA synthetase one, it is clear that there is no significant basis of evidence for considering this possibility seriously. Obviously, much more work needs to be done on both ALA and AA synthetases to understand their nature.

CHAPTER 10
GENERAL DISCUSSION

The work described in this thesis was a study of ALA synthetase in different biological systems. It was necessary to develop a more sensitive assay method other than the colorimetric methods commonly in use and to obtain the enzyme in a more purified state. In doing this a number of interesting and possibly valuable lines for future work were indicated and preliminary studies of these are also included in the thesis.

The main achievement of the work in this thesis has been the development of a new method of assay for ALA synthetase which permits experiments of a new type to be carried out, such as the quantitative measurement of ALA synthetase levels in tissue culture systems. It is hoped that this will enable more detailed information to be obtained, particularly of the early stages of enzyme induction which in turn may lead to a better understanding of this process. Since the completion of the work in this thesis further experiments in this laboratory by other workers suggest that the new assay method can be successfully applied to such studies (A.E. Edwards and W.H. Elliott, personal communication).

The finding that ALA synthetase is virtually undetectable in all normal cells studied (photosynthetic bacteria and reticulocytes excluded) simply underlines the remarkable control of this enzyme. The negative findings in normal liver do conflict with those of other workers who have detected ALA synthetase activity by the colorimetric procedures. The conflicting results may be due to two factors. Firstly, the "normal" level may fluctuate according to species, strain, physiological state, sex and age. Secondly, some of the reported traces of activity in normal liver could be due to failure to correct completely for the relatively large amount of AA produced by normal tissues. The discovery of an "inhibitor" in liver mitochondria is consistent with the failure to detect ALA synthetase activity in normal liver preparations. Whether or not this "inhibitor" has physiological significance is not known.

The enzyme purification studies have proved disappointing in that even with pyridoxal 5-phosphate stabilisation, the enzyme is very unstable in partially purified preparations. To obtain any significant amount

of enzyme, drug induction must be used, at least in the guinea pig. Whether other species are preferable as sources of the enzyme is not known, nor whether the stability of the enzyme may vary according to species. The recent work of Hayashi, Yoda and Kikuchi (1969) does indicate however, that the properties of ALA synthetase induced in rat liver by ALA may be different from those found in the present work for ALA synthetase induced in guinea pig liver by DDC.

While the main emphasis in the present work related to ALA synthetase, reference may be made to two points of particular interest concerning another enzyme, AA synthetase. One was the finding that two peaks of AA synthetase activity are eluted from Sephadex G-100, and the other that the level of AA synthetase activity in liver is reduced by treatment of animals with a drug capable of inducing the synthesis of ALA synthetase.

REFERENCES

Abbott, L.D. and Rudolph, S. (1961). Fed. Proc. 20: 376.

Ackner, B., Cooper, J.E., Kelly, M. and Nicholson, D.C. (1961). Lancet, 1: 1256.

Adamson, S.D., Herbert, E. and Godchaux, W. (1968). Arch. Biochem. Biophys. 125: 671.

Adamson, S.D., Herbert, E. and Kemp, S.F. (1969). J. Mol. Biol. 42: 247.

Arnon, D.I. (1949). Plant Physiol. 24: 1.

Avison, A.W.D. (1955). J. Chem. Soc. 1: 732.

Bertles, J.F. and Beck, W.B. (1962). J. Biol. Chem. 237: 3770.

Boardman, N.K. and Anderson, J.M. (1964). Aust. J. Biol. Sci. 17: 86.

Borst, P. (1966). Regulation of Metabolic Processes in Mitochondria, BBA Library, 7: 454, Elsevier Publishing Co. Amsterdam, London, N.Y.

Bottomley, S.S. and Smithee, G.A. (1968). Biochim. Biophys. Acta, 152: 27.

Bruns, G.P. and London, I.M. (1965). Biochem. Biophys. Res. Commun. 18: 236.

Bull, M.J. and Lascelles, J. (1963). Biochem. J. 87: 15.

Burnham, B.F. and Lascelles, J. (1963). Biochem. J. 87: 462.

Cohen-Bazire, G., Sistrom, W.R. and Stanier, R.Y. (1957). J. Cell. Comp. Physiol. 49: 25.

de Matteis, F., Prior, B.E. and Rimington, C. (1961). Nature, 191: 363.

de Matteis, F. and Prior, B. (1962). Biochem. J. 83: 1.

de Matteis, F. and Rimington, C. (1962). Lancet, 1: 1332.

de Matteis, F. and Rimington, C. (1963). J. Dermatol. 75: 91.

de Matteis, F. (1967). Pharmacol. Rev. 19: 523.

Dent, C.E. (1948). Biochem. J. 43: 169.

Donald, G.F., Hunter, G.A., Rowan, W. and Taylor, A.E.J. (1969). Brit. J. Dermatol. In press.

Dowdle, E.B., Mustard, P., Eales, L. and Schuur, G. (1967). Quoted by Neuberger, A. (1968). Proc. Roy. Soc. Med. 61: 191.

Elliott, W.H. (1959). Nature, 183: 1051.

Elliott, W.H. (1960a). Biochem. J. 74: 90.

Elliott, W.H. (1960b). Biochem. J. 74: 478.

Ephrussi, B. and Slonimski, P.P. (1950). Biochim. Biophys. Acta, 6: 256.

Feigelson, P. and Greengard, O. (1962). J. Biol. Chem. 237, 1908.

Firkin, F. and Linnane, A.W. (1968). Proc. Aust. Biochem. Soc. p.95.

Gajdos, A. and Gajdos-Török, M. (1963). Bull. Soc. Chim. Biol. 45: 1227.

Gajdos, A. and Gajdos-Török, M. (1965). Nature, 207, 640.

Gajdos, A., Gajdos-Török, M., Palma-Carlos, A. and Palma-Carlos, M.L. (1966). Bull. Soc. Chim. Biol. 48: 803.

Gajdos, A. (1966). Acta Medica Scandinavica Suppl. 445.

Gajdos, A., Plainfosse, B. and Scringe, P. (1967). La Presse Medicale, 75: 545.

Gajdos, A., Gajdos-Török, M., Palma-Carlos, A. and Palma-Carlos, L. (1967). Nature, 213, 1022.

Gajdos, A. and Gajdos-Török, M. (1968). Biochem. Symposia 28: 45, Ed. T.W. Goodwin, Academic Press, London.

Gassman, M., Pluscec, J., Bogorad, L. (1968). Plant Physiol. 43, 1411.

Gibson, K.D. (1955). Ciba Foundation Congr. Porphyrin Biosynthesis and Metabolism, Ed. G.E.W. Wolstenholme and E.C.P. Millar, J. & A. Churchill Ltd., London, p.27.

Gibson, K.D. (1958). Biochim. Biophys. Acta, 28: 451.

Gibson, K.D., Laver, W.G. and Neuberger, A. (1958a). Fed. Proc. 17: 228.

Gibson, K.D., Laver, W.G., and Neuberger, A. (1958b). Biochem. J. 70, 71.

Gibson, K.D., Matthew, M., Neuberger, A. and Tait, G.H. (1961). Nature, 192: 204.

Gibson, K.D., Neuberger, A. and Tait, G.H. (1962a). Biochem. J. 83: 539.

Gibson, K.D., Neuberger, A.and Tait, G.H. (1962b). Biochem. J. 83: 550.

Glowacki, E.R. and Millette, R.L. (1965). J. Mol. Biol. 11: 116.

Goldberg, A. (1954). Biochem. J. 57: 55.

Goldberg, A. (1959). Quart. J. Med. 28: 183.

Goldberg, A. and Rimington, C. (1955). Proc. Roy. Soc. B, 143: 257.

Goldberg, A. and Rimington, C. (1962). Diseases of Porphyrin Metabolism, Springfield, Ill. Charles C. Thomas Coy. pp.8-9.

Goldberg, A. (1968). Proc. Roy. Soc. Med. 61: 193.

Goto, K., Higuchi, M., Sakai, H. and Kikuchi, G. (1967). J. Biochem. 61: 186.

Granick, S. and Vanden Schriek, H.G. (1955). Proc. Soc. Exptl. Biol. and Med. N.Y. 88: 270.

Granick, S. (1961). Plant Physiol. 33: xlviii.

Granick, S. and Urata, G. (1963). J. Biol. Chem. 238: 821.

Granick, S. (1963a). Fed. Proc. 22: 410.

Granick, S. (1963b). J. Biol. Chem. 238: PC 2247.

Granick, S. and Levere, R.D. (1964). Progr. in Haematol. 4: 1.

Granick, S. (1964). J. Am. Med. Assn. 190: 475.

Granick, S. (1965). Ann. N.Y. Acad. Sci. 123: 188.

Granick, S. (1966). J. Biol. Chem. 241: 1359.

Granick, S. and Gibor, A. (1967). Progr. Nucleic Acid Res. Mol. Biol. 6: 143.

Granick, S. and Kappas, A. (1967a). Proc. Nat. Acad. Sci. 57: 1463.

Granick, S. and Kappas, A. (1967b). J. Biol. Chem. 242: 4587.

Grayzel, A.I., Horchmer, P. and London, I.M. (1966). Proc. Nat. Acad. Sci. 55: 650.

Green, M. and Elliott, W.H. (1964). Biochem. J. 92: 537.

Halvorson, H.O. (1960). Advan. Enzymol. 22: 99.

Hayashi, N., Yoda, B., Kikuchi, G. (1968). J. Biochem. 63: 446.

Hayashi, N., Yoda, B., Kikuchi, G. (1969). Arch. Biochem. Biophys. 131: 83.

Hutner, S.H., Bach, M.K., Ross, G.I.M. (1956). J. Protozool. 3: 1011.

Higuchi, M., Goto, K., Fujimoto, M., Namiki, C. and Kikuchi, G. (1965). Biochim. Biophys. Acta, 95: 94.

Higuchi, M., Ohba, T., Sakai, H., Kurashima, Y. and Kikuchi, G. (1968). J. Biochem. 64: 795.

Irving, E.A. and Elliott, W.H. (1969). J. Biol. Chem. 244: 60.

Kabat, D. and Attardi, G. (1967). Biochim. Biophys. Acta, 138, 382.

Kappas, A. and Granick, S. (1968). J. Biol. Chem. 243: 346.

Kappas, A., Song, C.S., Levere, R.D., Sachson, R.A. and Granick, S. (1968). Proc. Nat. Acad. Sci. 61: 509.

Kariban, D. and London, I.M. (1965). Biochem. Biophys. Res. Commun. 18: 243.

Kaufman, S. (1955). Methods in Enzymol. 1: 719.

Kikuchi, G., Kumar, A., Talmage, P. and Shemin, D. (1958). J. Biol. Chem. 233: 214.

Kikuchi, G., Kumar, A. and Shemin, D. (1959). Fed. Proc. 18: 259.

Labbe, R.F., Kurumada, T. and Onisawa, J. (1965). Biochim. Biophys. Acta, 111: 403.

Labbe, R.F. (1967). Lancet, 1: 1362.

Lang, P.A., Levine, H., Jones, C.C., Mills, G.C. (1968). Texas Reports on Biol. and Med. 26: 525.

Lascelles, J. (1955). Ciba Foundation Congr. Porphyrin Biosynthesis and Metabolism. Ed. G.E.W. Wolstenholme and E.C.P. Millar, J. & A. Churchill Ltd., London, p.265.

Lascelles, J. (1956). Biochem. J. 62: 78.

Lascelles, J. (1959). Biochem. J. 72: 508.

Lascelles, J. (1960). J. Gen. Microbiol. 23: 487.

Lascelles, J. (1964). Tetrapyrrole Biosynthesis and its Regulation, W.A. Benjamin, Inc. N.Y. and Amsterdam.

Lascelles, J. (1966). Biochem. J. 100: 18A.

Laver, W.G., Neuberger, A. and Udenfriend, S. (1958). Biochem. J. 70: 4.

Levere, R.D. and Granick, S. (1965). Proc. Nat. Acad. Sci. 54: 134.

Levere, R.D. and Granick, S. (1967). J. Biol. Chem. 242: 1903.

Lewis, M., Lee, G.R., Cartwright, G.E. and Wintrobe, M.M. (1967). Biochem. Biophys. Acta, 141: 296.

Limmane, A.W., Vitols, E., and Newland, P.G. (1962). J. Cell. Biol. 13: 285.

London, I.M., Drums, G.P. and Kariban, D. (1964). Medicine, 43: 789.

Lottsfeldt, F.I., Labbe, R.F. and Aldrich, R.A. (1961). J. Am. Med. Assn. 178: 928.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193: 265.

Marriott, J., Neuberger, A. and Tait, G.H. (1969). Biochem. J. 111: 385.

Marver, H., Forloth, M., Tschudy, D.P. and Collins, A. (1965). Fed. Proc. 24: 721.

Marver, H.S., Tschudy, D.P., Perloth, M.G., Collins, A. and Hunter, G. (1966a). Anal. Biochem. 14: 53.

Marver, H.S., Tschudy, D.P., Perloth, M.G. and Collins, A. (1966b). J. Biol. Chem. 241: 2803.

Marver, H.S., Collins, A., Tschudy, D.P. and Rechcigl, M. (1966c). J. Biol. Chem. 241: 4323.

Marver, H.S., Tschudy, D.P., Perloth, M.G. and Collins, A. (1966d). Science, 154: 501.

Marver, H.S., Collins, A., Tschudy, D.P. (1966). Biochem. J. 99: 31C.

Marver, H.S., Schmid, R. and Schlitzel, M. (1968). Biochem. Biophys. Res. Commun. 33: 969.

Matsuoka, T., Yoda, B. and Kikuchi, G. (1968). Arch. Biochem. Biophys. 126: 530.

Mauzerall, D. and Granick, S. (1956). J. Biol. Chem. 219: 435.

Merchante, A., Wajchenberg, B.L. and Schwartz, S. (1957). Proc. Soc. Exptl. Biol. and Med. 95: 221.

Milner, H.W., Lawrence, N.S. and French, C.S. (1950). Science, 111: 633.

Monod, J., Changeux, J. and Jacob, F. (1963). J. Mol. Biol. 6: 306.

Moyed, H.S. and Umbarger, H.E. (1962). Physiol. Rev. 42: 444.

Nakao, W., Wada, O., Kitamura, T., Uono, K. and Urata, G. (1966). Nature, 210: 838.

Narisawa, K. and Kikuchi, G. (1966). Biochim. Biophys. Acta, 123, 596.

Neuberger, A. (1961). Biochem. J. 78: 1.

Neuberger, A. (1968). Proc. Roy. Soc. Med. 61: 191.

Nishimura, M. and Chance, B. (1963). Biochim. Biophys. Acta, 66: 1.

Ockner, R.K. and Schmid, R. (1961). Nature, 189: 499.

Onisawa, J. and Labbe, R.F. (1963). J. Biol. Chem. 238: 724.

Perloth, M.G., Tschudy, D.P., Marver, H.S., Berard, C.W., Ziegel, R.F., Rechcigl, M. and Collins, A. (1966). Am. J. Med. 41: 149.

Peters, H.A., Woods, S., Eichman, P.L. and Reese, H.H. (1957). Ann. Int. Med. 47: 889.

Peters, H.A., Eichman, P.L. and Reese, H.H. (1958). Neurology, 8, 621.

Peters, H. (1961). Fed. Proc. 20: Suppl. 10.

Peters, H.A., Johnson, S.A.M., Cam, S., Oral, S. Müftü, Y. and Ergene, T. (1966). Am. J. Med. Sci. 251: 104.

Petres, J., Schröder, K., Consbruch, U., Ladner, H.A. (1968). Germ. Med. Monthly, 13: 73.

Porra, R.J. and Jones, O.T.G. (1963). Biochem. J. 87: 181.

Porra, R.J. and Lascelles, J. (1965). Biochem. J. 94: 120.

Porra, R.J. and Lascelles, J. (1968). Biochem. J. 108: 343.

Ramsley, R.F., Bridger, W.A., Moyer, R.W., Boyer, P.D. (1967). J. Biol. Chem. 242: 4287.

Rechcigl, M. and Heston, W.E. (1967). Biochem. Biophys. Res. Commun. 27: 119.

Roman, W. (1967). Enzymologia, 32: 37.

Rowley, P.T. and Morris, J.A. (1967). J. Biol. Chem. 242: 1533.

Rubenstein, D., Ottolenghi, P. and Denstedt, O.F. (1956). Canad. J. Biochem. and Physiol. 34: 222.

Schmid, R. and Schwartz, S. (1952). Proc. Soc. Exptl. Biol. and Med. 81: 685.

Schmid, R., Figen, J.F. and Schwartz, S. (1955). J. Biol. Chem. 217: 263.

Schmid, R. (1960). New Eng. J. Med. 263: 397.

Schmid, R. (1966). The Metabolic Basis of Inherited Disease, Ed. J.B. Stanbury, J.B. Wyngarden and J.S. Frederickson, McGraw Hill, New York, pp.813-870.

Schneck, D.W., Racz, W.J., Hirsch, G.H., Bubbar, G.L. and Marks, G.S. (1968). Biochem. Pharmacol. 17: 1385.

Schneider, W.C. and Hogeboom, G.H. (1950). J. Biol. Chem. 183: 123.

Schriefers, H., Keck, B. and Otto, N. (1965). Acta Endocrinol. 50: 25.

Schwartz, S. and Watson, C.J. (1957). Proc. Soc. Exptl. Biol. and Med. 95: 216.

Shanley, B.C., Zail, S.S., and Joubert, S.M. (1968). Lancet, 1: 70.

Shemin, D. and Wittenberg, J. (1951). J. Biol. Chem. 192: 315.

Shemin, D. and Kumin, S. (1952). J. Biol. Chem. 198: 827.

Shemin, D. and Russell, C.S. (1953). J. Am. Chem. Soc. 75, 4873.

Shemin, D., Abramsky, T. and Russell, C.S. (1954). J. Am. Chem. Soc. 76: 1204.

Shemin, D., Russell, C.S. and Abramsky, T. (1955). J. Biol. Chem. 215: 613.

Shemin, D., Kikuchi, G. and Bachman, B.J. (1958). Fed. Proc. 17: 310.

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

Simpson, C.F. and Kling, J.M. (1968). J. Cell. Biol. 36: 103.

Solomon, H.M. and Figge, F.H. (1959). Proc. Soc. Exptl. Biol. and Med. 100: 583.

Solomon, H.M. and Figge, F.H. (1960). Proc. Soc. Exp. Biol. and Med. 105: 484.

Spencer, D. and Possingham, J.V. (1960). Aust. J. Biol. Sci. 13: 441.

Spencer, D. and Wildman, S.G. (1964). Biochemistry, 3: 954.

Stadtman, E.R. (1955). Methods in Enzymol. 1: 596.

Stadtman, E.R. (1965). Advances in Enzymol. 28: 41.

Sutherland, E.W. and Wasilait, W.D. (1956). J. Biol. Chem. 218: 459.

Tschudy, D.P., Rose, J., Hellman, E., Collins, and Rechcigl, M. (1962). Metabolism, 11, 1287.

Tschudy, D.P., Welland, F.H., Collins, A. and Hunter, G. (1963). Lancet, 2: 660.

Tschudy, D.P., Welland, F.H., Collins, A. and Hunter, G. (1964). Metabolism, 13: 396.

Tschudy, D.P. (1965). J. Am. Med. Assn. 191: 718.

Tschudy, D.P., Perloth, N.G., Marver, H.S., Collins, A. and Rechcigl, B. (1965). Proc. Nat. Acad. Sci. 53: 841.

Tschudy, D.P., Marver, H.S. and Collins, A. (1965). Biochem. Biophys. Res. Commun. 21: 480.

Tschudy, D.P., Waxman, A. and Collins, A. (1967). Proc. Nat. Acad. Sci. 58: 1944.

Tsui, C. (1948). Amer. J. Bot. 35: 172.

Urata, G. and Granick, S. (1963). J. Biol. Chem. 238: 811.

Wada, O., Yano, Y., Urata, G. and Nakao, K. (1968). Biochem. Pharmacol. 17: 595.

Wainwright, S.D. and Wainwright, L.K. (1967). Canad. J. Biochem. 45: 344.

Waldenström, J. and Haeger-Aronsen, B. (1967). Progr. in Med. Genetics, 5: 58.

Walsh, R.L. and Elliott, W.H. (1969). Proc. Aust. Biochem.

Watson, C.J. (1960). New Eng. J. Med. 263: 1205.

Watson, C.J., Runge, W., Tuddeini, L., Bossenmaier, I. and Cardinal, R. (1964). Proc. Nat. Acad. Sci. 52: 478.

Waxman, A.D., Collins, A. and Tschudy, D.P. (1966). Biochem. Biophys. Res. Commun. 24: 675.

Welland, F.H., Hellman, L.S., Gaddis, E.M., Collins, A., Hunter, G.W. and Tschudy, D.P. (1964a). Metabolism, 13: 232.

Welland, F.H., Hellman, L.S., Collins, A., Hunter, G. and Tschudy, D.P. (1964b). Metabolism, 13: 251.

Wintersberger, E. (1966). Regulation of Metabolic Processes in Mitochondria, BBA Library 7: 439, Elsevier Publishing Co., Amsterdam, London, N.Y.

Woods, S.M., Peters, H.A., and Johnson, S.A.M. (1961). Arch. Dermatol. 84: 82.

Zucker, W.V., Schulman, H.M. (1968). Proc. Nat. Acad. Sci. 59, 582.

Zuyderhoodt, F.M.J., Borst, P., Huijing, F. (1969). Biochim. Biophys. Acta, 178, 408.

APPENDIX I.

After this thesis was prepared for submission it was noted that Lascelles and Hatch (1969) now consider that previous results (Lascelles, 1960; Goto *et al.*, 1967) indicating haem repression of ALA synthetase production in *R. sphaeroides* were due to contamination of enzyme preparation with haem and consequent inhibition of enzyme activity during assays. While it is still thought that haem does act as a corepressor, the evidence for this is rather more indirect and is based on studies with mutant strains of the bacterium (Lascelles and Hatch, 1969).

Furthermore, ALA itself had no repressing effect on enzyme production; when its conversion to haem was hindered either by mutation or methionine, enzyme production was not inhibited.

Lascelles, J. and Hatch, T.P. (1969). J. Bacteriol. 98: 712.

APPENDIX II

This proposal is not supported by later work (Lascelles and Altshuler, 1969) in which mutant strains of R. spheroides were used; these lacked either ALA synthetase or enzymes necessary for the conversion of ALA to haem or bacteriochlorophyll.

Lascelles, J. and Altshuler, T. (1969). J. Bacteriol. 98: 721.

APPENDIX III

The activation of ALA synthetase in crude extracts of light-anaerobically grown R. spheroides cells observed by Tuboi, Kim and Kikuchi (1969) was thought to be due to inactivation of an extremely labile inhibitor of ALA synthetase activity.

Tuboi, S., Kim, H.J., Kikuchi, G. (1969). Arch. Biochem. Biophys. 130:92.

APPENDIX IV

Several relevant points of interest arise from the recent publication of Scholnick, Hammaker and Marver (1969).

(1) These workers have purified ALA synthetase from the soluble fraction of cells from the livers of ALA treated rats, thus confirming the work of Hayashi, Yoda and Kikuchi

(1969). On the basis of their studies they propose that ALA synthetase is formed on the ribosomes, passes into the soluble fraction of the cells and thence migrates to specific sites within the mitochondria.

(2) They also found that the inhibition by normal rat liver mitochondria of ALA synthesis in mitochondria isolated from the livers of ALA treated rats could be overcome by a fourfold increase in the amount of succinyl CoA synthetase included in incubations. They suggest that the inhibitory effect of normal liver mitochondria on ALA synthesis is mediated through interference with succinyl CoA generation and that the deacylase activity of normal liver mitochondria could be responsible. However, since in the present work heat or acid treated debris from normal liver mitochondria (see p. 122) is still active as an inhibitor of ALA synthesis, other explanations are possible.

(3) It was established that the activity of ALA synthetase purified from the soluble fraction of liver cells is inhibited by haem, but not by myoglobin or cytochrome C. Furthermore, the degree of inhibition caused by haem is approximately twice that obtained in this laboratory using purified preparations of the intramitochondrial

enzyme.

Scholnick, P.L., Hammaker, L.E. and Marver, H.S. (1969). Proc. Nat. Acad. Sci. 63:65.