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Heme biosynthesis in *Escherichia coli*: Regulation of 5-aminolevulinic acid synthesis and the purification and characterization of 5-ALA dehydratase

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HEME BIOSYNTHESIS IN *ESCHERICHIA COLI*: REGULATION OF 5-AMINOLEVULINIC ACID SYNTHESIS AND THE PURIFICATION AND CHARACTERIZATION OF 5-ALA DEHYDRATASE

by

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ABSTRACT

HEME BIOSYNTHESIS IN ESCHERICHIA COLI: REGULATION OF 5-AMINOLEVULINIC ACID SYNTHESIS AND THE PURIFICATION AND CHARACTERIZATION OF 5-ALA DEHYDRATASE

by

Ormond Brathwaite

Adviser: Professor Charlotte S. Russell

A. A *hemA* mutant of *E. coli*, strain JL1268, containing a multi-copy plasmid which complemented the mutation, excreted ALA into medium and accumulated uroporphyrinogen III when grown under aerobic or anaerobic conditions. Superaerobic growth inhibited ALA excretion and the cell extract failed to synthesize ALA from glutamate and ATP. The synthesis of ALA by two sonicate fractions which had been processed by gel filtration and dialysis, was dependent on glutamate, ATP, NADPH, glu-tRNA^{glu} and PLP. Glu-tRNA^{glu} stimulated ALA synthesis in a concentration-dependent manner. Pretreatment with RNase lowered this stimulation. These results confirmed that *E. coli* synthesizes ALA by the C_5 pathway from the intact five-carbon chain of glutamate.

E. coli RP523, a strain which cannot utilize ALA because there is a mutation in the structural gene for the next enzyme in the pathway, ALA D, accumulated ALA under aerobic and anaerobic growth conditions. The amount of ALA accumulated in the medium of RP523 decreased when glucose or hemin was added to the medium

and the glucose effect was reversed in strain C600 by cAMP (W. Chen, W. Xiao, C. S. Russell and S. Cosloy unpublished). RP523 had more *hemA* specific mRNA when RP523 was grown anaerobically than aerobically or superaerobically. Strain JL1268 synthesized *hemA* mRNA under all growth conditions. The glutamyl tRNA synthetase, *gltX*, mRNA is highest in RP523 grown anaerobically; and also JL1268 grown aerobically and anaerobically. These results and an analysis of the upstream region the *hemA* gene suggest that the glucose effect is positively mediated by cAMP-CRP binding to a promoter region of *hemA*. *In vivo* high expression of the *hemA* gene product may stimulate *gltX* gene expression.

B. 5-Aminolevulinic acid dehydratase (ALA D, E.C. 4.2.1.24) catalyses the fourth step of the heme biosynthetic pathway in *E. coli*. This enzyme which is over-produced in a strain of *E. coli* containing a multi-copy plasmid harboring *hemB,* was purified 1000-fold to near homogeneity by a four step procedure including ammomium sulfate precipitation, ion exchange chromatography, hydrophobic chromatography and affinity chromatography. On Sepharose 6B the native enzyme had a molecular weight of 275,000 daltons. The nearly-homogeneous enzyme was isolated by electrophoresis on a native polyacrylamide tube gel and the band corresponding to ALA D was eluted and then run on SDS-PAGE. A single band corresponding to a subunit molecular weight of 39,000 daltons was observed. This data suggest the the *E. coli* ALA D is heptameric.

ALA D was activated by Cd^{++}, Mg^{++}, and Zn^{++}, ß-mercaptoethanol and dithiothreitol . The K_m for ALA was 0.82±0.06

mM. The enzyme was inhibited by lead, sulfhydryl-directed reagents and metal ion chelators. Both 4,6-dioxoheptanoic acid (succinylacetone ,SA) and succinlyacetone-aminolelulinic acid pyrrole were competitive inhibitors with K_i of 1.38 mM and 0.04 mM respectively. Levulinic acid (LA) was a weak competitive inhibitor of the enzyme, with a K_i of 16.34 mM. The enzyme was inhibited by uroporphyrin I and III; coproporphyrin I and III; protoporphyrin IX; and the corresponding porphyrinogens.

Inhibition of ALA D by hemin was media dependent. We compared our spectra of hemin in various media to those previously reported and observed some differences. The comparison of the spectra of hemin dispersed in Tween-80 and hemin dispersed in dextran to previously reported spectra, suggested that hemin in Tween-80 is less polymeric than hemin in dextran. In Tween-80 and dextran 45 and 72% inhibition respectively of *E. coli* ALA D at 1.39 mM hemin was observed. Hemin in poly(N-vinylpyrrolindone) and polyethylene glycol inhibited ALA D by 8 and 12% respectively. The spectra of a solution of hemin arginate in propylene glycol/ethanol/water suggested that it is probably monomeric. Hemin arginate did not inhibit *E. coli* ALA D.

These results demonstrate that *E. coli* ALA D is heptameric; activated by metals; activated by thiols, inhibited by lead and mercury and inhibited by γ-keto inhibitors. These properties are similar to those for the enzyme from other bacteria and mammals. The high specific activity of *E. coli* ALA D and its sensitivity to inhibition by porphyrins and porphyrinogens make the *E. coli* enzyme different from the enzyme from other sources.

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ABBREVIATIONS

ALA	5-Aminolevulinic acid
ALA D	5-aminolevulinic acid dehydratase
ALA S	5-aminolevulinic acid synthetase
amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
ß-ME	ß-Mercaptoethanol
cAMP	cyclic adenosine monophosphate
CN-Br	cyanogen bromide
CoA	coenzyme A
copro	coproporphyrin
coprogen	coproporphyrinogen
CRP	cAMP receptor protein
DCC	3,5-dicarbethoxy-1,4-dihydrocollidine
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	dioxyribonucleic acid
DOVA	4, 5-dioxovalerate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)cardodiide HCl
EDTA	ethylenediaminetetraacetic acid
Glns	glutaminyl-tRNA synthetase
gltX	glutamyl tRNA synthetase gene
Glu	glutamate

GSA	glutamyl-tRNA reductase
HAT	2-hydroxy-3-amino-tetrahydropyran-1-one
hemA	glutamyl tRNA reductase gene
hemB	ALA D dehydratase gene
HMB	hydroxymethylbilane
IAc	iodoacetic acid
IAm	iodoacetamide
kb	kilobase
LA	levulinic acid
MMTS	methyl methanethiosulfonate
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
PAGE	ployacrylamide gel electrophoresis
PCMB	parachloromercuribenzoate
PEG	polyethylene glycol
PHMB	parahydroxmercuribenzoate
PLP	pyridoxal-5-phosphate
proto	protoporphyrin
protogen	protoporphyrinogen
PVA	poly(vinyl alcohol)
PVP	poly(N-vinylpyrrolindone)
RNA	ridonucleic acid
SA	succinylacetone
SA-ALA	succinylacetone-aminolevulinic acid
SDS	sodium deodecyl sulfate

TBE	Tris-borate-EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
tet	tetracycline
Tween	polyoxyethylene sorbitan monooleate
uro	uroporphyrin
urogen	uroporphyrinogen

Chapter
1

5-AMINOLEVULINIC ACID SYNTHESIS IN *ESCHERICHIA COLI*

1. INTRODUCTION

Porphyrins and tetrapyrroles are ubiquitous molecules in nature being found in both prokaryotic and eukaryotic organisms. The biological functions that they serve include those related to the processes of energy capture and utilization, oxygen transport and coenzyme activity of many oxidative enzymes. The cyclic tetrapyrroles include hemes, chlorophylls, corrins, chlorins and the nickel tetrapyrrole, factor F_{430} (Warren and Scott, 1990) (Fig. 1.1). The linear tetrapyrroles include the bilins, a major class of light-harvesting photosynthetic pigments of plants, algae, and cyanobacteria and also phytochromes, plant hormones. The carbon skeleton of all tetrapyrroles is derived from 5-aminolevulinic acid (ALA) and uroporphyrinogen (Urogen) III is the common biosynthetic intermediate (Castelfranco and Beale, 1983) in this pathway (Fig. 1.2). The facultative bacterium, *Escherichia coli* synthesizes cyclic tetrapyrroles as the prosthetic groups of its cytochromes, catalase, nitrite and sulfite reductase, coenzyme B (vitamin B_{12}) and hydroperoxidases (Kranz et al., 1984).

1.1 THE BIOLOGICAL FUNCTIONS OF TETRAPYRROLES

The essential role of heme in the physiology of prokaryotic and eukaryotic organisms is affirmed by its function as the prosthetic group of a variety of important hemoproteins that are vital for cellular processes. Heme aids in oxygen transport as the prosthetic group of hemoglobin and myoglobin; in the transfer of electrons as the prosthetic group of cytochromes. Hemoglobin and myoglobin function mainly in the transport of oxygen (O_2) in the circulating erythrocytes and muscle tissues respectively. Oxygen binds to the heme Fe^{2+} at the sixth coordinate position. The cytochromes, which are classified according to the type of apoprotein and its covalently bound form of heme, function mainly as electron carriers. This is the process of energy transduction in the inner mitochondrial and cytoplasmic membrane of eukaryotes and bacteria respectively. Heme also has a co-catalytic role in tryptophan pyrrolase. Heme (protoheme IX) is also involved in the enzymatic decomposition of H_2O_2 as the prosthetic group of catalase and peroxidase; inactivation of oxygen radicals as the prosthetic group of mitochondrial cytochrome P_{450}; and in the synthesis and metabolism of hormones and drugs biotransformation by microsomal cytochrome P_{450} (Tsuneo and Sato 1963a, b). Cytochrome P_{450} contains heme a with an iron that can either be +2 or +3, and can accommodate the binding of O_2 while the iron is in the +2 oxidation state (Brown et al., 1990). Cytochrome P_{450} is a monooxygenase and along with auxiliary phase I enzymes of the liver carries out its hydroxylation reactions necessary for drug activation and detoxification (Abraham et al., 1983). Hemin has been shown to be a

multi-functional regulator of cellular activity in mammals, yeasts and other fungi and plants. Several reviews are available on this subject (Abraham et al., 1989; Padmanaban et al., 1989; Rimington, 1989). It has been shown to be a regulator of protein synthesis and to control efficiency of translation of globin and apo-cytochrome synthesis (Freedman et al,. 1976; Hunt, 1976; Padmanaban et al.,1989). Heme has also been demonstrated to be required for erythroid cell development and differentiation (Ross and Sautner, 1976; Ibraham et al., 1982).

The chlorophylls and bacteriochlorophylls, magnesium containing cyclic tetrapyrroles (Fig. 1.1), serve as the light harvesting and transformation pigments of plants, algae, and photosynthetic bacteria respectively. In plants the chlorophylls are found in the thylakoid membrane, while in bacteria they are in the cytoplasmic membrane.

Vitamin B_{12} (Fig. 1.1) which contains the corrinoid core with a central cobalt atom attached, serves as the prosthetic group of several enzymes including mammalian methylmalonyl-CoA mutase and homocysteine methyltransferase. *E. coli* and *Salmonella typhimurium,* both prokaryotic, facultative anaerobes, use vitamin B_{12} (coenzyme B_{12}) as the prosthetic group of two enzymes, ethanolamine ammonia-lyase and homocysteine methyltransferase. Methylmalonyl-CoA isomerase, an important enzyme in many organisms is also a coenzyme B_{12}-dependent enzyme. Some microorganisms can synthesize their own coenzyme B_{12}, while other organisms must ingest it (Menon et al., 1967). Vitamin B_{12} is made only by micoorganisms (Jeter et al., 1987; Stryer, 1988). *S.*

typhimurium, Propionibacterium shermanii and probably *E. coli* synthesize Vitamin B_{12} only anaerobically (Elliott and Roth, 1989; Jeter et al., 1984; Jeter et al., 1987; Menon et al., 1967). It was demonstrated that both ALA S and ALA D activities are reduced concomitantly with vitamin B_{12} synthesis in *P. shermanii* grown aerobically (Menon et al., 1967). This suggest that oxygen regulates these two enzymes of heme biosynthesis in this bacteria.

Plants, *E. coli, S. typhimurium*, and many prokaryotic photosynthetic species contain siroheme (Fig. 1.1) as the prosthetic group of nitrite and sulfite reductase, enzymes involved in biosynthesis of cysteine from inorganic sulfate and nitrate respectively (Zumft, 1972; Murphy et al., 1974; Ingledew et al., 1984; Elliott et al., 1989).

The methanogenic bacteria contain the Nickel-tetrapyrrole cofactor, Factor F_{430} (Fig. 1.1), as the prosthetic group of methyl-S-coenzyme M reductase which catalyzes the ultimate methane-yielding reaction in these bacteria (Wackett et al., 1987, Warren and Scott, 1990).

1.2 THE ENZYMES OF HEME BIOSYNTHESIS AND THEIR CELLULAR LOCALIZATION

1.2.1 Formation of 5-Aminolevulinic Acid

The steps in heme biosynthesis in *E. coli* are outlined in Fig. 1.2. Synthesis of 5-aminolevulinic acid (ALA), the first ubiquitous precursor of this pathway, involves the cooperation of three enzymes, glutamate, $tRNA^{Glu}$, ATP, NADPH (NADP) and possibly PLP or PALP (Li et al, 1989b; O'Neill et al., 1989; Avissar et al., 1989).

Glutamate is first activated by ligation to $tRNA^{Glu}$ by glutamyl-tRNA synthetase. This step requires ATP. The activated glutamate is then reduced by glutamyl-tRNA reductase (glutamyl-tRNA dehydrogenase) to glutamyl semialdehyde (GSA). GSA is then transaminated to ALA by GSA aminotransferase.

In animals, yeast, and some bacteria, ALA synthase catalyzes the synthesis of ALA from glycine and succinyl CoA (Fig. 1.3).

1.2.2 Formation of Porphobilinogen: ALA Dehydratase

5-Aminolevulinic acid dehydratase (EC 4.2.1.24) (ALA D) participates in porphyrin/heme biosynthesis by catalyzing the condensation and cyclization of two molecules of ALA to porphobilinogen (PBG) the first pyrrole of the pathway (Gidson et al., 1955). ALA D will be discussed extensively in Chapter 2.

1.2.3 Formation of Uroporphyrinogen III: PBG Deaminase and Uroporphyrinogen III Cosynthase

Four molecules of PBG are condensed to yield the first tetrapyrrole in the pathway, uroporphyrinogen III (urogen III). This reaction is catalyzed by PBG deaminase [EC 4.3.1.8] (urogen I synthase) (PBG D) and urogen III cosynthase [EC 4.2.1.75], resulting in rearrangement of one PBG molecule (ring D in Fig. 1.1 and 1.2). This enzymatic reaction has been studied extensively (Bogorad, 1958; Mauzer, 1960), but the exact mechanism for the inversion of one PBG unit which is catalyzed by urogen III cosynthase (Davies et al., 1973; Fryman et al., 1974) has not been clearly determined. Four molecules of PBG are first joined by a head-to-tail fashion to

generate a linear tetrapyrrole, hydroxymethylbilane (HMB) (Battersby et al., 1979). The order in which these four PBG rings are added to the deaminase are ring A, followed by B, C, and finally D, which then cyclizes to form urogen III after rearrangement of ring D by urogen III cosynthase. In the absence of the cosynthase, HMB cyclizes spontaneously to form urogen I. PBG D was shown to have covalently-bound PBG derived-dipyrromethane as cofactor (Jordan et al., 1981). The activity of *E. coli* PBG D *in vivo* was dependent on the availability of the substrate, PBG (Umanoff et al., 1988; Umanoff, 1990). A mutant with a lesion in ALA D has no PBG D activity and PBG D activity could be restored when the mutant is grown in media containing PBG. PBG is therefore a cofactor for its enzyme (Jordan et al., 1981; Umanoff, 1990).

1.2.4 Formation of Coproporphyrinogen III: Uroporphyrinogen Decarboxylase

Urogen III decarboxylase [EC 4.1.37] converts the four acetic acid side chains of urogen III to methyl groups to yield coproporphyrinogen III (Coprogen III). The reaction sequence is ordered, proceeding clockwise around the cyclic tetrapyrrole starting with the acetate group on ring D (Irving et al., 1976). The hepta-, hexa-, and penta-carboxylic porphyrinogen intermediates are not released into solution under normal conditions.

1.2.5 Formation of Protoporphyrinogen IX: Coproporphyrinogen Oxidase

Coprogen III oxidase [EC 1.3.3.3] oxidatively decarboxylates one of the two propionic acids on each of ring A and B of coprogen III to a vinyl group to yield protoporphyrinogen IX (protogen IX) (Porra et al., 1964). In mammals and yeast this reaction requires molecular oxygen (Porra et al., 1964; Batlle et al., 1965; Poulson et al., 1974), while in *E. coli*, grown anaerobically, nitrate and fumarate can replace oxygen (Jacobs et al., 1971; Jacobs et al.,1976; Jacobs et al.,1977).

1.2.6 Formation of Protoporphyrin IX: Protoporphyrinogen Oxidase

Protogen IX oxidase [EC 1.3.3.4] is responsible for the removal of six hydrogens from protogen, the substrate, to yield protoporphyrin IX (proto IX).

1.2.7 Formation of Heme: Ferrochelatase

The final step in heme biosynthesis is the insertion of ferrous iron into proto IX by ferrochelatase (heme synthetase) [EC 4.9.1.1], to form protoheme (heme). The enzymatic reaction of iron insertion into proto is specific for Fe^{2+} rather than Fe^{3+}, but it appears that zinc and cobalt can also be inserted into proto IX by ferrochelatase in animals fed zinc or cobalt (Lamola et al., 1974).

1.2.8 Cellular Localization and Evolutionary Conservation of Heme Biosynthetic Enzymes

In *E. coli* all of the enzymes except coprogen oxidase, protogen oxidase and ferrochelatase appear to be cytoplasmic. The latter three enzymes are associated with the inner surface of the cytoplasmic membrane (Jacobs et al., 1971; Jacobs et al., 1977; Jacobs et al., 1979). The heme pathway as it is currently understood in *E. coli* and *S. typhimurium*, has a major branch at urogen III leading to cobalamin (vitamin B_{12}) and siroheme (Fig. 1.2). This branch has not been demonstrated in the heme biosynthetic pathway of both plants and animals. The genes coding for the enzymes of the heme biosynthetic pathway in *E. coli* and *S. typhimurium* are dispersed throughout the *E. coli* genome (Fig. 1.4).

Many of the enzymatic steps in the heme biosynthetic pathway are conserved among microorganisms (both prokaryotic and eukaryotic), plants and animals. However there are some steps that are unique to groups of organisms. The major difference lies in the synthesis of the first common precursor, ALA, which as previously stated, can be synthesized via two dissimilar routes. In the C_4 pathway, the condensation of succinyl-CoA and glycine to form ALA, is catalyzed by ALA synthase. This enzyme has been found in mammals, yeast, *Euglena* and some bacteria (Kikuchi et al., 1958; Gibson et al., 1958; Warnick et al., 1971; Ohashi et al., 1982; Dzelzkalns et al., 1982; Borthwick et al., 1983; Volland et al., 1984). The second pathway (C_5) of ALA synthesis described above for *E. coli* also occurs in other bacteria, algae, *Euglena* and plants (Kannangara and Gough et al., 1977; Weinstein and Beale, 1983;

Weinstein and Beale 1984; Wang et al., 1984; Avissar et al., 1989b). The evolution of these two pathways is shown in Fig. 1.5.

In animal cells the enzymes of heme biosynthesis are segregated between the mitochondria and the cytoplasm (Fig. 1.6). In plant cells heme synthesis occurs in both the mitochondria and the chloroplast. However heme synthesis in plant mitochondria is segregated as in animal cells. The chloroplast unlike the mitochondria appears to have all the enzymes for *de novo* biosynthesis of protochlorophyllide from glutamate (Fuesler et al., 1984; Huang et al., 1986). 5-Aminolevulinic acid synthase (ALA S) and ferrochelatase are found exclusively in the mitochondrion; in the inner membrane and loosely bound to it respectively (McKay et al., 1969; Zuyderhoudt et al., 1969; Patton et al., 1973). Coproporphyrinogen (coprogen) oxidase is loosely bound to the external face of the mitochondrial inner membrane (Grandchamp et al., 1978). Protoporphyrinogen (protogen) oxidase has been shown to be associated with the mitochondrial membranes (Poulson, 1976; Poulson et al., 1974). The other enzymes are found in the cytoplasm.

1.3 REGULATION OF HEME SYNTHESIS BY HEME

Heme is the end product of a multistep biosynthetic pathway and therefore might be expected to regulate its own synthesis. It has been shown that glutamyl-tRNA reductase of *Chlamydomonas* is subject to feed-back inhibition by hemin (Wang et al., 1987). The first enzyme of heme biosynthesis in liver, 5-aminolevulinic acid synthase (ALA S), is the rate limiting enzyme of this pathway. Hemin has been shown to be an inhibitor of liver ALA S, exerting a

negative feedback control upon the whole biosynthetic pathway (Granick et al., 1975; Sassa et al., 1979; Sassa et al., 1981). Erythroid ALA S activity is not subject to feedback regulation by hemin and its molecular weight is different from that of the liver enzyme; although their antibodies showed a partial cross reactivity. These two species of ALA S have been shown to be coded for by separate genes (Riddle, 1986). ALA S activity of *Rhodopseudomonas spheroides, Spirillum itersonii, P. shermanii, Neurospora crassa* and *Saccharomyces cerevisiae* are feedback-inhibited by hemin (Burnham et al., 1962; Burnham et al., 1963; Ho et al., 1971; Nandi, 1978; Jordan et al.,1972; Shemin, 1972) .

In addition hemin is thought to inhibit ALA S mRNA synthesis in hepatic tissue; inhibit a post-translational step; and inhibit ALA S transfer from the cytoplasm to the mitochondrial matrix (Abraham et al., 1983). In addition to being a feedback inhibitor of ALA S activity in *R. spheroides*, hemin suppresses transcription of the ALA S structural gene (Jordan, 1990).

ALA dehydratase (ALA D), the next enzyme of the heme pathway, has been proposed as a possible site of regulation in heme biosynthesis. Loss of this enzyme activity results in an almost total lack of activity of subsequent enzymes in the pathway in *S. cerevisae* (Urban-Grimal et al., 1981). In addition ALA D from several sources is inhibited by hemin. This will be discussed in Chapter 2.

In lymphocytes hemin has been shown to inhibit ferrochetalase, which catalyzes the final step in the heme biosynthetic pathway (Rossi et al., 1990)

1.4 5-AMINOLEVULINIC ACID SYNTHESIS

5-Aminolevulinic acid (ALA), the first committed intermediate in the heme biosynthetic pathway, is synthesized by two major routes. One pathway, the C_4 pathway, involves the condensation of glycine and succinyl-CoA to yield ALA and carbon dioxide (Fig. 1.3) (Shemin and Russell, 1953; Shemin et al., 1955; Kikuchi et al., 1958; Gibson et al.,1958). In the second pathway (Fig. 1.3), the C_5 pathway, the 5-carbon skeleton of glutamate is converted to ALA in three steps (Beale and Castelfranco, 1974a, b; Beale et al., 1975; Kannangara and Gough, 1977). These two pathways appear to be mutually exclusive with *Euglena gracilis* is the exception having both C_4 and C_5 pathways (Weinstein and Beale, 1983; for a review see Kannangara et al., 1988; O'Neill and Soll,1990).

A minor route of ALA synthesis may involve a transamination reaction of 4,5-dioxovaleric acid (DOVA) to ALA by 4,5-dioxovalerate transaminase in bovine mitochondria (Varticovski et al., 1980). This enzyme has been shown to be present in a number of other organisms including plants, algae and bacteria (Dornemann et al., 1980; Elliott et al., 1990). However the arguments in support of this minor route are weakened by the fact that DOVA inhibited ALA D and therefore porphyrin synthesis in *Clostridium tetanomorphum; Erythrobacter Sp.* (Shioi et al., 1988a); and *Scenedesmus obliquus* (Kotzabasis et al., 1989) with a K_i similar to the K_m of transamination and less than K_m of ALA D for ALA. This implies that DOVA will bind equally well to the transaminase and to ALA D, and this binding is better than binding of ALA to ALA D. Therefore in high

concentration of DOVA, porphyrin biosynthesis will be constantly inhibited.

1.4.1 C_4 Pathway

The enzyme for the synthesis of ALA from glycine and succinyl-CoA was reported simultaneously by Shemin's group in bacterial extracts (Kikuchi et al., 1958) and by Neuberger and his group in avian preparations (Gibson et al., 1958). However the role of ALA as precursor for heme was reported first by Shemin et al., (1955). The enzyme is 5-aminolevulinic acid synthase (EC 2.3.1.37), and has been purified from many sources including; *R. spheroides* (Burnham, 1970; Warnick et al., 1971); rat liver (Ohashi et al., 1979); *E. gracilis* (Dzelzkalns et al., 1982); chicken liver (Borthwick et al., 1983); and yeast (Volland and Felix, 1984). The properties of the enzyme have been reviewed elsewhere (Jordan et al., 1972). As previously stated eukaryotic ALA S is found exclusively in the mitochondria (Patton et al.,1973). However evidence has been provided that ALA S is synthesized as a precursor which is subsequently processed and imported into the mitochondria (Abraham et al., 1983). Mitochondrial ALA S activity can be induced by 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) (Granick, 1963). This compound induces cytochrome P450 synthesis. The regulation of hepatic ALA S by heme has been previously discussed.

1.4.2 C_5 PATHWAY

This pathway first demonstrated in greening etiolated cucumber cotyledons that the carbon skeleton of glutamate but not glycine

and succinyl-CoA was preferentially incorporated into ALA (Beale, et al., 1974a,b). Cell-free extracts of *Chlorella vulgaris* demonstrated that the formation of ALA from glutamate is dependent on ATP, Mg^{2+}, NADPH, two or more enzymes and tRNA (Weinstein and Beale, 1985a, b; Weinstein et al., 1987; Schneegurt and Beale, 1988). In *in vivo* experiments with various organisms, including blue-green algae (Avissar, 1980; Kipe-Nolt et al., 1980; Meller et al., 1978), red algae (Jurgenson et al., 1976), green algae (Meller et al., 1978) and higher plant tissues (Beale et al., 1974b; Beale, 1975; Meller et al., 1975) in the presence of levulinic acid, the label from glutamate or α-ketoglutarate is specifically transferred to ALA. Castelfranco and Jones (1975) showed that the ALA formed from labeled glutamate is subsequently incorporated into both protoheme and chlorophyll in greening barley. Similarly, ^{14}C-labeled α-ketoglutarate but neither glycine nor succinate is preferentially incorporated into phycocyanobilin in growing cultures of *Anacystic nidulans* (laycock et al., 1981). ^{13}C NMR experiments performed with chlorophyll labeled with ^{13}C-glycine or ^{13}C-glutamate established that glycine gives rise only to the methoxyl group adjacent to the isocyclic ring, while glutamate contributes label in a fashion consistent with the exclusive, operation of the C_5 pathway (Oh-hama et al., 1982; Porra et al., 1983; Oh-hama et al., 1986a,b).

Three enzymes are required for the conversion of glutamate to ALA (Fig. 1.6). The first step, which requires ATP and magnesium ions (Mg^{2+}), is the esterification of the α-carboxyl group of glutamate to $tRNA^{Glu}$ (Kannangara et al., 1984). In the second step

the esterified α-carboxyl group of the charged glutamyl-tRNA^{Glu} is reduced by glutamyl-tRNA reductase (GTR), an NADPH (NADH) requiring enzyme, to generate a second intermediate, the structure of which is still debated in the literature (Hoober et al., 1988). Houen et al., (1984) has proposed glutamate 1-semialdehyde (GSA) as the structure of this intermediate, while Dornemann and his colleagues (Breu and Dornemann,1988; Breu et al., 1988; Hoober et al., 1988) have proposed 4,5-dioxovalerate (DOVA) as the structure. A third possible structure, 2-hydroxy-3-amino-tetrahydropyran-1-one (HAT) has been proposed as the structure of this intermediate (Jordan et al., 1989). Jordan, (1990) has argued that HAT, a cyclic form of GSA, is more consistent with the NMR data of an *in situ*-generated intermediate than GSA or DOVA. The intermediate is then isomerized to 5-aminolevulinic acid by a third enzyme, an aminotransferase. In higher plants, ALA is synthesized in the chloroplast stroma in cooperation with the nuclear genome products. The tRNA^{glu} used to activate the glutamate is coded for by the chloroplast genome, while the aminoacyl-tRNA synthetase, glutamyl-tRNA^{glu} reductase and aminotransferase are encoded by nuclear genes and are synthesized as precursors in the cytoplasm (Kannangara et al., 1988).

Each of the three enzymes has been fractionated from various sources including *C. vulgaris* (Weinstein et al., 1987; Avissar and Beale, 1988); *E. gracilis* (Mayer et al., 1987); barley seedlings (Wang et al., 1981; Grimm, 1990); *Chlamydomonas reinhardtii* (Wang et al., 1985; Chen et al., 1990a,b). Glutamyl-tRNA synthetase has been purified from wheat (Ratinaud et al., 1983), barley (Bruyant et al.,

1983), *Chlamydomonas* (Wang et al., 1987; Chen et al., 1990a), *Chlorella* (Weinstein et al., 1987) *Bacillus subtilis* (Proulx et al., 1983; Proulx and Lapointe, 1985; Breton et al., 1986) and *E. coli* (Lapointe and Soll 1972; Kern et al., 1979; Lapointe et al., 1985). The glutamyl-tRNA synthetase gene (*gltX*) of *E. coli* including the upstream region has been cloned and the primary nucleotide structure determined, and regulation of the gene studied (Breton et al., 1986; Brun et al., 1990a, b). There is considerable homology between *gltX* and glutaminyl-tRNA synthetase (*glnS*) (Yamao et al., 1982) both at the nucleotide and amino acid level (Breton et al., 1986). It was shown by a combination of northern hybridization and S_1 nuclease mapping that the *gtlX* gene transcript is monocistronic. The mRNA is initiated from one of three promoters and the major transcript arises from RNase E processing. The level of *gltX* mRNA increases with growth rate; three times more *gltX* mRNA is produced than the glutamyl-tRNA synthetase activity (Morgan et al., 1977; Brun et al., 1990).

Glutamyl-tRNA reductase (also called dehydrogenase) has been purified from *Chlamydomonas* and shown to catalyze the conversion of Glu-tRNA^{glu} to GSA in the presence of NADPH (Chen et al., 1990b). Barley GTR has been shown to discriminate between several glu-tRNA^{glu} or glu-tRNA^{gln} prefering the glu-tRNA^{glu} of its chloroplast (Peterson et al., 1988). The tRNA from bacteria, yeast, and other higher plants are not recognized by the barley glu-tRNA^{Glu} reductase (Kannangara et al 1984). The single tRNA^{Glu} of *E. coli* K-12 contains the UUC anticodon (Komine et al., 1990) which appears to be the only RNA anticodon that is capable of supporting ALA

formation from glutamate (Schneegurt and Beale, 1988). *E. coli* glu-tRNA^{glu} supported ALA synthesis in *Chlamydomonas* but not in other organism with the C_5 pathway. The tRNA^{glu} from *E. coli* (Fournier, M. J., and Ozeki, H., 1985) and barley chloroplast (Kannangara et al., 1988) has been cloned and sequenced and shown to be the same tRNA involved in protein synthesis. Hackett and Bragg (1983a,b) showed that in *E. coli* tRNAs and their amino acid tRNA synthetases activity varied with growth phase, carbon source, and strains. It has been shown that the *Chlamydomonas* glu-tRNA^{glu} reductase activity is subject to feed-back inhibition by hemin (Wang et al., 1987). This may be one of the control points of tetrapyrrole biosynthesis in this algae.

Glu-tRNA^{glu} reductase activity is encoded for by the *hemA* gene in *E. coli.* The *hemA* gene has been cloned and the nucleotide sequence determined (Li et al., 1989a; Drolet et al., 1989; Verkamp and Chelm, 1889). A strain of *E. coli* with a chromosomal mutation in *hemA* and complemented with a multicopy plasmid bearing this gene overproduces ALA in the medium. Cell free extracts of *E. coli* have been shown to preferentially incorporate ^{14}C labeled glutamate in ALA and the formation of ALA was dependent on tRNA, glutamate, ATP, NADPH and PLP (Li et al., 1989a,b; Avissar and Beale, 1989; O'Neill et al., 1989). The enzyme has recently been purified from *E. coli* and shown to be two different monomeric Glu-tRNA^{glu} reductase activities which differ in their molecular weights (Jahn et al., 1991). Like the barley enzyme (Peterson et al., 1988) the *E. coli* enzyme show sequence-specific recognition of tRNA; *E. coli* Glu-tRNA$_2^{glu}$ is a good substrate while *C. reihhardtii, B. subtilis* and

Synnechocystic Glu-tRNAglu species are poorly recognized (Jahn et al., 1991).

The third enzyme, glutamate-1-semialdehyde aminotransferase (GSA-AT), catalyzes the conversion of GSA into 5-aminolevulinic in a reaction which requires no transaminating amino acid donor (Hoober et al., 1988). This enzyme has been purified from *Chlamydomonas* (Wang et al., 1984) and barley (Grimm et al., 1989). Recently this gene has been cloned and the nucleotide sequence determined from barley nuclear DNA (Grimm, 1990) and *S. typhimurium* (Elliott et al., 1990).

1.4.3 ALA Synthesis via the C_5 Pathway in *E. coli*

An *E. coli* gene which complements the mutation in HU227, an ALA requiring mutant, whose mutation was mapped to min 27 designated the *hemA* locus and encodes a protein, the amino acid sequence of which (Li, 1989; Li et al., 1989a) shows no homologies with any reported ALA S sequences including human (Bawden et al., 1987); mouse (Schoenhaut et al., 1986); chicken liver (Borthwick et al., 1985); *Saccharomyces cerevisiae* (Urban-Grimal et al., 1986); and *Bradyhrizobium japonicum* (McClung et al., 1987) although all of these sequences have certain regions of significant homology. A number of ALA-requiring mutants of *E. coli* have been reported (Table 1.0); SASX41B, and SHSP19 (Sasarman et al., 1968), map at min 27 on the genome. Both were complemented by pJL68 (Li, 1989; Li et al., 1989a). The other, *popC*, was maps at min 4 (Powell et al., 1973; Wulff, 1967). This mutant is probably the one for GSA aminotransferase, one of the three enzymes involved in ALA

synthesis from glutamate. In *S. typhimurium*, a close relative of *E. coli*, this gene, designated *hemL* maps to min 5 and has recently been cloned and the nucleotide and amino acid sequences determined (Elliott et al., 1989; Elliott et al., 1990). The amino acid sequence determined from the nucleotide sequence for *S. typhimurium* GSA aminotransferase shows 58% homology to that of barley (Elliott et al., 1990).

It was demonstrated that *E. coli* grown in medium containing levulinic acid, and [1-^{14}C]glutamate or [2-^{14}C]glycine preferentially incorporates [1-^{14}C]glutamate in ALA isolated from the medium (Avissar and Beale, 1989a; Li, 1989; Li et al., 1989b; O'Neil et al., 1989). Li et al., (1989b) results further demonstrates that [1-^{14}C]glutamate is incorporated into ALA by an *E. coli* strain JL1268, a hemA mutant complemented with a multicopy plasmid harboring an *E. coli* hemA^{+} gene. The ALA so synthesize is labeled preferentially in C-5 of ALA. Had the C_4 pathway been operating, then this label would have been lost when glutamate was converted to α-ketoglutarate and then to succinyl-CoA (Schneegurt et al., 1986). When uniformly-labeled glutamate is used as a precursor, the label incorporated into ALA is about 20% of that incorporated when all the label is in C-5 of glutamate. In addition, while the structural gene for ALA S from *R. sphaeroides* could complement a *hemA* mutant, the complemented strain does not utilize glutamate for ALA synthesis (Li, 1989; Li et al., 1989b). These results prove that *E. coli* normally synthesizes ALA by the C_5 pathway but can use the C_4 pathway if the ALA S gene is introduced.

1.5. OXYGEN, CARBON SUBSTRATE AND cAMP EFFECT

1.5.1 Aerobic versus Anaerobic Metabolism

Facultative anaerobes can respond to changes in their invironment by synthesizing different types of respiratory pathways. These pathways include the terminal oxidases, which are a diverse collection of heme proteins containing protoheme IX (heme b) and/or chlorin (heme d) as prosthetic groups (Anruku et al., 1987). Ishida et al., (1972), pointed out that the effect of oxygen on heme and cytochromes in *E. coli* is manifold; oxygen enhances the formation of the enzymes for succinyl-CoA synthesis; formation of enzymes in some step between ALA and protoheme; the reaction of preformed enzymes in some step between ALA and protoheme (probably the conversion of coproporphyrinogen to protoporphyrin) and possibly the formation of ALA synthetase and apo-cytochromes. The various effects of oxygen on *E. coli* metabolism are proposed to be coordinately regulated by a global modulon, which is regulated by the ArcA protein. The term modulon describes a group of operons and or regulons that are under the modulation of a common pleiotropic regulatory protein (possibly even a specialized sigma (σ) protein) (Iuchi et al., 1988). There is much conflict in the literature as to which type of cytochrome is made under different growth conditions. In addition to oxygen, the carbon source and strain affect the cytochrome content. *E. coli* protoheme content is shown to vary directly with cytochrome b_1 for both aerobic and anaerobic cells (Ishida et al., 1972; Hino et al., 1973). Additionally the ratios of different cytochromes vary in aerobically and anaerobically grown *E. coli* (Shipp,1972; Rice et al., 1978). However it was demonstrated

that the NADH-dependent-cytochrome b apoprotein is synthesized in the absence of heme (Haddock et al., 1973a, b). Gray et al., (1966a, b) observed that cytochromes a_1, a_2 and b_1 are made in the same amount for both aerobic and anaerobic cells. However it was subsequently shown that aerobically grown cells of *E. coli* contains several times as much cytochrome b_1, d (cyt a_2) and protoheme as anaerobically grown cells (Ishida et al., 1972). Growth of *E. coli* under conditions of high oxygen tension results predominantly in formation of cytochromes b_{556}, b_{562} and o. When the oxygen tension was low, cytochromes b_{558} and d are induced in addition to others (Hackett et al., 1983a, b; Ingledew et al., 1984). Aerobic cytochrome b_{556} is shown to be immunologically unrelated to anaerobic cytochrome b_{556} (Kranz et al., 1984). From the above evidence it can be concluded that *E. coli* synthesizes cytochromes under aerobic and anaerobic conditions. It was shown that oxygen cause the conversion of coproprophyrinogen to protoporphyrin in extracts of *E. coli* which had been grown aerobically and anaerobically (Jacobs et al., 1971; Jacobs et al.,1976; Jacobs et al.,1979). However oxygen could be replaced by nitrate or fumarate in extracts from cells grown anaerobically on nitrate, indicating that these late steps in heme synthesis, coprogen to protogen occur anaerobically with an alternate electron acceptor (Jacobs et al., 1971; Jacobs et al.,1976; Jacobs et al.,1977). In liver mitochondria, the molecular oxygen required for this conversion cannot be replaced by alternate electron acceptors (Porra et al., 1964; Batlle et al., 1965). Heme-deficient strains of *E. coli* can grow anaerobically in rich media containing nitrate or fumarate as electron acceptors and glycerol as carbon

source or on glucose fermentatively (Ingledew et al., 1984). In contrast to *E. coli,* anaerobic cultures of *Staphylococcus epidermidis* has been shown to contain 10 to 15% of the amount of protoheme and cytochromes of aerobic cultures. Resting anaerobic suspensions of *S. epidermidis* transferred to glucose and ALA media show a fourfold increase in protoheme content in 60 min. When the ALA concentration is high these cells accumulate coproporphyrin (copro) and uroporphyrin (uro) but fail to accumulate protoporphyrin. Nitrate is reduced to nitrite anaerobi-cally but does not cause an increase in protoheme content, demonstrating that one of the late steps of heme biosynthesis in this bacteria requires oxygen as substrate (Jacobs et al., 1969).

1.5.2 Nitrate and Fumarate Reductase

Nitrate and fumarate can serve as electron acceptors through the nitrate and fumarate reductases respectively. These complexes are positively regulated by anaerobiosis via the *nirR* and *fnr* modulons respectively (for review see Ingledew et al., 1984). The *fnr* gene product, FNR protein, has been shown to function as an anaerobic repressor of cytochrome o and d complexes. Cytochrome o oxidase appears to be produced only under oxygen-rich growth conditions, while cytochrome d oxidase is expressed moderately under aerobic conditions and is elevated yet further when oxygen tension decreases (Cotter et al., 1990). The FNR protein shares sequence homology to the catabolite regulatory protein (CRP) of *E. coli* and has been proposed to bind to a FNR recognition sequence located 5' of the RNA polymerase site of the *fnr* gene. The amino-

terminal end of the FNR protein has been shown to be important in this oxygen-sensitive regulatory role (Melville and Gunsalus, 1990).

Anaerobic nitrate respiration is regulated at the level of gene transcription (Stewart,1982) or subsequent to gene expression at the level of nitrate transport. Anaerobiosis causes a derepression of the synthesis of the proteins involved in nitrate respiration, and the expression of these genes is further enhanced by the presence of nitrate (Stewart, 1988). It has been proposed the oxygen inhibited nitrate uptake by diverting electrons away from the nitrate reductase complex (Denis et al., 1990).

1.5.3 Catabolite Repression

Some cytochromes and some Kreb cycle enzymes are repressed when *E. coli* is grown on glucose. The Krebs cycle enzymes are also repressed anaerobically (Gray et al., 1966a, b; Kranz et al., 1984; Cotter et al., 1990). Glucose also lowers catalase activity in *E. coli* (Kranz et al., 1984). However not much is known about the effect of glucose on individual enzymes of *E. coli* heme biosynthesis. In bacteria, glucose (and its analogs) affect cellular metabolism usually through (1) cAMP-mediated regulation and is referred to as catabolite repression (Magasinik,1961; Pastan et al., 1976) (2) transient repression (Magasinik, 1970), and (3) inducer exclusion (Ullmann and Danchin, 1983; Magasanik and Neidhardt, 1987 for review).

Glucose and its analogs lower intracellular cAMP levels, causing a reduction in the expression of a set of catabolite-sensitive operons including *lac, ara* and *mal*. In these operons cAMP

and its receptor protein (CRP) act as a positive effector for transcription. Alternatively, it has been shown that cAMP-CRP can repress transcription of another set of genes (Movva et al., 1981; Aiba, 1983). In addition, this complex seems to be involved in modulating transcriptional termination in certain polycistronic operons (Ullmann et al., 1978; Guidi-Rontani et al., 1980). DNase I footprinting showed that cAMP-CRP specifically interacts with a unique site containing a consensus CRP binding sequence (deCrombrugghe et al., 1984; Ebright et al., 1984; Barber and Zhurkin, 1990). cAMP-CRP has been shown to negatively regulate the levels of adenylate cyclase and therefore the cellular levels of cAMP. A CRP^- mutant has been shown to have high levels of cAMP (Botsford et al., 1978; Majerfeld et al., 1981; Aiba, 1985).

An *E. coli* strain RP523 (Li et al., 1988) cannot utilize ALA because there is a mutation in the structural gene for ALA D and therefore it accumulates ALA. It has been shown that this accumulation was lowered when RP523 was grown on glucose-supplemented rich media than when grown without glucose (W. Chen, C. S. Russell, S. Cosloy, unpublished). It was further demonstrated that a wild type strain of *E. coli*, C600, made less *hemA* mRNA when grown on glucose than without glucose. This effect could be reversed by cAMP (W. Xiao, C. S. Russell, S. Cosloy, unpublished).

1.6 PURPOSE OF THIS STUDY

A. This report investigates the effects of various cofactors on the synthesis of ALA via the C_5 pathway in *E. coli.* Levels of *hemA* transcription and ALA production production by different *E. coli* strains grown under different conditions and with different supplements was investigated.

1.7 EXPERIMENTAL

1.7.1 MATERIALS and METHODS

1.7.1.1 Instruments and Equipment

Spectrometers: Lambda 3B (or Lambda array 3840) UV/Vis spectrophotometer, (Perkin-Elmer, Ridgefield, CT); Spektralphotometer 600 PM6, (Zeiss, Oberkochen, West Germany); Klett-Summerson electric colorimeter 800-3, (Klett MFG Co., New York, NY). Densitometer LKB 2222-010 UltroScan XL (LKB Produkter AB, Bromma, Sweden). Incubators and Shakers: Gyrotory Shaker model G-33 and Gyrotory Shaker water bath model G-76, Controlled Environment Incubator Shaker model G-25, (New Brunswick Scientific Inc. New Brunswick, NJ); Lab-Line high density fermentor, model 29500 (Lab-Line Instruments, Inc., Melrose Park, IL). Centrifuges: Eppendorf 5415; MicrofugeB 338720, (Beckman, Pala Alto, CA); Sorval RC 5B; Sorval RC70 (plus SW40T1 rotor); Sorval RT 6000 (Dupont, Wilmington, DE). Buchi Rotavapor <R>, (Glasapparatefabrik, Flawil, Switzerland). Electrophoresis apparatus: Polyanalyst 431 7000, (Haake/Buchler Instruments, Inc., Saddle Brook, NJ.); Protein-II (BioRad Labs, Richmond, CA.). Blotting apparatus: Vacusystem 1.3, (American Bionetics, Inc., New Haven, CT). Bio-Dot SF microfiltration apparatus, 170-6542, (BioRad, Richmond, CA). Ultrasonic cell disruptor 378 or 385 (Heat System Inc, Farmingdale, NY). Stratalinker UV Crosslinker 2400 (Stratagene, La Jolla, CA). Anaerobic jars and GasPaks, (BBL, Division of Becton, Dickinson & Co., Cockeyville, MD).

1.7.1.2 CHEMICALS AND ENZYMES

Restriction enzymes were purchased from Boehringer Mannheim (BMB) (Indianapolis, IN), and International Biotechnologies (New Haven, CT). DNA molecular weight markers II (λ DNA-Eco RI) and 1 kb DNA ladder were purchased from Boehringer Mannheim, and Bethesda Research Laboratories Inc., (BRL) (Gaithersburg, MD) respectively. The Genius system of nonradioactive detection was purchased from Boehringer Mannheim. DNase I, and ribonuclease were purchased from Worthington Biochemical Corp. (Freehold, NJ) and Bethesda Research Laboratories Inc. Lysozyme was purchased from Sigma (St. Louis, MO). 5-Aminolevulinic acid, uroporphyrin, coproporphyrin, protoporphyrin and porphyrin standards were obtained from Sigma (St. Louis, MO) and Porphyrin Products (Logan, UT). Hemin, dimethylaminobenzaldehyde, Trizma base, Dowex 50W8, $tRNA^{Glu}$, RNase, ATP, ampicillin, tetracycline, chloramphenicol, diethylpyrocarbonate (DEPC), TEMED, and l-glutamate were obtained from Sigma (St. Louis, MO). NADPH was obtained from Pharmacia (Piscataway, NJ). Ethyl acetoacetate and succinylacetone (2,4-pentanedione) were obtained from Aldrich, (Milwaukee, WI). Nylon membranes were purchased from GeneScreen Plus NEN Research Products, (Boston, MA). Media were prepared from Difco products (Detroit, MI). All other chemicals were reagent grade or better.

Table 1.0. Bacterial Strains and Plasmid

	Relevant genotype and or phenotype†	Source/(reference)
E. coli strains		
C600	Hem$^+$	*CGSC
RP523	C600, hemB$^-$, hemin-permeable	Li et al., (1988)
JL1002	RP523/pJL2, Amp^r	Li et al., (1988)
SASX41B	HfrPO2A hemA41 metB1 relA1	B. Bachman, CGSC
HU227	SASX41B, but hemin-permeable	Umanoff et al., 1988
JL1268	hemA$^+$, hemin permeable HU227/pJL68, Amp^r	Li et al., (1989)
§*Rhodobacter spheroides TB1(pU1563)*	hemA$^-$ but hem$^+$	
Plasmid		
pJL2	Hem+ amp (from pTZ18U)	Li, (1989)
pJL68	Hem$^+$ (from pTZ19U)	Li et al., (1989)

† other genetic markers for C600 thr-1, leuB6, thi-1, tonA21, SupE44 λ^-, F$^-$

*CGSC: *E. coli* Genetic Stock Center, Yale University, New Haven, CT

§A gift from Mark Moore, University of Illinois

1.7.2 BACTERIAL STRAIN AND GROWTH CONDITIONS

The bacterial strains used in this study are listed in Table 1.0 Bacteria were grown in LB (Ausubel et al., 1989) and media supplements and/or antibiotics added as stated below and in figure legends.

1.7.2.1 Aerobic Growth

E. coli JL1268 which contains a multicopy plasmid harboring a gene which complements the *hemA* mutation in SASX41B, was grown as follows. After growth for 24 h at 37^{0}C, cells (5 x 10^8 cells per ml) were washed with fresh medium and the cell paste was suspended in 20 ml of fresh medium as above but containing levulinic acid (4 mM), an inhibitor of ALA D. For the preparation of cell extracts of JL1268 and for the growth of TB1(pU1553), an *E. coli* strain which harbors the structural gene for ALA S from *R. spheroides*, which was kindly supplied by Mark Moore, 1 ml of an overnight culture of JL1268 was inoculated into 100 ml of LB containing ampicillin (50 µg/ml). The cultures were placed on a Gyrotory shaker model G-33 or in a Gyrotory Shaker water bath model G-76 and grown overnight to saturation (>2 X 10^9 cells per ml) at 37^{0}C.

To monitor the accumulation of ALA in the medium and for the preparation of RNA, *E. coli* JL1268, RP523, and *R. spheroides* were grown in 100 ml LB in 500 ml flasks or a proportionate ratio. JL1268 was grown in 50 µg/ml ampicillin and supplements as stated in figure legends. RP523 was grown aerobically with 4 µg/ml

hemin, 1% glycerol, and 55 mM fumarate. Supplements were added as stated in figure legends.

1.7.2.2 Superaerobic Growth

A superaerobic growth condition was achieved by growing cultures in a gyrotory type Controlled Environmental Incubator Shaker. This chamber has a closed lid and air is circulated through the chamber by a fan. The ratio of media to inoculant and supplements for superaerobic growth were the same as aerobic growth. Cultures were inoculated as stated above and shaken in the chamber between 175 to 290 rpm at 37^0C either overnight or for approximate 6 h.

1.7.2.3 Anaerobic Growth

Anaerobic conditions were achieved by placing cultures in anaerobic jars (GasPak 100) and either purging the jars several times with prepurified nitrogen gas or by the use of GasPak hydrogen + CO_2 Generator Envelope (BBL). In the latter case the sealed jars were allowed to stand for 45 to 60 min before shaking at 37^0C.

1.7.3 PREPARATION OF BACTERIAL EXTRACTS

Ten 100-ml cultures of *E. coli* JL1268 were grown for 16 h at 37^0C in LB with ampicillin (50 µg/ml) in a Gyrotory Shaker water bath shaking at approximately 175 rpms. Cells (>2 $X10^9$ cells per ml) were harvested by centrifugation at 4,300 rpm at 4^0C, washed with 0.1 M Tricine (pH 7.9), 25 mM MgCl2 , 5 mM DTT, suspended in 30 ml of the same buffer, and sonicated in 3-ml portions for 60 s each

(10-s intervals, 10-s on and 10-s off). This and all subsequent preparation was performed at 4°C. The sonicate was designated the crude extract (protein concentration, 15.8 mg/ml). The supernatant obtained after centrifugation (10,000 rpm, 10 min, 4°C) was designated S. The pellet was washed with 40 ml of Tricine buffer, suspended in 6 ml of buffer, and sonicated for 30 s as previously stated. This suspension was dialyzed against buffer for 3 h with two changes of Tricine buffer and designated P (protein concentration, 16.7 mg/ml). Supernatant S was passed through a column of Sephadex G-25 (100-ml bed volume, equilibrated with the same buffer) in two 13-ml portions. Fractions of 12 ml each were collected. Fractions 2 and 3 from both runs were combined, concentrated to 26 ml by ultrafiltration, and designated fraction S(1) (protein concentration, 8.3 mg/ml) This process was repeated to yield S(2) (protein concentration, 6.0 mg/ml) and again to yield S(3) (protein concentration, 5.9 mg/ml).

1.7.4 ENZYME ASSAY FOR ALA SYNTHESIS

ALA synthesis was determined by several methods. (a) When Succinate and glycine were used as substrates, the method of Burnham, (1970) was followed, except that 0.2 ml of crude extract contained levulinic acid (5 mM, final concentration) and succinyl-CoA (25 μM, final concentration) was used. The incubation time was 10 min at 37°C. The blank contained crude extract and buffer.

With glutamic acid as the substrate the method of Oh-hama and co-workers (1988) was followed except that the assay was done aerobically and 0.4 ml of crude extract was used. The blank

contained crude extract and buffer and the reaction was quench at zero time.

1.7.5 DETERMINATION OF ALA

Two methods for ALA determination were employed. (a) For crude extracts, the Burnham, (1970) method was used with the following modifications. After incubation, 50 µl of 70% perchloric acid was added to stop the reaction and the incubation mixture were spun down. 1 ml aliquots of the supernatants were mixed with 2 ml of sodium acetate, pH 4.7, and 50 µl of acetylacetone. After the mixtures were boiled for 15 min and cooled. 3 ml of modified Ehrlich reagent was added to each sample, and the spectrum from 450 to 630 nm was recorded after 10 min.

(b) The method of Weinstein and Beale (1985) was used to determine ALA for all other assays, The quenched incubation mixture which was first spun down was applied to a Dowex 50X-8-400 column (0.9 by 0.5 cm). The column had been prewashed with 2 ml of 1 N NaOH and 2 ml of 0.05 N sodium citrate (pH 3.01). The incubation tube was rinsed with 2 ml of water, which was also applied to the column. The column was washed with 0.05 M sodium citrate (pH 3.01) in 25% methanol (vol/vol). ALA was eluted with 0.5 M sodium phosphate, pH 6.8, in 1-ml fractions. Ethyl acetoacetate (40 µl) was added to each fraction, and the shaken mixtures were placed in a boiling water bath for 15 min. After cooling an equal amount of modified Ehrlich's reagent (Mauzerall et al.,1956) was added, and the spectrum from 450 to 650 nm was recorded. The ALA formed was quantitated from either a standard curve for ALA obtained in the

same way, in which the A_{553} was plotted against ALA concentration or using $\varepsilon = 7.2 \times 10^4\ M^{-1}cm^{-1}$ (Mauzerall et al., 1956). The results were corrected for endogenous ALA by a control blank using enzyme extract to which no assay mix had been added and the reaction quenched at zero time.

ALA excreted into the medium was determined as previously stated (Lien et al., 1982) with some modifications. 343 µl of medium was acidified with 257 µl of 10% TCA. To this solution 300 µl of 10% acetylacetone in 1 M Na-acetate was added and the solution mixed. The pH of the solution was adjusted to pH 4.6 with NaOH solution (pH 10). The tubes were capped with marbles and heated at 80^0C for 10 min. After cooling the pH was adjusted to 7.0 with 100 µl of 0.5 M Na_2HPO_4, 1M NaOH. To each tube 2 ml of methylene chloride was added and each tube was vigorously vortexed for 10 s. This was followed by gentle centrifugation for 2-3 min. To 500 µl of the aqueous layer (upper) an equal volume of modified Ehrlich's reagent was added, and the tubes spectra were recorded after 10 min as before. The ALA concentration was quantified by using $\varepsilon = 5.8 \times 10^4\ M^{-1}\ cm^{-1}$ for ALA-acetylacetone pyrrole (Sassa et al.,1979).

1.7.6 ASSAY OF PROTEIN CONCENTRATION IN SOLUTION

Protein concentration of all bacterial extracts were determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA), which is a modification of the protein dye binding method of Bradford, (1976) .

1.7.7 PREPARATION OF PLASMID DNA CARRYING *HEMA* GENE AND PROBE DNAs

1.7.7.1 Plasmid Preparation

E. coli JL1268, which carries the plasmid pJL68, was grown overnight in 250 ml of LB containing 50 µg/ml ampicillin. The cell pellet was collected by centrifugation at 5,000 rpm (Sorvall, GSA rotor) at 4^0C for 15 min. The cell pellet was suspended in 6 ml of freshly prepared 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme and incubated on ice for 20 min. Following incubation 12 ml of 0.2 M NaOH, 1% SDS was added; the suspension was gently mixed; and incubated in ice for 10 min. After this interval, to the suspension 7.5 ml of 3 M Na-acetate, pH 4.6 was then added and the contents of the tube mixed by inversion and incubated on ice for 20 min. The resulting suspension was then centrifuged at 15,000 rpm (Sorvall, SS-34 rotor) for 15 min. The supernatant was removed and 50 µl of RNase A (1mg/ml) was added and the solution incubated at 37^0C for 20 min. The solution was then twice extracted with an equal volume of phenol:chloroform (1:1 v/v) and the DNA precipitated with an equal volume of ethanol. The DNA pellet was dissolved in 1.6 ml of water and 400 µl of 4 M NaCl and 2 ml of 13% PEG was added and the solution mixed. The resulting solution was incubated for 60 min on ice. The resulting suspension was centrifuged at 10,000 rpm (SS-34 rotor) for 10 min. The supernatant was removed and the pellet washed with 70% ethanol and dried by Speedvac. The plasmid DNA was dissolved in 200 µl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Plasmid DNA concentration was determined by A_{260}/A_{280} ratio and electrophoresed on a 0.7%

agarose gel (6.2 X 10.4 X 0.465 cm), containing ethidium bromide (0.5 µg/ml) and visualized by UV light (Maniatis et al., 1982).

1.7.7.2 Preparation of *hemA* 933 bp Fragment

Plasmid DNA (100 µg) was digested with restriction endonuclease Hinc (Hind) II (100 units) at 37^{0}C for 1-2 h in the buffer supplied by the company. The efficiency of the digestion was assessed in a 0.7% agarose gel electrophoresis in 1 X TBE buffer as previously stated. The fragment sizes were determined relative to DNA molecular markers (DNA markers II, BMB and 1 kb DNA ladder, BRL). The observation of six fragments (Table 1.1) established that pJL68 was properly digested with Hinc II (Fig. 1.7). 80µl of the digestion solution was loaded into a preparative well in the agarose gel and electrophoresis continued at 80 volts for 2 h. The 933 bp DNA fragment was then excised from the agarose gel and the slice placed in dialysis tubing (130 mm i.d.). Electroelution was performed as recommended by Maniatis et al., (1982) in 0.5 X TBE buffer at 100 volts for 45 min (or until the DNA had move out of the gel slice). The electrodes were reversed for 2 min before the DNA solution was removed from the tubing and the tubing washed with a small volume (500 µl) of 0.5 X TBE buffer. The solution was then extracted with an equal volume of chloroform/phenol (1:1 v/v) and the DNA precipitated overnight with an equal volume of ethanol at -70^{0}C. The DNA pellet was collected by centrifugation, 30,000 rpm for 30 min at 4^{0}C (Sorvall RC70, SW40T1 rotor). The pellet was dissolved in 60µl of TE.

1.7.7.3 DNA Labeling of *hemA* and *gltX* Probes

The 933 bp DNA fragment was labeled by the random primer method using the Genius kit and incorporating digoxigenin-labeled deoxyuridine-triphosphate (Dig-dUTP) as a non-radioactive label. Linearized, purified DNA was incubated with a random hexanucleotide mixture, dATP, dCTP, dTTP, Dig-dUTP and the Klenow enzyme. The hexanucleotides can hybridize to the DNA and serve as primers for the Klenow enzyme to synthesize DNA using the linear DNA as template. Dig-dUTPs get incorporated into the newly synthesized DNA which will serve as the probe. The Dig-dUTP is antigenic (digoxigenin is the hapten) and will bind an antibody to which alkaline phosphatase has been conjugated. The reaction mixture was incubated overnight and the labeled probe purified by ethanol precipitation.

An oligonucleotide (21-mer,5'-CTGGTGCGTCTGGGCTGGTCC-3') of the glutamyl tRNA synthetase gene (*gltX*) (Breton et al., 1986) was purchased from Research Genetics (Huntsville, AL). This oligonucleotide was labeled with Dig-dUTP using terminal transferase. This enzyme in the presence of $CoCl_2$ catalyzes a template-independent addition of Dig-dUTP to the 3'-OH ends of double or single stranded DNA. An incubation time of 1 h or overnight gave the same amount of labeling. The labeled oligomer was not further purified but added directly to the hybridization solution.

1.7.8 PREPARATION OF TOTAL RNA

Total RNA from *E. coli* and *R. spheroides* was prepared by the method of Ausubel et al., (1987) with minor modifications. The bacteria, grown as previously stated, under various growth conditions, were harvested at both mid-logarithmic and stationary phases by centrifugation in a Sorvall RT6000, at 10,000 rpm with a H1000B rotor (or 8,500 rpm in Sorvall, SS-34 rotor) for 20-30 min. The cell pellet was resuspended in 0.5 ml lysis buffer (30 mM Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% SDS, with proteinase K to 100 ug/ml added just before use) transferred to microtubes and frozen on dry ice. The cell suspension was thawed at 37°C and sonicated for 20 min (Cup Horn Sonicator 431A, Heat System, pulse, 60% duty cycle, OPC=7, OPP=30%) at 4°C. The homogenate was incubated at 37°C for 1 h. It was then extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). To the aqueous phase 19 µl of 5 M NaCl was added and the microtube filled with ethanol. The contents of the tube were mixed and the tube incubated overnight at -20°C or 1 h on dry ice. The RNA was pelleted in an Eppendorf microcentrifuge for 15 min at 4°C, rinsed with 70% ethanol and dried in a Speedvac. The RNA pellet was redissolved in 95 µl of DNase digestion buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) containing 10 µg of RNase-free DNase and incubated for 1 h at 37°C. This was followed by extraction with 100 µl phenol/chloroform/isoamyl alcohol (25:24:1 v/v). The organic phase was again extracted with 100 µl TE buffer. The pooled aqueous phase was then extracted with 200 µl chloroform/isoamyl alcohol

(24:1 v/v). To the aqueous phase 10 µl of 5 M NaCl and 600 µl of ethanol was added and the RNA precipitated overnight at -20^{0}C or 15 min in a dryice/ethanol bath. The pellet was collected, rinsed, dried, and redissolved in 100 µl of DEPC-treated water. The concentration was determined from the A_{260}/A_{280} ratio.

In an alternative rapid isolation procedure, the *E. coli* cells were harvested and the cell pellet suspended in 10 ml of protoplasting buffer (15 mM Tris-HCl, pH 8.0, 0.45 M sucrose, 8 mM EDTA) containing 4 mg lysozyme. This suspension was incubated on ice for 15 min. The protoplasts were collected by centrifugation for 5 min at 7000 rpm in an SS-34 rotor and gently resuspended in 0.5 ml gram-lysing buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM Na-citrate, 1.5% SDS) containing 15 µl DEPC. This mixture was then incubated for 5 min at 37^{0}C, chilled on ice and 250 µl of saturated NaCl added. This mixture was mixed and incubated on ice for an additional 10 min, followed by centrifugation for 10 min at 9000 rpm with a SS-34 rotor. The supernatant was removed to two clean microtubes, 1 ml of ethanol was added to each tube and the RNA was precipitated for 30 min on dry ice or overnight at -20^{0}C. The pellet was collected, rinsed, dried, redissolved and the concentration determined. The latter procedure gave lower A_{260}/A_{280} ratios than the former procedure. The RNA was stored at -70^{0}C until used. All solutions used in these experiments were treated with diethylpyrocarbonate (DEPC) (Ausubel et al., 1988).

1.7.8.1 Transfer of RNA to Nylon Membrane

RNA was diluted to the concentrations specified in figure legends by buffer solution consisting of 2.75 mM formamide, 37.5 mM Na_2HPO_4, pH 8.0. DEPC-treated water was added to adjust the final formamide and Na_2HPO_4 concentrations to 0.73 mM and 6.88 mM, respectively, pH 8.0. The solutions were then heated to 65^0C for 10 min and rapidly cooled on ice/salt bath. Each tube was then serially diluted into the same buffer. Prior to applying the RNA sample to the nylon membrane (GeneScreen Plus) the membranes were soaked in DEPC-treated water for about 15 min. The membranes (8.5 X 11 cm) were placed into the blotting apparatus and the apparatus assembled as specified by the manufacturer (Vacusystems 1.3, ABN). The RNA solutions were applied to the membranes either during or followed by light vacuum (15-20 inches Hg) until the solutions were aspirated from the membranes, using a vacuum pump. After aspiration the membranes were allowed to air dry. The membranes were then placed on filter paper moistened with buffer and irradiated (120,000 μjoules/cm^2, Stratalinker) for 3 min to covalently crosslink the RNA to the membrane. *R. spheroides* RNA was used as a negative control since this bacteria uses the C4 pathway and therefore does not contain the GTR gene (*hemA*). RNA prepared from *E. coli* JL1268 was used as the positive control.

1.15 PREHYBRIDIZATION AND HYBRIDIZATION TO *hemA* AND *gltX* PROBES

1.7.8.1 *hemA* Probe

Prehybridizations and hydridizations were performed essentially as specified in the Genius manual (BMB, Cat. No. 1093 657) with some modifications. Membranes to be hybridized to *hemA* specific DNA were prehybridized for 1 h at 42^{0}C in 20 ml of prehybridization solution-A (5 X SSC; blocking reagent {Genius kit reagent} 5%; N-lauroylsarcosine, Na-salt, 0.1%; SDS, 0.02% {w/v}; formamide, 50%) in sealed plastic bags (Thomas Sci., Cat. No. 1312A48). The prehybridization-A was removed and replaced by 5-8 ml of hybridization solution-A (5 ml prehybridization solution-A + 10µl *hemA* gene probe). Hybridization was carried out at 42^{0}C overnight. The membranes were washed 2 X 5 min at room temperature with 50 ml of 2 X SSC; SDS, 0.1% (w/v) and 2 X 15 min at 68^{0}C with 0.1 X SSC; SDS, 0.1%. Both prehybridization and hybridization solutions were saved and reused.

1.7.8.2 *gltX* Oligonucleotide Probe

Membranes to be hybridized to the *gltX* oligonucleotide probe were prehybridized for 1 h at 45^{0}C in 20 ml of prehybridiztion solution-B (5 X SSC; blocking reagent 2%; N-lauroylsarcosine, Na-salt, 0.1%; SDS, 0.02% {w/v}; formamide, 20%) in sealed plastic bags. This was followed by hybridization in 5-8 ml of hybridization-B solution (5 ml prehybridization solution-B + 22 µl *gltX* gene probe) at 45^{0}C overnight. The membranes were washed 2 X 5 min at room

temperature with 50 ml of 2 X SSC; SDS, 0.1% (w/v) and 10 min at 35^0C with 0.1 X SSC; SDS, 0.1%.

1.7.8.3 Immunochemiluminescent Detection and Semiquantitation

Immunochemiluminescence detection was performed essentially as specified by the Genius detection system using the Lumi-Phos reagent. After hybridization and washing the membranes were incubated at room temperature in a solution containing an anti-digoxigenin antibody-conjugated to alkaline phosphatase. The antibody recognizes and binds to digoxigenin incorporated into the probe DNA. After removal of excess antibody the membranes were incubated in a solution of Lumi-Phos 530, a 1,2-dioxetane. Lumi-Phos 530 is chemiluminescent substrate for alkaline phosphate which dephosphorylated this compound to produce an unstable intermediate. As the unstable intermediate decomposes, it emits light in direct proportion to the amount of alkaline phosphatase present. The membranes were exposed to X-ray film in the dark for 5 to 60 min until a good signal was obtained.

The X-ray film was scanned in the LKB densitometer and the peak heights were normalized by the instrument. Growth under aerobic conditions was taken as 100% and the results for the other conditions were calculated relative to it and presented as Table 1.8.

1.7.10 ALA SYNTHESIS

1.7.10.1 Effects of Cofactors, tRNA, RNase, Levulinic and Gabaculine on ALA Synthesis

To study the effects of cofactors, tRNA, RNase, and gabaculine, on ALA synthesis *in vitro* the sonicate was processed as described above and assayed with selective exclusion of substrates, cofactors, or inhibitors from the incubation mixture. Processing of the enzyme extract was described above. The reaction mixture contained 50 µl of P (cell homogenate), 250 µl of S (supernatant) run through Sephadex once or twice, 100 µl of assay mix, buffer and additions (i.e., tRNA) to bring it up to 500 µl, containing, for the complete system, 100 mM Tricine, pH 7.9, 25 mM $MgCl_2$, 5 mM DTT, 5mM ATP, 1 mM NADPH, 3 mM glutamate, 5mM levulinic acid, and 20 µM pyridoxal phosphate. The blank contained S, P, and buffer. After 2 h at 37^{0}C, 50 µl of 1 M citric acid and 0.5 ml of 10% sodium dodecyl sulfate were added to terminate the reaction. The mixtures were boiled for 2 min and cooled, and ALA accumulation was determined as described above.

1.7.11 ACCUMULATION OF PORPHYRINOGEN

E. coli JL1268 excreted ALA but not porphyrins into the medium. The cultures were grown overnight, harvested and washed as stated elsewhere. Prior to sonication the cell pellet was observed under a long wave UV lamp. The cell pellet was solubilized in 2-4 ml of extraction buffer and sonicated. Immediately after sonication the homogenate was observed again under the long wavelength UV lamp. The homogenate was then allowed to stand on ice exposed to the air

for 3-4 h. The homogenate was again observed under the UV. The homogenate was then acidified with 2-8 drops of 1 N HCl, vortexed and the supernatant removed by centrifugation in a table top centrifuge. The visible spectrum of the porphyrins present was taken followed by an analysis by thin-layer chromatography.

1.7.11.1 Thin-layer Chromatography of Porphyrins

Porphyrins were identified using the method of Jensen, (1962) and the modifications suggested by Zhu, (1987). This procedure separates the porphyrin acids based on the number of carboxylic acid groups. The porphyrin with the least number of carboxylic groups move the farthest up the chromatogram. This system can also resolve copro I and copro III. The developing solvent was a mixture of 2,6-lutidine and water (5:3.5 v/v) and separate beakers of 2,6-lutidine, water and 7N NH_4OH were placed in the bottom of the tank. These were renewed daily. The tank was closed and allowed to equilibrate. Porphyrin acid standards (2, 4, 5, 6, 7, 8 COOH's) as well as uro I , uro III, copro I, copro III, and proto IX standards (Porphyrin Products) and samples were spotted onto TLC sheets (10 X 10 cm, Eastman Kodak Comp., Rochester, NY) until fluorescence could be observed. The chromatograms were then developed for approximate 2.5 h, until the solvent front was approximately about 1 inch from top of sheet.

1.7.11.2 Visible Spectra of Porphyrins

Dilute solutions of uroporphyrin III coproporphyrin III and cell extracts were prepared in 0.5 N HCl (Falk, 1964) and the visible

transmittance and/or absorbance spectra taken from 650 to 300 nm. The spectrum of uroporphyrin III and coproporphyrin III were compared to that of the cell extract of JL1268. The absorption maxima of each spectrum were tabulated.

1.8 RESULTS

1.8.1 Overproduction of ALA in the Medium

E. coli RP523 accumulates ALA because it has a mutation in the structural gene for ALA D and therefore cannot utilize ALA. The accumulation of ALA in the medium is dependent on growth conditions: anaerobic minus hemin> anaerobic plus hemin> aerobic plus hemin> superaerobic plus hemin (Table 1.2).

JL1268 excreted ALA into the medium (Fig. 1.8, Table 1.2). The growth of *E. coli* JL1268 in LB with and without glutamate and the excretion of ALA into the medium was monitored for up to 25 h. At approximately 6 h it was observed that this strain, grown aerobically without glutamate, started to excrete ALA into the medium (Fig. 1.8). Under superaerobic conditions ALA did not appear in the medium for 9 h even though the cells grew equally well under both conditions. When either 0.1% or 0.3% glutamate was added to the medium of superaerobic cultures ALA excretion was observed at an earlier time period. 0.1% glutamate was more effective than 0.3% glutamate in restoring this excretion at 6 and 7 h respectively (Fig. 1.8). Aerobic cells excreted 6.9 times as much ALA as superaerobic cultures; 1.4 times that of superaerobic in 0.1% glutamate; 3.1 times that of superaerobic in 0.3% glutamate (Table 1.3). The specific activity of ALA excretion under superaerobic growth in 0.1% glutamate approached that of aerobic growth (Fig. 1.8). Crude cell free extracts of JL1268 grown aerobically and superaerobically in 0.1% glutamate synthesized ALA from glutamate via the C5 pathway (Table 1.3). It was observed that extracts of *E. coli* JL1268 grown superaerobically in the absence and presence of 0.3% glutamate did

not synthesize ALA in an *in vitro* assay (Table 1.3). Less ALA accumulated in the medium when JL1268 was grown under the latter two conditions (Figure 1.8 and Table 1.3).

The cell pellet of a culture that accumulated ALA in the medium developed fluorescence only at the surface in contact with the air when it was left exposed to the air for 2-3 h. After sonicating the homogenate become fluoresent and the intensity of the fluoresence increased when the extract was allowed to incubate on ice for another 3-4 h

These observations led us to examine the effects of aeration on the transcription of the *hemA* gene of *E. coli*.

1.8.2 Effect of Small Molecule Cofactors on ALA synthesis

The soluble and pellet portions of sonicates of JL1268 each had very low ALA synthesis compared with the whole sonicate, but when they were mixed, activity was restored. The pellet preparation may contain active but insoluble aggregates or membrane-bound species of the cloned protein or other enzymes of the C_5 pathway which is essential for ALA synthesis. Dialysis of the washed and sonicated pellet did not affect its activity. The maximum synthesis of ALA by the combined S and P fractions was dependent on glutamate, NADPH, ATP, and pyridoxal phosphate when S was processed through a Sephadex G-25 column twice to remove small molecules (Weinstein et al., 1985). These results are shown in Table 1.4.

1.8.3 Effect of $tRNA^{glu}$

E. coli $tRNA^{glu}$ stimulated ALA synthesis in a concentration dependent fashion (Fig. 1.9), while nonspecific *E. coli* tRNA was only marginally stimulatory (Table 1.4). Preincubation of $tRNA^{glu}$ with RNase and then addition of extract lowered synthesis of ALA (Fig. 1.10). When it was added to a mixture of $tRNA^{glu}$ and extract, the effect was very weak.

1.8.4 Uroporphyrinogen Accumulation

TLC analysis of the porphyrins formed by air oxidization or by acidification of crude cell free extracts of JL1268 revealed that it was a uroporphyrin (Fig. 1.11) present in the extract. The Rf value of porphyrin in the extract was the same as those for the uroporphyrins (Table 1.5). The visible transmittance spectrum of extract from JL1268 is similar to the spectra of uroporphyrin III (Fig. 1.12) and different from that of coproprophyrin III. The maxima of each spectrum was tabulated (Table 1.6) and demonstrate that the maxima of JL1268 were the same as those of uroporphyrin III.

This data taken along with the absence of fluorescence in the intact cells indicates that JL1268 probably accumulated the uroporphyrinogen under aerobic growth conditions.

1.8.5 Hybridization Studies with a *hemA* Probe

Anaerobic cultures of RP523 grown in hemin to midlog or stationary phase synthesized the greatest amount of *hemA*-specific mRNA (Fig. 1.13 and Fig. 1.14 band C), 170 and 506% of aerobic cultures (Fig. 1.13 and Fig 1.14 band A) respectively (Table 1.8). RP523 grown anaerobically, without hemin to stationary phase made twice as much (200% of aerobic cells, Table 1.8) message as aerobic cells, which was only 40% of anaerobic cells grown with hemin (Fig. 1.13 band D and Table 1.8). At midlog phase, the *hemA* specific mRNA of aerobic cells and anaerobic cells without hemin were approximately equal, 100 and 98% respectively (Fig. 1.16, band A and D, Table 1.8). The presence of ALA in the growth medium of cells grown to midlog under anaerobic conditions lowered the amount of *hemA* mRNA from 98% to 64% (relative to aerobic cells) (Fig. 1.16, band E, Table 1.8). The least amount of message was synthesized under superaerobic conditions in both midlog and stationary phase cells, 66 and 42% (Table 1.8) of aerobic cells respectively (Fig. 1.15 and Fig. 1.16, band B). The effect of exogenous ALA on stationary phase cultures was not determined since this strain excreted ALA into the medium in stationary phase (Table 1.8)

E. coli JL1268 grown under aerobic, superaerobic and anaerobic conditions to both midlogarithmic (6 h) and stationary phases (>16 h) synthesized *hemA* specific mRNA under all three conditions (Fig. 1.13 and Fig. 1.14). Superaerobically more *hemA* mRNA was synthesized in both midlog and stationary cultures, (Fig. 1.13 and Fig. 1.14 band B), 242 and 176% of aerobic cultures (Fig. 1.13 and Fig. 1.14 band A) respectively (Table 1.8). JL1268 made the

least *hemA* mRNA anaerobically, 93 and 67% (Table 1.8) of aerobic cultures for midlog and stationary cultures respectively (Fig. 1.13 and Fig. 1.14 band C). These results are not compatible with the of pattern of accumulation of ALA in the medium of stationary phase cells where the greatest amount of ALA accumulated in the anaerobic cultures and least in the superaerobic cultures.

1.8.6 Hybridization Studies with the *gtlX* Probe

When the oligoprobe for the glutamyl tRNA synthetase gene *(gltX)*, was hybridized with RNA from JL1268 grown under different conditions the strongest signal was obtained with RNA from anaerobic cells and the weakest with RNA from superaerobic cells (Fig. 1.18, bands E-G). Very weak signals were also detected for RNA from RP523 grown anaerobically with (Fig. 1.18, band C) and without hemin (Fig. 1.18, band D). However under our conditions we failed to detect any signal from aerobic or superaerobic cultures.

1.9 DISCUSSION AND CONCLUSIONS

1.9.1 ALA Synthesis Via the C_5 Pathway in *E. coli*

Crude sonicates of JL1268 make appreciable quantities of ALA. The observation that dialyzed P and gel-filtered S fractions were both required for ALA synthesis means that at least two kinds of macromolecules, possibly two enzymes, are required in this pathway. When S was processed through Sephadex G-25 in order to remove small molecules and cofactors, the production of ALA became dependent on glutamate, ATP, NADPH, pyridoxal phosphate, and $tRNA^{glu}$. Levulinate was also required to prevent further utilization of ALA. $tRNA^{glu}$ stimulated ALA synthesis in a concentratin-dependent manner. It is interesting that *E. coli* tRNA type II acts as a specific inhibitor of the C5 ALA-forming system in *Euglena gracilis* (Mayer et al., 1987). Pretreatment of $tRNA^{glu}$ with RNase added after the tRNA had been allowed to interact with proteins in the extract still supported some ALA synthesis. This suggests that when the tRNA is bound it is protected from RNase. Elimination of pyridoxal phosphate from the assay mixture lowered ALA synthesis only partially. The crude and S(1) and S(2) extracts are not very sensitive to gabaculine (data not shown). These results suggest that bound rather than free pyridoxal phosphate or pyridoxamine (Hoober et al., 1988) may be acting as a cofactor.

The patterns for uptake of labeled precursors (LI, 1989; Li et al, 1989), the requirement for at least two kinds of macromolecules, the requirement for cofactors and $tRNA^{glu}$ and the recent purification of *E. coli* glu-$tRNA^{glu}$ reductase (Jahn et al., 1991)

provide compelling evidence that ALA synthesis in *E. coli* follows the C_5 pathway by the series of steps shown in Fig. 1.3.

1.9.2 The Effects of Oxygen on *E. coli hemA* Gene Transcription and ALA accumulation

The effects of oxygen tension on the cytochrome content of the inner membrane of *E. coli* has been well documented (Lascelles, 1964; Shipp, 1972; Ingledew et al., 1984). However not much is known about the effects of oxygen tension on the biosynthesis of the heme prosthetic of the cytochromes. It is clear that wild type *E. coli* synthesizes heme under both aerobic and anaerobic conditions of growth (Jacobs et al., 1969; Shipp, 1972; Hino and Ishida, 1973). In addition *E. coli* synthesizes Vitamin B_{12} under anaerobic conditions (Jeter et al., 1984; Jeter et al., 1987).

We have shown that transcription of the glutamyl-tRNA reductase gene, *hemA*, is regulated by oxygen tension. More *hemA* transcript is present in a hemin-permeable strain, RP523, and a wild type strain, C600, (W. Xiao, C. S. Russell and S. Cosloy, unpublished) under anaerobic conditions as compared to aerobic and superaerobic conditions. This effect is observed for both midlog and stationary phase cultures. The *hemA* transcript of *E. coli* strain, JL1268, which has a *hemA* mutation on the chromosome and is complemented by *hemA* on a multicopy plasmid, did not show regulation by oxygen. It is not customary to study regulation of a gene in a strain with a multicopy plasmid since this does not reflect wild type regulation. However we examined the *hemA* transcript in JL1268 because we observed the absence of ALA synthesis activity in extracts of this

strain grown superaerobically. It was previously demonstrated that under conditions of high density growth, that the plasmid copy number increased in strains of *E. coli* carrying multicopy plasmids (Horn et al., 1990). The presence of the *hemA* transcript in superaerobic cells of JL1268 may reflect an increase in plasmid copy number. Under superaerobic conditions JL1268 excreted less ALA into the medium and extracts of this strain did not synthesis ALA from glutamate. This suggest that the *hemA* gene is transcribed but not efficiently translated under superaerobic growth.

The measurement of ALA accumulation *in vivo* and in the medium in the absence and presence of inhibitors of heme biosynthesis has provided an important technique for studying the physiology and biochemistry of ALA synthesis in higher plants and algae (Beale, 1978). We have used the accumulation of ALA in the medium of *E. coli,* strain RP523, to assess ALA synthesis in this bacterium. More ALA is present in the media of stationary phase, anaerobic cultures of RP523 as compared to aerobic and superaerobic cultures. The least ALA is present in superaerobic cultures (Table 1.2). This result agrees with the *hemA* transcript being highest in anaerobic cultures compared to aerobic and superaerobic cultures each grown with hemin. The mechanism for oxygen control of *hemA* transcription may be mediated by a regulatory protein. It has been reported (Ingledew et al., 1984; Thomas et al., 1972) that when *E. coli* is grown at high partial pressures of oxygen, lower levels of cytochromes are synthesized as compared with anaerobic growth. This may be because at high aeration, ATP synthesis is much more efficient. This observation

may reflect hemin synthesis and suggest that ALA synthesis would therefore be turned down.

1.9.3 The Effect of ALA and Hemin on *E. coli hemA* Transcription and ALA accumulation

The presence of hemin in the medium of RP523 lowers the amount of ALA present in stationary phase, anaerobic cultures as compared to anaerobic cultures grown without hemin (Table 1.3). However we have observed that the *hemA*-specific message is greater when hemin is present in the media under anaerobic conditions (Tables 1.7 and 1.8). This may reflect the fact that the cells grow better (higher Klett units, Table 1.2) in the presence of hemin and therefore make more message. Thus hemin may be exerting its effect at some point post-transcriptionally to lower the amount of ALA synthesized and excreted into the medium. If ALA is acting as a repressor of one of the genes involved in its synthesis then a decrease in ALA may lead to derepression of *hemA* gene. The addition of ALA to the growth medium of anaerobic cultures of RP523, without hemin lowers the amount of *hemA* specific message by 34% for midlog phase cells. This result, taken together with the effect of hemin, suggest that expression of the *hemA* gene is slightly repressed by high levels of exogenous ALA.

1.9.4 The Effect of Carbon Source on ALA Biosynthesis in *E. coli*

The recovery of ALA in the medium of JL1268 grown under superaerobic conditions in 0.1% glutamate may reflect a stringent

response (Grunberg-Manago, 1987) where the availability of glu-tRNA^{glu} may be the molecule regulating the rate of ALA synthesis. Under superaerobic growth conditions glutamate may be rate-limiting and therefore is utilized more economically for protein synthesis and need heme synthesis

1.9.5 Regulatory Features of the Upstream Region of *E. coli hemA* Gene

The upstream region of the sense strand of the *hemA* gene (Li, 1989; Li et al., 1989a) has some interesting features which are comparible with the experimental results.

(a) There are two promoters and two initiation of transcription sites (Verkamp et al., 1989; Mulligan et al., 1986). P1 is at nt 1257-1262, TTTATT and nt 1279-1284, TATGAT. P2 is at nt 1148-1163, TTATAACCCTTAAGC and nt 1175-1188, TCCGCTATCGTC TA. There is an inverted repeat at nt 1217-1233, TAACCGGCTTTCGGCAAT. There are two stem loop structures: stem loop 1 at nt 1014-1029, CCGGGCTTTGCCAGG and stem loop 2 at nt 1025-1043, CCAGGACCTTTGGGCGTGG. Stem loops 1 and 2 both contain the sequence, CCAGGA, but at different sides of the stem. In stem loop 1, this sequence is on the downstream side of the stem and in stem loop 2, it is on the upstream side. There is significant homology between these loops and a stem loop from tRNA^{glu} (CCAGGACACCGCCCUUUCACGGCGGUA) and an upstream sequence in glutamyl-tRNA synthetase (GTR) (CCAGGATTTGCCGGTTGTCCGGCA). Brun et al (1990) observed this homology for GTR and tRNA^{glu} and suggest that it may reflect coordinate regulation of protein

synthesis and heme synthesis. There is strong evidence (Schneegurt et al., 1989; Peterson et al., 1988) that glutamyl-tRNA reductase (GTR) recognizes the UUC anticodon of $tRNA^{glu}$. It is also evident that glutamyl-tRNA synthetase (GTS) binds tRNAGlu. There may therefore be binding of GTS and GTR to their own respective transcripts (self-regulation) or to each other's transcript (reciprocal regulation).

There is a putative cAMP binding site at nt 915-919 with the consensus sequence, ACACT with a less conserved inverted repeat, TGAGA at nt 929-933. A third putative promoter (Mulligan et al., 1986) can be found downstream from these sequences at nt 959-974 (-35), TGATACTGATTAAGAT and nt 985-998 (-10), TTGCTGATGCTGAC. A stringent response sequence, GTCTGA, is at nt 956-961. The complementary strand in this region has an open reading frame (nt 1116-646) transcribed in the opposite direction. Initiation of translation is at nt 1330.

The cAMP-CRP consensus sequence suggests that full expression of hemA requires cAMP-CRP and that the "glucose effect" reflects this.

The inverted repeat suggests binding of a regulatory protein. The oxygen effect may be mediated by a protein whose concentration and or structure is regulated by oxygen (Storz et al., 1990; Cotter et al., 1990).

The stem-loops are downstream from the cAMP-CRP consensus sequence. This also suggests another upstream promoter and initiation of transcription site. It may be that several transcripts of different lengths are made from different promoters which are

under different kinds of control (Verkamp and Chelm, 1989). A longer transcript would incorporate the stem loops and be under the control of GTR and or GTS which might bind to them because of their resemblance to a stem loop of $tRNA^{glu}$.

Table 1.1 The cutting sites of Hinc II on ¥hemA insert gene of *pJL68

Fragment number	Fragment size	Start	End
1	329	+1	+329
2	466	+2	+795
3	674	+796	+1469
4	933	+1470	+2402
5	521	+2403	+2923

¥The hemA gene is 1255 bp (Li et al., 1989a).
*The size of pJL68 is 5787 bp; 2863 bp are vector sequences and 2924 bp are insert sequences.

Table 1.2 Accumulation of ALA by JL1268 and RP523 grown to stationary phase in LB medium

Strain and Growth Condition	Hemin (4 μg/ml)	Growth (Klett)	ALA (mmole/mg protein)
*RP523			
Superaerobic	+	245	1.57 ± 0.12
Aerobic	+	208	1.03 ± 0.08
Anaerobic	+	145	2.27 ± 0.27
Anaerobic	-	104	4.46 ± 0.30
ΨJL1268			
Superaerobic	-	299	0.32 ± 0.12
Aerobic	-	193	1.47 ± 0.39
Anaerobic	-	88	6.54 ± 0.74

Each value represents the average of 4 determinations.
*RP523 was grown glycerol (0.1%) and fumarate (50mM).
ΨJL1268 was grown with 50 μg/ml ampicillin.

Table 1.3 Excretion of ALA into the medium by *E. coli* JL1268 and ALA synthesis from glutamate by cell free extracts

Growth Condition	ALA Excreted (μM)	ALA Synthesis by Extract (μM)
Aerobic	10.30	2.71
Superaerobic	1.50	0.00
Superaerobic + 0.1% glu	7.44	0.52
Superaerobic + 0.3% glu	3.35	0.00

Each value is the average of two determinations

Table 1.4. Effects of cofactors on synthesis of ALA by an extract of *E. coli* JL1268 processed through Sephadex G-25[a]

Assay mix	ALA synthesized nmol/reaction mix	% of Control
Complete	1.80	45
Complete + $tRNA^{Glu}$(0.5 U) (control)	4.00	100
Complete + $tRNA^{Glu}$(0.5 U)		
-Glutamate	1.13	29
-ATP	0.40	10
-NADPH	1.12	28
-Levulinic acid	1.56	39
-Pyridoxal phosphate	2.00	50

[a]The reaction mixture contained 250 µl of S(2) fraction (passed through Sephadex G-25 twice) (1.5 mg of protein), 50 µl of P fraction (0.8 mg of protein), 100 µl of assay mix, and buffer and additions to make 500 µl. The complete reaction mixture contained 100 mM Tricine, pH 7.9, 25 mM levulinic acid and 20 µM puridoxal phosphate. The mixtire was incubated for 2 h. ALA was determined by the method of Weinstein and Beale, (1985).

Table 1.5 R_f values of porphyrin standards from TLC

Type of Porphyrin	R_f
Uro I	0.025
Uro III	0.025
Copro I	0.200
Copro III	0.320
Proto IX	0.420
Extract	0.025

Each value is the average of 4 determinations

Table 1.6 Absorption maxima of uroporphyrin III, coproporphyrin III an extract from JL1268

Absorption maxima (nm)		
Copro III	Uro III	JL1268 extract
798	831	
591	595	595
549	553	552
402	408	408

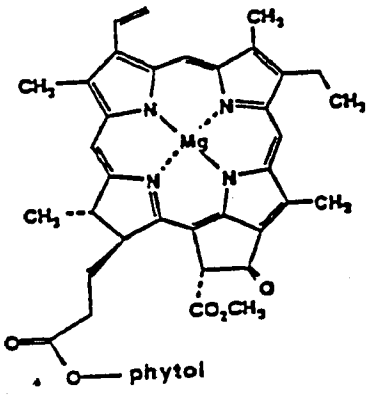
Table 1.7 Densitometry results -area under each peak- of X-ray film that was exposed to chemiluminescence

		Band (AU x mm)			
Growth conditions	Hemin	1	2	3	4
RP523 mid log phase cells					
Aerobic	+	4.09	1.30	1.21	0.76
Superaerobic	+	2.15	0.90	0.55	0.24
Anaerobic	+	4.17	1.94	1.85	1.23
Anaerobic	-	2.27	1.54	1.30	0.99
Anaerobic + ALA	-	1.68	1.29	1.03	0.46
RP523 stationary phase cells					
Aerobic	+	1.25	0.70	0.33	0.23
Superaerobic	+	0.89	0.55	0.36	0.23
Anaerobic	+	7.15	5.82	5.43	3.89
Anaerobic	-	4.32	2.19	1.98	0.43
JL1268 mid log phase cell					
Aerobic	-	3.02	2.91	2.31	1.82
Superaerobic	-	7.39	6.39	5.67	4.85
Anearobic	-	2.90	2.53	2.42	1.45
JL1268 mid log phase cell					
Aerobic	-	2.04	2.91	2.31	1.24
Superaerobic	-	5.75	5.02	2.03	1.71
Anearobic	-	1.95	2.57	1.85	1.05

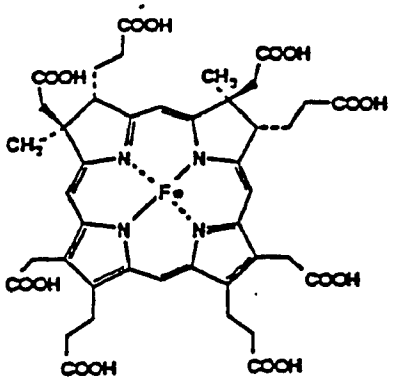
Table 1.8 Densitometric quantitation of slot blots

Strain and Growth Condition	Hemin (4 μg/ml)	Growth Phase: Mid log (% of aerobic signal)	Growth Phase: Stationary (% of aerobic signal)
RP523			
Aerobic	+	100	100
Superaerobic	+	66	42
Anaerobic	+	170	506
Anaerobic	-	98	200
Anaerobic + ALA	-	64	ND*
JL1268			
Aerobic	-	100	100
Superaerobic	-	242	176
Anaerobic	-	93	67

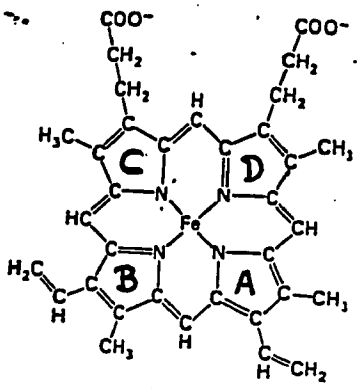
The data is the result of two experiments and each value is the average of eight signals (Table 1.7) for the two experiments.
*The effect of exogenous ALA on anaerobic stationary cell was not determined because this strain excretes ALA in the stationary phase.



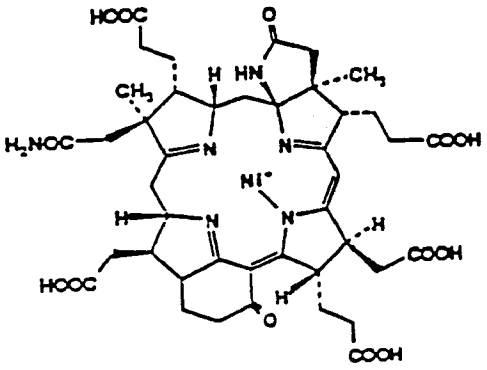
Chlorophyll -*a*



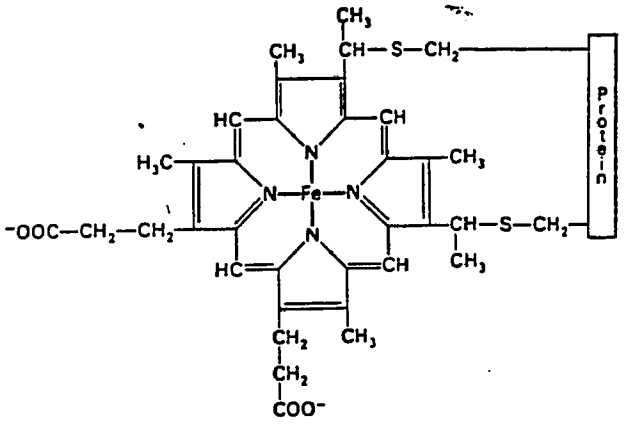
Sirohaem



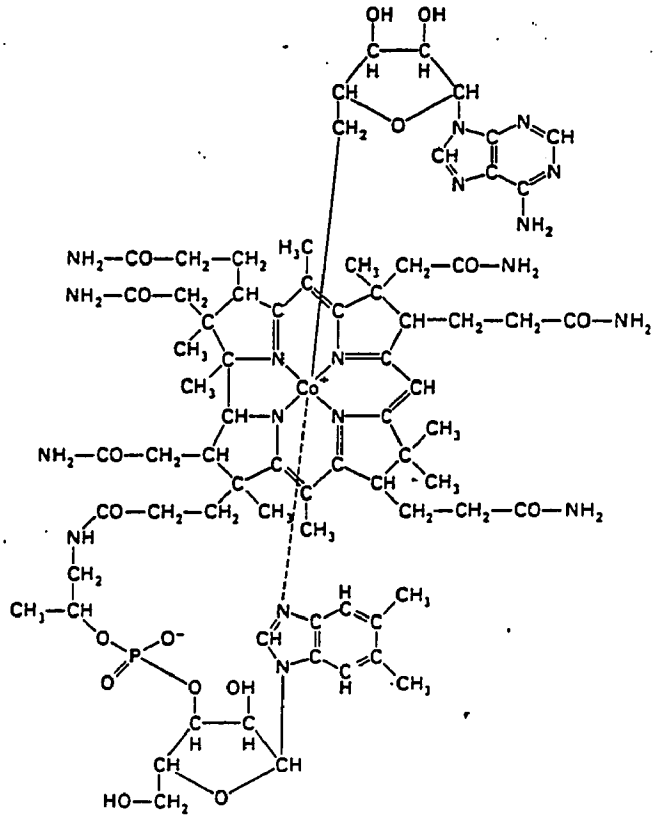
Heme
(Fe-protoporphyrin IX)



Factor F_{430}



Cytochrome C



Vitamin B_{12}

Figure 1.1 Structure of various biological cyclic tetrapyrroles (adapted from Styrer, 1988 and Warren and Scott, 1990).

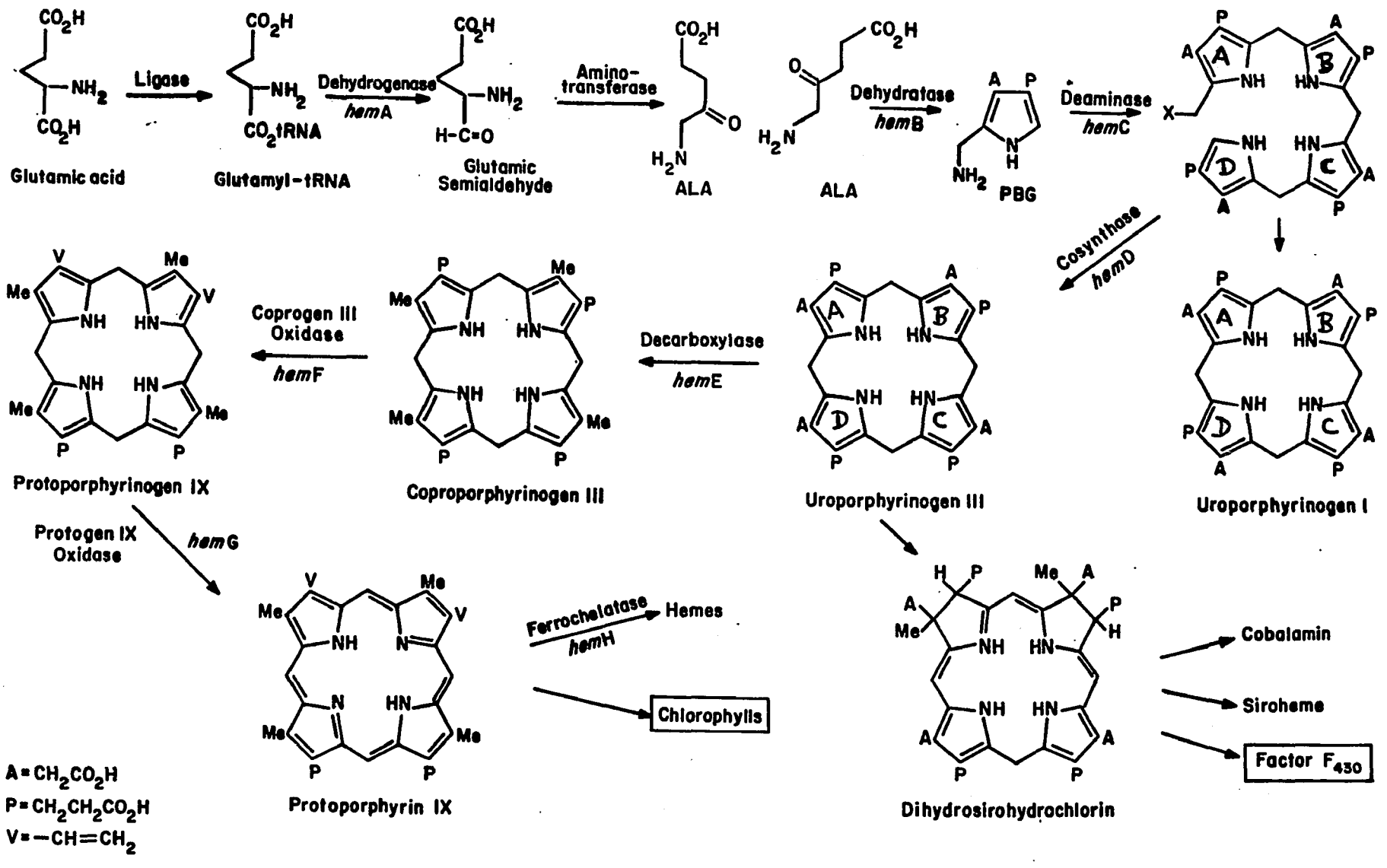


Figure 1.2 The heme biosynthetic pathway of *E. coli* and *S. typhimurium* as it is currently known. The boxed areas are end products of the pathway which are not found in these bacteria.

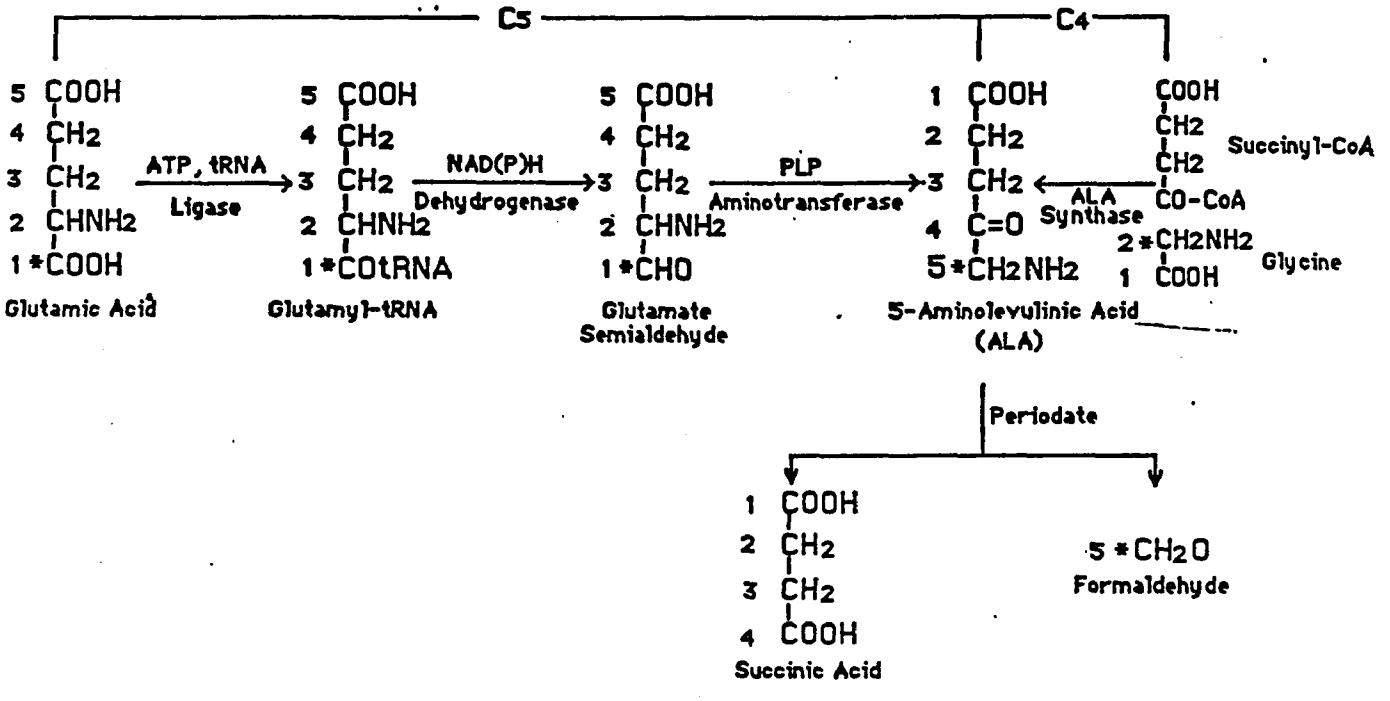


Figure 1.3 5-Aminolevulinic acid biosynthesis via the C_4 and C_5 pathways.

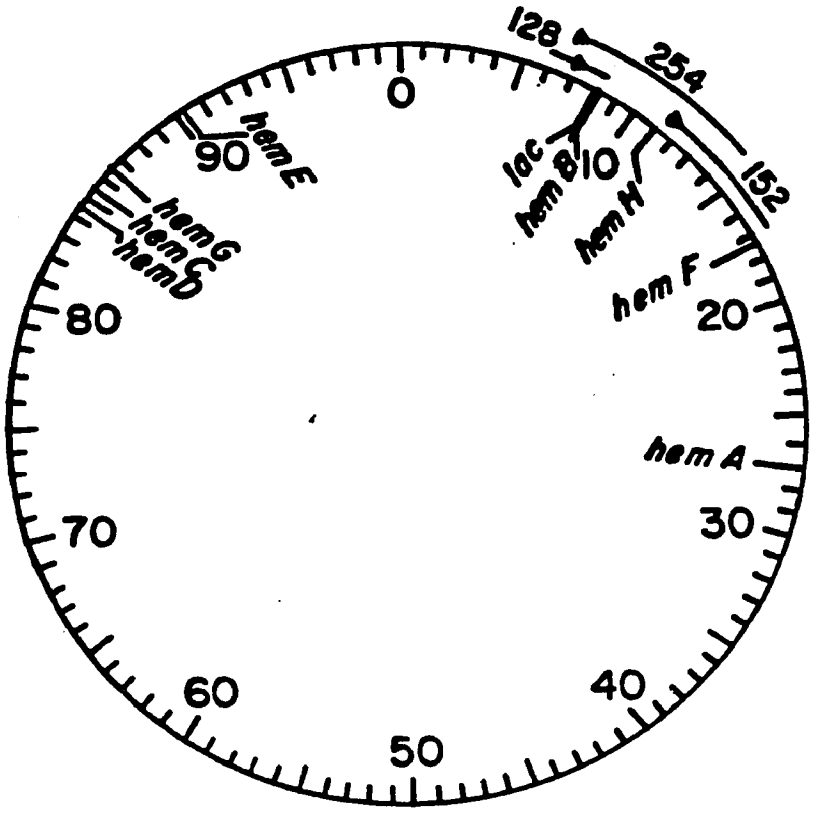


Figure 1.4 Location of heme biosynthetic genes on the *E. coli* K-12 linkage map (Adapted from B. Bachman, 1987).

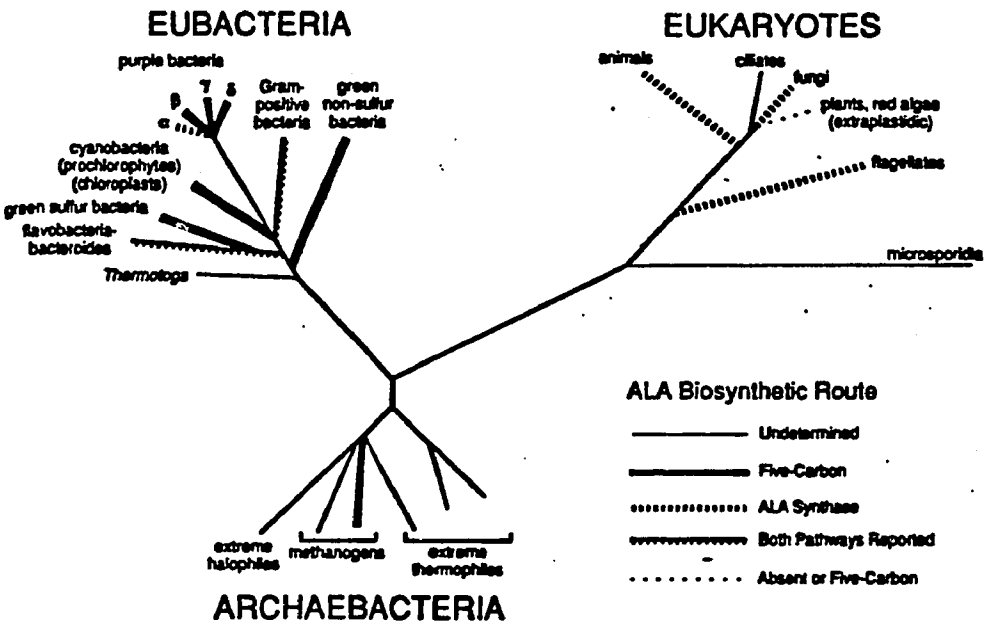


Figure 1.5 Universal phylogenic tree (adapted from Woese, 1987) illustrating the distribution of the two ALA biosynthesis routes.

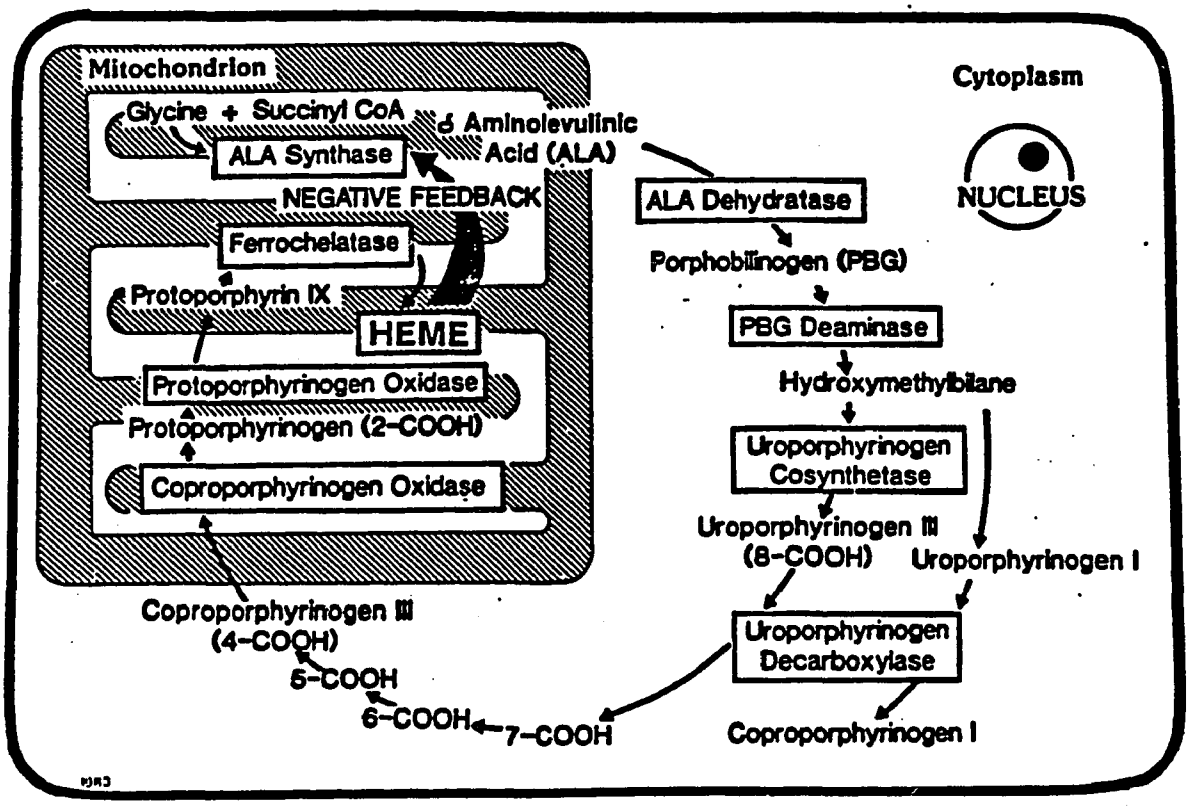


Figure 1.6 Segregation of the heme biosynthetic pathway in eukaryotic animal cells (adapted from Dierks, 1990).

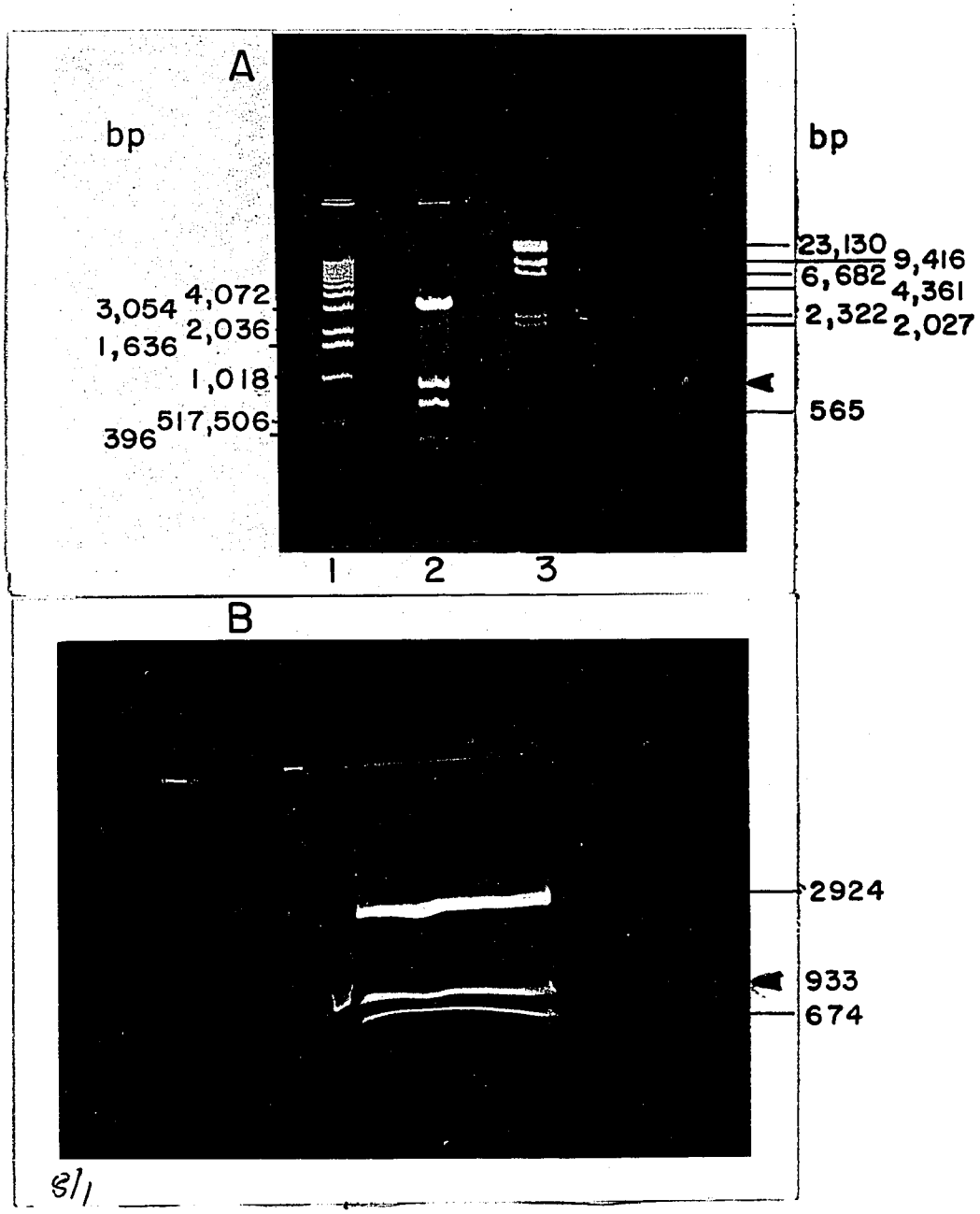


Figure 1.7A Agarose gel electrophoresis of Hinc II digestion of plasmin PJL68. The digestion and electrophoresis was performed as stated in the materials and methods. Lane 1, 1Kb marker DNA ladder (BRL). Lane 2, PJL68 digested with Hinc II showing the 5 fragments expected from *hemA* (Table 1.) and a sixth fragment, the vector sequence of pJL68. Lane 3, marker II DNA (BMB). Arrow indicate 933 base pair fragment.

Figure 1.7B Preparative DNA agarose gel electrophoresis of 933 base pair fragment (arrow). 80 µl of PJL68 digestion mixture was added to the agarose and electrophoresis was performed as stated in materials and methods.

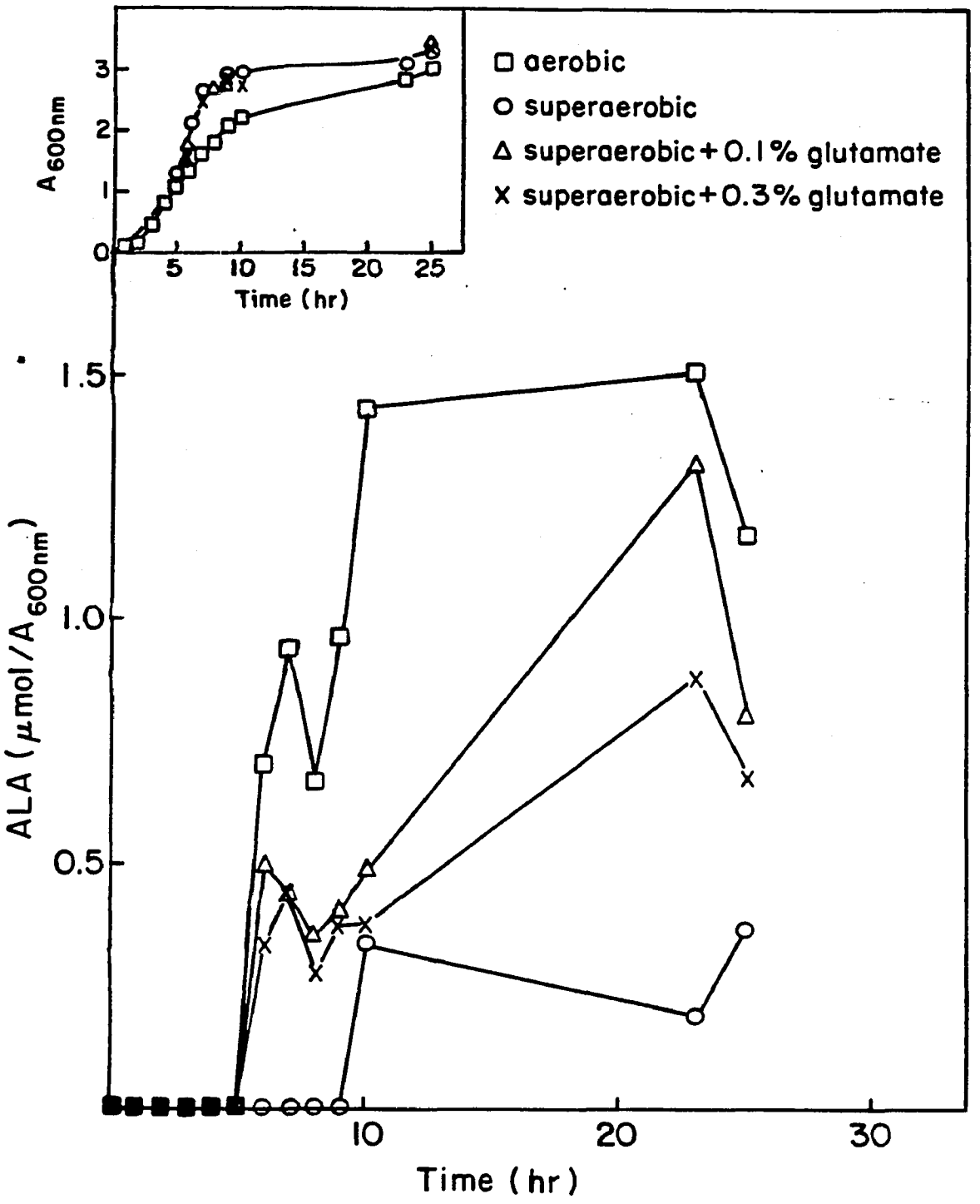


Figure 1.8 The excretion of ALA in to the medium by *E. coli* JL1268 grown under different conditions. Inset the bacterial growth curve. ALA in the medium was determined as stated in materials and methods.

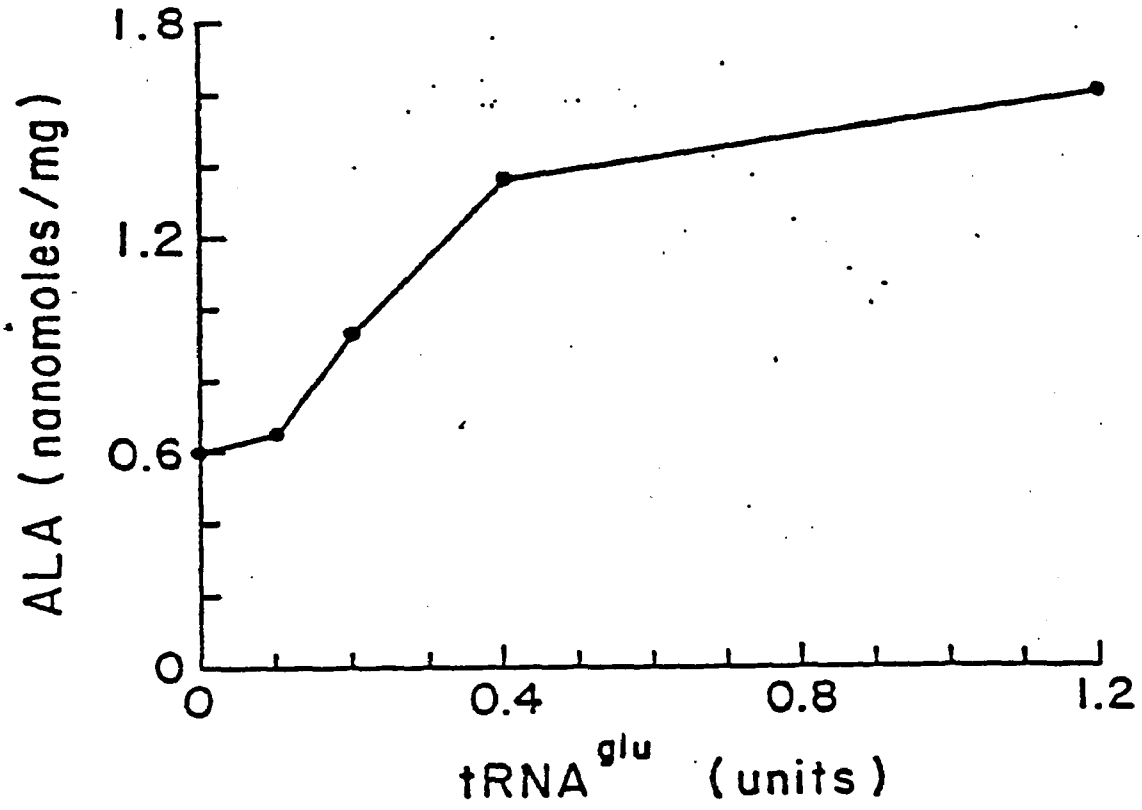


Figure 1.9 The effect of $tRNA^{Glu}$ on the synthesis of ALA by extracts of JL1268. The extracts were processed and the experiment preformed as stated in materials and methods.

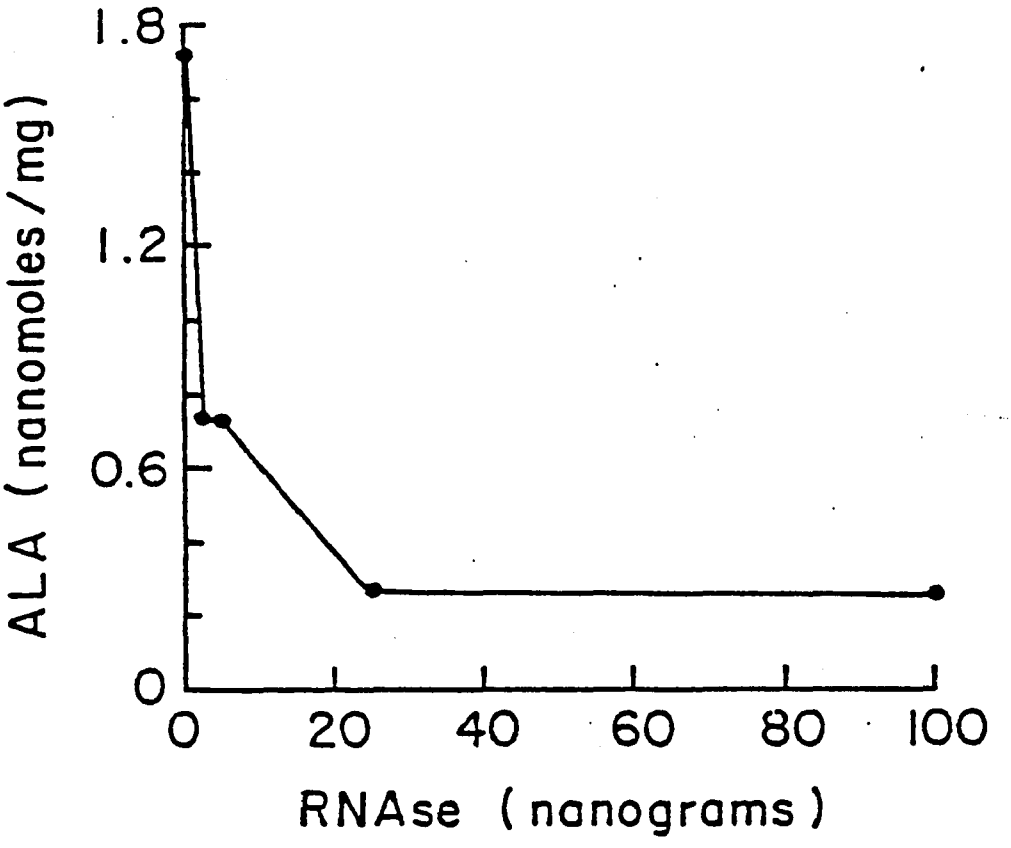


Figure 1.10 The effect of tRNase on the synthesis of ALA by extracts of JL1268. The extracts were processed and the experiment performed as stated in materials and methods.

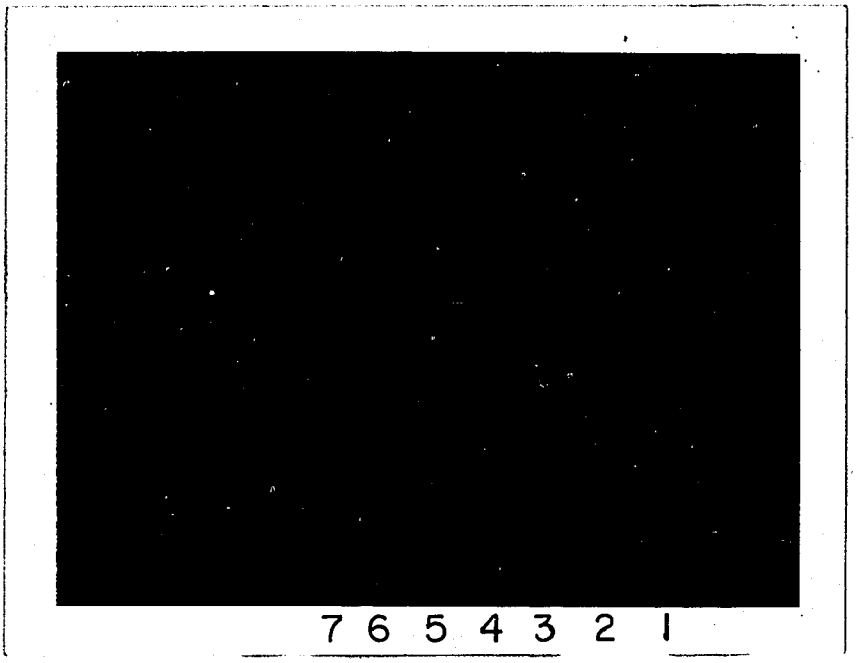


Figure 1.11 Thin layer chromatography of porphyrin acid standards and cell free extracts from *E. coli* JL1268. The porphyrins were spotted and the chromatograph developed as stated in materials and methods. Lanes: (1) uro I (2) uro III , (3) copro I, (4) copro III, (5) proto IX, (6) and (7) JL1268 extract.

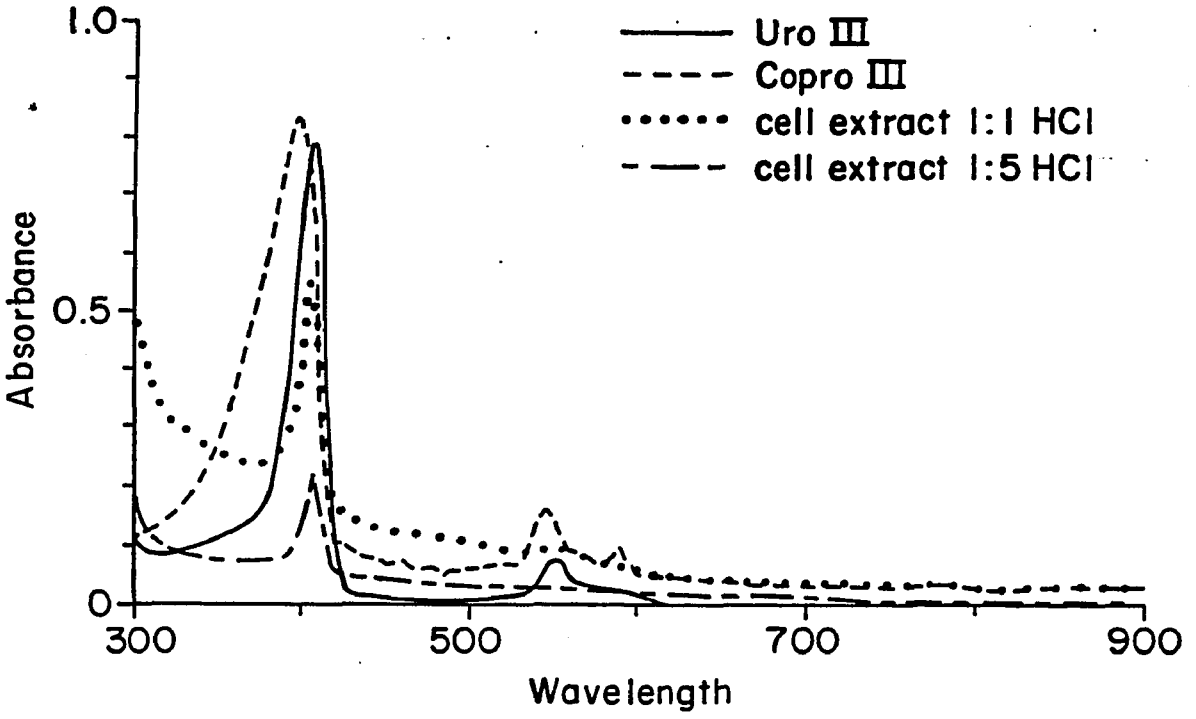


Figure 1.12 Absorbance spectra of coproporphyrin III, uroporphyrin III and extract from JL1268 in 0.5 N HCl.

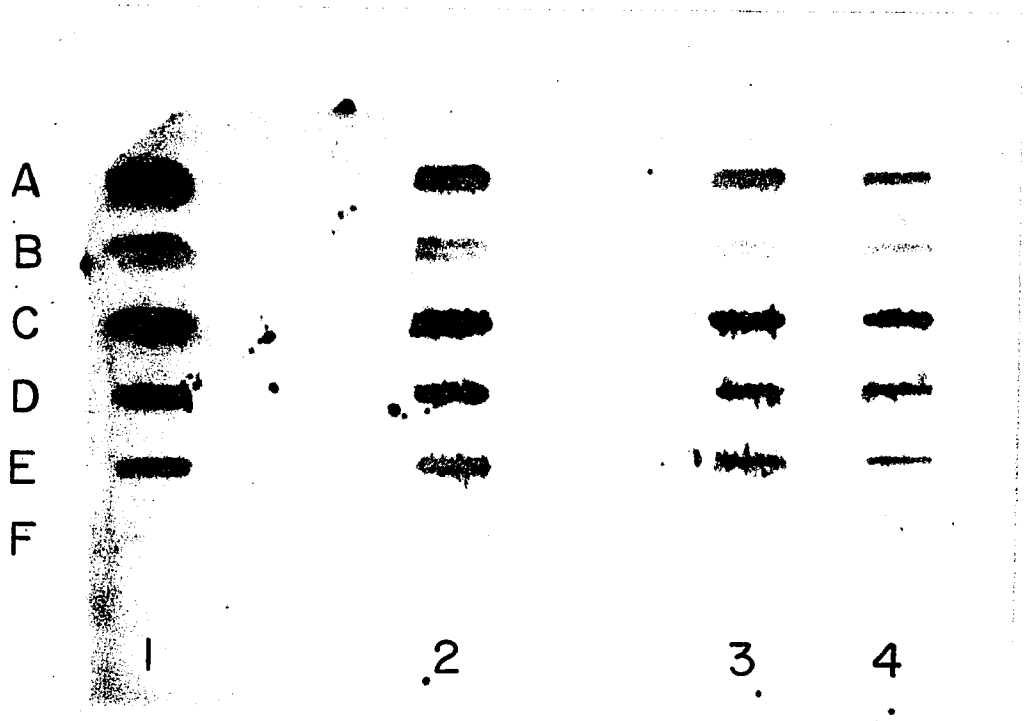


Figure 1.13 Slot blot of RNA from *E. coli* RP523 hybridized to the 933 base pair *hemA* gene probe. Cells were grown in LB to mid log phase and total RNA was prepared as stated in materials and methods. Growth conditions (A) aerobic + hemin; (B) superaerobic + hemin; (C) anaerobic + hemin; (D) anaerobic; (E) anaerobic + ALA and (F) *R. spheroides* (negative control). The μg of RNA applied to the nylon membrane are as follows: (1) 50; (2) 25; (3) 12.5 and 6.25 μg.

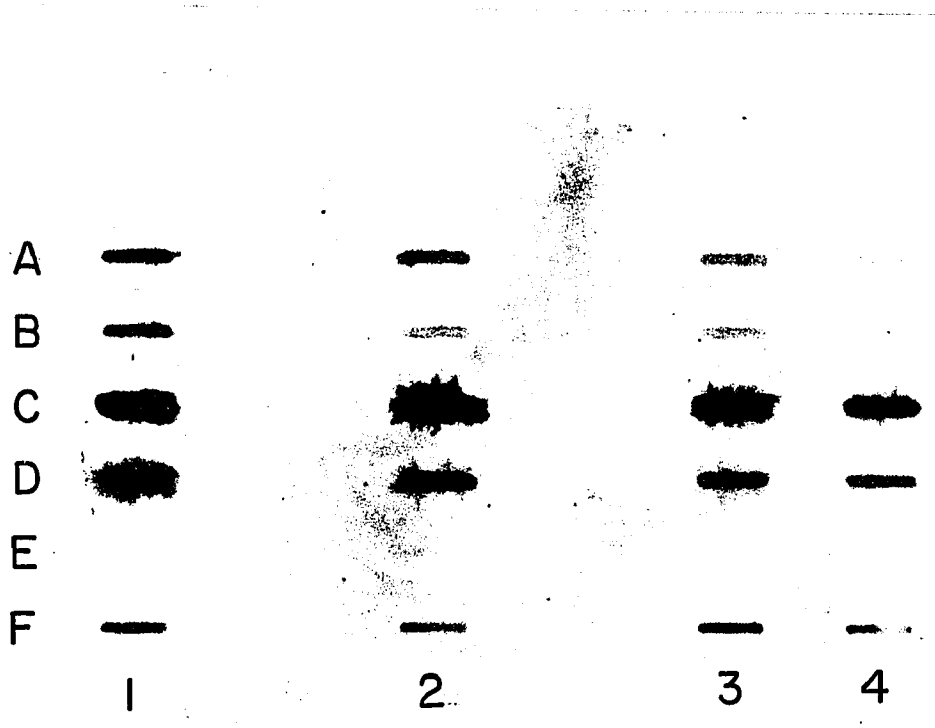


Figure 1.14 Slot blot of RNA from *E. coli* RP523 hybridized to the 933 base pair *hemA* gene probe. Cells were grown in LB to stationary phase and total RNA was prepared as stated in materials and methods. Growth conditions (A) aerobic + hemin; (B) superaerobic + hemin; (C) anaerobic + hemin; (D) anaerobic; (E) *R. spheroides* (negative control); and (F) JL1268 (positive control). The μg of RNA applied to the nylon membrane are as follow: For RP523 and *R. spheroides* (1) 50; (2) 25; (3) 12.5 and 6.25 μg. For JL1268 the corresponding amounts were 10, 5, 2.5 and 1.25 respectively.

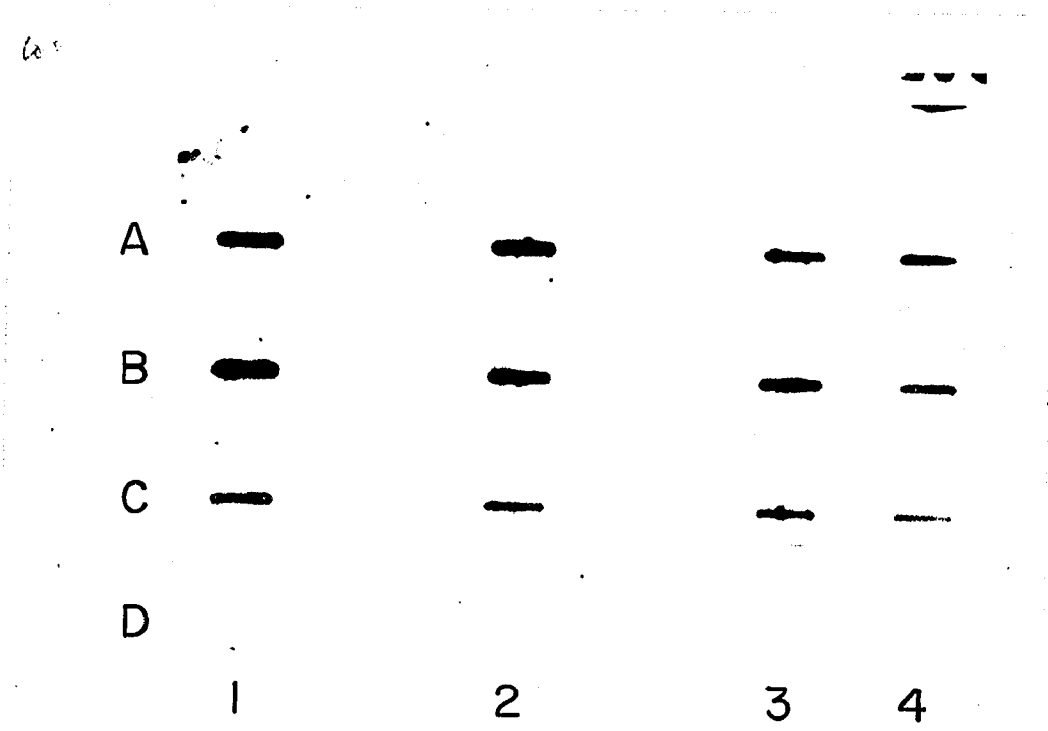


Figure 1.15 Slot blot of RNA from *E. coli* JL1268 hybridized to the 933 base pair *hemA* gene probe. Cells were grown in LB to midlog phase and total RNA was prepared as stated in materials and methods. Growth conditions (A) aerobic; (B) superaerobic; (C) anaerobic and (D) *R. spheroides* (negative control). The µg of RNA applied to the nylon membrane are as follow: (1) 10; (2) 5; (3) 2.5 and 1.25 µg.

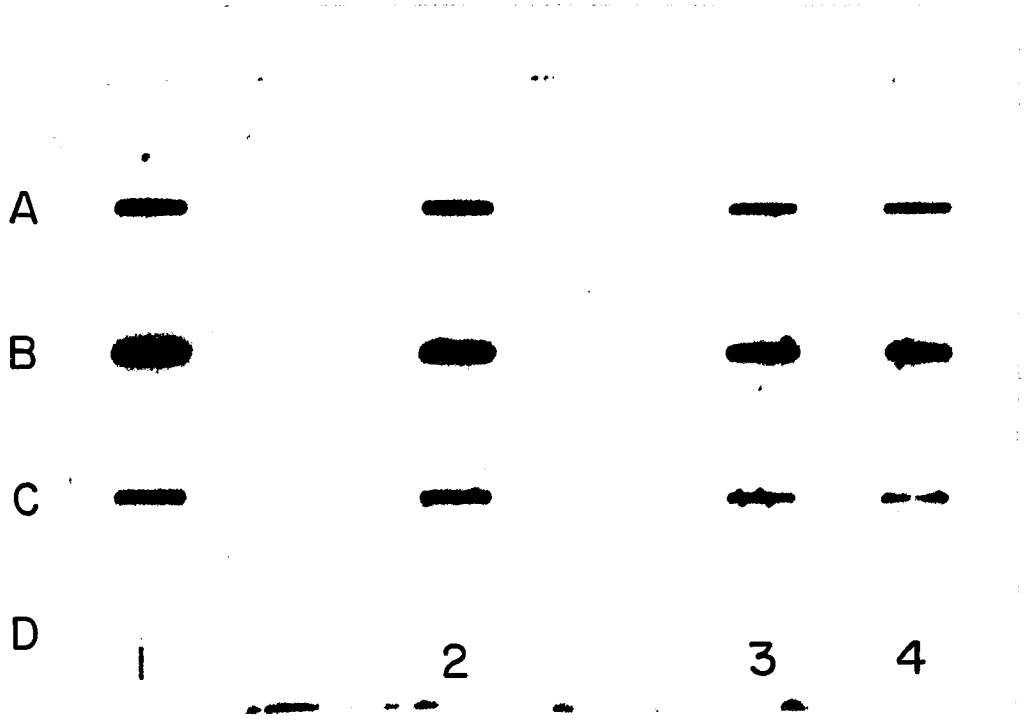


Figure 1.16 Slot blot of RNA from *E. coli* JL1268 hybridized to the 933 base pair *hemA* gene probe. Cells were grown in LB to stationary phase and total RNA was prepared as stated in materials and methods. Growth conditions (A) aerobic; (B) superaerobic; (C) anaerobic and (D) *R. spheroides* (negative control). The μg of RNA applied to the nylon membrane are as follow: (1) 10; (2) 5; (3) 2.5 and 1.25 μg.

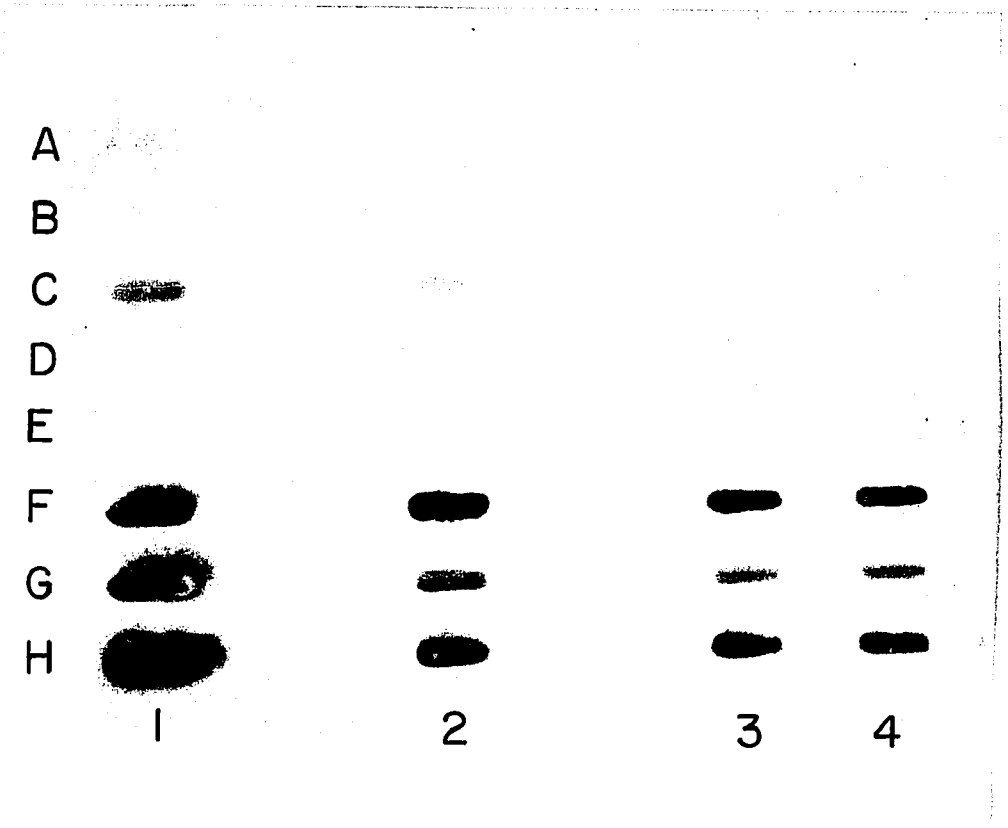


Figure 1.17 Slot blot of RNA from *E. coli* RP523 and JL1268 hybridized to the *gltX* gene probe. Cells were grown in LB to midlog phase and total RNA was prepared as stated in materials and methods. Growth conditions A-E RP523 (A) aerobic + hemin; (B) superaerobic + hemin; (C) anaerobic + hemin; (D) anaerobic; (E) anaerobic + ALA; and F-H JL1268 (F) aerobic; (G) superaerobic and (H) anaerobic . The µg of RNA applied to the nylon membrane are as follow: For RP523 (1) 50; (2) 25; (3) 12.5 and 6.25 µg. For JL1268 the corresponding amounts were 10, 5, 2.5 and 1.25 µg RNA respectively.

Chapter
2

5-AMINOLEVULINIC ACID DEHYDRATASE

2. INTRODUCTION

2.1 General Properties of ALA D

5-Aminolevulinic acid dehydratase (ALA D) (porphobilinogen synthase; 5-aminolevulinate, hydro-lyase, EC 4.2.1.24) catalyses the dimerization of two molecules of 5-aminolevulinic acid in a Knorr condensation with the elimination of two molecules of water to form porphobilinogen (PBG) (Gibson et al.,1955). Lascelles (1956) showed that *R. spheroides* converted ALA into porphyrins and bacteriochlorophyll anaerobically but only in the light and aerobically in the dark. The enzyme, ALA D, is ubiquitous in nature and has been purified from a variety of sources including *R. spheroides* (Nandi et al., 1968), *C. tetanomorphum* (Brumm et al., 1981), *Erythrobacter Sp.* (Shioi and Doi, 1988) and yeast (Borralho et al., 1990), bovine liver (Gibson et al., 1955; Schearer, 1972; Wu et al., 1974; Shemin, 1976; Stella et al., 1977; Hasnain et al., 1985), human erythrocytes (Gibbs et al., 1985) (Table 2.1). Several general properties of ALA D from various sources are summarized in Table 2.1. The enzyme is cytoplasmic and is obtained in the soluble fraction, but in *E. gracilis* a small amount is bound to the chloroplast (Ebbon and Tait, 1969). ALA D isolated from *R. spheroides* (Nandi et

al., 1968); yeast (Borralho et al., 1990); mouse liver (Coleman, 1966); bovine liver (Gibson et al., 1955; Schearer, 1972; Wilson et al., 1972;) and human erythrocytes (Gibbs et al., 1985) is a relatively thermostable enzyme which allows a heat-treatment step in purification. ALA D from many sources has subunit structure, being composed of six to eight subunits (Shemin, 1972; Wu et al., 1974; Jaffe et al., 1984). The native enzyme from *R. spheroides,* which is oligomeric, forms aggregates (dimers, trimers, and tetramers) in the presence of K^+ with specific activity being highest when K^+ is present (Nandi and Shemin, 1968a). The plot of enzyme activity versus ALA concentration for the enzyme is hyperbolic with some exceptions. The enzyme from *R. spheroides* assayed in the absence of K^+ (Nandi and Shemin, 1968a), and the enzyme from *Neurospora* (Muthukrishnan et al., 1972) both gave sigmoidal curves. The V_{max} of enzyme activity for the *R. spheroides* ALA D in the absence of K^+ is only half of the same enzyme assayed when K^+ is present (Nandi and Shemin, 1968a).

5-Aminolevulinic acid dehydratase from most sources is a metalloenzyme, and zinc (Zn^{2+}) appears to be the metal of choice for *E. coli,* yeast, rat, bovine and human (Finelli et al., 1974; Finelli et al., 1975; Wu et al.,1974). Other metal ions have been found associated with the ALA D from other sources; Mg^{++} and K^+ in *M. phlei* (Yamaski and Moriyama, 1971); Mg^{++} and Mn^{++} in *S. itersonnii* (Ho and Lascelles, 1971); K^+ in *R. spheroides* (Nandi et al., 1968; Heyningen and Shemin, 1871) and Mg^{++} in bajra seedlings (Prasad et al., 1988). Mouse liver ALA D showed the unusual property of being

stimulated by Hg^{+2} and Fe^{+2} at μM concentrations and by EDTA (Coleman, 1966).

The mammalian enzyme is a homo-octamer with molecular weight of 242,000 to 285,000 and subunit size of 31,000 to 35,000 (Table 2.1) and appears to contain eight zinc atoms per enzyme molecule (Cheh et al., 1973; Shemin, 1976). This finding was confirmed by Jaffe et al., (1984) who showed that methylmethanethiosulfonate-modified apo-ALA D contained less than 0.1 Zn^{+2}/mole modified apo-ALA D. Only four Zn^{2+} ions were shown to be necessary for maximum catalytic activity (Bevan et al., 1980; Jaffe et al., 1984). It was further demonstrated that sulfhydryl groups are necessary for binding of Zn^{2+} to the enzyme (Bevan et al., 1980). The replacement of zinc by cadmium in bovine ALA D resulted in an active enzyme with the effect on the enzyme being dependent on the pH profile (Schlosser and Beyerman, 1987). The quarternary structure of the enzyme is thought to be either a cube with a uniform edge length (Wu et al., 1974) or a less symmetrically-structured square arrangement of four stacks composed of two monomeric subunits (Pilz et al., 1988). The kinetics of dissociation of the quaternary ALA D immobilized on a solid support and subsequent reassociation of the subunits of the subunits have been studied (Batlle et al., 1978; Gurne et al., 1977). It was demonstrated that half of the enzyme remained bound to the support and was active. This data support the proposal of "half of sites reactivity". Two cysteines per dimer have been proposed to be sufficiently close in the quaternary structure to form a disulfide bond which is important in the regulation of the enzyme (Barnard et

al., 1977). These cysteine residues are alkylated by thiol active site-directed reagents (Wu et al., 1974; Seehra and Jordan, 1981). There are four active sites per octameric enzyme molecule (Wu et al., 1974). Seehra et al. (1981) have proposed that there are four sulfhydryl groups per mole of subunit; two involved in catalysis or substrate binding and two that are structural. It has been proposed that in the native enzyme zinc is four-coordinated to three cysteine sulfurs and one nitrogen or oxygen (Hasnain et al., 1985). These zinc atoms are thought to be located close to the active site and to influence the ionization of essential amino acid residues (Schlosser et al., 1987). However neither zinc nor reduced sulfhydryl groups are required for the formation of the first intermediate, the Schiff base (Jaffe et al., 1986; Jaffe et al., 1987), proposed for the enzyme mechanism (Nandi and Shemin 1968b) (Fig., 2.1). Two types of zinc binding sites have been proposed. Site A contains two or three histidine ligands and one or two additional oxygen-donating groups from protein or solvent. This is proposed to be the catalytic zinc, the site that is required for full catalytic activity of ALA D. Site B is the cysteine rich site and is proposed to have a structural role (Dent et al., 1990). All the current data therefore suggest a catalytic role for zinc once the enzyme has formed a Schiff base.

The bovine enzyme was reversibly inhibited by the metal ion chelators, EDTA, 1,7-phenanthroline and 1,10-phenanthroline (Bevan et al., 1980; Jaffe et al., 1984). EDTA has been shown to inhibit the enzyme from many sources, the exceptions being the enzyme from S. itersonii (Ho et al., 1971) Rhodopseudomanas (Nandi et al., 1967; Heyningen et al., 1971; Nandi and Shemin, 1973) and spinach

(Liedgens et al., 1983) (Table 2.1). In contrast and as previously stated the ALA D from mouse liver shows the unusal property of being activated by EDTA (Coleman, 1966). Lead (Pb^{+2}), an important environmental pollutant is a potent inhibitor of ALA D from many sources including *R. capsulata* (Nandi and Shemin, 1973); yeast (Borralho et al., 1990); wheat (Nandi et al., 1967); guinea pig (Weissberg and Voytek, 1974); rat (Fujita et al., 1981; Goering et al., 1987); bovine (Gibson et al., 1955); and human (Anderson et al., 1979; Gibbs et al., 1985). The synthesis of ALA D protein was increased in rats fed Pb^{+2} in their diets; however the enzyme activity of ALA D was decreased (Fujita et al., 1981). The assay of ALA D activity and the accumulation of ALA in blood and urine has been used as a sensitive indicator of lead poisoning (Meredith and Moore, 1978; Sassa, 1978). Other heavy metal inhibitors include Cu^{+2}, Hg^{+2}, Ag^{+2} and Co^{+2} (Coleman et al., 1966; Gibson et al., 1955; Nandi et al., 1967).

Other known inhibitors of ALA D are sulfhydryl-directed reagents and γ-keto compounds containing a succinyl moiety (Fig. 2.2). Included among the sulfhydryl reagents are iodoacetic acid (IAc), iodoacetamide (IAm), p-chloromercuribenzoate (PCMB), N-ethylmaleimide (NEM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). IAc, IAm, PCMB, and NEM had been shown to inhibit the ALA D from *R. capsulata* (Nandi and Shemin, 1973); bajra seedlings (Prasad et al., 1988); wheat leaves (Nandi et al., 1967) bovine liver (Gore et al., 1976), and human erythrocytes (Anderson et al., 1979; Barnard et al., 1977; Gibbs et al., 1985). Spinach ALA D was shown to be insensitive to inhibition by IAm and to have a low cysteine

content (Liedgens et al., 1983). The product of ALA D, PBG, partially protected the bovine enzyme from inactivation by IAm (Barnard et al., 1977)

The γ-keto related inhibitors include levulinic acid (LA), succinylacetone (SA, 4,6-dioxoheptanoic acid), succinylacetone-ALA (SA-ALA) pyrrole, and 4,5-dioxovaleric acid (DOVA). Succinylacetone has been shown to be a metabolite in the serum of patients with hereditary tyrosinemia, severely inhibiting ALA D in these patients (Lindblad et al., 1977). These γ-keto related compounds have been shown to inhibit the ALA D from *R. spheroides* (Nandi and Shemin, 1968b); *R. capsulata* (Nandi and Shemin, 1973); *S. itersonii* (Ho and Lascelles, 1971); *C. tetanomorphum* (Brumm et al., 1981); *Erythrobacter* (Shioi and Doi, 1988a); mouse liver (Ebert et al., 1979); bovine (Gibson et al., 1955) and human (Lindblad et al., 1977; Gibbs et al., 1985).

Unlike two other enzymes of the heme biosynthetic pathway, ALA S and PBG D, there are no known isozymes of ALA D. However a recent report demonstrated that in rat, ALA D from hepatic tissue may be a different molecular specie having a different K_m, pH optimum, and response to succinylacetone, than that of renal tissue (Roth et al., 1990). ALA D synthesis in mouse liver is regulated by a single nuclear gene at the levulinate (*Lv*) locus, which has at least two co-dominant alleles (Russell et al., 1963; Coleman, 1966; Doyle et al., 1969).

2.2 Nature of the Active Site and Mechanism of Action of ALA D

Eight molecules of ALA must bind to the active site of ALA D before four molecules of PBG are formed by an asymmetric condensation of two ALA molecules (Fig. 2.1). The mechanism of action of ALA D first proposed by Nandi and Shemin (1968b) showed that the first molecule of ALA, and some substrate analogues, formed a Schiff base at the active site of the enzyme. This ALA molecule was proposed to become the acetic (A) side of PBG, while the second molecule become the propionic (P) side. A mixed pyrrole was formed between ALA and levulinic acid when ALA was the first molecule demonstrating that the amino group of the substrate was necessary for Schiff base formation (Nandi and Shemin 1968b). More recently, single-turnover experiments have shown that the first ALA molecule forms a Schiff base at the P side (Fig. 2.1) and the second ALA forms the A side (Jordan et al., 1980a, b; Jordan et al., 1985). These results have been confirmed and extended by NMR experiments on ^{13}C, ^{15}N-labeled ALA trapped on apo-ALA D and holo-ALA D by reduction of the Schiff base with sodium borohydride. The first ALA forms an imine tautomer with E stereochemistry with C5 trans to the lysine methylene group (Fig. 2.1B) (Jaffe et al., 1990). The octameric enzyme however trapped only four moles of substrate or analogue per mole of enzyme, confirming four active sites per octameric enzyme or four active sites in ALA D (Seehra et al., 1981). There are therefore two substrate molecules per active site, that is half the sites reactivity (Wu et al., 1974; Batlle et al., 1978; Jaffe et al., 1984). The enzyme group involved with the binding and Schiff

formation of ALA to the bacterial, bovine and human enzyme has been identified as a lysine residue (Nandi, 1978; Gibbs and Jordan, 1986). There have been several proposals for the other amino acid residues that are present at the active site of ALA D; a histidine residue in maize (Maralihall et al., 1985); a cysteine in bovine (Chaudhry and Jordan, 1976; Gore et al., 1976; Jordan et al., 1976; Tsukamoto et al., 1979); and an arginine residue in spinach (Liedgens et al., 1983) have all been proposed to be present at the active site and are thought to be essential for binding of ALA to the enzyme. It has been suggested the a positive charge present at the active site is important for the binding of ALA (Maralihall et al., 1985).

2.3 Nucleotide Sequence of Structural Gene of ALA D and Amino Acid sequence of the Enzyme

The amino acid sequences of ALA D from the *E. coli hemB* gene (Echelard et al., 1988; Li et al., 1988); yeast *hem2* gene (Myers et al., 1987); rat liver (Bishop et al., 1986); and human (Wetmur et al., 1986a, b) derived from the nucleotide sequences of the cloned structural genes show considerable homologies. The amino acid sequence of *E. coli* ALA D is 36, 40, and 40% homologous to yeast, rat and human, respectively. Shorter amino acid sequences of stronger homology (84.2%) in the above four sequences include the zinc binding site (Berg, 1986; Berg, 1988; Vallee et al., 1990) and the active site lysine (Li et al., 1989). The amino acid sequence surrounding the active site lysine is highly conserved (Li, 1989; Jordan, 1990). The four sequences gave similar hydrophilic and

hydrophobic segments with the zinc binding-site and the active site lysine being both in hydrophobic segments (Li, 1989).

2.4 Porphyrias in Humans Related to ALAD

The porphyrias are a group of disorders of heme biosynthesis resulting in specific patterns of overproduction of heme precursors associated with characteristic clinical features. Each porphyria is the result of a specific decrease in the activity of one of the enzymes of the heme biosynthetic pathway. The porphyria associated with ALA D is termed Doss porphyria after Doss et al., (1979) who first described it in two young adults. This form of acute hepatic porphyrias was characterized by a large increase in excretion of ALA and coproporphyrin (mainly copro III) in urine; PBG was only moderately elevated; fecal excretion of porphyrins was normal, but erythrocyte porphyrins were slightly increased. Thunell et al., (1987) more recently described a case of this porphyria in a much younger patient who had suffered several attacks including nervous palsey during the first two years of life. The patient showed moderate increases in fecal porphyrins (mainly harderoporphyrin, a tricarboxyporphyrin which represents an intermediate step between copro and proto). The pattern of overproduction of heme precursors in Doss porphyria closely resembles that of severe lead poisoning. However it can be differentiated by normal blood and urine levels of lead and the activity of ALA D cannot be restored by dithiothreitol.

2.5 Regulatory Role of ALA D

Heme is one of the end products of the heme biosynthetic pathway (Fig.1.2). Therefore it may act as a feedback regulator of one of the early steps of this pathway. Hemin has been shown to be a strong inhibitor of ALA D from bovine liver (Schearer, 1972), mouse liver (Coleman, 1966; Doyle & Shimke, 1969; Coleman,1970), human erythrocytes (Calissano et al., 1966), *R. spheroides* (Burnham and Lascelles, 1963, Nandi et al., 1968) and a weak inhibitor of *R. capsulata* ALA D (Nandi and Shemin, 1973). A summary of the effects of hemin on the the ALA D from various sources is included in Table 2.1.

The possible regulatory role of ALA D, as the rate limiting enzyme of the heme biosynthetic pathway, in *P. shermanii* has been proposed. Like *E. coli, P. shermanii* also synthesizes vitamin B_{12} only anaerobically. When anaerobic cultures of *P. shermanii* were shifted to aerobic growth it was demonstrated that both ALA S and ALA D activity were reduced concomitantly with decreased synthesis of vitamin B_{12} (Menon et al., 1967). This suggests regulation by oxygen of these two enzymes of heme biosynthesis in this bacteria. The enzyme has been proposed to have a regulatory role in *N. crassa* (Muthukrishnan et al., 1968); *E. gracilis* (Ebbon and Tait, 1969); *S. cerevisiae* (Jayaraman et al.,1971; Labbe-Bois et al., 1977); and in higher plants (Rebeiz et al., 1973; Tchuinmogne et al., 1989). In *N. crassa* ALA D activity was decreased in cultures grown in iron deficient medium and could be restored when Fe^{+3} was added. Hemin had little or no effect when added to the growth medium but protoporphyrin IX decreased ALA D activity by 63 percent. This

suggested that ALA D synthesis is repressed by protoporphyrin and that iron relieves this repression (Muthukrishnan et al., 1968; Muthukrishnan et al., 1969). Subsequent work on the purified enzyme showed that *in vitro* neither protoporphyrin nor hemin inhibited the enzyme but coproporphyrin III and coproporphyrinogen III were strong inhibitors (Muthukrishnan et al., 1972). These authors proposed the presence of a small molecule activator and a protein inhibitor of ALA D in *Neurospora*. ALA D activity increased concomitantly with increase oxygen uptake during the derepression phase (glucose depletion of the medium) in *S. cerevisiae*. The enzyme was repressed by growth on glucose (Jayaraman et al., 1971). Zinc (Zn^{2+}) added to the medium of *U. sphaerogena* stimulated the synthesis of ALA D in this organism (Komai et al., 1968).

In vivo and in an in *vitro* transcription-translation system, hemin had no effect on *E. coli* ALA D activity and the level of translation of *hemB* respectively (Umanoff, 1990).

2.6 PURPOSE OF THIS STUDY

This report demonstrates the purification and characterization of 5-aminolevulinic acid dehydratase from Escherichia coli. The effects of various activators, inhibitors and the kinetics of inhibition of some of the inhibitors are discussed.

2.7 EXPERIMENTAL

2.7.1 MATERIALS AND METHODS

Tween-80 PEG (15-20,000), PVP (40,000), dimethylamino-benzaldehyde, 5-Aminolevulinic acid, SDS molecular weight standards, DTT, MMTS, iodacetamide, PHMB (sodium salt) SA, LA, hemin, and Trisma base, were obtained from Sigma (St. Louis, MO). Ammonium persulfate, DEAE (cellex D), EDAC, protein concentration standard and color reagent were obtained from BioRad, (Richmond, CA). Acrylamide and Bisacrylamide were obtained from National Diagnostic (Manville, NJ). ß-mercaptoethanol was obtained from J. T. Baker (Phillipburg NJ). CN-Br-activated Sepharose 4B, Sephadex G-25, and Sepharose 6B were obtained from Pharmacia, (Piscataway, NJ). 1,2-diaminoethane, ethyl acetoacetate and (2,4-pentanedione) were obtained from Aldrich, (Milwaukee, WI). TSK Butyl Toyopearl was obtained from Supelco Inc., (Bellefonte, PA). Dextran (75,000) was obtained from Refined Syrups and Sugars Inc. (Yonkers NY).

2.7.1.1 Low Density Growth

E.*coli*, strain JL1002 (Li et al., 1988) (Table 1.0) was cultured by inoculating 2-5 ml of cells from an overnight culture into 1 liter of LB containing 30-40 mg per liter ampicillin in a 4 liter flask. The cells were incubated at 37^{0}C on a gyratory type shaker platform in the warm room.

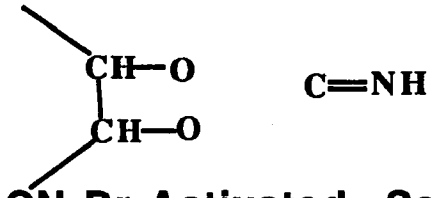
2.7.1.2 High Density Growth

Alternatively, *E. coli* JL1002 cells were grown in a Lab-Line high density fermentor using a modified previously specified medium (Grubmeyer et al. 1986) containing, per liter, 20 g of Bacto-tryptone, 10 g of yeast extract, 5 g NaCl, 5 ml of 0.1 M $CaCl_2$, 5 ml of 1 M $MgSO_4$, 1 ml of 10 mM $Fe(NH_4)(SO_4)_2$, 7.19 g of KH_2PO_4, 60.48 g K_2HPO_4 and 10 ml of glycerol. 25 ml aliquots were removed at time intervals and assayed for ALA D specific activity. Each time an aliquot was removed 100 ml of 50% glycerol was added. The pH of the medium was maintained between 6.5 and 7.2 with 1 M KOH. The cells in each aliquot were pelleted, washed, sonicated, centrifuged and assayed for enzyme activity and protein concentration. At the end of the growth period (15 h), depending on the volume of the cells, they were either harvested at 8,000 rpm (Sorval RC5B) or 9,000 rpm (Sorval SS-3 4) between 0-4°C. The cell pellet was washed twice with an equal volume of 0.9% NaCl and the cells stored at -20°C until further use. The cell pellet collected from the fermentor had a wet weight of 175 g/4 L medium.

2.7.1.3 Preparation of Affinity Column

Sepharose amidoethyl-ALA was prepared as previously reported (Stella et al., 1977; Stella et al., 1978) with some modifications. CN-Br-activated Sepharose 4B (Pharmacia) (7 g) was swollen for 15 min and washed on a glass filter with 1 mM HCl (2 L). The ligands were then added according to Scheme I (p. 94).

Scheme I. Preparation of an Affinity Column

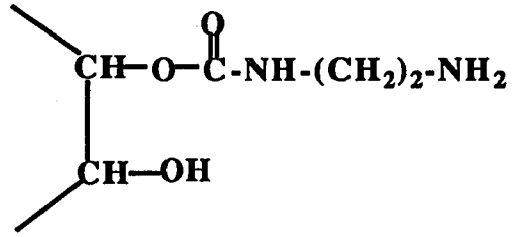


CN-Br-Activated Sepharose 4B

Wash with 1 mM HCl (2 L) for 15 min

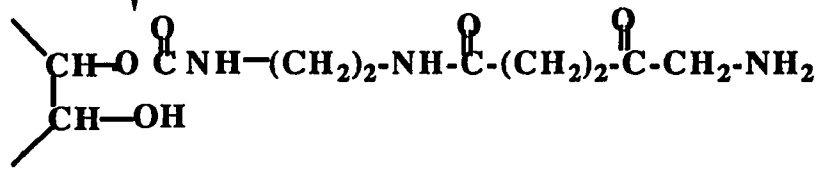
Swollen CN-Br-Activated Sepharose 4B

1,2-diaminoethane (H_2N-$(CH_2)_2$-NH_2) (70 mg) in 25 ml 0.1 M $NaHCO_3$ pH 10 with 0.5 M NaCl; mix 20 h at 4°C



Amino ethyl-Sepharose 4B

Wash with 3.5 L cold deionized water and resuspend in 25 ml of buffer. Add ALA ($HOOC$-$(CH_2)_2$-CO-CH_2-NH_2) in $NaHCO_3$ buffer (234.64 mg in 25 ml) adjust to pH 4.5. Add EDAC in water (665 mg in 25 ml); mix 13 h at 4°C.



5-Amino levulinate amido ethyl-Sepharose 4B

2.7.1.4 Synthesis of Succinylacetone-aminolevulinic Acid Pyrrole

The SA-ALA pyrrole was synthesized as previously described (Brumm, 1981) and quantified with Ehrlich's reagent (Mauzerall et al 1956) using $\varepsilon = 53 \times 10^4\ M^{-1}cm^{-1}$ (Brumm, 1981). The final ALA-SA pyrrole concentration was 0.131 ± 0.009 mM in 0.05 M Tris, pH 7.6.

2.7.1.5 Enzyme Assay for ALA D

ALA D was assayed as previously stated (Sassa 1982) with some modifications. 20 µl of enzyme was incubated with 200 µl of 8.8 mM ALA in 0.05 M Tris, pH 7.6, at 37°C for 30 min to 1 h in 1.5 ml polypropylene tubes. The reaction was quenched by adding 600 µl of 6% TCA containing 0.01 M $HgCl_2$ and the tube centrifuged for 5 min in a table top nonrefrigerated microfuge (Beckman). 600 µl of the supernatant was removed and added to an equal volume of modified Ehrlich's reagent (Mauzerall et al., 1956; Sassa 1982). After 10 min the resulting solution was scanned from 650-450 nm. ALA D activity, measured as the µM PBG formed, was quantified by absorbance at 553 nm by using the extinction coefficeint of 6.1 X $10^4\ M^{-1}\ cm^{-1}$ (Mauzerall et al.,1956).

2.7.1.6 Molecular Weight Determination by Sepharose 6B Gel Filtration Chromatography

Sepharose 6B (Pharmacia) was prepared as suggested by the manufacturer. The column (2.6 dia. X 40.6 cm, flow rate 7.2 ml cm^{-2} h^{-1}, 38 ml h^{-1}) was equilibrated with 0.05 M Tris pH 7.6. The

column was calibrated using Blue Dextran 2000 (1 mg/ml); thyroglobulin, 669,000 (10 mg/ml); ferritin, 440,000 (2 mg/ml); catalase, 232,000; and aldolase, 158,000, (20 mg/ml) (Fig. 2.11 B).

To the above column, 4 ml of affinity-purified ALA D was applied and the column eluted with 0.05 M Tris buffer pH 7.6. Fractions of 3.5 ml were collected and assayed for enzyme activity.

2.7.1.7 Native and SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as previously described (Laemmli, 1970; O'Farrell, 1975) with some modifications. Enzyme from the affinity column was resolved on a 7% acrylamide (T), 2.7% Bisacrylamide (C) resolving gel. The stacking gel was 7.5% T and 2.0% C. The system used was the Mighty Small system (model SE250; Hoefer Scientific Instruments, San Francisco, CA), with 1.5 mm thick gels. Electrophoresis was carried out at 15 mAmps for about 3 h or until the tracking dye reached about 0.5 cm from the bottom of the gel. After electrophoresis the gel was immersed in 8.8 mM ALA for 1 h. Then 6% TCA and modified Ehrlich's reagent were added. Enzyme activity appeared as a pink band on the gel. The gel was then stained with Coomassie blue as stated in the Hoefer manual.

The enzyme from the affinity column was also resolved on tube gels consisting of 7% acrylamide (T), 2.7% Bisacrylamide (C) resolving gel. The stacking gel was 7.5% T and 2.0% C. The system used was the Polyanalyst 431 7000. The gels were 0.5 (diameter) X 7.5 cm. Each gel tube received 1.175 ml of resolving gel and 0.2 ml

of stacking gel. To each gel 40 µl of enzyme preparation (10 mg/ml) was added. Electrophoresis was carried out at 5 mAmps per gel for about 6 h or until the tracking dye reached about 0.5 cm from the bottom of the gel. After electrophoresis, one gel was sliced into 5 mm slices, another gel was sliced into 2.5 mm slices and both sets of slices were assayed for enzyme activity. The third gel was stained with Coomassie Blue as stated in the Hoefer manual (Hoefer Scientific Instruments, San Francisco). The slices corresponding to the peak of enzyme activity coincided with an intensely Coomassie Blue-stained band. These slices were eluted into 2 X SDS-treatment buffer and prepared for SDS-PAGE.

The apparatus used for SDS-PAGE was the Protein-II (BioRad). The resolving gel was 10% T and 2.7% C, while the stacking gel was 4% T and 2.7% C. The gel had the following dimensions: 1.5 mm thickness and 16 X 16 cm. 20 µl of a mixture of low molecular weight markers (Sigma cat. # MW-SDS-70L) was applied to the gel. The gel also received 130-180 µl of SDS-treated ALA D eluted from the non-denaturing gel. Electrophoresis was carried out as follows: 25 mAmps stacking gel for 3 h and 35 mAps resolving gel for 8 h. The gel was then stained with Coomassie blue.

2.7.1.8 pH Studies of ALA D in Phosphate and Tris Buffers

The pH of the substrate, 8.8 mM ALA in both 0.01 M phosphate and 0.05 M Tris was varied from pH 5 to 10 by approximately 0.5 pH units at 37°C. At each pH interval, 200 µl of substrate and 20 µl of enzyme preparation, previously equilibrated at the corresponding pH,

was mixed. The reaction was incubated for 30 min at 37°C, and ALA D activity was determined as previously stated. The concentrations of the buffers were not adjusted for their differences in ionic strength.

2.7.1.9 Activation by Thiols and Divalent Cations

ALA D was purified to near homogeneity without added Mg^{++}, Zn^{++}, and thiols. The enzyme was then assayed in the absence and presence of Mg^{++}, Zn^{++}, Cd^{++}, DTT and ß-ME.

2.7.1.10 Enzyme Kinetics

The initial velocities of ALA D, in the absence and presence of LA, SA ALA-SA pyrrole, uro III, copro I, copro III and proto IX were determined using the substrate concentrations range from 0.125 mM to 4 mM and the inhibitor concentration range specified in Tables 2.4 to 2.6. Substrate, enzyme and inhibitors were maintained at 37°C and mixed at zero time. Aliquots of 220 µl were removed from the reaction mixtures at various time intervals and added to 600 µl of 6% TCA containing 0.01 M $HgCl_2$. Enzyme activity was determined as previously stated.

2.7.2 Purification of ALA D

2.7.2.1 Crude Extract

The cell pellet from *E. coli* JL1002 was resuspended in a minimal volume of 0.05 M Tris pH 7.6 containing 3.3 mM $MgCl_2$, 10 µM $ZnCl_2$, 0.01 M ß-mercaptoethanol, 0.02% NaN_3. The cell suspension was sonicated for 5 min using 5 sec pulses with a duty

cycle setting of 50% and a corresponding off cycle of 1/2 of a second. (Sonicator Cell Disruptor, model W385, using the microtip, 1/4-20 stub). This and all subsequent procedures were carried out at 4^{0}C. The resulting homogenate was then centrifuged at 20,000 rpm (Sorvall SS-34) for 30 min and the supernatant collected. The pellet was sonicated twice more and the supernatants pooled. Ammonium sulfate was added to 35% saturation and the solution stirred for 30 min. This was followed by centrifugation and the supernatant was brought to 55% ammonium sulfate and stirred for 30 min. The resulting supernatant was discarded and the pellet was resolubilized in a minimal volume of the above Tris buffer and dialysed for 3 h against 400 times volume of the same buffer with 2 changes of buffer. The resulting preparation was then applied to a DEAE column.

2.7.2.2 Ion Exchange Chromatography on DEAE Cellulose

Cellex-D cellulose was prepared according to manufacturer's (BioRad) directions. 10 to 20 g of cellex D was suspended in 500 ml of 0.25 M NaOH and allowed to swell for 30 min. Vigorous or prolonged stirring was avoided to prevent generation of fine particles. The suspension was filtered and rinsed with deionized water . The DEAE was then resuspended in 500 ml of 0.25 M HCl and allowed to stand for 10 min. It was then filtered and rinsed and resuspended in 0.25 M NaOH . The cellulose was then equilibrated with 0.05 M Tris, pH 7.6, and poured into a glass column. To the DEAE cellulose column (2.9 dia. X 19 cm, flow rate 11.31 ml cm^{-2} h^{-1}, 75

ml h^{-1}) approximately 32 ml of enzyme preparation was applied and the column was washed with 410 ml of buffer. This was followed by a second wash of 234 ml of 0.01 M potassium phosphate pH 7.6 containing 3.3 mM $MgCl_2$, 10 µM $ZnCl_2$, 0.01 M ß-ME and 0.02% NaN_3. Protein (as detected at 254 nm) but no enzyme activity was eluted during this wash. ALA D was partially-eluted with a linear gradient of 0.0 to 0.27 M KCl in 0.01 M phosphate pH 7.6, 225 ml per chamber. The remainder of the enzyme was eluted with a second gradient of 0.27 to 0.3 M KCl in phosphate buffer; 122 ml per chamber. Active fractions were pooled and concentrated by ultrafiltration using an Amicon PM 10 membrane.

2.7.2.3 Hydrophobic Adsorption Chromatography on TSK Butyl Toyopearl 650M

The concentrated enzyme fractions from the DEAE cellulose column was dialysed against Tris buffer, pH 7.6, containing 20% ammonium sulfate. TSK Butyl Toyopearl 650M was prepared as suggested by the supplier (Supelco, Bellefonte, PA) and packed into a column under pressure using a peristaltic pump (Buchler model 2-6100, setting 2(1/2). The column (1.5 X 12.6 cm, flow rate 44.7 ml $cm^{-2}h^{-1}$, 78.9 ml h^{-1}) was equilibrated with 0.05 Tris M buffer pH 7.6, containing 20% ammonium sulfate. The latter enzyme preparation (20 ml) was applied unto the column by gravity and the column was washed under pressure with 120 ml of the same buffer. The enzyme was subsequently eluted with a linear gradient of 20% to 0% ammonium sulfate in Tris buffer pH 7.6. Active fractions were

pooled and concentrated as before followed by dialysis against 0.01 M phosphate buffer.

2.7.2.4 Affinity Chromatography

To the affinity column (1.5 X 17.8 cm; flow rate 47 $cm^{-2}h^{-1}$, 83 ml h^{-1}) of hydrophobic chromatography-purified ALA D was applied. Both the column and the enzyme preparation were previously equilibrated with 0;01 M potassium phosphate pH 7.6 buffer. The column was washed with 60 ml of buffer. The enzyme was eluted with a linear gradient of 0 to 0.25 M KCl in buffer, 140 ml of the corresponding solution per chamber.

2.8 RESULTS

2.8.1 Growth of *E. coli* JL1002

E. coli strain JL1002 showed normal logarithmic growth when grown in both a dry air incubator (low density) and in a high density fermentor at 37°C (Fig. 2.3 and Fig. 2.4). The specific activity of ALA D from the dry air culture reached a maximum at 39 hours and decreased by 20% at 55 hours (Fig. 2.3). The culture in the high density fermentor behaved differently in that maximum specific activity of ALA D was reached at only 15 hours and showed a sharp decrease by 43% at 25 hours (Fig. 2.4). The wet weight of the cell paste obtained from the high density fermentor represented greater than a 10-fold increase over 10 liters of culture from a dry air incubator.

2.8.2 Purification of ALA D from Cell-free Extracts of *E. coli* JL1002

Ion Exchange Chromatography

E. coli ALA D was eluted off DEAE cellulose as a single peak (Fig. 2.5) which resulted in an 11-fold purification as shown in Table 2.2.

Hydrophobic Chromatography

ALA D eluted off TSK Butyl Toyopearl (Fig. 2.6) had a specific activity of 3870 units/mg protein. This represented a 308-fold purification and a yield of 36 percent (Table 2.2). This was the novel step in our purification.

Affinity Chromatography

At a phosphate concentration of 0.01 M, ALA D was retained on the Sepharose-amidoethyl-ALA column. The enzyme eluted off this column (Fig. 2.7) was purified 1051-fold with a 30 percent yield (Table. 2.2).

Table 2.2 summarizes the steps in the purification of ALA D. The two most effective steps were (1) the hydrophobic chromatography step on TSK Butyl Toyopearl and (2) the affinity step which resulted in 3- and 100-fold increases in specific activity respectively over the DEAE step (Table 2.2).

On a Coomassie blue-stained nondenaturing polyacrylamide gel the affinity purified enzyme appears as a dark band (Fig. 2.8b) which had enzyme activity when the gel was assayed for enzyme activity as stated (Fig. 2.8a and Fig. 2.12). There were a few minor bands still present even at this level of purification.

2.8.3 Effect of ALA on ALA D Activity

A plot of ALA D activity versus ALA concentration gave a hyperbolic curve (Fig. 2.9a) that shows typical Michaelis-Menten kinetics (Fig. 2.9b). This indicates that there is no cooperative effect between the active sites of the enzyme. This result is similar to that obtained for the ALA D from *R. spheroides* (assayed in the presence of K^+) (Nandi et al., 1968) and more recently from yeast (Borralho et al., 1990).

2.8.4 Stability at -20°C and 4°C

Figure 2.10 shows that ALA D from *E. coli* lost enzyme activity even when it is stored at -20°C for a short period. This loss of enzyme activity was accelerated when the enzyme was kept at 4°C. Storage at -20°C is crucial to our work because this is our normal method of storage. We therefore had to find a more effective method of maintaining enzyme activity during storage. At a high protein concentration the enzyme was more stable (data not shown). The JL1002 cell pellet could be kept indefinitely at -20°C without lost of ALA D activity (data not shown).

2.8.5 Molecular Weight and Subunit Size of ALA D

ALA D eluted off a Sepharose 6B column had a molecular weight of 275,000 daltons (Fig. 2.11). When active ALA D was eluted from a non-denaturing tube gel (Fig. 2.12) and run on SDS-PAGE a single Coomassie Blue-stained band of molecular weight 39,000 daltons was observed (Figures 2.13). This data taken along with the native molecular weight of 275,000 daltons obtained from Sepharose 6B chromatography suggest that the *E. coli* enzyme is a heptamer. This is different from the octameric enzyme isolated from other sources (Gibson et al., 1955; Schearer, 1972; Wu et al., 1974; Shemin, 1976; Stella et al., 1977; Nandi et al., 1968; Gibbs et al., 1985 and Shioi et al., 1988).

2.8.6 The Effect of pH ALA D Activity

The pH of optimum activity for *E. coli* ALA D is pH 7.5 - 7.6 in both 0.01 M phosphate and 0.05 M Tris buffer (Fig. 2.14). However the activity is slightly higher in Tris buffer than in phosphate buffer, which may be due to an ionic strength effect or to chemical nature of the buffer ion.

2.8.7 Activation of ALA D by Divalent Cations and Thiols

The data presented in Table 2.3 and Figure 2.15 showed that ALA D was activated by Mg^{++}, Zn^{++}, and Cd^{++}. Cadmium appeared to be as good an activator as zinc even though zinc is thought to be the natural metal ion. Schlosser and Beyersman (1987) had previously shown that Cd^{++} could replace Zn^{++} in bovine ALA D and that the effect was on K_{cat}/K_m.

Both DTT and ß-ME activate (400%) *E. coli* ALA D (Table 2.3 and Figure 2.14). However the enzyme prepared in the absence of both of these reagents was active (Figure 2.15). This shows that unlike the enzyme from *R. spheroides* (Nandi et al. 1968), *R. capsulata* (Nandi and Shemin, 1973), yeast (Borallho et al., 1990), mouse liver (Coleman, 1970), bovine liver (Barnard et al., 1977), and human erythrocytes (Gibbs et al., 1985) all of which required thiols; reducing agents were not essential for *E. coli* ALA D activity. The enzyme from two other bacteria, *S. itersonii* (Ho et al.,1971) and *Erythrobacter* (Shioi and Doi, 1988b) and from guinea pig (Weissberg et al., 1974), and spinach (Nandi and Waygood, 1967) also did not require addition thiols for full activity.

2.8.8 Determination of Kinetic Parameters of ALA D

The the kinetic data were analysed by Lineweaver-Burke and by Eadie-Scatchard plots using two computer packages for simple regression (Cricket graph 1.2.3, Cricket Graph SoftWare, Malvern, CA and Stat View 512+, BrainPower, Inc, Agoura Hill, CA). Both treatments of the data gave a K_m value of 0.83 ± 0.095 mM for ALA (Fig. 2.9a) and a V_{max} = 3.44 ± 0.3 μM PBG h^{-1}. The initial velocity was linear for the substrate concentration range chosen for up to 15 min (Inset Fig. 2.9a).

2.8.9 Effect of Inhibitors on ALA D

Levulinic acid, succinylacetone (SA), and aminolevulinic acid-succinylacetone (ALA-SA) pyrrole were all competitive inhibitors of *E. coli* ALA D (Fig. 2.16a,b, Fig. 2.17a,b and Fig. 2.18a,b respectively). The mixed pyrrole proved to be the most effective (K_i = 0.04 ± 0.001 mM) of the three γ-keto-inhibitors. SA (K_i = 1.38 ± 0.029 mM) was a better inhibitor than LA (K_i = 16.43 ± 1.25 mM) (Table 2.4).

E. coli ALA D was inhibited by the sulfhydryl-directed reagents iodoacetamide, *p*-hydroxymercuri-benzoate and methyl methanethiosulfonate (MMTS) (Table 2.5). Preincubation of the enzyme with iodoacetamide greatly increased its inhibitory effect. MMTS appear to be the best-sulfhydryl directed inhibitor. MMTS is thought to be capable of removing zinc from the bovine holo-enzyme thus creating an apo-ALA D (Jaffe et al., 1984). The apo-enzyme, after removal of MMTS, could subsequently be reactivated by the

addition of Zn^{++} (Jaffe et al., 1984). The *E. coli* enzyme was also inhibited Pb^{++}, 100% at 10 mM (Table 2.4).

The chelating agent EDTA which is capable of removing Zn^{++} from the enzyme, inhibited the enzyme (Table 2.4). 1,10-phenanthroline but not 1,7-phenanthroline inhibited the *enzyme* (Table 2.4). Both metal ion chelators are inhibitors of the enzyme from *Neurospora* (Mythukrishnan et al., 1972) and bovine liver (Bevan et al., 1980; Dent et al., 1990).

2.8.10 The Effect of Porphyrinogens and Porphyrins on ALA D

Coproporphyrin I and III were competitive inhibitors of the enzyme (Fig. 2.19a,b and Fig. 2.20a,b) with K_i values of 0.04 ± 0.002 mM and 0.02 ± 0.001 mM respectively (Table 2.6). Uroporphyrin III and protoporphyrin IX were both mixed type inhibitors with K_i of 0.001 ± 0.0005 mM and 0.39 ± 0.09 mM respectively (Fig. 2.21a,b and Fig. 2.22a,b respectively, Table 2.6). Uroporphyrin III therefore appears to the be the most effective inhibitor of the porphyrins examined. Uroporphyrinogen III, coprophyrinogen III, and protoporphyrinogen IX all inhibited *E. coli* ALA D (Table 2.6). Coprophyrinogen III had the greatest inhibitory effect (95% inhibition), then uroporphyrino-gen III (77% inhibition) and protoporphyrinogen IX is the least inhibitory (35% inhibition) (Table 2.6).

2.9 DISCUSSION AND CONCLUSION

2.9.1 Growth of *E. coli* JL1002

E. coli JL1002 grew well under both high and low density conditions and ALA D activity increased with cell density. The extraction of ALA D from the cell pellet required more than one sonication to release most of the activity. This may be due to the fact that this strain carries a cloned gene on a high copy plasmid (Li et al., 1988) and may be producing protein aggregates. It was previously demonstrated that plasmid copy number in *E. coli* increases with high density growth (Horn et al., 1990) and that the protein produced from such plasmids can form aggregates within the cell (Schein, 1989).

2.9.2 Comparison of *E. coli* ALA D to ALA D from Other Sources

E. coli ALA D has been purified 1000-fold to near homogeneity, as shown by both native and SDS-PAGE, by utilizing a four step procedure including an affinity chromatography step. The method of using an affinity chromatography as a protein purification step was first proposed by Cuatrecasas, (1970), and has since found numerous applications. The specific activity of the *E. coli* ALA D (217 µmole PBG/mg h) is higher than that reported for the enzyme from most sources (Table 2.1), but lower than that of another bacteria *Erythrobacter* (452 µmole PBG/mg h) (Shioi and Doi, 1988b). Enzymes with high specific activities are generally not thought to be rate-limiting in a biosynthetic pathway, but ensure maximum utilization of their substrates. This may be the case in *E. coli*

where the accumulation of ALA does not occur in wild type strains under normal conditions. Yeast (ALA D specific activity 16.2 μmole PBG mg^{-1} h^{-1}, Table 2.1) and *Neurospora* under certain normal growth conditions these fungii accumulate ALA (Muthukrishnan et al., 1972; Labbe-Bois et al., 1977). Thus supporting the proposal that ALA D is rate-determining and therefore has a regulatory role in the latter two organisms.

2.9.3 Molecular Weight and Subunit Size of ALA D

The homogeneous enzyme eluted from a native PAGE gel and run on SDS-PAGE gave a subunit molecular weight of 39,000 daltons (Fig 2.13). As previously stated this data taken with the native molecular weight of 275,000 daltons shows that the enzyme is oligomeric. From our data we have calculated that the enzyme is heptameric. This subunit weight data is supported by Li et al., (1988) who found a subunit molecular weight of 38,000 daltons by the maxicell procedure and 35,506 to 36,763 from the amino acid sequence derived from the nucleotide sequence of *hemB* using two open reading frames (Li et al., 1989).

2.9.4 Thiol Reagents and Metal Ions Activated ALA D

Our studies on the thiol dependency (Table 2.3) and of sulfhydryl directed inhibitors (Table 2.5) indicate that *E. coli* ALA D, like all other ALA D studied may have cysteine residues at its active site. This was supported by the deduced amino acid sequence which showed conserved cysteines at the proposed active site of *E.*

coli ALA D (Li, 1989; Li et al., 1989; Echelard et al., 1988). The *E. coli* enzyme was also inhibited by Pb^{+2}, and Hg^{+2} two heavy metals thought to react with active cysteine residues. However the enzyme prepared in the absence of added thiols was partially active.

The inhibition of *E. coli* ALA D by EDTA and 1, 10-phenanthroline, and its activation by Zn^{+2} and Cd^{+2} demonstrate that the enzyme is a metalloezyme. The amino acid sequence of *E. coli* ALA D deduced from the nucleotide sequence show the presence of a highly conserved zinc binding sequence (Li, 1989; Echelard et al., 1988; Li et al., 1988).

2.9.5 γ-Keto Compounds Inhibited ALA D

γ-Keto acids having the succinyl moeity are well known competitive inhibitors of ALA D (Brumm et al., 1981; Meller & Gassman 1981, Shioi et al., 1985). The inhibitors, levulinic acid (LA) and succinylacetone (SA) are similar in structure to the substrate aminolevulinic acid (Figure 2.23) and might be expected to be competitive inhibitors. In agreement with the prediction, *E. coli* ALA D was competitively inhibited by both compounds. However both LA (K_i = 16.43 mM) and SA (k_i = 1.38 mM) are weaker inhibitors of the *E. coli* enzyme than the enzyme from bovine liver (Barnard et al., 1977), mouse erythrocyte (Ebert et al., 1979), barley leaves (Meller et al., 1981), *R. spheroides* (Nandi & Shemin, 1968), *R. capsulata* (Nandi & Shemin, 1973) and *M. Phlei* (Yamasaki, 1971). The mixed pyrrole of ALA and SA was a much better competitive inhibitor (K_i = 0.04 mM) than SA alone (K_i = 1.38 mM). Brumm et al., (1981) suggested that, in spite of its being a pyrrole, its

resemblance to the substrate rather than to the product that accounts for this inhibition. SA is found as a metabolite in patients with hereditary tyrosinemia where it causes excretion of ALA in these patients (Lindblad et al., 1977). This SA can combine spontaneously with ALA to produce the mixed pyrrole which can result in severe inhibition of ALA D in these patients.

2.9.6 Porphyrins and Porphyrinogens Inhibited ALA D

It had been previous demonstrated that ALA D was inhibited by some of the porphyrinogen and/or porphyrin intermediates of the heme biosynthetic pathway (Muthukrishnan et al., 1972). *E. coli* ALA D was inhibited by the porphyrins; uro III, copro III, and proto IX, and by the corresponding porphyrinogens. Under normal conditions of growth, the heme biosynthetic pathway in *E. coli* follows that outlined in Fig. 1.2. In this pathway the porphyrinogens are the physiological intermediates. Protoporphyrin IX is the only porphyrin in this pathway (Fig. 1.2). Wild type *E. coli* does not accumlate these intermediates under normal growth conditions. However urogen III and uro I accumulate when ALA is present in the growth medium (Sasarman et al., 1968a). From our studies we were unable to determine whether the inhibition of *E. coli* ALA D by the porphyrinogen may reflect some physiolocal regulation in this bacteria. Proto IX has been proposed as a physiological repressor of the ALA D gene in *Neurospora* (Muthukrishnan et al., 1972). Proto IX inhibited the ALA D in this fungus only slightly, while the enzyme was strongly inhibited by copro III and coprogen III. Like the enzyme

of *Neurospora* the *E. coli* ALA D was only slightly inhibited by proto IX and protogen IX but strongly inhibited by copro III and coprogen III. However a regulatory role for this porphyrin and/or porphyrinogen on heme biosynthesis in *E. coli* could not be established.

2.9.7 Conclusion

In conclusion the *E. coli* ALA D shares many properties with the enzyme isolated from other sources and does not appear to play a regulatory role in heme biosynthesis in this bacteria.

Table 2.1 COMPARISON OF THE PROPERTIES OF OF 5-AMINOLEVULINIC ACID DEHYDRATASE PURIFIED FROM VARIOUS ORGANISMS

Source of the Enzyme	Properties											
	Purification (fold)	Molecular weight (daltons)	Subunit weight (daltons)	pH Optimum	Specific activity (µmole PBG /mg/hr)	Km (mM)	Metal ion requirement	Added Thiol requirement	Inhibition EDTA	Inhibition Proto IX (%)	Inhibition Hemin (%)	Refer.
BACTERIA												
E. coli	1000	275,000	39,000	7.5-7.6	217	0.87	Mg^{++},Cd^{++} Zn^{++}	-	+	27[a]	72[b]	1
M. phlei	160	NR	NR	9.0-9.2	0.7	0.077	Mg^{++},K^{+}	NR	+	NR	0[c]	2
Spirillum itersonii	160	NR	NR	8.5	2.2	0.1	Mg^{++},Mn^{++}	-	NR	33[d]	19[e]	3
R. spheroides	270	250,000	39,000	8.5	121	0.7	Mg^{++},Mn^{++} Na^{+},K^{+}	+	-	NR	100[f]	4-5
R. capsulata	400	260,000	39,000	8.0-8.6	97	0.7	none	+	-	8[g]	9[g]	6
Erythrobacter sp.	7,405	260,000	40,000	8.2	452	0.29	none	I	-	27[e]	13[h]	7
YEAST												
S. cerevisiae	193	275,000	37,000	9.8	16.2	0.359	Zn^{++}	+	+	NR	NR	8
PLANT												
Bajra seedlings	137	592,000	80,000	NR	17.8	0.5	Mg^{++}	I	NR	65[g]	92[g]	9
Spinach		324,000	50,000	8.2	14.5	NR	Mg^{++}	-	NR	NR	NR	10
Wheat leaves	100	NR	NR	7.5-7.6	15.1	1.0	none	NR	-	NR	NR	11

Table 1 continued:

MAMMALS												
Guinea Pigs:												
Adult liver	371	NR	NR	6.5-7.5	2.60	0.72	NR	-	NR	NR	30[i]	12
Fetal liver	106	NR	NR	6.5-7.5	1.60	0.92	NR	-	NR	NR	30[i]	12
Adult erythrocytes	188	NR	NR	6.5-7.5	3.58	0.4	NR	-	NR	NR	70[i]	12
Fetal erythrocytes	92	NR	NR	6.5-7.5	2.75	0.4	NR	-	NR	NR	50[i]	12
Mouse liver	1180	270,000	NR	6.3-6.7	11.8	0.4	Mg^{++},Mn^{++} Zn^{++},Ba^{++} Co^{++},Fe^{3+}	+	A	50[j]	50[j]	13-15
Rat	1350	280,000	35.000	NR	26.0	NR	Zn^{++}	+	NR	NR	NR	16
Rabbit	1350	280,000	35,000	NR	26.6	NR	Zn^{++}	+	NR	NR	NR	16
Bovine liver		242,000-289,000	34,900-42,000	6.3-6.7	18.0	0.1	Zn^{++},Cu^{++}	+	+	NR	NR	17-20
Human erythrocytes	34600	285,000	31,000-35,000	6.3-6.8	22-24	0.287	Zn^{++}	+	+	NR	NR	21-22

Key: [hemin or proto IX], a=0.69 mM; b=1.39 mM; c=1 mM; d=0.2 mM; e=0.1 mM; f=0.33 mM; g=0.05 mM; h=5 mM; i=0.06 mM; j=0.5 mM. I=inhibitor; A=activator; NR = not reported.

References:

1 This study
2 Yamaski et al 1971
3 Ho et al., 1971
4 Nandi et al., 1967
5 Heyningen et al.,1971
6 Nandi & Shemin, 1973
7 Shioi & Doi,1988b
8 Borralho et al., 1990
9 Prasad et al., 1988
10 Liedgens et al., 1983
11 Nandi & Waygood, 1967
12 Weissberg et al.,1974
13 Coleman, 1966
14 Doyle et al, 1969
15 Coleman, 1970
16 Fujita et al., 1981
17 Schearer, 1972
18 Wu et al., 1972
19 Jaffe et al., 1984
20 Stella et al., 1977
21 Anderson et al., 1979
22 Gibbs et al., 1985

Table 2.2 PURIFICATION OF ALA D FROM *ESCHERICHIA COLI*

	PROTEIN		ENZYME					
	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude Extract from Sonication	33	32.2	1063	6.64	219.1	0.21	100	1.0
Precipitate from 55 % ammonium sulfate	20	17.3	346	7.62	152.5	0.44	70	2.1
DEAE cellulose chromatography	14	4.1	57	9.02	126.3	2.20	58	10.7
Hydrophobic chromatography (TSK-Toyopearl Butyl 650M)	11.2	0.11	1.23	6.97	73.1	63.40	36	308.0
Affinity chromatography (AlA-amidoethylsepharose)	2.0	0.15	0.30	32.52	65.0	216.80	30	1051.0

One unit = 1 μmole porphobilinogen per h.

Table 2.3 Effects of various activators on ALA D activity

Additions	Concentration (M)	Activity (%)	
None	0.00	100	
		Preincubation	
		No	Yes
Thiols			
ß-Mercaptoethanol (ME)	1.0×10^{-2}	417	429
Dithiothreitol	1.0×10^{-2}	375	394
Metal Ions			
Mg^{++}	3.3×10^{-3}	134	ND
Cd^{++}	3.3×10^{-3}	124	ND
Zn^{++}	1.0×10^{-5}	144	ND
Metal Ions Plus Thiols			
Mg^{++} (+ 10 mM ß-ME)	3.3×10^{-3}	382	ND
Cd^{++} (+ 10 mM ß-ME)	3.3×10^{-3}	409	ND
Zn^{++} (+ 10 mM ß-ME)	1.0×10^{-5}	412	ND

Table 2.4 Effects of various inhibitors on ALA D activity

Additions	Concentration (M)	Activity Remaining (%)	K_i (mM)	Type of Inbibitor
None	0.00	100		
Lead (Pb^{+2})	1.00×10^{-3}	24		
	1.00×10^{-2}	0		
Levulinic Acid	6.80×10^{-3}	100	16.43 ± 1.25	com†
	1.30×10^{-2}	70		
	2.73×10^{-2}	57		
	4.54×10^{-2}	36		
4,6-Dioxoheptanoic Acid (Succinyl Acetone)	3.41×10^{-4}	96	1.38 ± 0.029	com
	1.14×10^{-3}	82		
	3.32×10^{-3}	58		
	6.80×10^{-3}	17		
	1.14×10^{-2}	13		
Succinyl Acetone-ALA Pyrrole	1.00×10^{-5}	88	0.04 ± 0.001	com
	1.04×10^{-4}	4		
EDTA	2.27×10^{-3}	100		
	4.54×10^{-3}	43		
	9.09×10^{-3}	39		
	1.14×10^{-2}	35		
Phenanthroline				
1,7 (meta)	4.00×10^{-4}	100		
	4.00×10^{-3}	100		
1,10 (ortho)	4.00×10^{-4}	95		
	4.00×10^{-3}	67		

†com is competitive inhibition

Table 2.5. Inhibition by Sulfhydryl Reagents

Additions	Concentration (M)	Activity Remaining (%)	
None	0.00	100	
		Preincubation*	
		No	Yes
p-Hydroxymercuri-Benzoate	1.50×10^{-5}	99	97
	1.50×10^{-4}	4	4
	2.25×10^{-4}	4	4
	3.30×10^{-4}	1	1
Iodacetamide	4.54×10^{-5}	82	6
	4.54×10^{-3}	21	1
	6.82×10^{-3}	17	1
	1.00×10^{-2}	9	1

*Preincubation was for 15 min at 37°C

Table 2.6. Inhibition by Various Intermediates Heme Biosynthesis

Additions	Concentration (M)	Activity Remaining (%)	K_i (mM)	Type 0 f inhibitor
None	0.00	100		
Uroporphyrin-I	1.05×10^{-4}	60		
	5.26×10^{-4}	31		
Uroporphyrin-III	1.48×10^{-4}	49	0.001 ± 0.0005	
	4.43×10^{-4}	32		mix¥
Uroporphyrinogen-III (prepared by Na-amalgam reduction)	6.70×10^{-4}	23		ND
Coproporphyrin-I	1.02×10^{-4}	87	0.04 ± 0.002	com
	3.07×10^{-4}	56		
Coproporphyrin-III	1.02×10^{-4}	91	0.02 ± 0.001	com
	3.07×10^{-4}	58		
	7.23×10^{-4}	42		
Coproporphyrinogen-III (prepared by Na-amalgam reduction)	6.36×10^{-4}	5		ND
Protoporphyrin IX	6.85×10^{-4}	73	0.39 ± 0.09	mix
Protoporphyrinogen IX prepared by Na-amalgam reduction)	1.90×10^{-4}	65		ND§

§ K_i values were not determine for the porphyrinogens because these compounds are readily oxidized by air

†com is competitive inhibition

¥mix is mixed-type inhibition

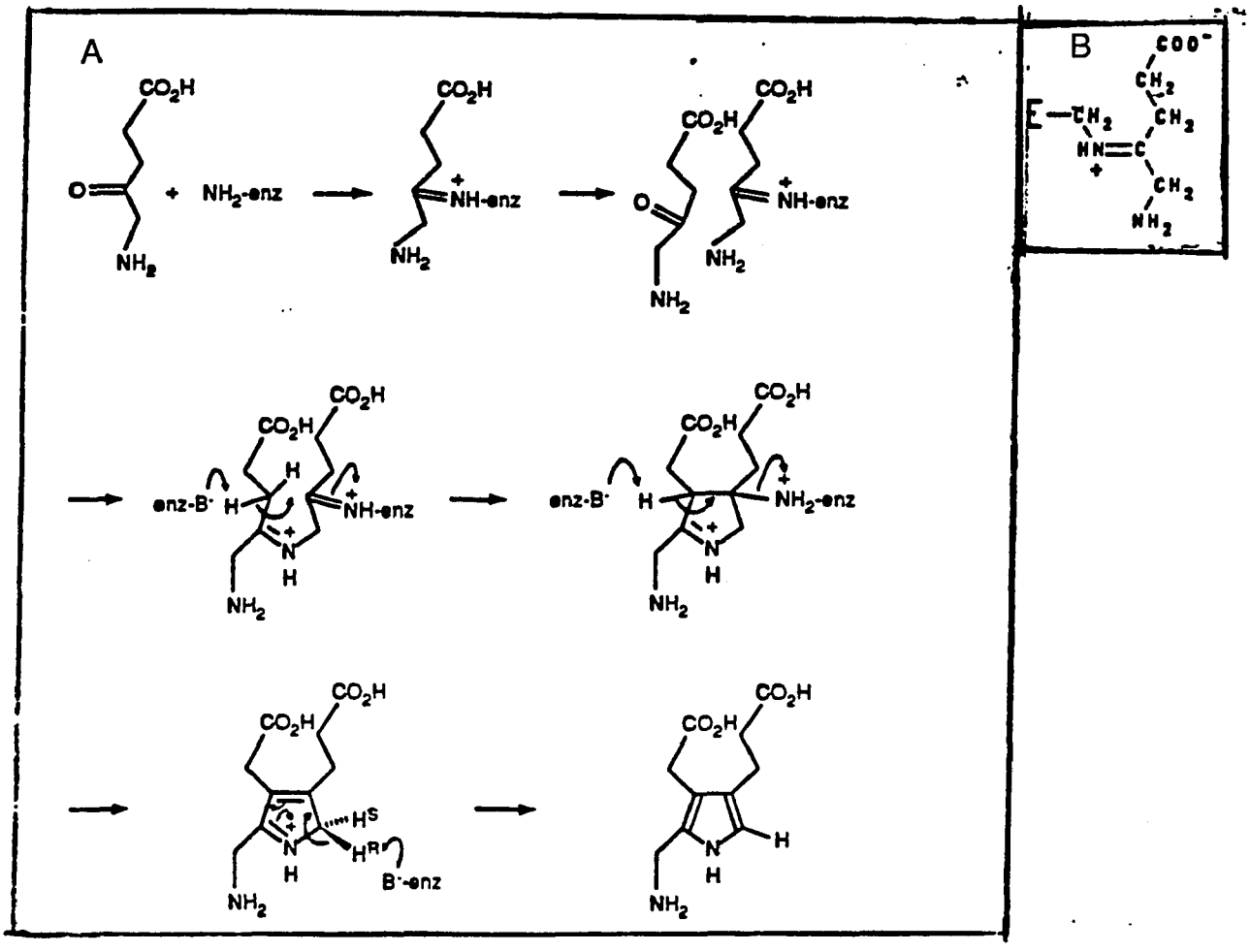


Figure 2.1 A: Mechanism of 5-aminiolevulinic acid dehydratase (Jordan 1990) and B: structure of the P-side ALA Schiff base intermediate showing E stereochemistry (Jaffe et al., 1990)

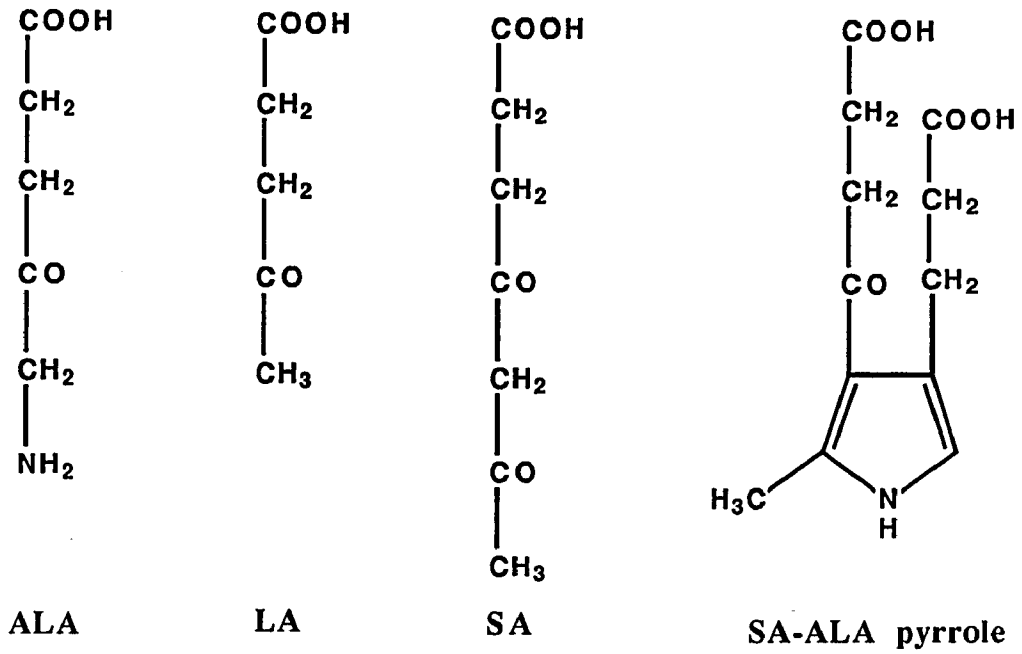


Figure 2.2 Comparison of the structure of the substrate of 5-aminolevulinic acid dehydratase with those of some inhibitors of the enzyme. ALA is 5-aminolelulinic acid, LA is levulinic acid, SA is succinylacetone and SA-ALA is succinylacetone-5-aminolevulinic acid pyrrole.

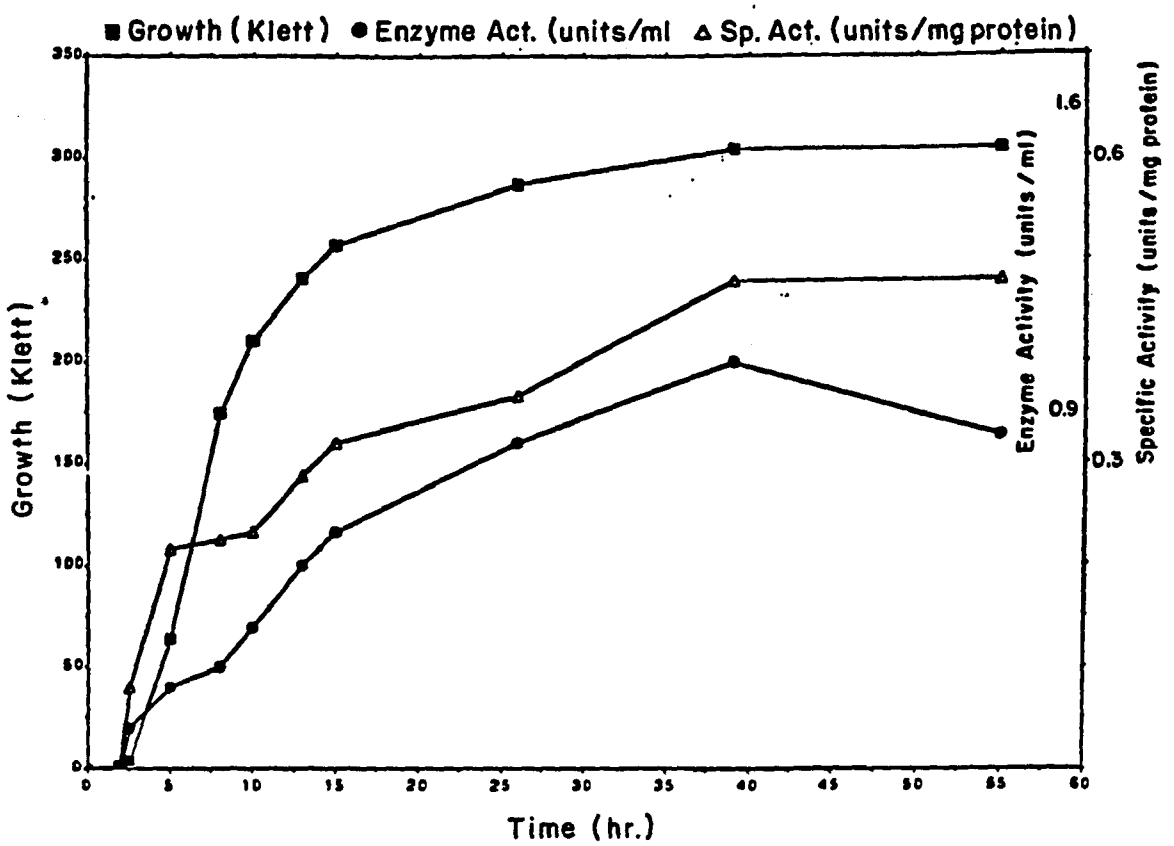


Figure 2.3 Growth of *E. coli* strain JL1002 in LB at low density. The culture was inoculated into LB media in a 4 liter flask and grown as stated in materials and methods.

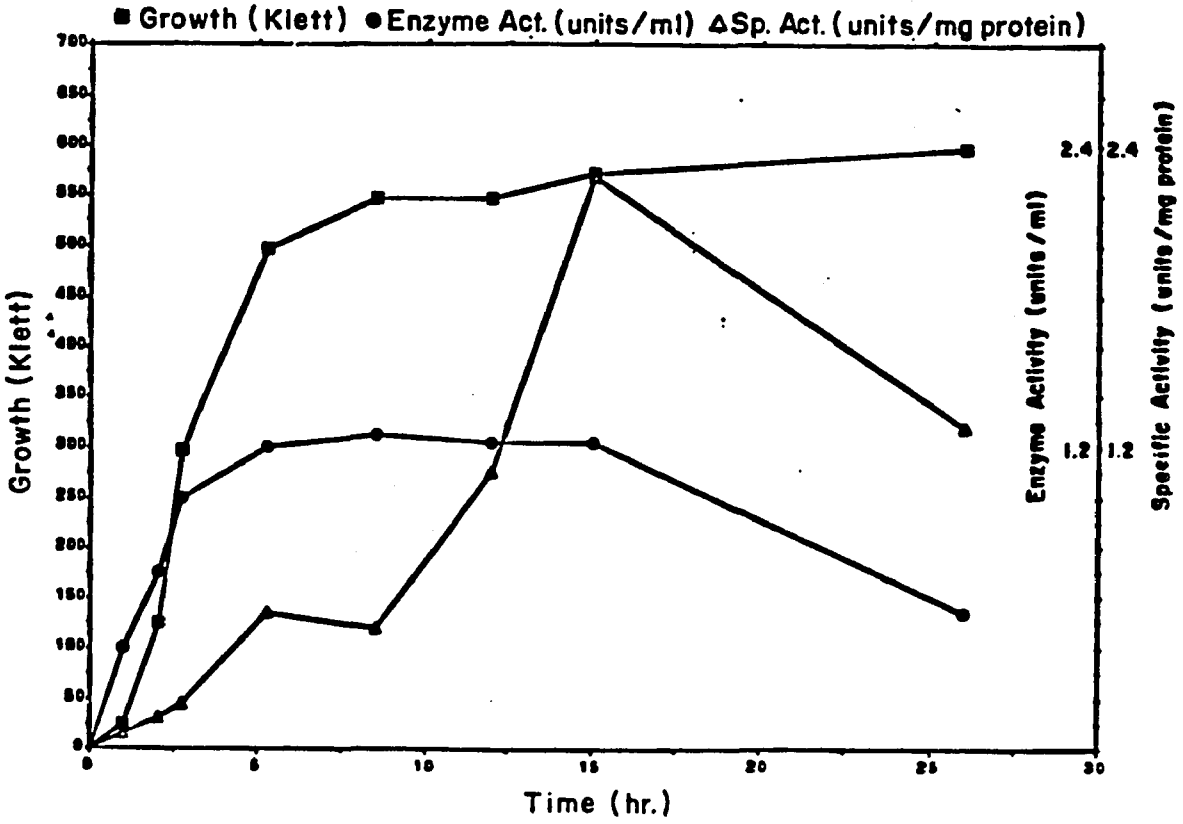


Figure 2.4 Growth of *E. coli* strain JL1002 in LB at high density. The culture was inoculated into the fermentor and grown as stated in materials and methods.

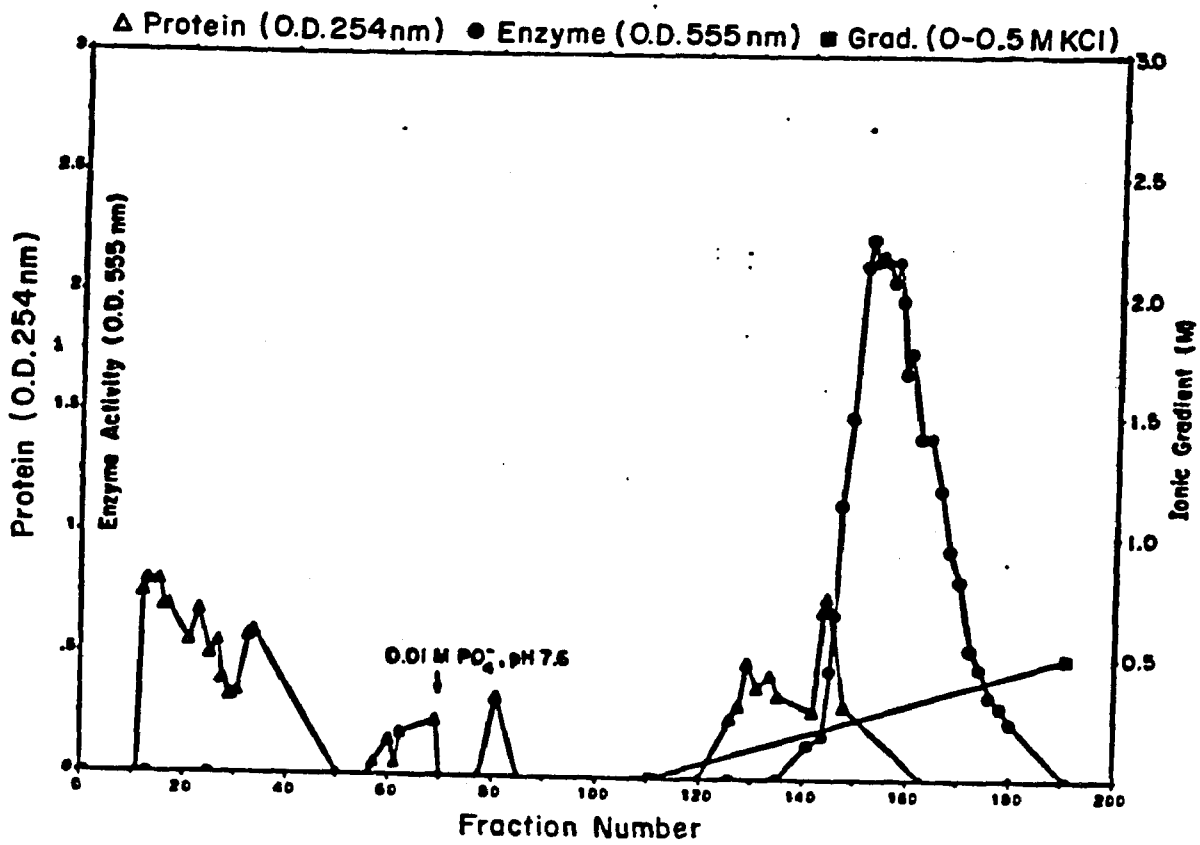


Figure 2.5 Ion exchange chromatography of *E. coli* ALA D on DEAE. The enzyme was applied to the column and eluted as stated in the materials and methods.

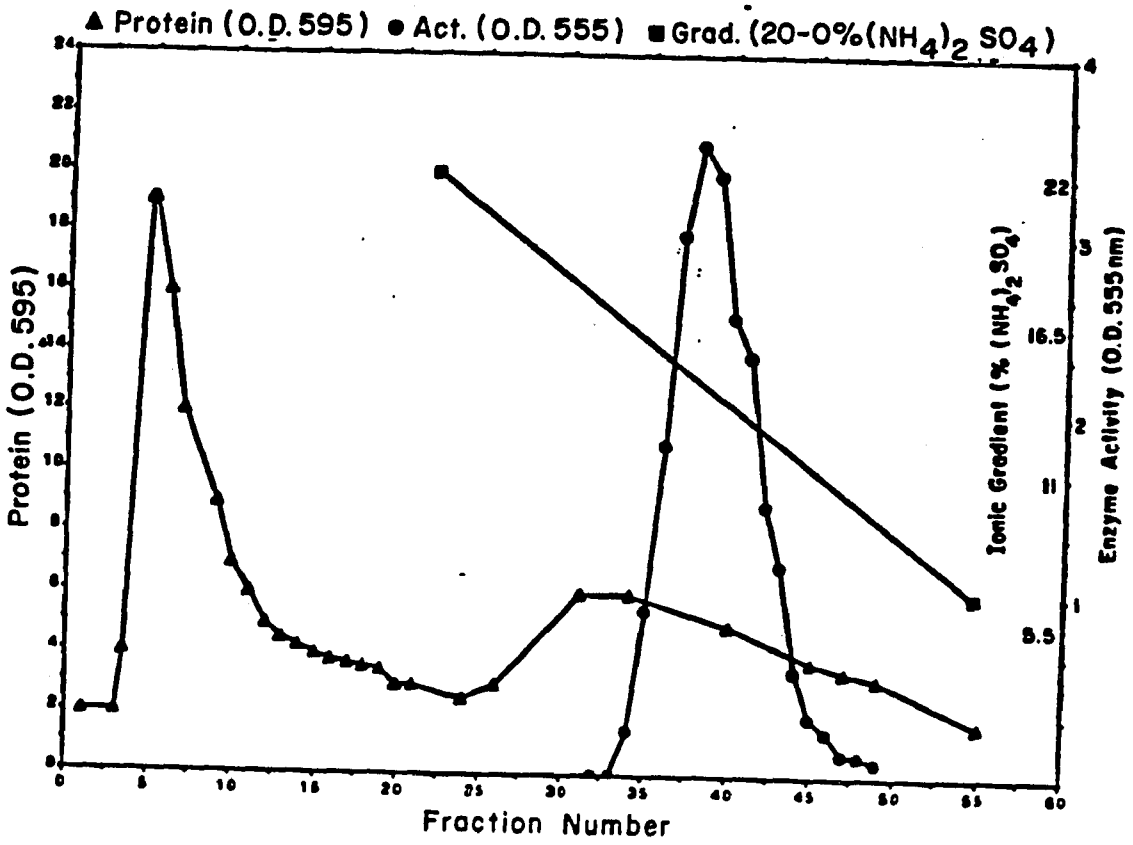


Figure 2.6 Hydrophobic chromatography of *E. coli* ALA D on TSK Butyl Toyopearl. The enzyme was applied to the column and eluted as stated in the materials and methods.

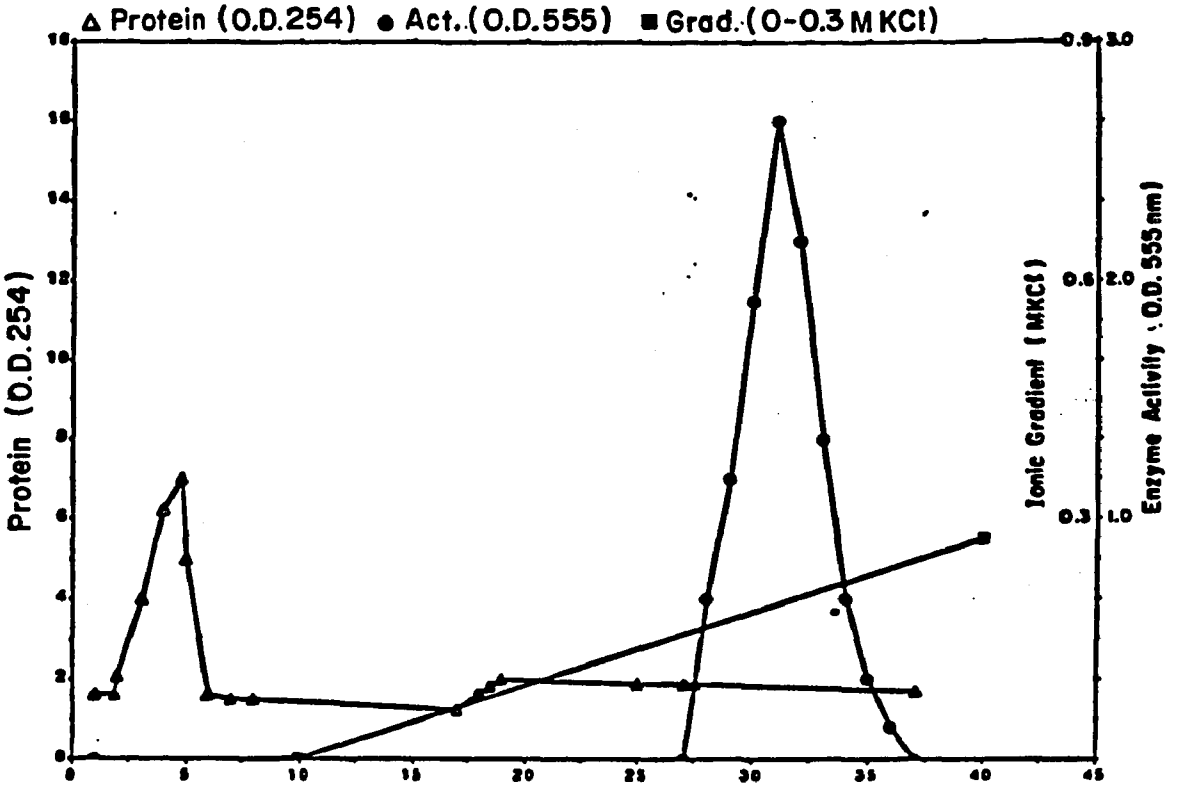


Figure 2.7 Affinity chromatography of *E. coli* ALA D on 5-aminolevulinate ethylene diaimide Sepharose 4B. The enzyme was applied to the column and eluted as stated in the materials and methods.

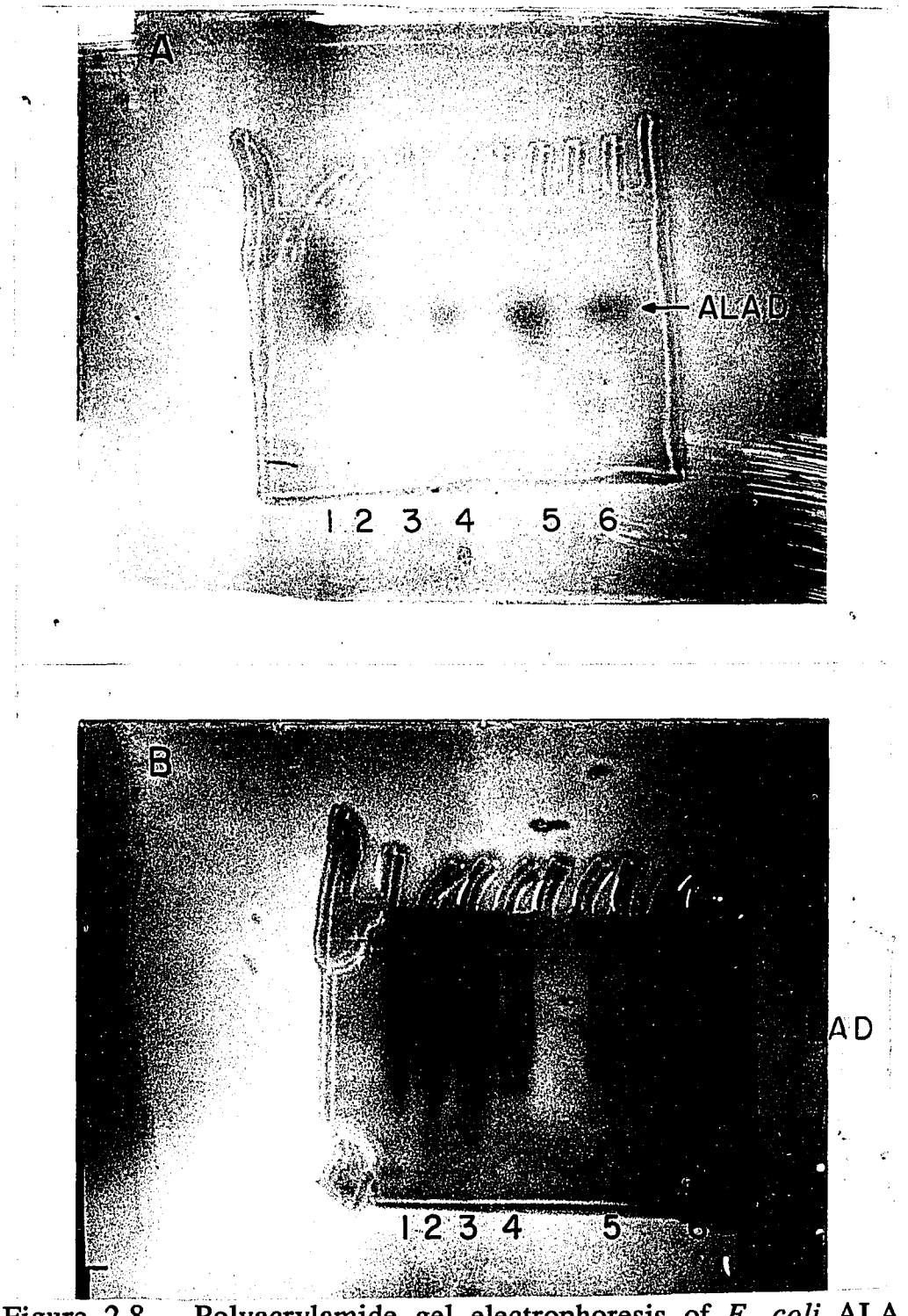


Figure 2.8 Polyacrylamide gel electrophoresis of *E. coli* ALA D from different steps in the purification. A. Assay for enzyme activity. B. Coomassie blue-stained following enzyme assay. Lanes:1, crude extract; 2, NH_4SO_4 precipitate; 3, ion exchange chromatography; 4, hydrophobic chromatography; 5 & 6 affinity chromatography.

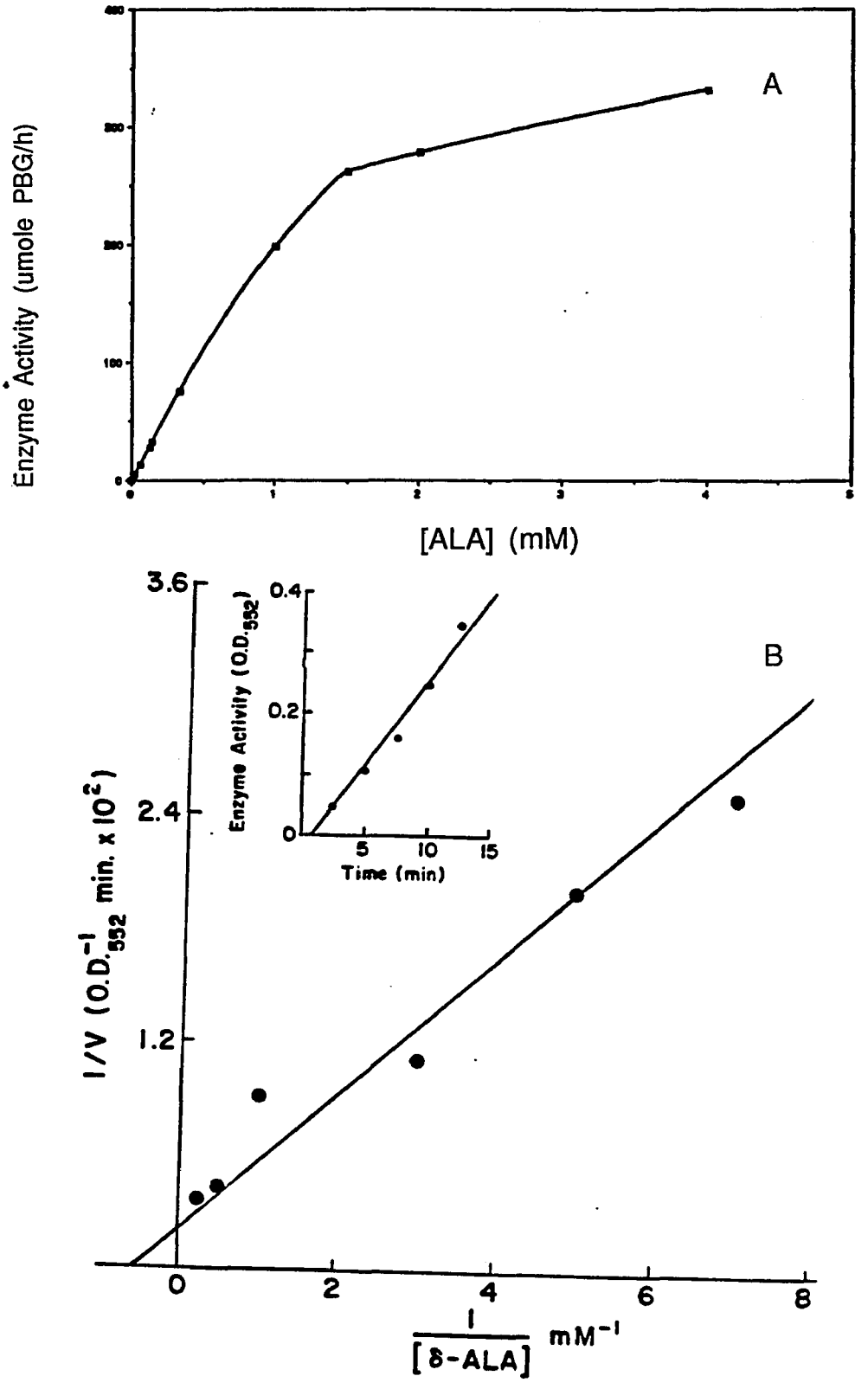


Figure 2.9 The effect of ALA of the activity of *E. coli* ALA D. (A) Plot of Enzyme activity versus ALA concentration. (B) Double reciprocal plot of *E. coli* ALA D. The ALA concentrations were the same as those shown in Fig. 1.15A. Inset Fig1.15B Plot of velocity of *E. coli* ALA D activity versus time.

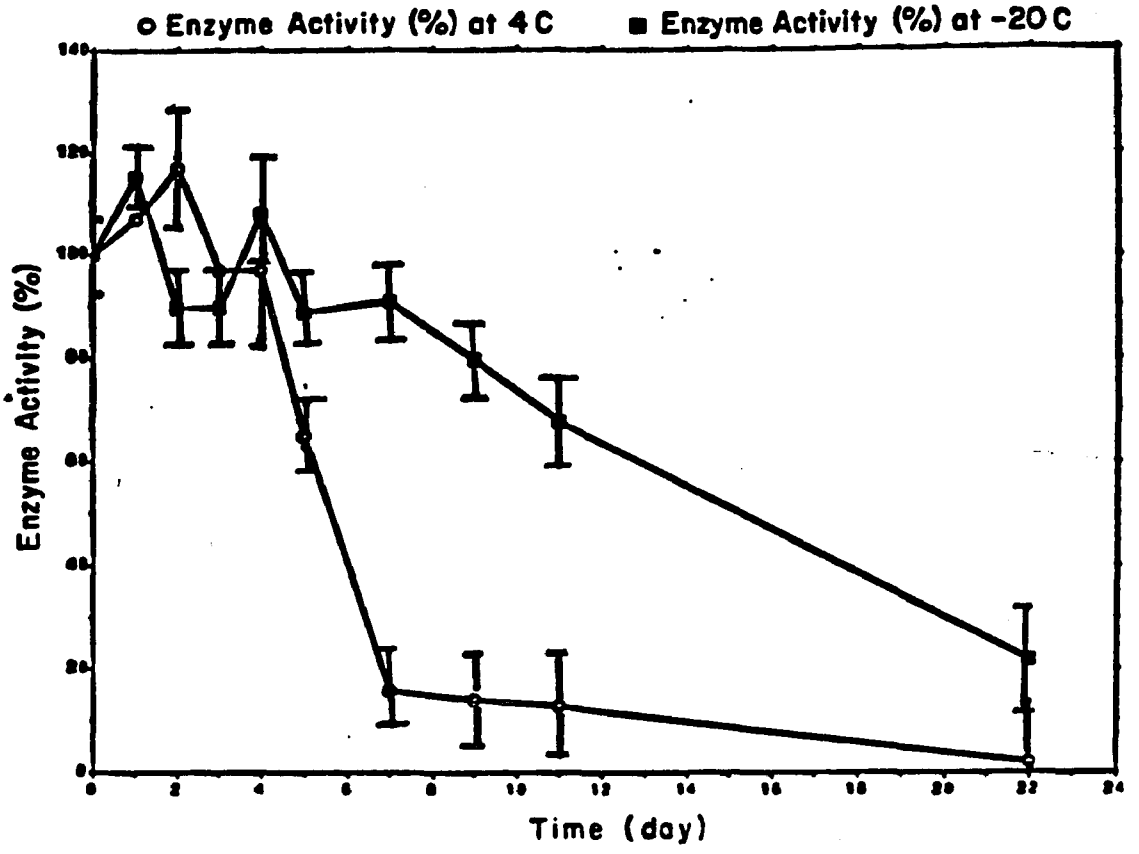
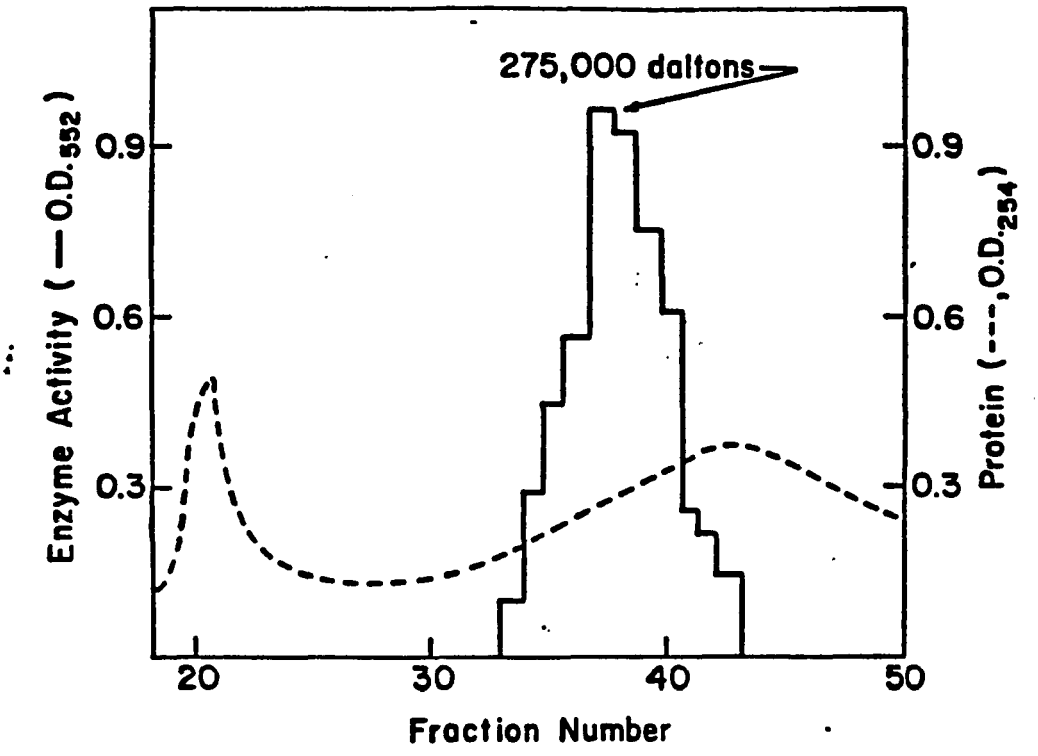


Figure 2.10 Stability of *E. coli* ALA D stored at 4 and -20°C. Aliquots of the enzyme were kept either at 4°C or -20°C. At the indicated time the enzyme was remove from storage and allowed to come to 37°C. The enzyme was then assayed as stated in materials and methods. The results represents the average of 3 determination with a 9% error in the enzyme assay.



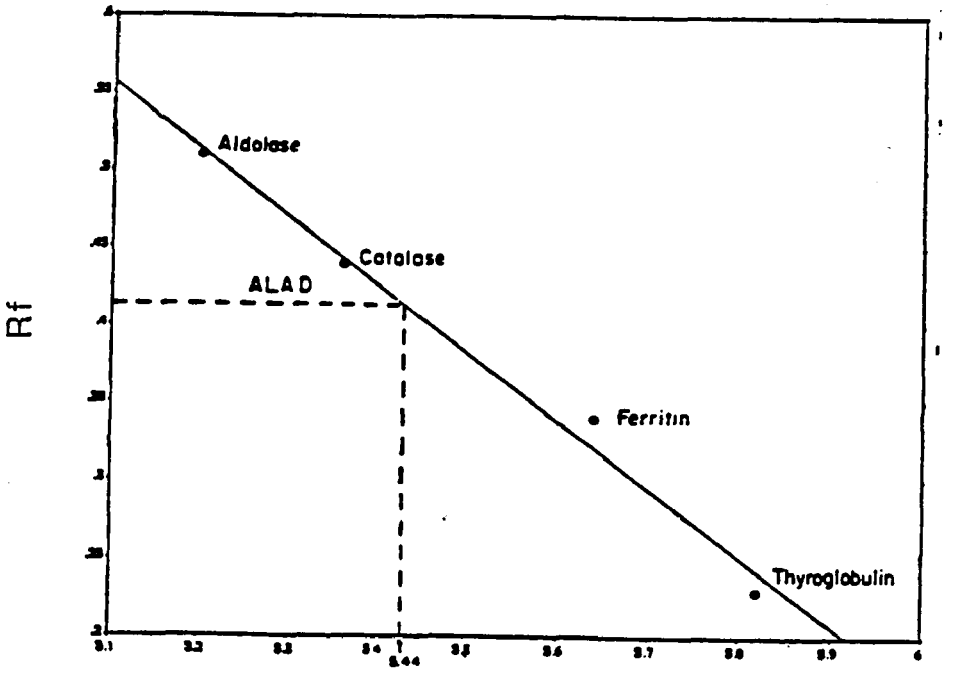


Figure 2.11 Gel filtration chromatography of *E. coli* ALA D of Sepharose 6B. A. Elution profile. B. Plot of K_{av} versus log. molecular of standard proteins.

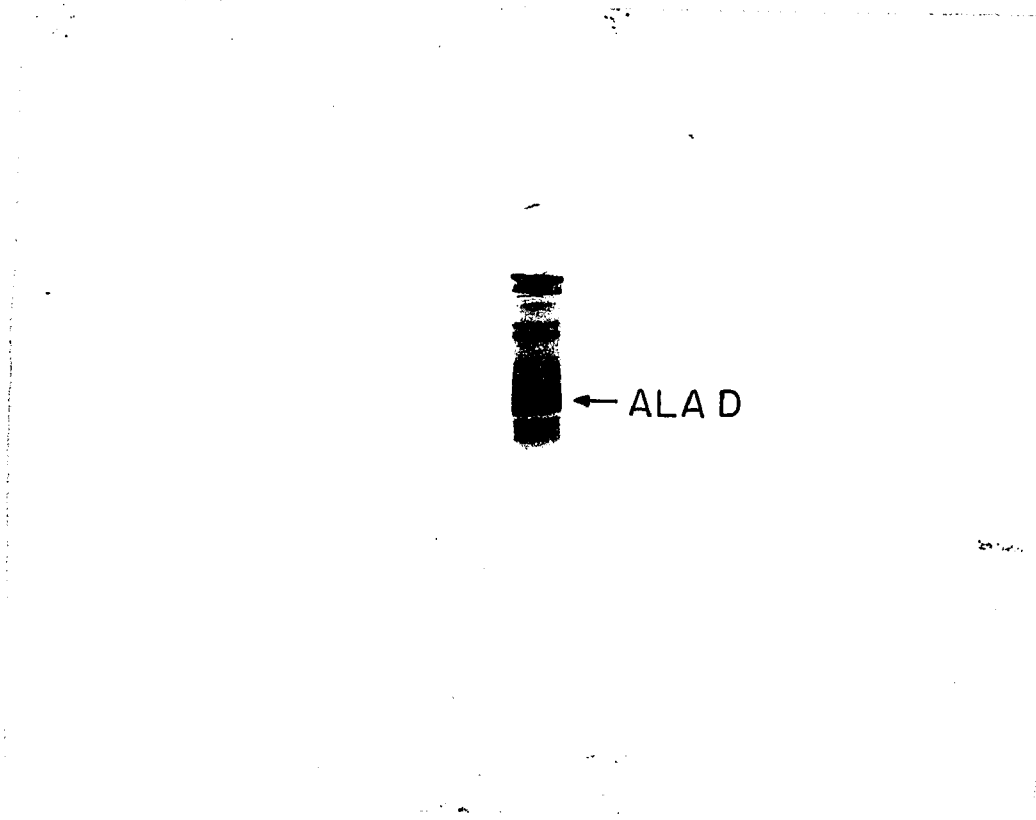


Figure 2.12 Preparative polyacrylamide gel electrophoresis. The enzyme preparation was applied to the tube gel and electrophoresis carried out as stated in materials and methods.

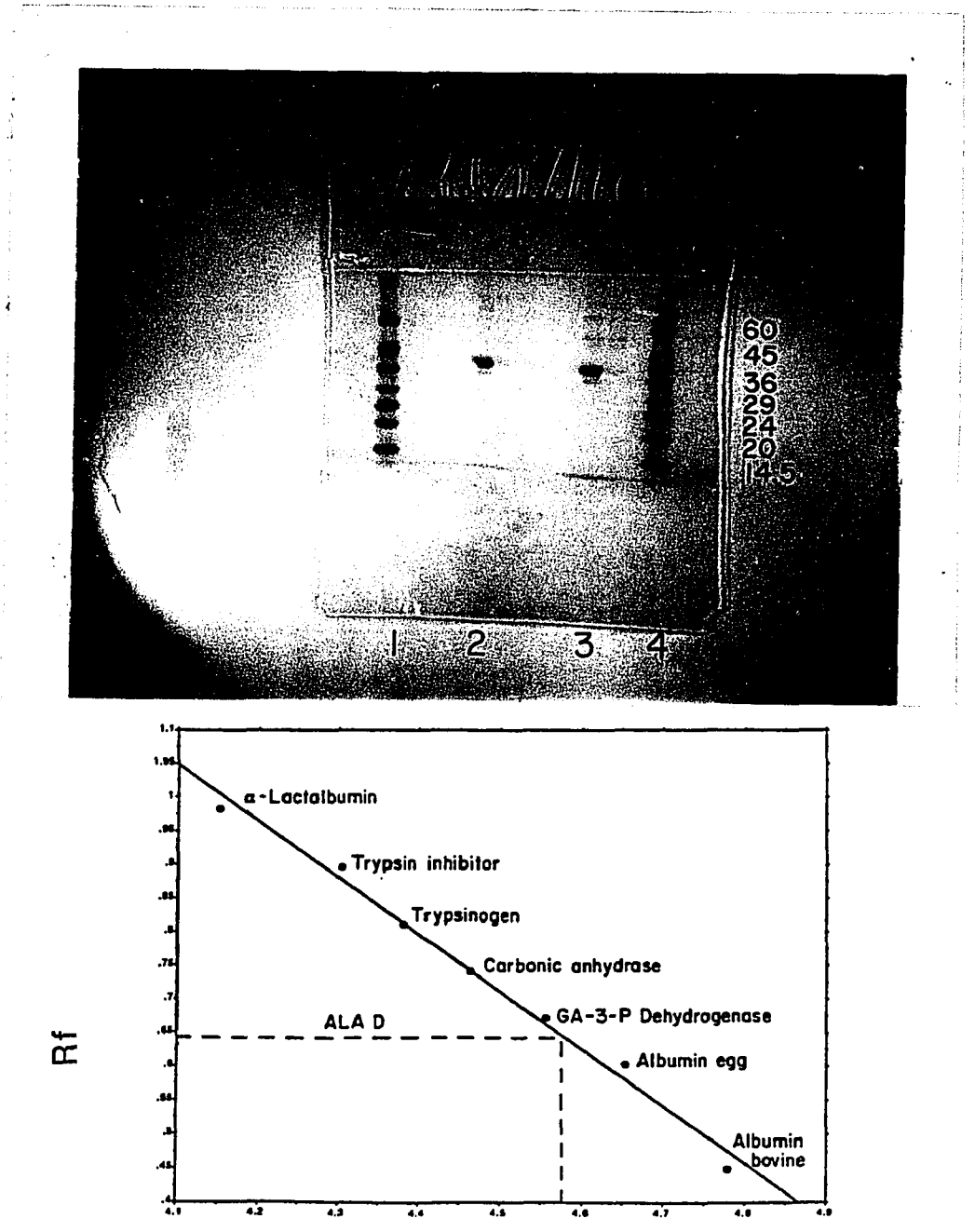


Figure 2.13 A. SDS polyacrylamide gel electrophoresis of *E. coli* ALA D eluted from the preparative PAGE. B. Plot of Rf versus log molecular weight of standard proteins.

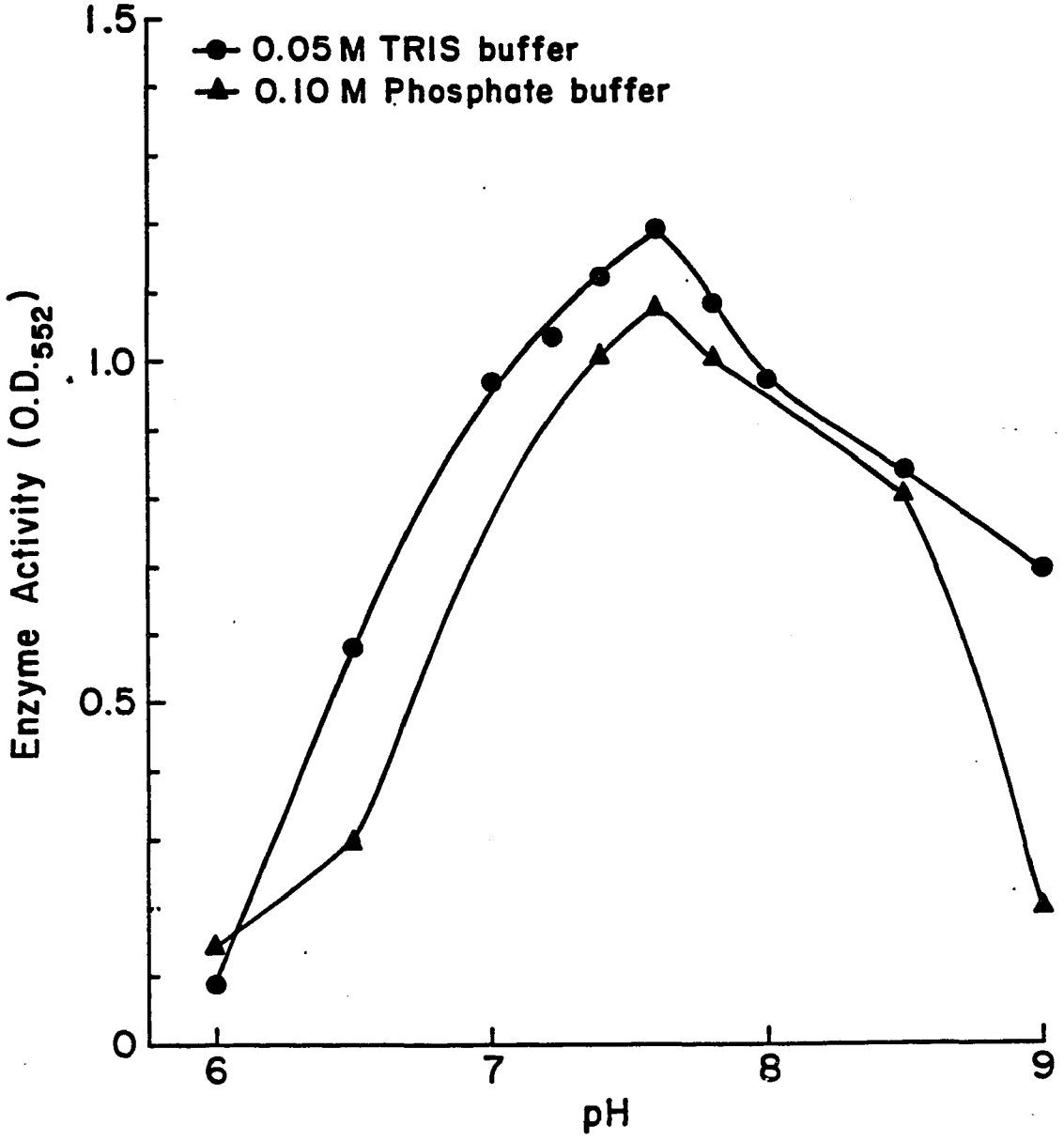


Figure 2.14 pH profile of *E. coli* ALA D in phosphate and Tris buffer.

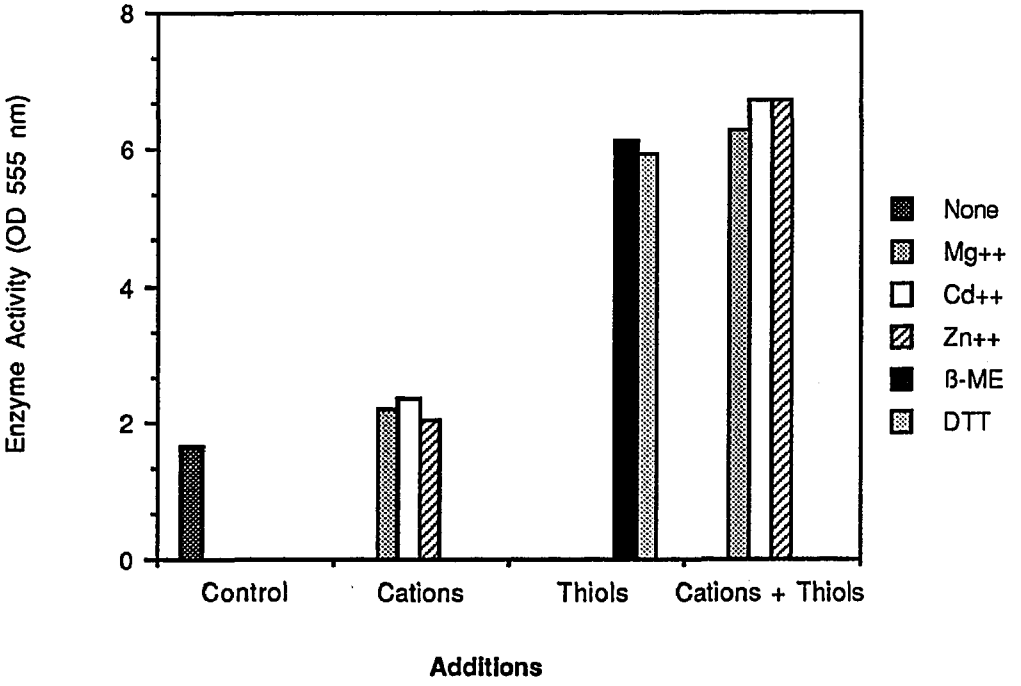


Figure 2.15 The activation of *E. coli* ALA D by Divalent cations and Thiol. Each column represents the average enzyme activity of two duplicate experments.

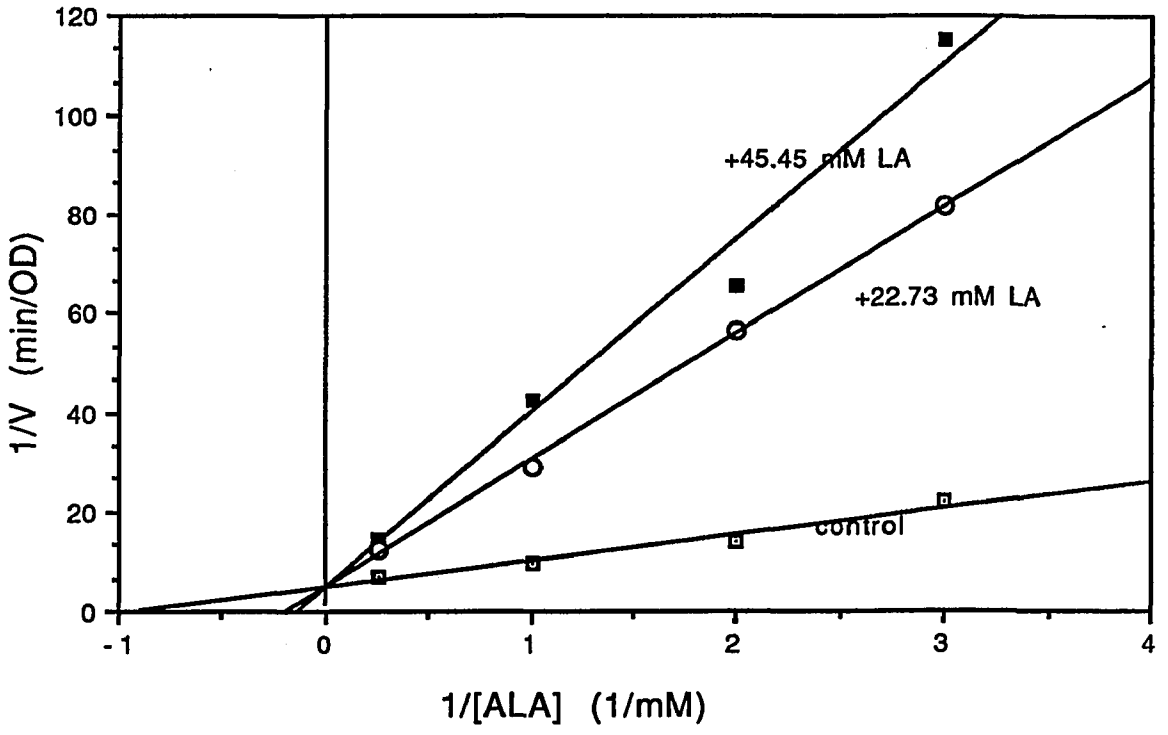


Figure 2.16A Double reciprocal plot of *E. coli* ALA D in the absence and presence of levulinic acid.

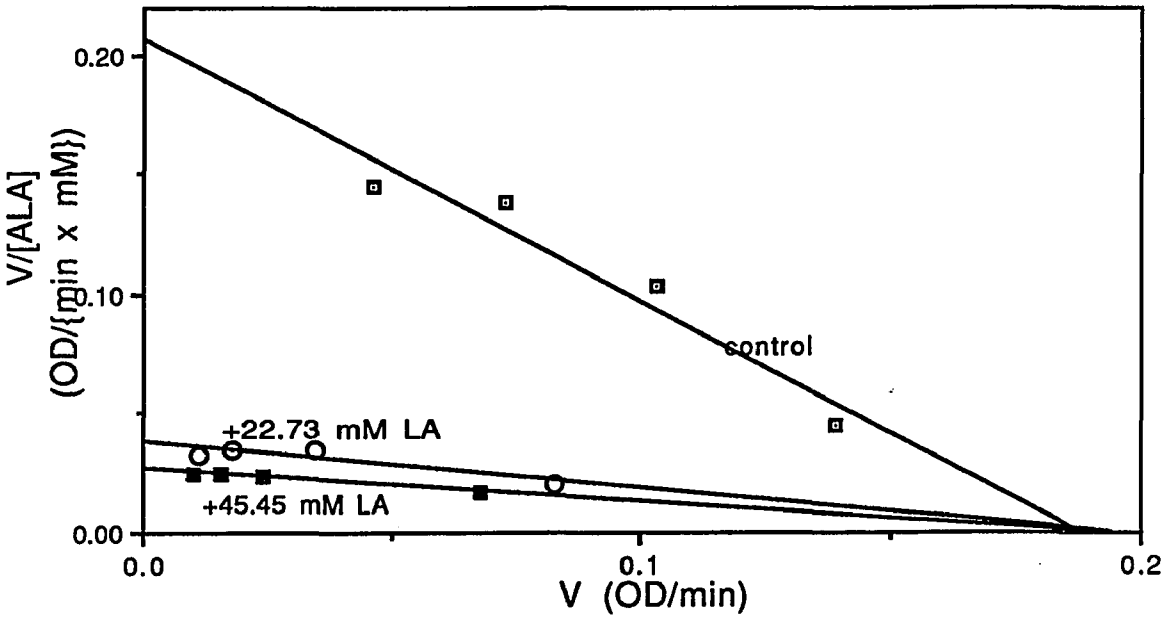


Figure 2.16B Eadie-Scatchard plot of *E. coli* ALA D in the absence and presence of levulinic acid. Replot of the data used in Fig. 2.16A.

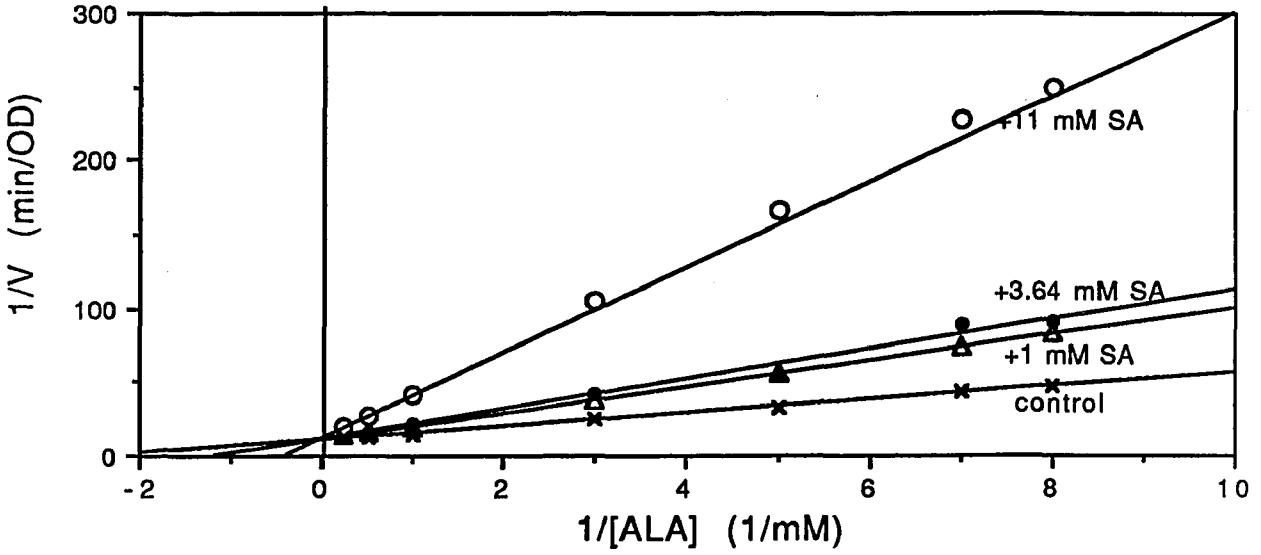


Figure 2.17A Double reciprocal plot of *E. coli* ALA D in the absence and presence of succinylacetone.

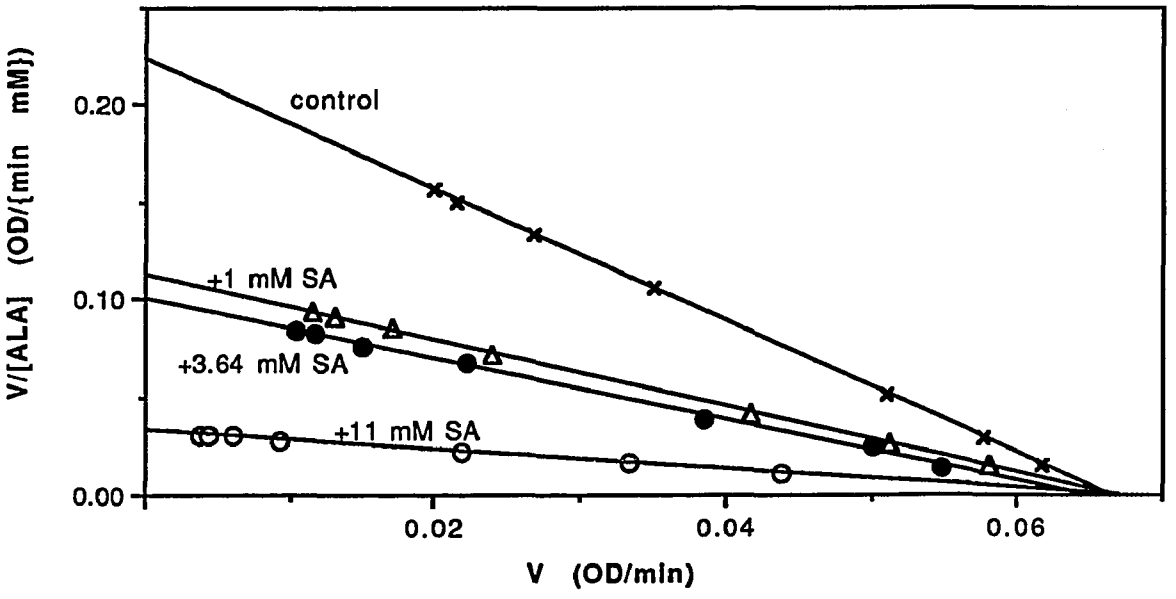


Figure 2.17B Eadie-Scatchard plot of *E. coli* ALA D in the absence and presence of succinylacetone. Replot of the data used in Fig. 2.17A.

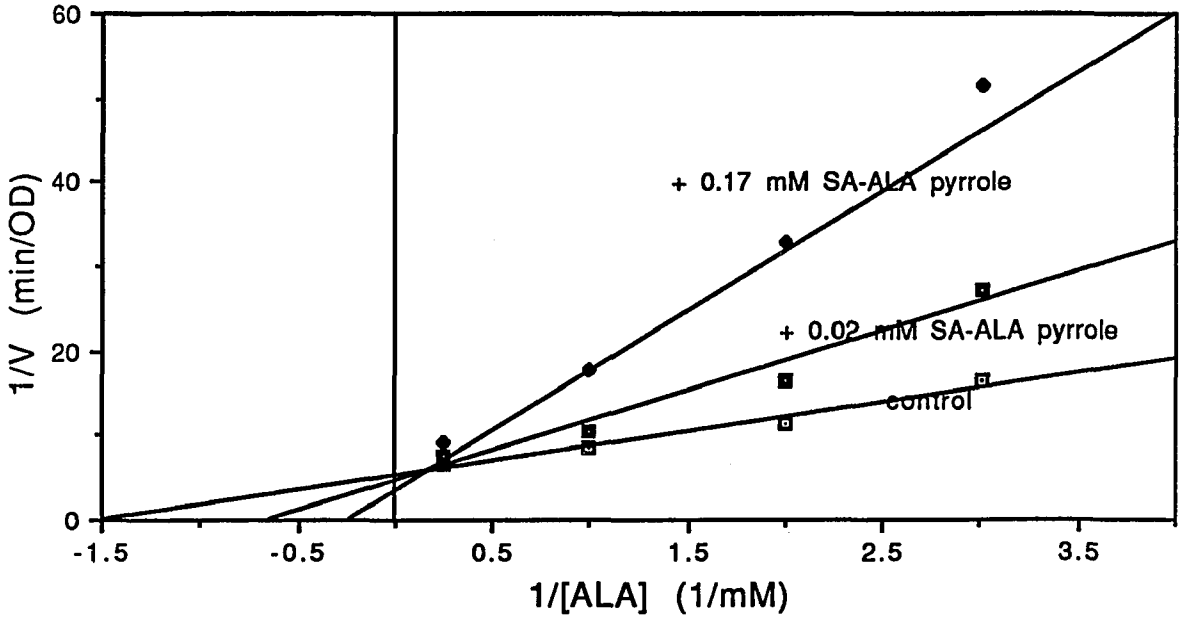


Figure 2.18A Double reciprocal plot of *E. coli* ALA D in the absence and presence of succinylacetone-aminolevulinic acid pyrrole.

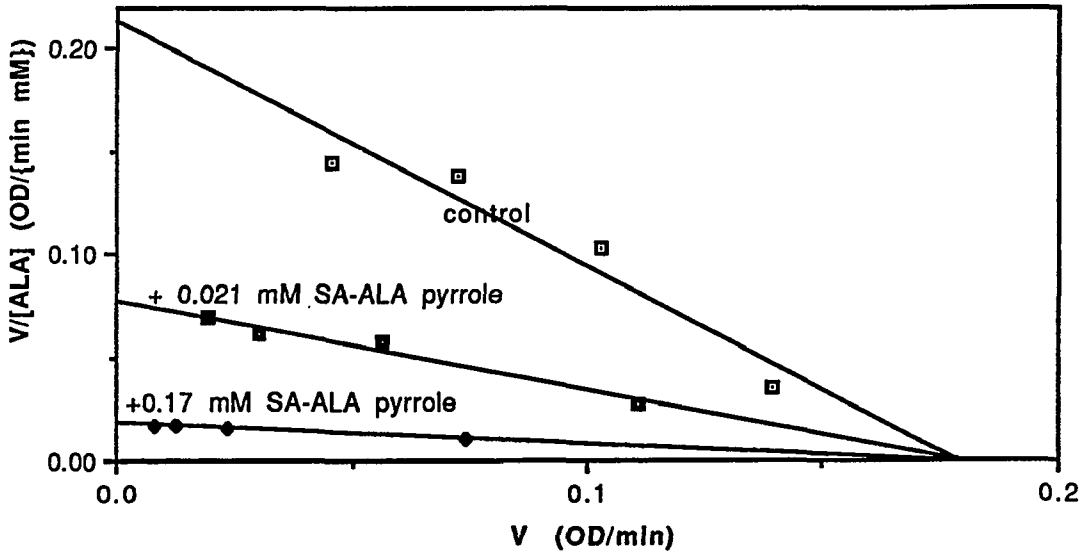


Figure 2.18B Eadie-Scatchard plot of *E. coli* ALA D in the absence and presence of succinylacetone-aminolevulinic acid pyrrole. Replot of the data used in Fig. 2.18A.

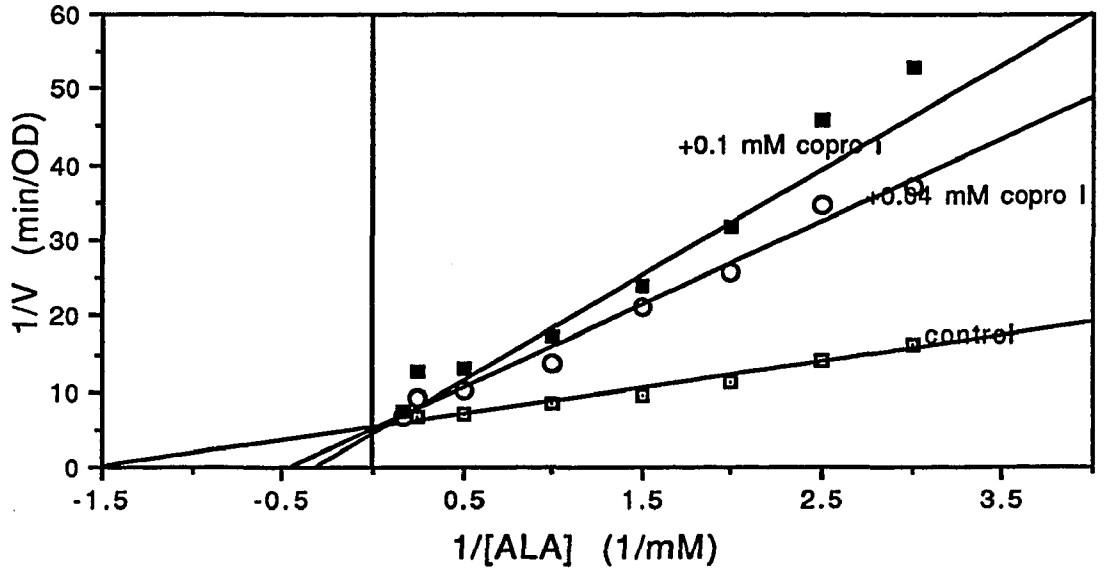


Figure 2.19A Double reciprocal plot of *E. coli* ALA D in the absence and presence of coproporphyrin I.

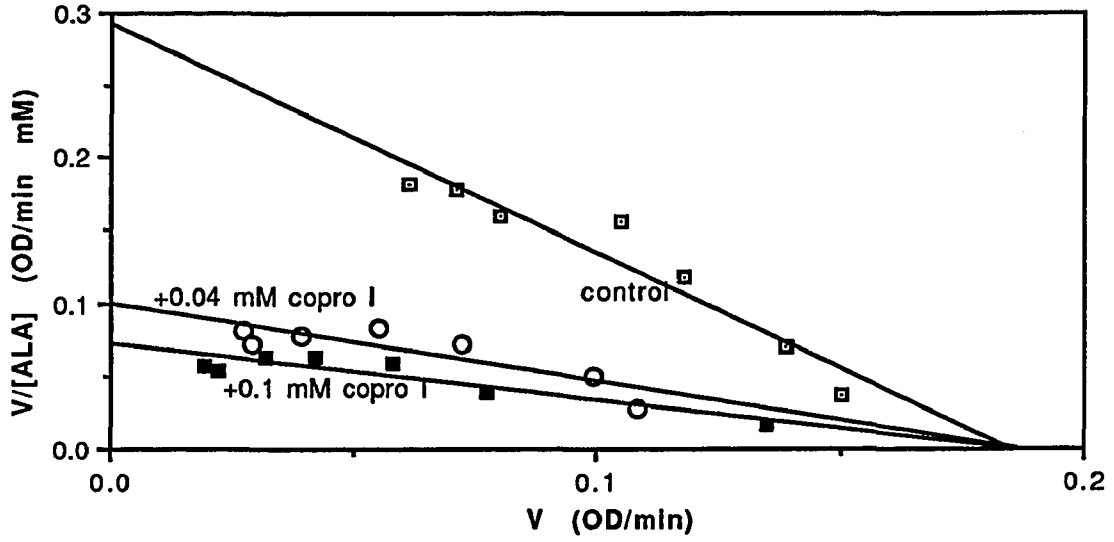


Figure 2.19B Eadie-Scatchard plot of *E. coli* ALA D in the absence and presence of coproporphyrin I. Replot of the data used in Fig. 2.19A.

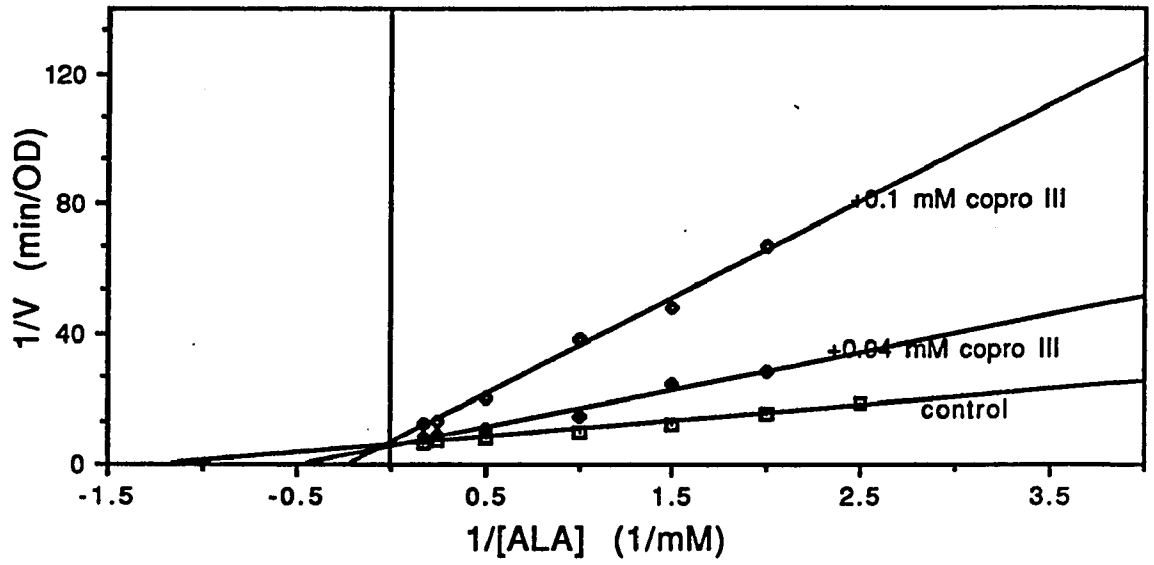


Figure 2.20A Double reciprocal plot of *E. coli* ALA D in the absence and presence of coproporphyrin III.

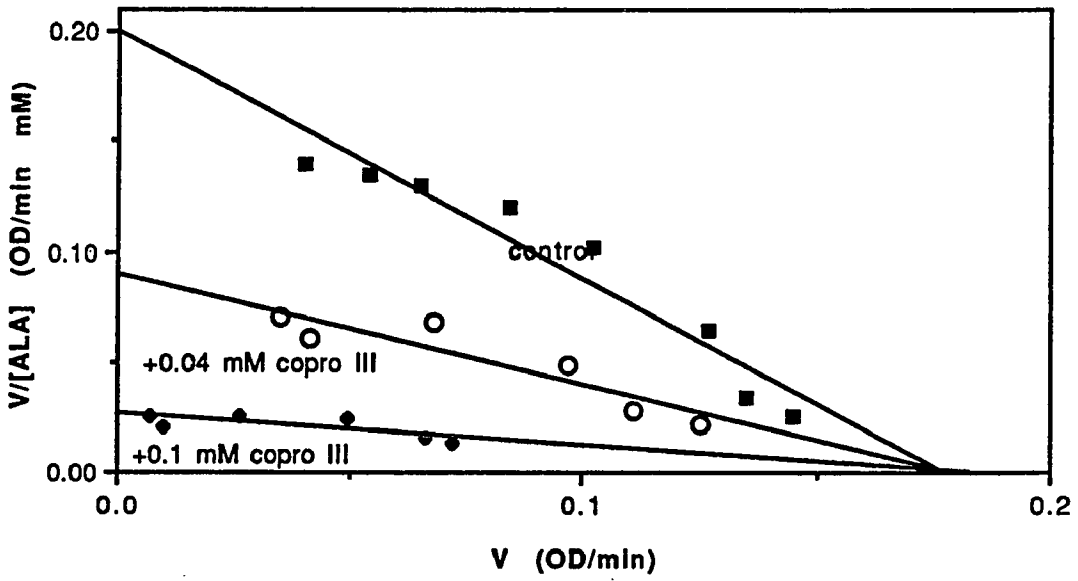


Figure 2.20B Eadie-Scatchard plot of *E. coli* ALA D in the absence and presence of coproporphyrin III. Replot of the data used in Fig. 2.20A.

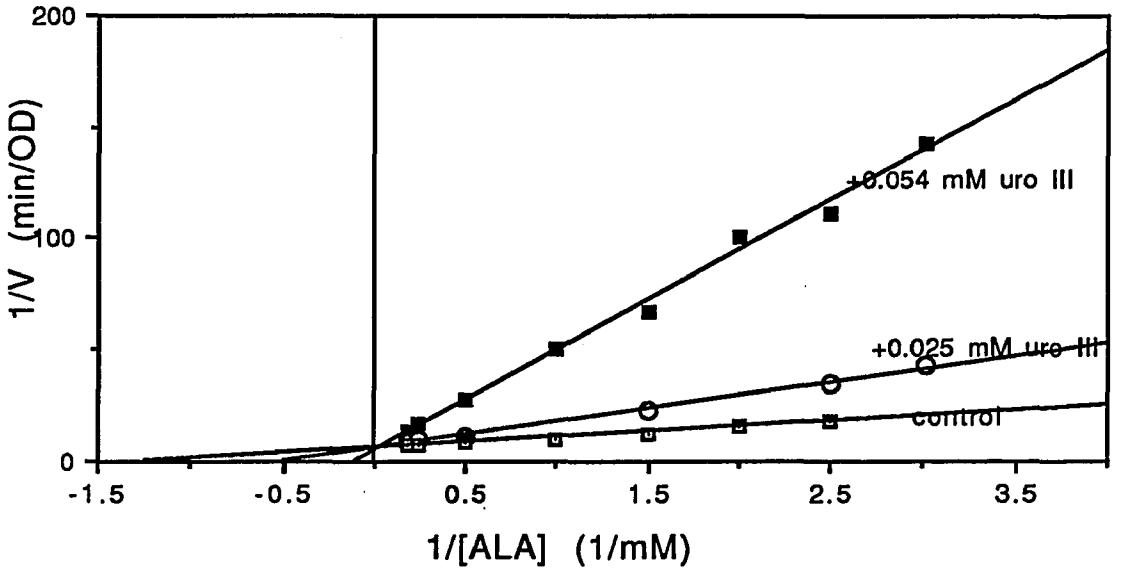


Figure 2.21A Double reciprocal plot of *E. coli* ALA D in the absence and presence of uroporphyrin III.

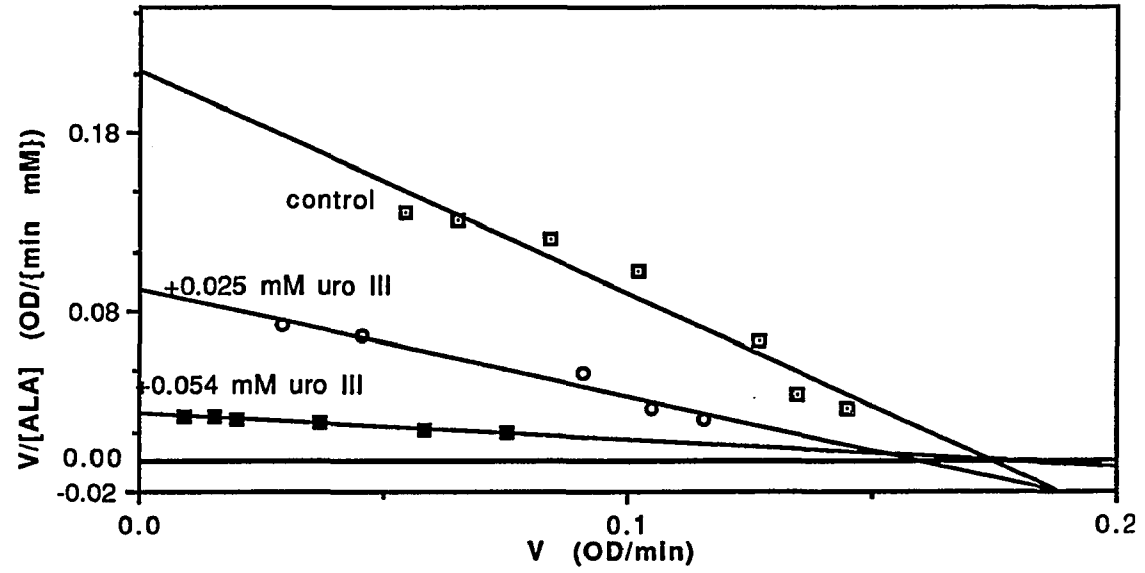


Figure 2.21B Eadie-Scatchard plot of *E. coli* ALA D in the absence and presence of uroporphyrin III. Replot of the data used in Fig. 2.21A.

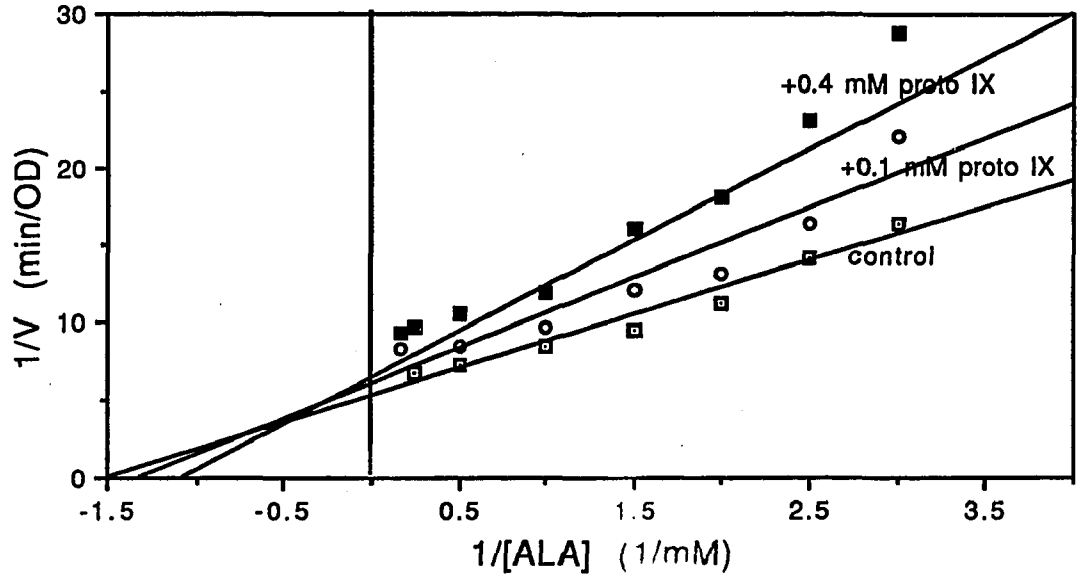


Figure 2.22A Double reciprocal plot of *E. coli* ALA D in the absence and presence of various concentration of protoporphyrin IX.

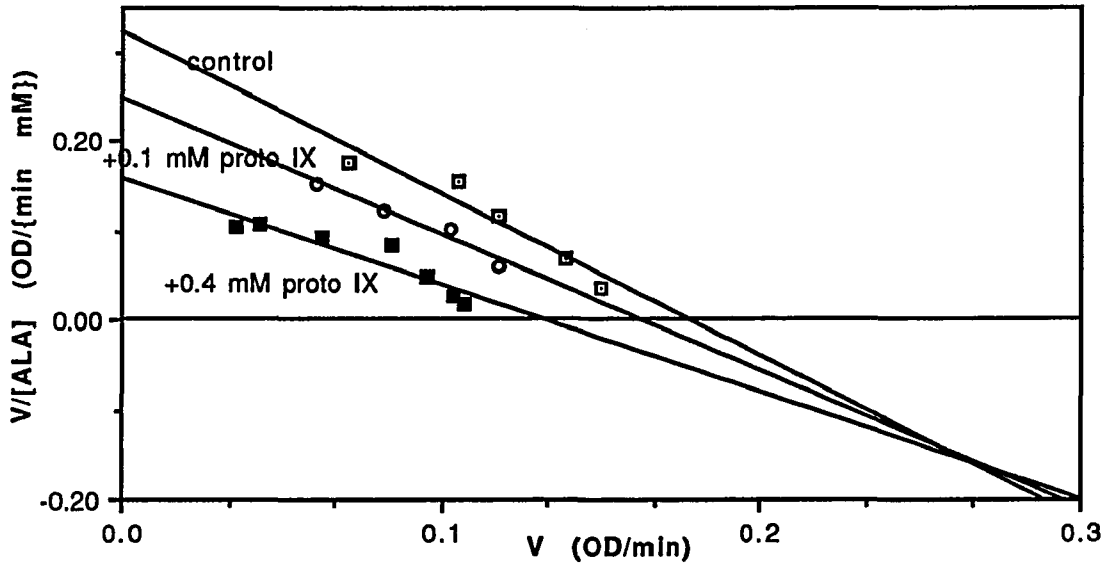


Figure 2.22B Eadie-Scatchard Plot of E coli ADA D in the absence and presence of protoporphyrin IX. Replot of the data from Figure 2.22A.

Table 2.7 Statistical treatment of kinetic data

Figure	X-intercept (OD/min)	Y-intercept (min/OD)	Vmax (OD/min)	K_m (mM)	K_i (mM)
2.16A		4.74 ± 0.23	0.21 ± 0.009	0.87	16.43 ± 1.25
2.16B	0.19 ± 0.003		0.91 ± 0.002	0.91	
2.17A		11.27 ± 0.8	0.09 ± 0.009	0.78	1.38 ± 0.029
2.17B	0.166 ± 0.001		0.166 ± 0.001	0.67	
2.18A		4.40 ± 0.87	0.24 ± 0.004	1.06	0.04 ± 0.001
2.18B	0.18 ± 0.001		0.18 ± 0.004	0.84	
2.19A		4.93 ± 0.22	0.20 ± 0.09	0.67	0.04 ± 0.002
2.19B	0.18 ± 0.001		0.18 ± 0.002	0.83	
2.20A		5.71 ± 0.49	0.18 ± 0.004	0.86	0.02 ± 0.001
2.20B	0.18 ± 0.004		0.18 ±0.007	0.89	
2.21A		5.34 ± 0.44	0.19 ± 0.006	0.86	0.001±0.0005
2.21B	0.16 ± 0.009		0.17 ± 0.04	0.80	
2.22A		6.06 ± 0.94	0.17 ± 0.04	0.84	0.39 ± 0.09
2.22B	0.18 ± 0.09		0.18 ± 0.006	0.84	

The average Km = 0.83 ± 0.095 mM
The data was analysed by Cricket graph 1.2.3, Cricket Graph SoftWare, Malvern, CA. and Stat View 512+, BrainPower, Inc, Agoura Hill, CA.

Chapter
3

HEME INHIBITION OF *E. COLI* ALA D. AGGREGATION BEHAVIOR OF HEMIN IN VARIOUS MEDIA

3.1 INTRODUCTION

Heme molecules have a planar cyclic tetrapyrrole core with four nitrogen atoms, derived from protoporphyrin IX, coordinated to an iron atom at the center (Fig. 1.1 Chapter 1). The iron atom may be Fe^{2+} or Fe^{3+} and has five to six coordinate bonds. The fifth and sixth coordinate positions are perpendicular to the ring. The hemes are classified as heme a, b, c, or d depending on the ring substituents (Stryer, 1988).

In hemoglobin and myoglobin the fifth position of the iron atom (Fe^{2+}) is covalently coordinated to histidine (His 93 (F8)) of the apoprotein. The sixth position is the oxygen binding site. Native cytochromes unlike the two previously mentioned proteins, contain a heme molecule with a ferrous iron atom which is reversibly oxidized to ferric iron (Fe^{3+}) and with both the fifth and sixth positions covalently coodinated to amino acid residues of the proteins.

Hemin contains an iron in the +3 oxidation state. In hemin chloride the fifth position is occupied by a chloride. In hematin the fifth position is occupied by an hydroxyl group. The behavior of hemin in solution is dependent on the medium in which it is dispersed. Hemin

is soluble in some organic solvents, alkaline aqueous solutions and aqueous detergent micelles but insoluble in neutral and acidic aqueous solutions (Brown et al., 1969; Simplicio, 1972; Fuhrhop, 1975). Hemin has been shown to undergo changes from monomeric to dimeric to polymeric in a concentration and pH dependent manner (Brown et al., 1970; Inamura et al., 1989). A specie called an oxodimer has a covalent oxo-bridge between the two iron atoms and has been proposed to occur only in the presence of alkali (Brown et al., 1969; Brown et al., 1970). In the nonionic detergent, sodium lauryl sulfate, hemin at μM concentration has been shown to exist as a monomer probably isolated in micelles (Simplicio, 1972). Soluble stable aqueous solutions of hemin as hemin arginate (1:3, hemin:arginine) have been obtained and used in hemin therapy (Tenhunen et al., 1987). Hemin arginate in solution (0.071 to 0.36 mM in 4:1:5 (w/w) propylene glycol:ethanol:water) shows characteristic maxima at 401 (Soret band), 506 and 621 nm (M. Hughes and C. S. Russell unpublished). Hemin arginate has been used as a media supplement for hemin-requiring mutants of *E. coli* and supports their growth (C. S. Russell et al., unpublished). ESR spectra of hemin arginate suggest polymeric structures at high concentration (38 mM), and oligomeric and dimeric structures at lower concentration (0.6 mM) (Tenhunen, 1986).

Hemin was dispersed in aqueous solutions of a number of low molecular weight (20-77 K) polymers such as poly(N-vinylpyrrolin-done) (PVP), polyethylene glycol (PEG), poly(vinylalcohol) (PVA) and dextran and shown to be soluble and proposed to be monomeric in PVP, PEG, and PVA, and dimeric in dextran (Inamura et al., 1989) (Fig. 3.1).

Hemin in solution prepared by first dissolving the crystals in 1N KOH and then diluting with buffer (Karibian and London, 1965) has been shown to inhibit the ALA D from several sources (Table 2.1 p. 112). The inhibition of ALA D from *R. spheroides* by hemin, prepared by the method of Karibian and London, (1965), was dependent on the sequence of addition of DTT and hemin to the reaction mixtures containing the enzyme. When the enzyme was preincubated with hemin (0.01 mM) prior to simultaneous addition of ALA and DTT greater inhibition (100% versus 82%) was observed than when the enzyme was first preincubated with DTT followed by simultaneous addition of ALA and hemin (0.01 mM) (Burnham and Lascelles, 1963; Nandi et al., 1968a).

The liver and red blood cell ALA D from both fetal and adult guinea pigs was noncompetitively inhibited by hemin (K_i= 3.4 to 44 μM). The only exception was the fetal red cell ALA D, which showed mixed-type inhibition by hemin in the presence of glutathione (Weissberg and Voytek, 1974). They did not described how the hemin solution was prepared

Hemin (0.1 mM) prepared by the method of Karibian and London, (1965) inhibited mouse liver ALA D by 80% (Coleman, 1966). In addition, a hemin preparation designated "hematin" without experimental details of preparation, was shown to be a competitive inhibitor of mouse liver enzyme with K_i= 7.1 μM (Doyle and Schimke, 1969). The K_m of mouse liver enzyme was 0.4 mM for ALA.

3.2 PURPOSE OF THIS STUDY

This report investigates the effect of hemin, dispersed in various media, as a possible feedback regulator on the ALA D from *E. coli*.

3.3 EXPERIMENTAL

3.3.1 Materials and Methods

Chemicals: 5-aminolevulinic acid, hemin, Tween-80 polyethylene glycol (PEG) (MW 15-20,000), poly(N-vinylpyrrolidone) (PVP) (MW 40,000), and Trisma base, were obtained from Sigma (St. Louis, MO). Branched dextran (MW 75,000) was obtained from Refined Syrups and Sugars Inc. (Yonkers, NY) a gift from Prof M. Fishman, City College. All other chemicals were reagent grade or better.

3.3.2 Preparation of Hemin Solutions and Hemin Spectra

Hemin (Sigma) in an aqueous solution of Tween-80 was prepared as followed: To 2.5 ml of Tween-80, 0.1 g of hemin and 22.5 ml of 0.05 M Tris pH 7.6, containing 3.3 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.01 M ß-ME, was added. The resulting solution was then titrated with 1 to 3 drops of 5 N NaOH until the hemin was completely dissolved. Finally the pH of the solution was adjusted to pH 7.6 for enzyme assay.

Hemin in aqueous solution of low molecular weight polymers was prepared as previously described (Inamura et al., 1989) with slight modifications. The polymer (0.4 to 1 g, dependent on recovery following re-precipitation) was dissolved in 0.05 M Tris pH 7.6, containing 3.3 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.01 M ß-ME (1 to 2 ml) in a 250 ml round bottom flask to form a paste. To the paste in the flask, 330 μl of 28% aqueous ammonia per gram of polymer, and 0.1 g of hemin were added. The flask was placed on a Rotavapor under vacuum until a thin dry film formed on the flask. To this film 25 ml of Tris buffer was added to give a hemin concentration of 4 mg per ml (6.136 mM). Each of the hemin/polymer solutions was then diluted to the

concentrations previously stated (Inamura et al., 1989) with the corresponding polymer solution in Tris buffer and the spectrum taken.

Hemin arginate solution was prepared as previously described (Tenhunen et al., 1987) with some modifications (M. Hughes and C. S. Russell unpublished). Propylene glycol (1,2-propanediol) (4 g) was mixed with ethanol (1 g), water (5 g) and arginine (2 g). The solution was stirred while hemin (0.25 g) was added. Stirring was continued for 4 to 5 h until the hemin was completely dissolved. This solution corresponded to a 25 mg/ml (38.34 mM) hemin solution. The hemin arginate solution was diluted with a solution of propylene glycol/ ethanol/water (4:1:5) and the ultraviolet-visible spectra were recorded.

Studies of the inhibition of *E. coli* ALA D by hemin were performed in the corresponding media in which the hemin was dispersed. The hemin solution was diluted with the appropriate buffer to the concentration specified in Table 3.1 and assay of ALA D was performed as stated in section 2.5.1.5, Chapter 2.

3.4 RESULTS

3.4.1 Inhibition of *E. coli* ALA D by Hemin

The data in Table 3.2 show that hemin (1.39 mM) in aqueous dextran (4%) had the greatest inhibitory effect (72%) on *E. coli* ALA D. However assay of ALA D in dextran gave non-linear kinetics (data not shown). *E. coli* ALA D was also inhibited by hemin dispersed in aqueous PVP (8%), PEG (12%) and Tween-80 (45%) (Table 3.2). Hemin arginate dissolved in either 0.05M Tris, pH 7.6 or propylene glycol:ethanol:water (4:1:5 w/w), pH 7.6, did not inhibit *E. coli* ALA D (Table 1.3). At micromolar concentrations of hemin no inhibition of the enzyme was observed.

The double reciprocal initial velocity plots of ALA D in the presence of hemin dispersed in Tween-80 was non-linear and the data points occurred in clusters for low and high ALA concentrations suggesting a biphasic type of inhibition (data not shown). We did not pursue this further, but this may be the result of different hemin species interacting with an enzyme which may change conformation in different concentrations of substrates. This warrants further study.

3.4.2 Visible Spectra of Hemin in Aqueous Solutions of Polymers and of Hemin arginate

The ultra violet-visible spectrum of hemin disperse in aqueous dextran (4%), aqueous Tween-80 (10%) and hemin arginate (in 4:1:5 propylene glycol:ethanol:water) were recorded from 300 to 700 nm (Fig. 3.2 and Fig. 3.3).

3.5 DISCUSSION AND CONCLUSION

The hemin preparations in Tween, dextran and the hemin arginate all supported growth of hem$^-$ mutants of *E. coli*. Tween (10%) and dextran (4%) do not lower ALA D activity and hemin in these media are the best inhibitors (Table 3.2). The other polymers maybe sequestering the hemin species. The spectrum of hemin dispersed in Tween-80 had a sharp Soret band (Fig. 3.2) demonstrating that hemin in Tween-80 was probably more monomeric. Hemin in Tween-80 was a less effective inhibitor of *E. coli* ALA D than hemin in dextran but more effective than hemin in PVP and PEG (Table 3.2). Based on the comparison of spectra of hemin arginate, and hemin in Tween-80 to that of monomeric hemin (Inamura et al., 1989) hemin arginate is probably more monomeric than hemin in Tween-80. A monomeric hemin-chloroquine complex or hemin-BSA complex was shown to lyse malaria parasites and cells, where hemin aggregates do not (Orjih et al., 1981; Balasubramanian et al. 1984). Both the inhibition studies (Table 3.2) and spectral data (Fig. 3.2 and Fig. 3.3) suggest that possibly hemin aggregates can inhibit *E. coli* ALA D, but not as effectively as for the enzymes from other sources. In the case of hemin which may be trapped in micelles in Tween-80 the hemin may be less accessible to inhibit the enzyme, while in dextran, which is branched and mesh-like, the hemin may be bound on the surface of the polymer making it more accessible to inhibit the enzyme. *E. coli* ALA D was not inhibited by hemin arginate.

Table 3.1 Absorption maxima of hemin dispersed in various media (adapted from Inamura et al., 1989)

Medium	Absorption maxima (nm)			
	Soret band	I	II	III
DMSO (100%)	405	498	575	623
NaOH (pH 10)	360 388	500		603
PVP (4%, pH 6.3)	401		568	608
PEG (4%, 7.1)	400	490		608
PVA (4%, pH 7.5)	389			600
Dextran (4%, pH 7.5)	365 389			608
Triton-X 100 (1%, pH 5.7)	401		573	600

Table 3.2. Activities of *E. coli* ALA D in various media with and without hemin (or hemin arginate)

Aqueous Medium (pH 7.6)	Hemin (1.39 mM)	ΨEnzyme Activity (%)	¥Activity Remaining (%)
Tris (0.05M)	-	100	100
Tween-80 (10%)	-	100	
Tween-80 (10%)	+		55
PVP (4%)	-	84	
PVP (4%)	+		92
PEG (4%)	-	75	
PEG (4%)	+		88
Dextran (4%)	-	120	
Dextran (4%)	+		28
	Hemin arginate (1.39 mM)		
Tris (0.05 M)/ 20% arginine	-	40	
	+		100
*PG:EtOH:H2O/ 20% arginine	-	27	
	+		100

ΨEnzyme activity in Tris was taken as 100%.

¥ For inhibition studies 100% activity was taken as ALA D assayed in each of the corresponding aqueous polymer solutions in the absence of hemin.

*PG:EtOH:H2O is propylene glycol:ethanol:water (4:1:5 w/w)

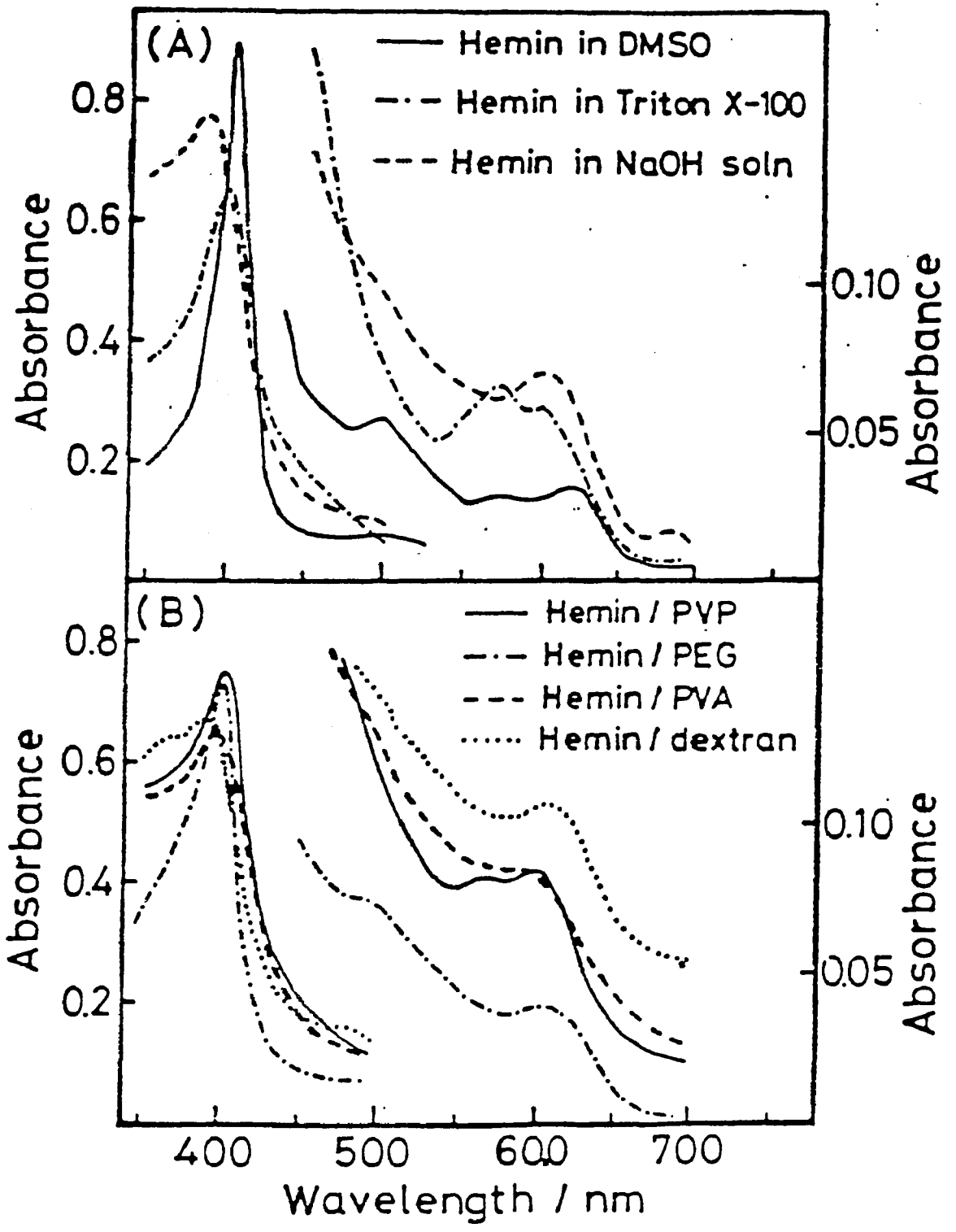


Figure 3.1 Absorption spectra of hemin in different media (adapted from Inamura et al., 1989)

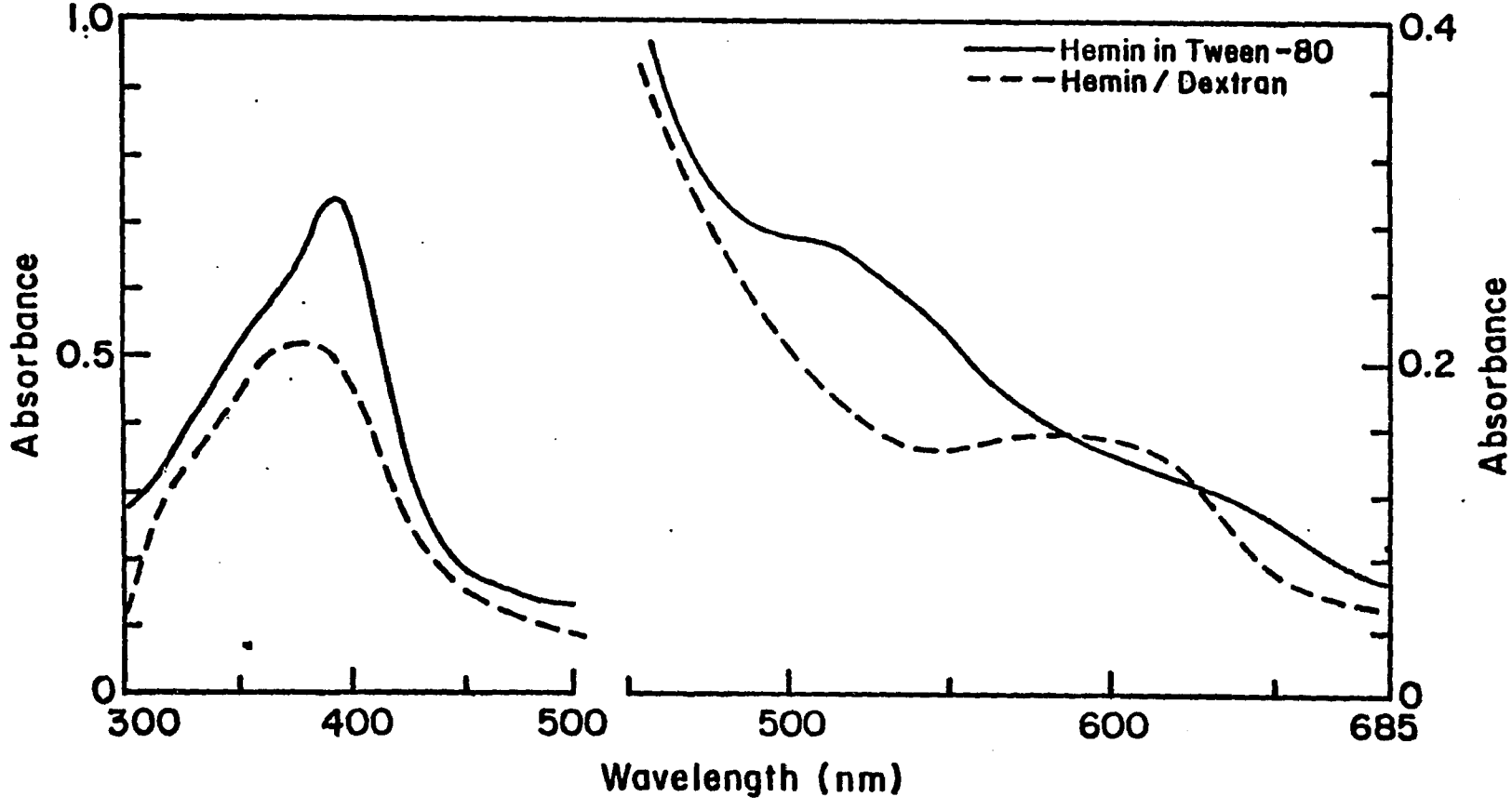


Figure 3.3 Absorption spectra of hemin (0.139 mM) dispersed in Tween-80 (10%, pH7.6) and Branched dextran (4%, pH 7.6).

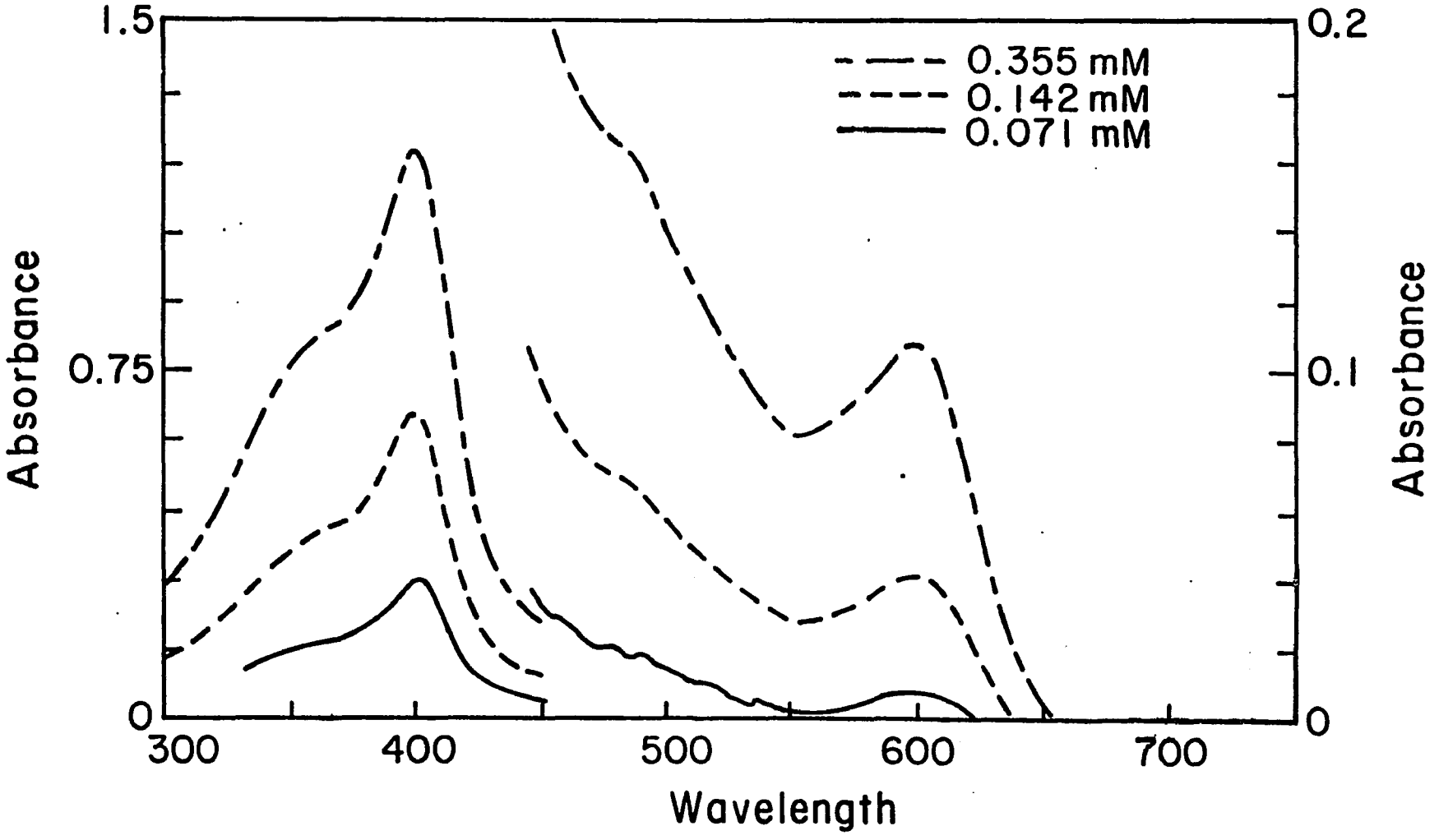


Figure 3.3 Absorption spectra of hemin arginate (0.071 to 0.355 mM) in propylene glycol/ethanol/water (4:1:5) solution.

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