Lien, Li-Fen Elizabath

STUDIES ON THE CHARACTERIZATION OF DELTA-AMINOLEVULINIC ACID SYNTHASE AND AN ASSOCIATED AMIDASE

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STUDIES ON THE CHARACTERIZATION OF δ-AMINOLEVULINIC ACID SYNTHASE AND AN ASSOCIATED AMIDASE

by

Li-Fen E. Lien

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ABSTRACT

THE CHARACTERIZATION OF δ- AMINOLEVULINIC ACID SYNTHASE AND AN ASSOCIATED AMIDASE

by

Li-Fen E. Lien

Advisor: Professor D.S. Beattie

The colorimetric assay and radiochemical assay for determining the activity of δ-aminolevulinic acid synthase (ALAS, E.C.4.2.1.24) were compared. A rapid and sensitive radioactive method for the assay of soluble ALAS has been developed based on the single column procedure of Ebert et al. It was concluded that the major contaminant is the cysteamine thioester of succinate. This contaminant was shown to be formed by the enzymatic cleavage of the amide bond between β-mercaptoethylamine and pantothenic acid of succinyl CoA by an amidase.

The product of the amidase is optimally formed in the absence of glycine and is isolated by stopping the reaction with acidic SDS. Optimal conditions for the measurement of this amidase activity are described.

The submitochondrial localization was compared for the δ-aminolevulinic acid synthetase (ALAS) and this amidase. It was found that the amidase is localized on the outer surface of the inner membrane while ALAS is localized in

the matrix.

The protease inhibitors, PMSF, TLCK or TPCK were without effect on the amidase, however, it was inhibited by o-phenantheoline and the inhibition was reversed by addition of either Co^{2+} or Mn^{2+} suggesting a metal requirement at the active site of the amidase.

Optimal conditions were established to assay both liver ALAS and skeletal muscle ALAS by using the improved radiochemical procedure. The enzymatic activities of both enzyme were investigated under conditions known to affect the heme biosynthetic pathway. Compared with controls, streptozotocin induced diabetic rats had a 56% decrease in ALAS activity in skeletal muscle mitochondria, while no change was found in ALAS activity in liver mitochondria. Administration of insulin to the diabetic animals partially reversed the effect of diabetes on skeletal muscle ALAS; however, administration of insulin to control animals caused a 21% decrease in skeletal muscle ALAS activity. By contrast, treatment with inducers of hepatic ALAS such as allylisopropyacetamide or 3,5-dicarbethoxy-1,4-dihydrocollidine had no effect on skeletal muscle ALAS. These results confirmed that ALAS activity is regulated in a tissue-specific manner.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

allylisopropylacetamide	AIA
acute intermittent porphyria	AIP
δ-aminolevulinic acid	ALA
δ-aminolevulinic acid dehydratase	ALAD
δ-aminolevulinic acid synthetase	ALAS
catalase	CAT
cytochrome	CYT
dexamethasone	DEX
3,5-dicarbethoxy-1,4 dihydrocollidine	DDC
Digitonin-soluble fraction	DS
dimethylaminobenzaldehyde	DMAB
dithiothreitol	DTT
ethylenediaminetetracetic acid	EDTA
hour(s)	hr
inner membrane	IM
isocitrate dehydrogenase	ICDH
Lubrol-soluble fraction	LS
minutes	min
molecular weight	Mr
N-acetylglucosominidase	NAGA
porphobilinogen	PBG
pyridoxalphosphate	PLP

sodium dodecyl sulfate	SDS
succinyl CoA synthase	SCS
succinate dehydrogenase	SDH
trichloroacetic acid	TCA
thin-layer chromatography	TLC
tosyl-leusinyl chloromethyl ketone	TLCK
tosyl-phenylalanyl chloromethyl ketone	TPCK

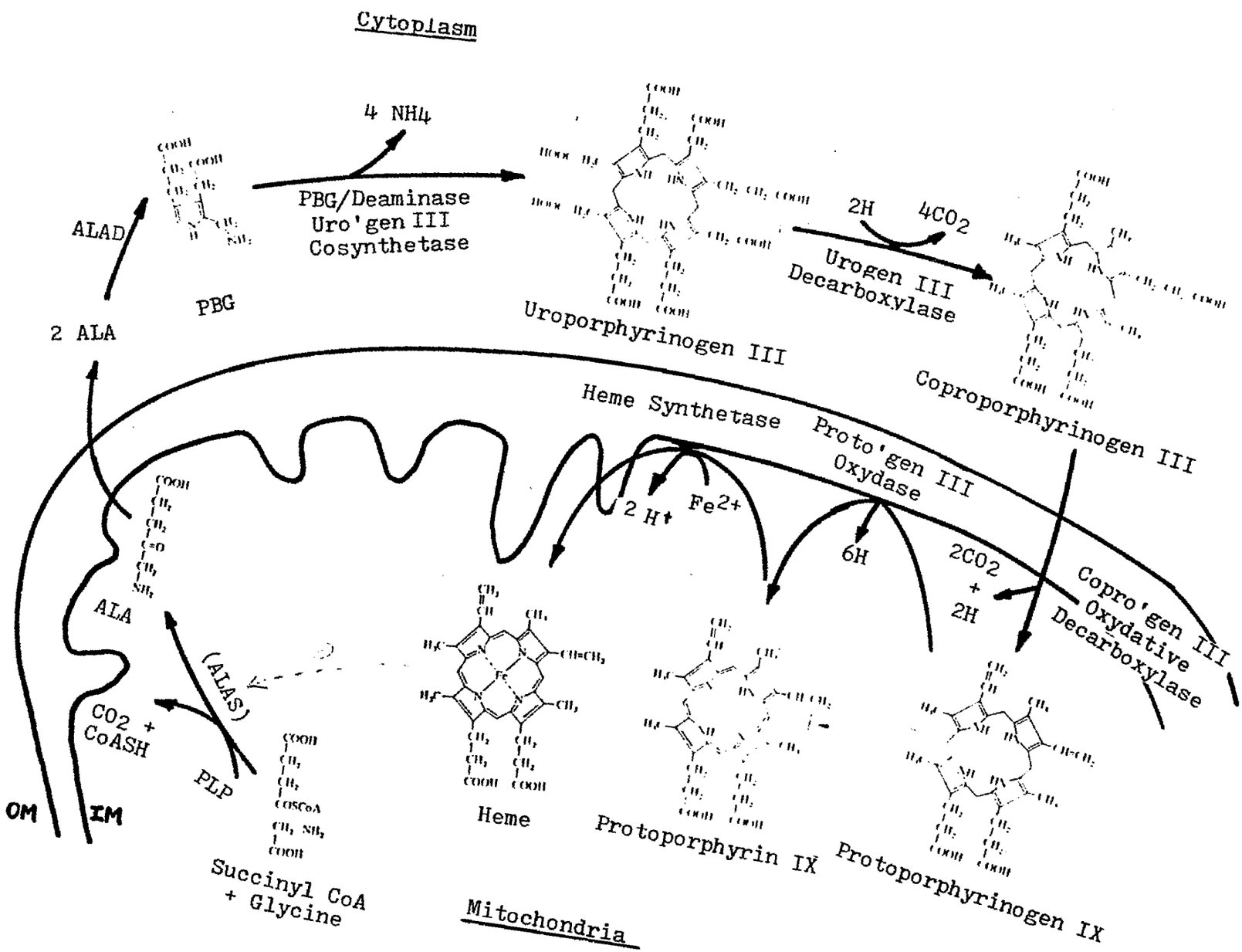
I. INTRODUCTION

A. Heme Biosynthesis

The heme biosynthetic pathway was originally elucidated by Shemin and others in a series of experiments in which radiotracers were used (1,2). The sequence of enzymatic steps from the condensation of glycine and succinyl CoA to form δ-aminolevulinic acid (ALA) to the final insertion of iron into protoporphyrin IX to yield heme is outlined in Fig. 1. This pathway is compartmentalized with a special distribution between the mitochondria and the cytosol. The first and rate-limiting step is catalyzed by the mitochondrial enzyme δ-aminolevulinate synthase (ALAS) (E.C.2.3.1.37) in mammalian, avian cells and photosynthetic bacteria (3,4). ALAS catalyzes the condensation of glycine and succinyl CoA to form ALA with the release of carbon dioxide and CoA. ALA then diffuses into the cytoplasm where ALA dehydrotase catalyzes the self-condensation of two molecules of ALA to form the pyrrole, porphobilinogen (PBG) (5,6). Porphobilinogen deaminase catalyzes the conversion of 4 moles of PBG to 1 mole of uroporphobilinogen I and 4 moles of ammonia. Uroporphyrinogen-III-cosynthetase then converts uroporphyrinogen I to its steroisomer, uroporphyrinogen III (7,8). Another cytoplasmic enzyme, uroporphyrinogen decarboxylase,

Figure 1. Biosynthesis of Heme

In the mammalian system, the first enzyme, ALAS, is a mitochondrial enzyme and is the rate-limiting enzyme for the entire biosynthetic pathway. ALAS was shown to be an allosteric enzyme which can be feedback inactivated by the end product heme. However, the concentration of heme required for the inactivation is considerably high (51). Recently, more evidence supports heme inhibiting the formation of ALAS at the transcriptional level. This pathway is compartmentalized with a special distribution between the mitochondria and the cytosol, as shown in the figure. The last three enzymes were shown to be localized with the mitochondrial inner membrane.


Cytoplasm
4 NH4
PBG/Deaminase
Uro'gen III
Cosynthetase
ALAD
PBG
2 ALA
Uroporphyrinogen III
2H
4CO2
Urogen III
Decarboxylase
Coproporphyrinogen III
Heme Synthetase
Proto'gen III
Oxydase
Copro'gen III
Oxydative
Decarboxylase
2 H+
Fe2+
6H
2CO2
+
2H
ALA
(ALAS)
CO2 +
CoASH
PLP
OM
IM
Heme
Protoporphyrin IX
Protoporphyrinogen IX
Succinyl CoA
+ Glycine
Mitochondria

converts the four acetic acid side chains to methyl groups to give coproporphyrinogen III. The remaining steps in heme biosynthesis occur in the mitochondria again. Coproporphyrinogen oxidase oxidatively decarboxylates the two propionic acid side chains on ring A and B to vinyl groups, forming protoporphyrinogen III. Protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen III to protoporphyrin IX. Finally, heme synthetase (ferrochelatase) catalyzes the insertion of Fe^{2+} into the protoporphyrin IX ring resulting in the formation of the end product heme (9,10).

There are two major types of cells that are responsible for synthesizing the bulk of heme in mammals: the erythropoietic cells and the liver parenchymal cells. However, the control of heme and hemoprotein biosynthesis in one cell is different from the other. The heme synthesized by erythropoietic cells is mainly incorporated into hemoglobin. By contrast, the heme synthesized by the liver cell serves as the prosthetic group for mitochondrial and microsomal cytochromes. The former cytochromes are for electron transport and the latter are for drug detoxification. Part of the heme which is synthesized by the liver is incorporated into peroxisomal hemoproteins, such as peroxidase and catalase. These hemoproteins play important roles for general liver functions. In erythropoietic cells, heme was shown to act at the translational level by stimulating globin synthesis (11,12).

By contrast, in liver, heme does not stimulate the synthesis of protein moieties of hemoproteins. The rate of synthesis of individual apoproteins changes in response to environmental changes, and the free heme pool (regulatory heme) is therefore changed; subsequently, the rate of heme biosynthesis is controlled (13,14). Since heme is a feedback inhibitor, it controls the rate-limiting enzyme, ALAS, at either translational or transcriptional levels (15,16,17). Therefore, heme can regulate its own sytnesis at the level of ALAS. Ohashi and Kikuchi (18) have reported that heme also controls the translocation of ALAS from the cytosol into the mitochondria; however, heme does not affect the transport of ALAS in embryonic chick liver cells (19). Heme can also control its own degradation by serving as an inducer of heme oxygenase (20), which converts the heme to biliverdin IX and carbon monoxide. Under normal conditions, liver mitochondrial ALAS is present in very low levels. Also, ALA and other intermediates of the pathway exist in small amounts. This indicates that the biosynthetic pathway is very efficiently regulated to provide the heme requirements of the cell without waste of intermediates. However, when the control mechanism breaks down, more intermediates are accumulated and excreted. These conditions are defined as porphyrias.

B. The Porphyrias

The hereditary porphyrias were recognized early in the 1920's as diseases of abnormal heme metabolism and were characterized by specific patterns of porphyrin excretion. The enzymatic deficiencies of these disorders were not identified until the 1970's. The porphyrias are classified into erythropoietic and hepatic types (21,22). The hepatic types of porphyria are interesting diseases, because their victims are healthy for most of their lives with the exception of acute attacks. Each type of hepatic porphyria (Table I) is associated with a specific enzyme deficiency in the heme biosynthesis. The acute attacks can be exacerbated by many drugs, such as phenylbutazone, barbituates, sedatives (23,24) and certain types of steroid hormones (25,26,27), etc. During the acute attack, the victims have a specific pattern of porphyrin or early precursor excretion. All of them, however, were found to have highly induced activities of ALAS.

Some drugs, such as Allylisopropylacetamide (AIA), 3,5-dicarbethoxy-1,4 dihydrocollidine (DDC), etc., can cause experimental porphyria in normal animals or humans. Those drugs are lipophilic and are defined as porphyrinogenic drugs. The experimental porphyria has always been an excellent tool for the study of the mechanism of porphyria formation. The mechanism of AIA induced porphyria has received intensive study. AIA was shown to be a very potent inudcer of ALAS and porphyria. Its induction involved a drug detoxification

Table 1. The Inherited Hepatic Porphyria

Each type of inherited hepatic porphyria is associated with a specific enzymatic deficiency in the heme biosynthetic pathway. During the acute attack, patients have a specific pattern of porphyrin on early precursor excretion. These inherited porphyrias are inherited as autosomal dominant traits.

Porphyria	Enzymatic Defect
Intermittent Acute Porphyria (IAP)	PBG Deaminase
Porphyria Cutanea Tarda (PCT)	Urogen Decarboxylase
Hereditary Coproporphyria (HCP)	Coprogen Oxidase
Variegate Porphyria (VP)	Protogen Oxidase

system. It was also shown to be the suicidal substrate for cytochrome P450 (28). Each cytochrome P450 turns over 230 AIA molecules and is then destroyed (29,30). The destructive process is still unknown, but it is clear that the prosthetic heme of cytochrome P450 is the exclusive target site (31). During the process, the prosthetic heme of cytochrome P450 is somehow alkylated and then removed from its apoprotein to form green pigment (32). The remaining apoprotein is ready to be reconstituted with another heme from the "free heme pool" (33). Therefore, the free heme pool is depleted. Subsequently, the repression of the synthesis of ALAS is released. While de novo synthesis of ALAS is highly increased (34,35), under these circumstances, other enzymes for heme biosynthesis become restricted, and intermediates of heme biosynthesis are accumulated and excreted.

The mechanism of ALAS regulation and its induction by porphyrinogenic drugs have been extensively studied in both rat liver and chick embryo liver (26,27,36). In the rat, ALAS has been identified in a variety of extrahepatic tissues, including adrenal (37), spleen (38), kidney (39), heart (40), brain (41), testes (42) and skeletal muscle (43), suggesting that ALAS is present in all tissues which synthesize porphyrins. ALAS activity appears to be regulated in a tissue-specific manner by a number of factors, including nutritional status (37,40), hormones (44,45), porphyrinogenic

drugs (36,46), ethanol (47) and metal ions (48,49,50).

The knowledge of ALAS has a significant contribution to the understanding of heme biosynthesis regulation and porphyria. In fact, it has been used as a life-saving therapy to give the porphyria patients heme in order to release them from an acute attack. This is an excellent example of how basic research could be applied to clinical therapy.

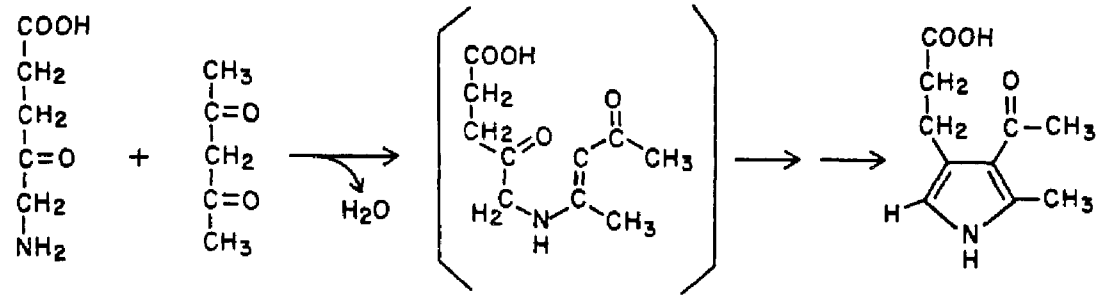
C. The Determination of ALAS

There are three major methods for the determination of ALAS activity. They are briefly described in the following sections.

1. Colorimetric Assay

The original method developed to measure ALA was a colorimetric assay of urine. It was first described by Mauzerall and Granick in 1956 (52). In this procedure, the product ALA is first converted to a pyrrole by the condensation of an aminoketone such as ALA with either a ketone or a keto ester to form a pyrrole in the presence of acetate (Equation 1). The resulting ALA pyrrole is then determined colorimetrically after reaction with a modified Ehrlich's reagent in which dimethylaminobenzaldehyde (DMAB) is the reactive component (52) (Equation 2). The colored salt is then quantitated at 552 nm in a spectrophotometer.

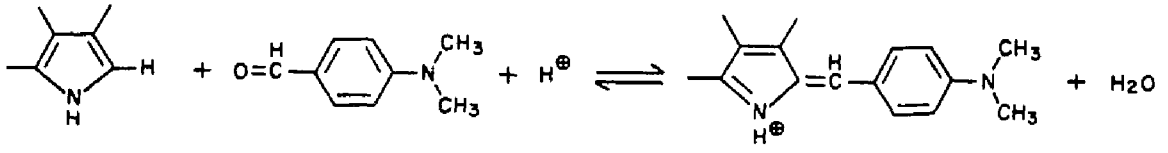
Eq 1.



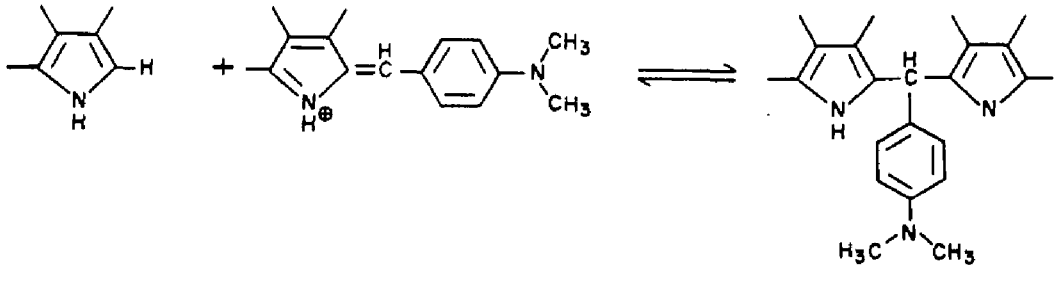
ALA Acetylacetone

ALA-Pyrrole
(2-methyl-3-acetyl-
4-propionic acid pyrrole)

Eq 2



P DMAB E



P E M

The major problem with the colorimetric assay is the formation of another compound, aminoacetone pyrrole, which also reacts with Ehrlich's reagent to form a colored salt with the same absorption properties as ALA pyrrole (53,54, 55). Thus, it is necessary to separate the aminoacetone pyrrole from the ALA pyrrole in order to obtain an accurate measurement of the ALAS activity. There are two types of methods: chromatography on Dowex 1 or organic solvent extraction. The first chromatographic method described for the quantitative separation and determination of aminoacetone pyrrole and ALA pyrrole involved multiple column steps to separate the aminoketones (53). This method was later modified to a single chromatographic procedure which is based on the different binding mechanisms between the anion exchange resin (Dowex 1) and either ALA-pyrrole or aminoacetone pyrrole (56). The disadvantage of this method is that it is time-consuming.

Alternately, the pyrroles of ALA and aminoacetone can be separated by differential solvent extraction. Either extraction has been used successfully to remove the aminoacetone pyrrole from the ALA-pyrrole in the reaction mixture by taking advantage of their different solubilities (57,58). Recently, it was found that aminoacetone pyrrole is readily separated from ALA-pyrrole by extraction with two volumes of dichloromethane, which removes 92 $\pm$ 5% of the aminoacetone pyrrole formed in the mixture (18,51,59).

The colorimetric assay has been widely used to assay ALAS in mammalian tissues, avian cells and bacteria such as Rhodopseudomonas spheroides (4). The major advantages of the colorimetric assay when compared to radiochemical assays are its simplicity, rapidity and low cost. The major disadvantage of the colorimetric assay is its insensitivity, which limits its use to tissues with high ALAS activity (0.56 units per ml of incubation mixture). Among the different methods described prior to colorimetric determination, extraction with dichloromethane is the easiest and most rapid method; however, extraction with ether is the preferred method because of its low background and high reproducibility.

2. Radiochemical Assay

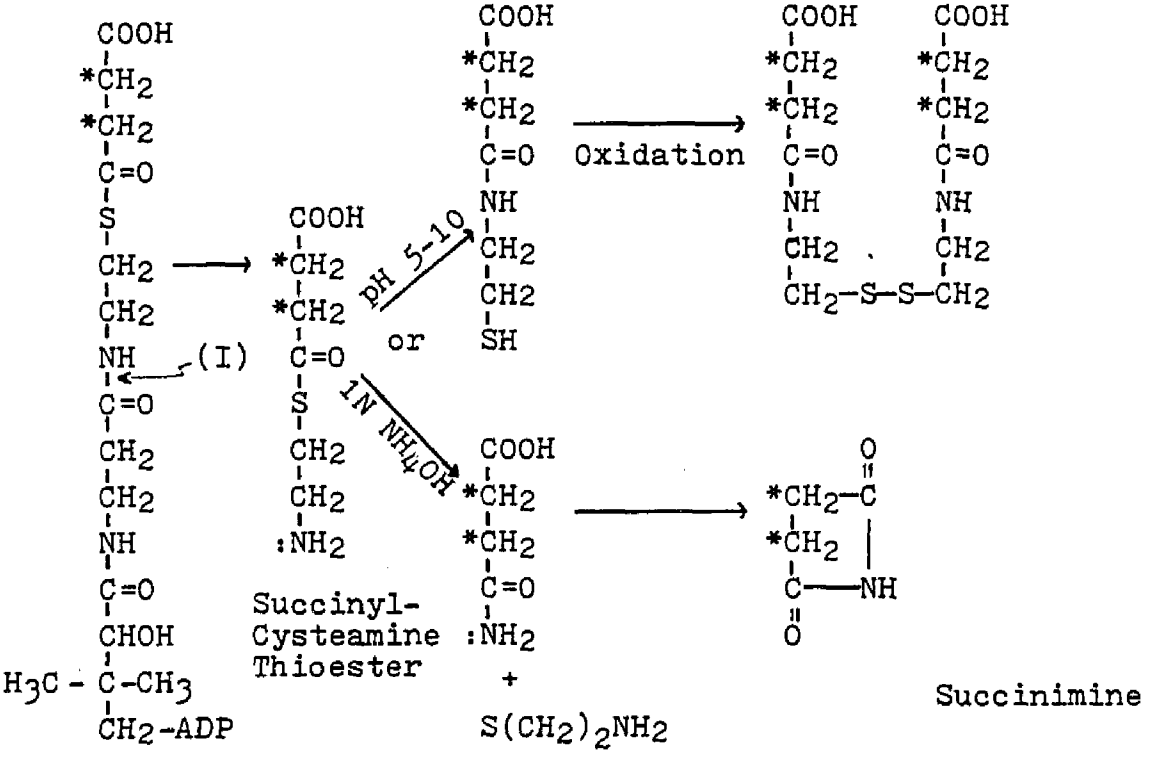
The radiochemical assay first developed by Ebert et al. (38) consists of the radiochemical detection of ALA, using (2,3,^{14}C)succinyl CoA as a tracer. The labeled ALA is selectively absorbed on Dowex 50 (Na^+), ion-exchange resin at pH 3.9 and subsequently eluted by a strong base. Unreacted succinate and some other byproducts formed through the Krebs-cycle should not be bound to the resin under these conditions. Because of its sensitivity and reproducibility, it has been widely used for the assay of ALAS either in intact mitochondria or in solubilized enzyme preparations. In recent years, several laboratories have reported that the

single-column methods lead to large errors in determining ALA concentration. The presence of contaminants with similar mobilities on Dowex 50 to that of ALA (60,61,62) has been reported. Bishop and Wood (61), using an amino acid analyzer, reported the presence of tyrosin-like and valine-like compounds in the column eluate in addition to ALA. Subsequently, Minaga et al. (60) proposed that in rat liver homogenates, there are succinyl CoA-degrading enzymes which may hydrolyze succinyl CoA through an endopeptidase-like activity. They suggested that succinyl CoA was cleaved at position 1 (see Equation 3) to form succinyl-cysteaminthioester which undergoes an internal nucleophilic rearrangement at pH 5-10 and forms N-succinylcysteamine. This thiol is further oxidized to the stable disulfide dimer.

Strand et al. (63) have developed a three-column radiochemical assay which involves two tandem columns to separate ALA from unreacted substrates, followed by conversion of ALA to ALA pyrrole which is then separated on Dowex 1 (acetate) columns. This method is specific but tedious, time-consuming and has higher errors due to the variability of recovery of the ALA-pyrrole.

When intact mitochondria are assayed, succinyl CoA is generated readily from the Krebs-cycle. When disrupted mitochondria are assayed, an exogenous succinyl CoA generating

Equation 3:



Succinyl CoA

system must be supplied. Succinyl CoA synthetase (succinyl CoA thiokinase) can be prepared either from an ALA requiring mutant of Rhodopseudomonas spheroides (66) or from Escherichia coli (68). The pig heart enzyme is also commercially available. Chemically synthesized succinyl CoA is also a suitable substrate (60,65).

3. Fluorometric Assay

This method is involved in the specific quantitation of ALAS activity by the enzymatic conversion of ALA to the highly fluorescent product, URO I through the heme biosynthetic pathway (66). The practicality of the coupled enzyme assay is based on the high-yield purification of ALA dehydratase (67) and PBG deaminase (68) from human erythrocytes. The bovine ALA dehydratase is commercially available, but PBG deaminase is not. Hopefully, in the near future, purified PBG deaminase will be commercially available. It was reported (66) to have a sensitivity for determining picomole quantities of ALA by this method. It was also shown to be a rapid and specific determination.

D. Mitochondrial Protein Degradation and Protease

1. Mitochondrial Protein Degradation

The mechanism of the degradation of mitochondrial pro-

teins has been an important and interesting problem.

Penn (69) first described a CoA-dependent neutral protease in rat liver mitochondria breaking down endogenous proteins. Alberti and Bartley (70,71,72) showed the production of amino acids by rat liver mitochondria. Based on those study, there are neutral protease activities residing within the mitochondria.

By contrast, Ferdinand et al. (73) questioned about this hypothesis. They compared the amino acid composition of the mitochondrial membrane and the amino acid produced during anaerobic incubation of mitochondria at pH 7.4 and found a lot of discrepencies. The other group showed the mitoplasts possess much less proteolytic activity than mitochondrial preparations. Yet, the digitonin soluble fraction contains high proteolytic activity at neutral pH (74). Therefore, they suggested that actually lysosomes are responsible for the protein degradation of mitochondria. Later on, a novel protease associated with mitochondrial inner membrane was reported (75).

Also, Dugue-Magalhaes and Ferreira (76) observed a cytochrome c degrading enzyme in rat liver mitochondria by means of a suitable zymographic technique.

Up to now, a definitive model of mitochondrial protein degradation remains unestablished, even though many protease-like activities were found, and still much is being made to

solve this question.

2. Mitochondrial Proteases

In a series of investigations of mitochondrial protein degradation (Table 2), a group-specific protease for apoproteins of pyridoxal enzymes was reported (77,78,79). This enzyme cleaves the apoproteins but not holo-enzymes of pyridoxal enzymes. It was characterized to be a serine protease. This group-specific protease has been isolated from various organs: rat livers, skeletal muscles and intestines. It has maximal activity at alkaline pH and has chymotrypsin-like properties. However, during the study of its characterization, it was found that the amino-terminal sequences and immunologic properties of this group-specific protease is identical with the chymase of peritoneal mast cells (80). That finding raised the question of the mitochondrial origin of this group-specific protease. In the meantime, a histone degrading enzyme was reported to be a mitochondrial inner membrane enzyme in rat liver (81,82,83). Shortly after its discovery, the mitochondrial histone degrading enzyme was then shown to be no different from group-specific protease (82). In fact, after a re-evaluation of its localization, it was found to be localized within the granules of "Atypical" mast cells (84). The "Atypical" mast cells are present in large numbers under the liver capsule and around the

Table 2. Literatures of Putative Mitochondrial Proteases

Enzyme	Properties	Ref.
Group-Specific Protease	Early Findings: 1. Isolated from rat liver mitochondria, specific for apoproteins of pyridoxal enzymes 2. pH optimal = 8.5 3. Associated with mitochondrial inner membrane	78,79.
	Actually: 1. It is mast cell enzyme	80.
Histone Degrading Enzyme	Early Findings: 1. Degrade histone, glucagon & non-histone chromosomal protein 2. pH optimal = 8.0 3. Molecular weight = 23K dalton 4. Probably associated with mitochondrial inner membrane	82, 83, 84.
	Actually: 1. It is identical to group-specific enzyme 2. It is not mitochondrial origin but mast cell enzyme	84.
Carboxy-peptidase -SH Type	1. It is a -SH requiring carboxypeptidase 2. pH optimal = 9.0 3. Molecular weight = 35 ~ 38K 4. Appeared to be inner membrane enzyme	83.
Cytochrome c Degrading Enzyme	1. pH optimal = 7.4 2. There are 3 different types of cytochrome c degrading enzymes: two membrane enzymes and one soluble enzyme 3. Each type has its own pattern of cleavage (examined by zymograph)	76.

hilum. Those mast cells were co-isolated with mitochondria, and during the submitochondrial fractionation, they were co-isolated with the inner member fraction.

During the past ten years, many other mitochondrial proteases were reported such as a protease which shows specificity for ALAS isolated from human bone marrow mitochondria (85), and a carboxyl protease which was also reported (82).

In spite of many frustrations in the study of mitochondrial protease, it is still a very promising area. Since some mitochondrial proteins are made as larger precursors in the cytoplasm, they need to be imported and processed to their mature size. Some types of proteases have to associate with mitochondria in order to operate the processing of mitochondrial precursor proteins.

E. Protein Process and Transportation

1. Vectorial Translation and Signal Peptidase

Numerous recent studies have vastly increased the knowledge of the synthesis of secretory proteins and polypeptide hormones. The secretory proteins and polypeptide hormones are synthesized on membrane-bound polyribosomes; their NH_2-terminal acts as a "signal" directing the nascent peptide chains to rough endoplasmic reticulum (RER) membranes (86), it is therefore a "vectorial translation" or so called "signal hypothesis" (86,87).

During peptide elongation, the growing polypeptide would be "driven" into and through the membrane (86,87), and therefore, it is "co-translational" transportation. The NH_2-terminal, which is a pre-segment (or signal peptide), is proteolytically removed during translation by a signal peptidase. Amino acid sequences of the signal peptides of many of these precursors have been partially or completely determined (88,89,90). All of the signal peptides are very hydrophobic, but there are no homologies among them. More strikingly, the COOH-terminal sections of the signal peptides are markedly variable. It is therefore very difficult to predict and design a synthetic substrate for studying the signal peptidase. Jackson and Blobel (91) used a cell-free translational system to study the signal peptidase and found its activity to be confined to the RER and is latent. A signal peptidase activity processing the pre-human placental lactogen was also characterized and found to act like chymotrypsin (92). Many integral membrane proteins are also found to be synthesized as precursors with a signal peptide at the amino terminus (93). The signal peptidase (leader peptidase) for M13 procoat protein from Escherichia coli was also purified and characterized (94,95). It was reported to be a membrane protein in both the inner and outer membrane of E. coli working either during or post-translation.

2. The Processing of Cytoplasmically-Made Mitochondrial Proteins

Most mitochondrial proteins are coded for by nuclear genome and synthesized in the cytoplasm. They have to be imported to their functional sites in the mitochondria. Many cytoplasmically-made mitochondrial proteins were shown to be initially made on free polysomes (96,97), and many of them are made as precursors which are between 2000 and 6000 daltons larger than the mature polypeptide (Table 3). For example, F_1 ATPase subunits α, β, γ (98), carbamylphosphate synthase (CPS) (97,109,110), cytochrome c1, subunit V of cytochrome bc1 complex (96), etc., are made extramitochondrially as larger precursors. Presumably, this class of cytoplasmically made proteins has to first bind to a "receptor" on the mitochondrial surface. Then, somehow they diffuse across one or both membranes and are converted to their mature form by a certain type of protease. The transport of these precursors into mitochondria is independent of concomitant protein synthesis (36,116), but it is probably energy dependent (101,113) and is very different from the "vectorial translation" (as described in the previous section).

In spite of the knowledge of signal process and signal peptidase, the import and process of mitochondrial proteins are still unknown. Raymond and Shore (110) have shown that the precursor of carbamyl phosphate synthase (pCPS) (105) is taken up by mitochondria and immediately followed by pro-

Table 3. Cytoplasmically-Made Mitochondrial Polypeptides

Organism	Protein	Molecular Weight Mature	Molecular Weight Precursor	Submitochondrial Location	Ref.
Yeast	α-F_1 ATPase	58K	64K		
	β-F_1 ATPase	54K	56K	M	98
	γ-F_1 ATPase				
	cyt. bc_1 complex	25K	27K	IM	99
	subunit V				
	cyt. c	12.6K	12.6K	IM	100
	cyt. oxidase			IM	100,
	subunit IV				102
	subunit V	each 2~6K larger			
	subunit VI				
	subunit VII	5K	5K		
	ADP/ATP translocator	32K	32K	IM	102
	cyt. c_1	31K	37K	IM	
	cyt. c peroxidase	33.5K	39.5K	IS	104
	Mn^{2+}-superoxide dismutase			M	100
Neurospora					
	citrate synthase	45K	47K	M	105
	ADP/ATP translocator	32K	32K	IM	106
	cyt. c	12K	12K	IM	107, 108
	cyt. c_1	31.5K	37K	IM	103

Table 3. Cytoplasmically-Made Mitochondrial Polypeptides Continued

Organism	Protein	Molecular Weight Mature	Molecular Weight Precursor	Submitochondrial Location	Ref.
Rat Liver	carbamyl-phosphate synthetase	160K	165.5K	M	109, 97, 110
	ornithine transcar-bamylase	39K	43K	M	111, 112, 113
	ALAS	51K	45K	M	35

Abbreviations: cyt.: cytochrome; M: matrix; IM: inner membrane; IS: intermembrane space.

cessing event. There is no pool of precursors that could be detected in association with mitochondria. The processing of pCPS occurs either coincident with or immediately following its transmembrane uptake by mitochondria. The other extramitochondrial-made mitochondrial precursor protein is rat liver ornithine transcarbamylase (OTCase), which was also extensively studied (112,113,117). In hepatocytes the pre-OTCase disappeared from cytoplasm with a half life time of 1-2 minutes (113). In this period of time, the pre-OTCase is transported deeply into the mitochondria and converted to mature size although not completely into the matrix space (117). These studies indicate that the proteolytic processing of pOTCase might occur on the surface of mitoplast. However, another study by Boehri et al. (100) reported a mitochondrial matrix protease which is responsible for processing F_1 ATPase α, β, γ subunits. The purification and characterization of the pre-segment protease is an area now.

F. The Biosynthesis and Degradation of Coenzyme A and Its Derivatives

CoA plays essential roles in the metabolism of carbohydrates and fatty acids as an acyl group activator. It is the cofactor form of pantothenic acid and was first reported in the late 1940's by Lipmann's group. The biosynthesis of

CoA has been studied by many investigators in various living systems (118). As shown in the past, CoA is synthesized from pantothenic acid, L-cysteine and ATP in the presence of Mg^{2+} in various organisms. The first step is catalyzed by pantothenate kinase in the presence of ATP and Mg^{2+} (120,121). The following step is the condensation between 4'-phosphopantothenic acid and L-cysteine to form 4'-phosphopantothenyl-L-cysteine. This enzyme, 4'-phosphopantothenyl-L-cysteine synthase is a Mg^{2+} requiring enzyme, and it can also catalyze the condensation between 4'-phosphopantothenic acid and β-mercaptoethylamine (120,121,122). 4'-phosphopantothenyl-L-cysteine is then decarboxylated to 4'-phosphopantetheine by a decarboxylase (121). Subsequently, a reversible enzyme, dephospho CoA pyrophosphorylase synthesizes the pyrophosphate bond between 4'-phosphopantetheine and 5'-AMP from ATP. Finally, the dephospho-CoA kinase forms CoA from dephospho-CoA and ATP. All of the enzymes involved in CoA biosynthesis are soluble enzymes.

Enzymatic degradation of CoA has been studied mainly in rat liver, rat kidney and horse kidney. In the rat liver, CoA is first dephosphorylated by lysosomal acid phosphatase to dephospho-CoA, which is then subjected to pyrophosphate bond cleavage to form 4'-phosphopantetheine and 5'-AMP by the action of a dephospho-CoA pyrophosphatase. This enzyme is located on the plasma membrane of microsomal

and nuclear fraction of rat liver. 4'-phosphopentetheine then undergoes dephosphorylation to pantetheine. Finally, the pantetheine is cleaved by pantethinase to form pantothenic acid and cysteamine. Pantothenic acid is a final product of CoA degradation in animals. Cysteamine is further oxidized to hypotaurine by a specific oxygenase.

The pantethinase was purified from horse kidney cortex (119). It requires the presence of a reduced thiol group and is highly specific for pantetheine. This enzyme cannot cleave the amide bond of other substrates: CoA, pantetheine thazoline, pantothenoly cysteine. Therefore, the degradation process of CoA could not start from the cleavage of this amide bond between cysteamine and pantethenic acid. The amide bond (or pseudopeptide bond) within pantetheine between cysteamine and pantothenic acid cannot be hydrolyzed by any protease, even with a broad specificity (as bacterial pronase and subtilism).

Interestingly, there are three major CoA pools: in the cytoplasm, in the intramitochondrial membrane space and in the mitochondrial matrix. The mitochondrial inner membrane is a barrier to many macromolecules and ions. It is also impermeable to CoA (123,124,125). Therefore, how could mitochondrial matrix obtain its CoA? This has become a very interesting question. Skrede and Halvorsen (126) had screened some possible substrates for mitochondrial CoA synthesis. They

found 4'-phosphopantetheine can be the initial precursor for mitochondrial CoA biosynthesis. Furthermore, they reported that mitochondria contains dephospho-CoA pyrophosphorylase and dephospho-CoA kinase in the inner membrane. Those findings suggest that mitochondria probably synthesize its own CoA.

Another question remains as to how the excess or unwanted mitochondrial CoA is degraded and whether or not the mitochondria contains its own CoA degrading system.

It has been reported that isolated mitochondria have the tendency to swell even in an iso-osmotic medium, and therefore, its endogenous CoA leaks out easily (123,124). This finding was not sufficient for proving that the mitochondrial CoA is normally leaking out for destruction in vivo. However, no mitochondrial CoA degrading system has been reported either.

II. OBJECTIVE

The major objective of this research is to study the characterization of ALAS and an associated amidase. In this dissertation, the following areas have been emphasized:

1) evaluate different colorimetric assays. Determine the maximun sensitivity of this assay for the measurement of ALAS activity. Use the colorimetric assay to characterize liver ALAS.
2) investigate the radiochemical assays, namely the single column and multiple column methods. Modify the single column method to avoid the high amounts of radioactive contaminant(s) which interfere with the measurement of ALA. Furthermore, identify the major radioactive contaminant and develop an optimal assay system for the amidase which is responsible for formation of the contaminant.
3) characterize both liver ALAS and amidase by using the assay systems established in this study. Compare the submitochondrial localization, physical properties, and biological regulation of ALAS and the amidase.
4) use the modified radiochemical assay to characterize ALAS activity in skeletal muscle. Investigate the tissue specific regulation of ALAS in skeletal muscle,

such as the effect of diabetes, fasting, glucocorticoid, and porphyrinogenic drugs.

III. MATERIALS

Reagents and Chemicals

Lubrol, pyridoxal 5'-phosphate, dithiothreitol, coenzyme A (lithium salt), trypsin, streptozotocin, GTP and ATP were purchased from Sigma Chemical Company. Digitonin was obtained from Sigma and recrystallized from hot ethanol prior to use. Succinic thiokinase (porcine heart) was from Sigma, and succinyl coenzyme A synthetase (porcine heart) was from Boehringer Mannheim. Succinyl CoA synthetase was also prepared from δ-aminolevulinic acid-requiring mutant of Rhodopseudomonas spheroides, which was a generous gift of Dr. June Lascelles, Department of Bacteriology, University of California, Los Angeles, California, according to the method of Scholmick et al. (64) and assayed according to the method of Kaufman (127). [2,3-^{14}C] Succinic acid (102.4 mCi/mmol) was purchased from Amersham and diluted with cold succinate to a specific activity of 1μCi/50 nmol. Labeled δ-amino [3,5^{3}H] levulinic acid (1.20 Ci/mmol) and δ-amino [4-^{14}C] levulinic acid (53.2 mCi/mmol) were obtained from New England Nuclear. Dowex AG 50w-X8 (200-400) mesh Dowex 1-X8 (200-400 mesh) and (100-200 mesh) and Bradford reagent were obtained from Bio-Rad. The Perkin-Elmer model 557 spectrophotometer was used for spectral determinations. Allylisopropylacetamide (AIA) was a gift from Hoffmann-

LaRoche, Inc. (Nutley, New Jersey). Acetylacetone (2,4-pentanedione) was from Fisher Scientific Company. Anesthesia grade ether was from Fisher. 3,5-dicarbethoxy-1, 4-dihydrocollidine (DDC) was from Eastman (Rochester, New York). Other chemicals were of the highest purity obtainable from commercial sources.

IV. METHODS

A. The Preparation of Liver Mitochondria and Submitochondrial Fractionations

Male Sprague-Dawley rats (175-225 g) were fasted for 24 hours prior to sacrifice (except for those experiments for diabetic rats). The livers were immediately removed after decapitation and transferred to the homogenization medium: 50 mM Tris-HCl pH 7.4, 0.25 mM, sucrose, 0.5 mM EDT (TSE). The livers were minced to small pieces with scissors and rinsed with the same buffer before homogenization in 4 volumes of TSE with a Potter-Elvehjem homogenizer at 4°C. The homogenization was performed very gently passing up and down 3 times. The volume was then adjusted to 10 volumes of the original liver wet weight and used for assay or for further isolation of mitochondria.

For preparation of mitochondria, the 10% liver homogenate was adjusted to 5% with TSE and centrifuged at 300 xg for 10 minutes. This low speed centrifugation prevents the loss of a considerable amount of mitochondria by entrapment in the tissue fibers. The supernatant is then centrifuged at 9,500 xg for 10 minutes. The sedimented mitochondria are resuspended in TSE to 10 volumes of the original liver wet weight and gently dispersed using a loose-fitting glass homogenizer. The suspension is then centrifuged at 600 xg for 10 minutes to remove any remaining large particles.

The mitochondria were then pelleted by centrifugation at 9,000 xg for 10 minutes. The pellet was resuspended in a volume of TSE approximately equal to the original liver wet weight prior to protein determination (128). A typical yield of mitochondria by this method is 25-35 mg mitochondrial protein per g of liver wet weight (43). Subsequently, mitoplasts were prepared from isolated mitochondria by treatment with digitonin according to the method of Schnaitman and Greenawalt (129) as modified. A 2% ice cold digitonin solution in TSE was added to the mitochondrial suspension (25-35 mg/ml) to a final concentration of 1.1 mg digitonin per 10 mg of mitochondrial protein. The subsequent procedures were identical (129) except that the digitonin-solubilized supernatant was not further centrifuged to obtain the outer membrane fraction. The mitoplasts were resuspended in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM pyridoxal 5'-phosphate, 0.1 mM dithiothreitol pH 7.5 (TEPD buffer) to 10 mg/ml and treated with the nonionic detergent Lubrol WX (130). Generally, a concentration of 1.05 mg Lubrol WX per 10 mg of mitoplast protein was used. After 15 minutes with gentle stirring, the suspension was centrifuged at 144,000 xg for 45 minutes. The resulting supernatant (Lubrol extract) comprised the "matrix" compartment. The sediment was resuspended in TEPD and comprised the inner membrane.

B. Preparation of Skeletal Muscle Mitochondria

Rats were killed by decapitation, and the skeletal muscles from the hind legs (gastrocnemius) and back were cleaned of fat and connective tissue, quickly removed and placed in ice cold medium M (210 mM mannitol, 10 mM Hepes, 70 mM sucrose, and 2 mM EDTA, pH 7.4). All subsequent operations were performed at 0-4°C. The muscles were finely minced with scissors and processed through an Edco hand tissue presser. Ten g of tissue in 100 ml of medium M were stirred with trypsin (2.4 mg/10 g tissue) for 30 minutes in an ice bath. The resulting suspension was centrifuged for 10 minutes at 650 xg to remove fibers and debris and the supernatant was filtered through a double layer of cheese cloth before centrifuging at 10,000 xg for 5 minutes to sediment the mitochondria (131). Liver homogenates and mitochondria were prepared as described previously (132).

C. Treatment of Animals

1. Porphyrinogenic Drugs (AIA, DDC) Treatment

The rats were fasted for 24 hours prior to injection (133). AIA (20 mg per ml in 0.154 M NaCl) was injected subcutaneously at a dose of 60 mg per 100 g body weight 16 hours prior to sacrifice. DDC was suspended in corn oil by sonication at a concentration of 25 mg per ml, and was injected intraperitoneally at a dose of 25 mg per 100 g

of body weight, also 16 hours prior to sacrifice. The control rats were injected with saline solution or corn oil in the relative amount respectively.

2. Streptozotocin Induced Diabetes

Diabetes was induced by the intravenous injection of streptozotocin (10 mg/100 g body weight) dissolved in citrate buffer (pH 3.8-4.2). Controls received an equivalent volume of buffer only (134). The rats were diabetic after 2-3 days with blood glucose levels greater than 400 mg% and generally sacrificed after 5 days. These rats were fed and given water ad libitum unless they were doing the fast experiments. The fasted rats (control and diabetic) were caged separately and deprived of food starting at 9:00 A.M. two days prior to sacrifice. The diabetic rats were fasted starting three days after receiving streptozotocin and sacrificed two days later.

3. Insulin and Dexamethasone Administration

When the effects of insulin were to be investigated, soluble insulin (5 units/ml in 0.9% saline) was administered subcutaneously at a dose of 1 unit/100 g body weight for 5 doses. The first dose was given at the end of the third day after streptozotocin administration, and the dose was repeated on the morning and evening of the fourth day. Two

doses of insulin were administered on the morning of the fifth day (135), and the animals were sacrificed two hours later. Determinations of blood glucose at the time of sacrifice as well as prior to the first insulin injection confirmed that the streptozotocin-induced hyperglycemia was reduced to control levels by insulin treatment. Dexamethasone was given by intramuscular injection at a dose of 50 mg /100 g body weight twice daily for two days.

D. The Colorimetric Assay for δ-ALAS Activity

1. Assay Procedures

To a 16 x 100 mm test tube were added 400 μl of stock assay mixture (150 mM Tris-HCl, pH 7.4, 250 mM Glycine, 25 mM Na Succinate, 12.5 mM EDTA, 0.1 mM Pyridoxal-5'-Phosphate) and 100 μl of stock succinyl CoA generating system (100 mM GTP, 1mM Coenzyme A, 50 mM $MgCl_2$ and suitable amounts of succinyl CoA synthase, which catalyzed the formation of 5 μmol of succinyl CoA per hour at 37°C). Enzyme (8-12 mg of mitochondria or 500 μl of a 10% homogenate or suitable amounts of soluble enzyme preparation) was added to a final volume of 1.0 ml. The final assay mixture contained 100 mM glycine, 10 mM succinate, 10 mM GTP, 0.1 mM CoA, 5 mM $MgCl_2$, 5 mM EDTA and 90 mM Tris-HCl. When the intact mitochondria or 10% homogenate were assayed, addition of the succinyl CoA generating system did not increase the activity. When necessary, the volume was

adjusted with TSE. When the soluble enzyme was assayed, the succinyl CoA generating system was required. The tubes were incubated in a shaking water bath at 37°C for 20 minutes. The reaction was stopped by the addition of 125 μl of 50% TCA to the 1 ml incubation mixture. The zero time assay contained the same amount of enzyme preparation to which TCA was added at time zero. The denatured protein was then pelleted in a clinical centrifuger at full speed for 5 minutes. A 0.6 ml aliquot of supernatant was transferred to another tube, 0.3 ml of 10% acetylacetone in 1 M Na acetate was added and the mixture shaken on a vortex mixer. The pH of the solution, which should be 4.6, was checked with short range pH paper. The tubes were capped with marbles and heated at 80°C for 10 minutes to convert the aminoketones to pyrroles. Next, the major contaminant, aminoacetone pyrrole, was removed from the mixture by one of the three following precedures.

2. Extraction with Dichloromethane (59,136,51)

After the tubes had been cooled, the pH of the mixture was adjusted to 7.0 with 100 μl of a mixture containing 1 volume of 0.5 M Na_2HPO_4, and 3 volumes of 1 N NaOH. The mixture was vigorously mixed with 2 ml of dichloromethane for exactly 10 seconds. This procedure removed 92% of the aminoacetone pyrrole formed in the mixture (51,136). For complete phase separation, the tubes were centrifuged for 3 minutes, then

700 µl of the aqueous phase (upper layer) were removed and mixed with an equal volume of Ehrlich's reagent.

3. Extraction with Ether (57,58, Pomeroy and Bonkowsky, Personal Communication)

The tubes were removed from the water bath and placed in an ice bath for 30 minutes. Then 25 µl of 10 N NaOH was added to adjust the pH to 8-8.5 and checked with short range pH paper. Two ml of equilibrated ether were added and shaken vigorously on a vortex for exactly 15 seconds. The tubes were placed in the ice bath to allow complete separation of the two phases, at which time the ether layer was aspirated. An aliquot containing 0.65 ml of the bottom aqueous layer was removed and placed in a tube containing 50 µl of glacial acetic acid. The mixture was shaken vigorously, and an equal volume of Ehrlich's reagent was added to allow color development and then was shaken. The steps involving an akaline akaline solution were performed as rapidly as possible to avoid high blank values.

4. Column Method (137,56)

After the tubes have cooled, the mixture was applied to a 0.8 cm x 2 cm column con taining Dowex 1 in the acetate form. The column was washed thoroughly with 20 ml water, then washed successively with 2 ml of 50% methanol, 2 ml

of 1 M acetic acid and finally with 2 ml of glacial acetic acid. The aminoacetone pyrrole was eluted exclusively in the methanol fraction, while the ALA pyrrole appears in the glacial acetic acid eluate. An equal amount of Ehrlich's reagent was next added for color development.

5. Ehrlich's Reaction

Fifteen minutes after the addition of the Ehrlich's reagent, the spectrum of the colored salt was obtained between 650-450 nm with a dual beam scanning spectrophotometer in a cuvette of 1 cm light path using a zero-time assay treated in the same manner as the reference sample. The ratio of absorbance at 525:552 nm should be approximately 0.69 (52). The difference in absorbance at 552 nm and 650 nm was used to determine the formation of ALA with an apparent extinction coefficient of 45 $nM^{-1}cm^{-1}$ after extraction with ether. One unit of ALAS activity was defined as one nmole of ALA formed per hour.

E. The Radiochemical Assay for ALAS Activity

1. Determination of Liver ALAS Activity

The soluble ALAS in the matrix fraction (50-200 μl) was assayed in an incubation mixture containing 50 mM Tris-HCl, pH 7.4, 100 mM glycine, 10 mM EDTA, 0.1 mM pyridoxal 5'-phosphate, 20 mM magnesium chloride, 1.0 mM coenzyme A,

1 mM GTP or ATP as required, 0.25 μCi of [^{14}C] succinic acid (50 μM), and 0.006 U of succinyl CoA synthetase in a final volume of 250 μl. The reaction mixture was incubated with shaking at 37°C for 20 minutes in a New Brunswick Gyrotory water bath and terminated by the addition of 0.125 ml of 25% trichloracetic or 1 ml of 10% sodium dodecyl sulfate (basic SDS). Intact mitochondria were assayed by the same procedure except that the succinyl CoA synthase, coenzyme A and nucleotide were omitted and the assays were performed for 15 minutes. A known quantity of δ-amino [^{3}H] levulinic acid was added to all samples to correct for losses during recovery and δ-amino [^{14}C] levulinic acid was added to control tubes to correct for isotopic exchange. Carrier succinate (1 μmol) and carrier ALA (100 nmol) were added to each assay. All samples were assayed in triplicate, and the mean and standard deviation of these values are presented.

Isolation of ALA was performed essentially by the Dowex 50 column method of Ebert *et al.* (38). To confirm these results ALA was converted to the pyrrole by adjusting the sample to pH 4.6 with 1 M acetate buffer and condensation with 200 ml acetyl acetone at 80°C for 20 minutes. The resulting ALA-pyrrole was isolated by the final Dowex 1 column procedure of Strand *et al.* (63). In some experiments [^{14}C] succinate was adsorbed to a Dowex 1 column in 0.1 M acetate buffer,

pH 3.9, prior to the Dowex 50 column of Ebert et al. (38). The ALA-pyrrole was also extracted into ethylacetate from the aqueous phase (60) and separated by chromatography directly on silica gel thin layer plates to assess purity of the isolated compound.

2. Determination of Skeletal Muscle ALAS Activity

ALAS was assayed at 37°C in tubes containing 2 mg of skeletal muscle mitochondrial proteins in a final volume of 1 ml containing 75 mM glycine, 75 mM Hepes, pH 7.4, 10 mM EDTA (disodium salt), 0.1 mM PLP, and 0.5 mM sodium succinate containing 2 μCi of [^{14}C] succinate. When the succinyl CoA generating system was added, 1 mM GTP, 0.1 mM coenzyme A, 20 mM $MgCl_2$ and 0.05 units of succinyl CoA synthetase were added. The reaction was terminated by the addition of 3 ml of 10% SDS. The reaction mixture was left at room temperature overnight prior to separation on Dowex 50 columns[4]. These modifications of the method of Ebert et al. (38) eliminate the presence of nonspecific labeled compound (s) which have been reported to co-elute with ALA (61,60). The purity of radioactive ALA obtained from this column was confirmed by formation of the ALA-pyrrole which was subsequently separated on Dowex 1 (acetate) columns (63). The radioactive ALA-pyrrole obtained from the Dowex 1 column was identified by thin-layer chromatography (138).

In order to correct the variation in recovery between different columns, an internal standard of [^{3}H] ALA (10 nmole containing 6,500 dpm) was added to each sample prior to loading on the Dowex 50 columns. In addition, standards containing [^{14}C] ALA and [^{3}H] ALA were also eluted from the columns to determine recovery. The recovery of [^{14}C] ALA from the Dowex 50 columns was 85-93%, and the recovery of [^{3}H]ALA was 90-98%. After conversion to the ALA-pyrrole and separation on the Dowex 1 columns, the recovery of the [^{14}C] ALA-pyrrole was 60-65% and the recovery of the [^{3}H] ALA-pyrrole was 35-40%. Specific activity was calculated for each sample using the recovery data. The results obtained were identical where the single column procedure was compared to the double column procedure after pyrrole formation (see Table 16).

The colorimetric method was also used to determine ALA. The reaction mixture was modified to include 10 mM succinate and 8-12 mg of mitochondrial protein. The reaction was terminated by addition of trichloracetic acid and ALA determined in the supernatant as described previously (43). After addition of Ehrlich's reagent, the spectrum of the colored salt was measured and the absorbance change between 552 nm and 650 nm was used to calculate ALA concentration using an extinction coefficient of 41 mM^{-1} cm^{-1} (43).

F. Standard Amidase Assay

The amidase activity in the soluble fraction was assayed in the absence of glycine so that no significant amount of ALA was formed. The assay medium contained 1 μCi of [^{14}C] succinate (50 μM), 0.5 mM coenzyme A, 1 mM GTP, 20 mM $MgCl_2$, 5 mM EDTA and 50 mM Tris-HCl (pH 7.4) and 0.025 U of succinyl CoA synthetase in a final volume of 1.0 ml. A freshly prepared assay mixture was preincubated for 10 minutes at room temperature at pH 7.4, the optimal pH for the succinyl CoA generating system. The mixture was adjusted to pH 8.2 with KOH and the reaction started by the addition of enzyme preparation (200-600 μg). Due to the latency of the amidase, the intact mitochondrial and mitoplast preparations were disrupted by either sonication or Lubrol treatment prior to assay. For each sample, a boiled enzyme preparation was used as a control to correct for the nonspecific degradation of succinyl CoA. The reaction was stopped by the addition of 1 ml of 10% SDS, pH 3.9 in 0.1 M sodium acetate buffer and applied to a Dowex 50 column (1 x 2 cm).

In order to achieve maximum recovery of the amidase product, the cysteamine thioester of succinate, the column elution profile for ALA was modified as follows. The wash of the Dowex 50 resin with 0.01 HCl was omitted since significant and variable amounts of the new product were

eluted in this wash. Hence, the Dowex 50 columns were washed three times with 20 ml of 0.1 M sodium acetate, pH 3.9, and then the product eluted with 3 ml of 1 M NH_4OH.

G. Partial Purification of ALAS and Amidase

1. DEAE-Cellulose Chromatography

A 3 cm x 20 cm column containing precycled DEAE-cellulose was thoroughly equilibrated with TEPD buffer. It is critical to precycle DEAE-cellulose in both acid and base before being used. The Lubrol extract was loaded on the top and 300 ml of salt gradient (0 mM - 800 mM NaCl) was pumped through the column at a flow rate of 3 ml/min. Each 1.5 ml aliquote of eluate was collected in a tube for further determination. The active fractions were pooled together and concentrated through an Ultrafiltration concentration unit (Amicon).

H. Product Identification

1. Silica Gel Thin-Layer Chromatography

Eastman silica gel G plate was used. The tank was saturated with the vapor of the running buffer by placing a Watman filter paper around the wall of the tank with running buffer for at least 2 hours before use. The plate was spotted with concentrated sample and dried before the

development. The chromatography was carried out in n-butanol-water-glacial acetic acid (4:5:1) at room temperature, and then dried with air. The dried plates were sprayed with modified Ehrlich's reagent to show the pink color of pyrrol. Sample lanes were divided into 0.5 cm sections, and the gel was scraped into scintillation vials and counted in Betaflour counting fluid.

2. Fixion Ion-Exchange Chromatography

Ion exchange TLC has an advantage over others since it is compatible with samples containing high concentrations of salts. When cation exchange TLC was applied, sodium citrate buffer at pH 3.3 (Na^+ = 0.4 M, citrate = 0.4 M) was used as elution buffer. The chromatography would obtain the best and most rapid result at 45°C, since we could find a warm room at 35°C, at which temperature the chromatography was completed in 3 hours. To prevent the uneven solvent front, the origin of the sample spot should be at least 1.5 cm above the solvent. The sample line should not be too close to the edge of the plate, since the solvent tends to adsorve and move faster on the edge of the plate. After the chromatography was complete, the plate was air-dried and examined under the UV light.

For detecting ALA-pyrrole, the plate was sprayed with modified Ehrlich's reagent. In the case of authentic succin-

imide, the plate was either developed by iodine vapor alone or developed by iodine vapor followed by the spraying with saturated starch solution. The succinimide spot showed a light brown color under iodine vapor, and it showed a blue color after the starch treatment.

To locate the labeled spot, each lane of the plate was cut off per 1 cm and put into vials with Betaflour for counting the radioactivity.

3. Paper Descending Chromatography

The big tank was equilibrated with the running solvent (butonol 1-ol-acetic acid-water; 5/2/3, V/V/V) for overnight. Whatman no. 3 paper was spotted with a different amount of samples which were dissolved in the same eluent (25 mg/ml). The chromatography was carried out at room temperature for 4.5 hours and dried with air.

To detect the SH-group, the dried paper was sprayed with Na-Nitropruside. The SH-containing compound should show a bright, purple red visible color which would disappear very fast. Therefore, the spot should be circled with a pencil when the color shows up. Each lane of the sample was also cut into 1 cm pieces and counted with Betaflour.

4. Preparation of Spraying Reagent

a. Modified Ehrlich's Reagent

Two stock solutions were prepared and kept in the cold room: 1.5% $HgCl_2$ in glacial acetic acid and 70% $HClO_4$. The reagent was always prepared freshly when it was needed: 15 ml glacial acetic acid, 5 ml 1.5% $HgCl_2$ in glacial acetic acid, 5 ml 70% $HClO_4$ and 500 mg dimethylaminobenzaldehyde (recrystallized).

b. Ninhydrin

The ninhydrin solution was simply prepared by dissolving 0.3 g ninhydrin in 100 ml n-butanol and 3 ml acetic acid. To obtain the result faster, the plate after spraying was heated at 110°C until the best color development was achieved. Using this method, the resulting color was not stable and faded gradually. For color preservation, 10 mg $SnCl_2$ was added into the mixture and was stirred for at least 5 minutes.

c. Nitroprusside

1.5 g Na-nitroprusside was dissolved in 5 ml NHCl and 95 ml methanol; then 10 ml of 25% NH_4OH was added. The solution was then filtered and kept in a dark bottle. It was stable for less than two weeks at room temperature.

5. Recrystallization and Rechromatography

A 195 mg of authentic succinimide (recrystallized) and 5 mg of radioactive sample was dissolved in 1.2 ml hot ethanol (60°C). The initial specific activity was detected by counting 40 ml of the solution. The solution was then kept at 4°C for 24 hours before it was filtered through a fine scintered glass filter (at 4°C). The crystal was air-dried on the filter and measured the dry weight. The crystals were again dissolved in hot ethanol at a concentration of 1 g/6 ml. A small quantity of solution was then counted to get the specific activity. Then the recrystallization was repeated 3 more times.

I. Other Enzyme Assays

Succinyl CoA synthase was assayed according to the method of Bridger *et al.* (140). N-acetyl-glucosaminidase was assayed as described by the method of Loomis (141). Succinate dehydrogenase was assayed spectrophotometrically according to Kim and Beattie (142). Isocitric dehydrogenase was determined by measuring the increase in optical density at 340 nm due to NADPH formation (143). Catalase was measured as described (144). The content of cytochrome *c* was determined by differential spectroscopy using the extinction coefficient of Rieske (145).

J. Protein Determination and Statistics

Protein concentrations were estimated by the method of Lowry according to Dully and Greive (146) and/or by using the dye binding method of Bradford (147) with bovine serum albumin as standard.

Data were analyzed by Student's t test in order to determine the significance of the difference between the means.

V. RESULTS & DISCUSSION

A. Colorimetric Determination and Characterization of Rat Liver ALAS

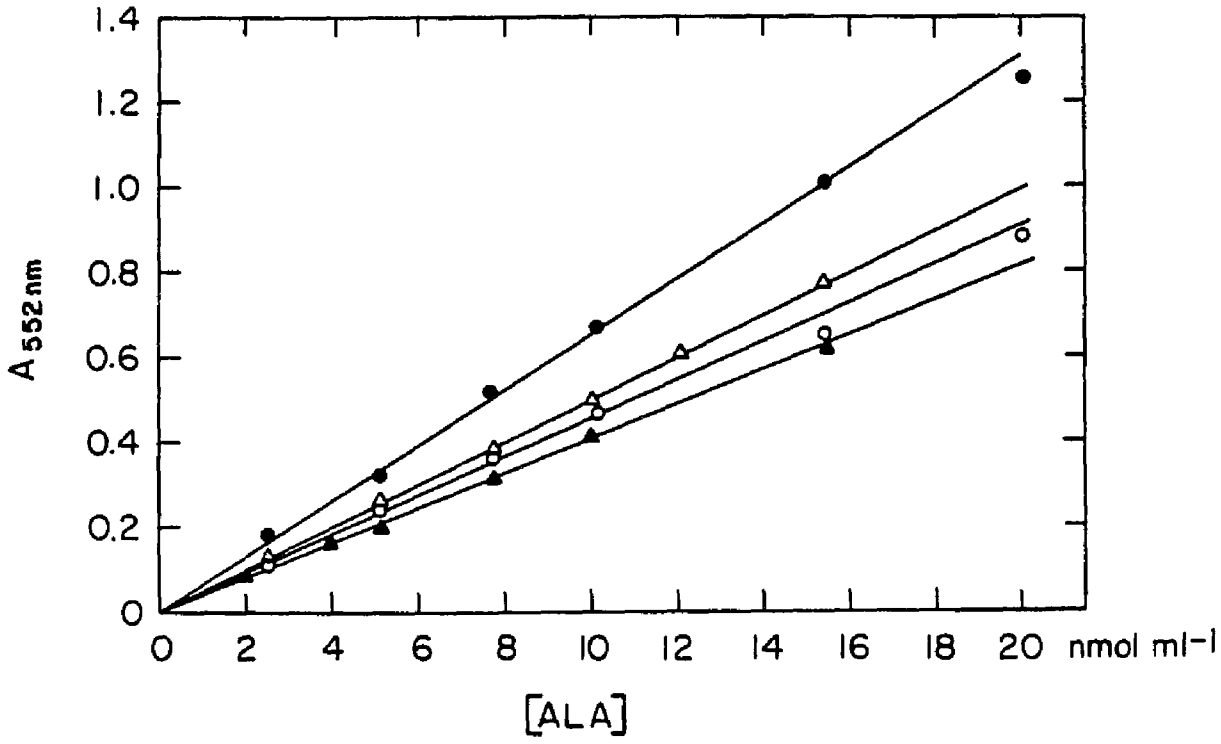
The colorimetric assay of ALAS was evaluated in this study. The δ-aminolevulinic acid formed was converted to a pyrrole with acetylacetone and quantitated spectrophotometrically at 552 nm after reaction with Ehrlich's reagent. Three methods, chromatography on Dowex-1, ether extraction and extraction with dichloromethane, was compared for the isolation of the pyrrole.

1. Results

The standard curves obtained for ALA-pyrroles formed and isolated by the different procedures outlined under Methods are plotted in Figure 2. When ALA was converted to pyrrole in distilled water, and extracted with ether or dichloromethane, and then allowed to react with an equal amount of Ehrlich's reagent, the pyrrole had an extinction coefficient at 552 nm of 65 mM^{-1} cm^{-1}. If ALA was converted to the pyrrole in the presence of assay mixture and then reacted with Ehrlich's reagent, the apparent extinction coefficient at 552 nm was reduced. For example, after extraction with dichloromethane, the apparent extinction coefficient was 41 mM^{-1} cm^{-1}, while after extraction with

Figure 2. Standard Curves of ALA-Pyrrole

The effect of assay mixture on color formation is shown. Authentic ALA was converted to pyrrole in distilled water (●——●) or in the assay mixture and purified by either column chromatography (Δ——Δ), dichloromethane extraction (▲——▲) or ether (○——○). The data are expressed as the final concentration of ALA after the addition of Ehrlich's reagent.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A_{552nm}
0
2
4
6
8
10
12
14
16
18
20
nmol ml-1
[ALA]

ether, it was 45 mM^{-1} cm^{-1}.

Experiments were next performed to determine which components of the assay mixture interfere with color formation. The results indicated that the only component of the assay mixture which decreased the extinction coefficient was glycine. In addition, the presence of glycine also caused high blank values. We have also found that the absorbance at 552 nm of the standards decreases more rapidly in the presence of assay mixture than in water alone. There are three possibilities to explain the inhibition of color formation by glycine: (1) Glycine may interact with ALA and form a cyclic product (suggested by R. Bonnett, personal communication) and thus, reducing the condensation efficiency of ALA with acetylacetone (2) Glycine may interact with the ALA-pyrrole such that the pyrrole is less available for forming color salts with Ehrlich's reagent (3) Glycine may react with Ehrlich's reagent to form a yellowish schiff's base which contributes to the high blank values and also prevents ALA-pyrrole from reacting with Ehrlich's reagent. When the ALA-pyrrole was purified by the column method, an apparent extinction coefficient of 49 mM^{-1} cm^{-1} was obtained. This lowered extinction coefficient may reflect the loss of ALA-pyrrole during the chromatography, since glycine would also be removed by this procedure.

The time course of the enzymatic reaction with either mitochondria or homogenate as source of enzyme is shown in

Figure 3. When intact mitochondria were assayed, ALA formation proceeded linearly for 30 minutes. When the homogenates were assayed, the linearity extended to 45 minutes.

A typical ALA-pyrrole spectrum obtained at various times during the incubation of intact mitochondria is shown in Figure 4. The major peak appeared at 552 nm with a shoulder at 525 nm. The absorbance at 650 nm was used as the reference. The lowest limit of this spectrophometric assay is approximately 560 pmole per ml of incubation mixture, a value corresponding to an absorbance change of 0.01. To obtain this amount of ALA in a 1 ml assay, 8-12 mg of control hepatic mitochondria is required. The ALAS activity was linear with protein from 2-14 mg in a 1 ml assay (Figure 5). Duplicate determinations of ALAS activity by the colorimetric assay were always within 5% of each other.

The activity of ALAS in the 10% homogenates of liver from male rats (150-175 g) determined in our laboratory using the ether extraction procedure is approximately 105 units per g liver (Table 4). This compares to results obtained in other laboratories of between 20 and 120 nmole ALA formed/hr/g liver (137,24,148). The activity of ALAS in intact mitochondria from male rats (150-175 g) determined in our laboratory is approximately 0.35 units per mg of protein (Table 4). One injection of the inducer allylisopropylacetamide increased ALAS activity to 2.6 units

Figure 3. Time Course of the Reaction

Liver mitochondria (o——o) at a concentration of 7 mg ml^{-1} or 500 μl of liver homogenate (●——●) obtained from rats induced with one injection of allylisopropyl-acetamide were incubated in the assay mixture described under Methods. ALA-pyrrole was purified by ether extraction method.

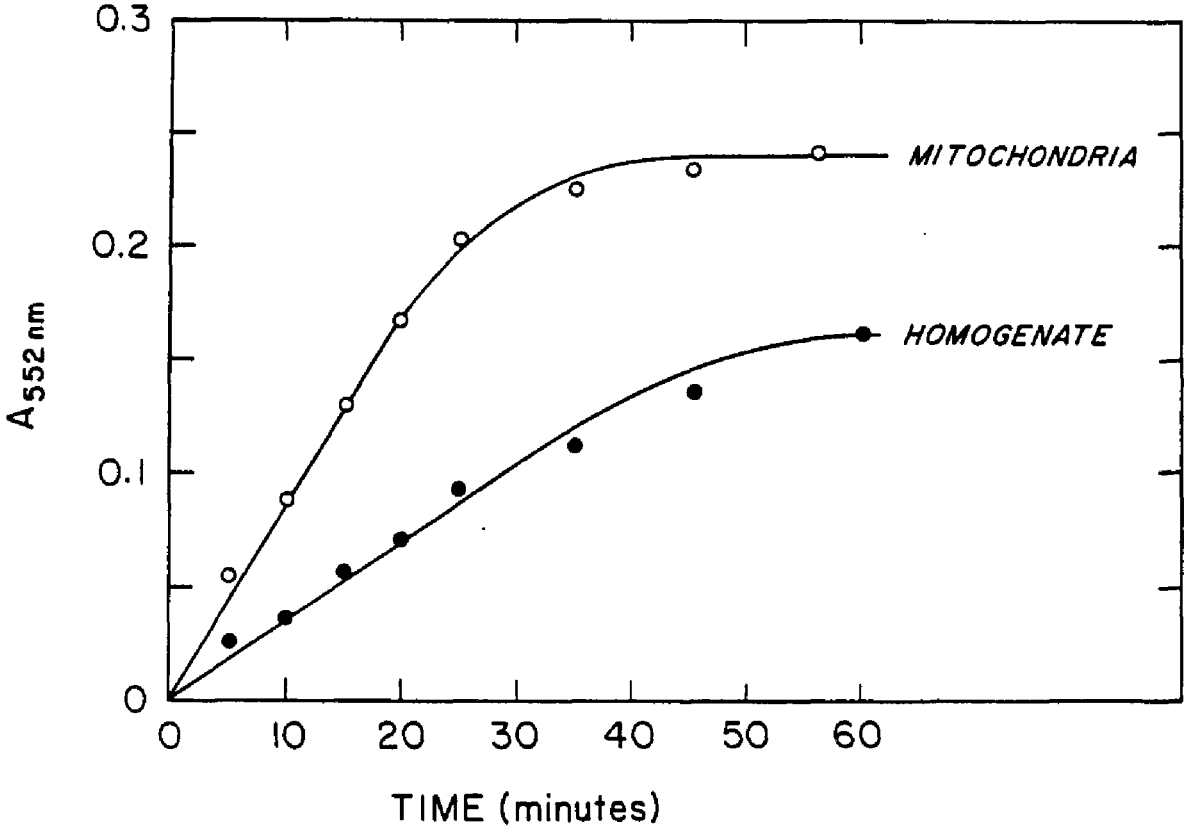
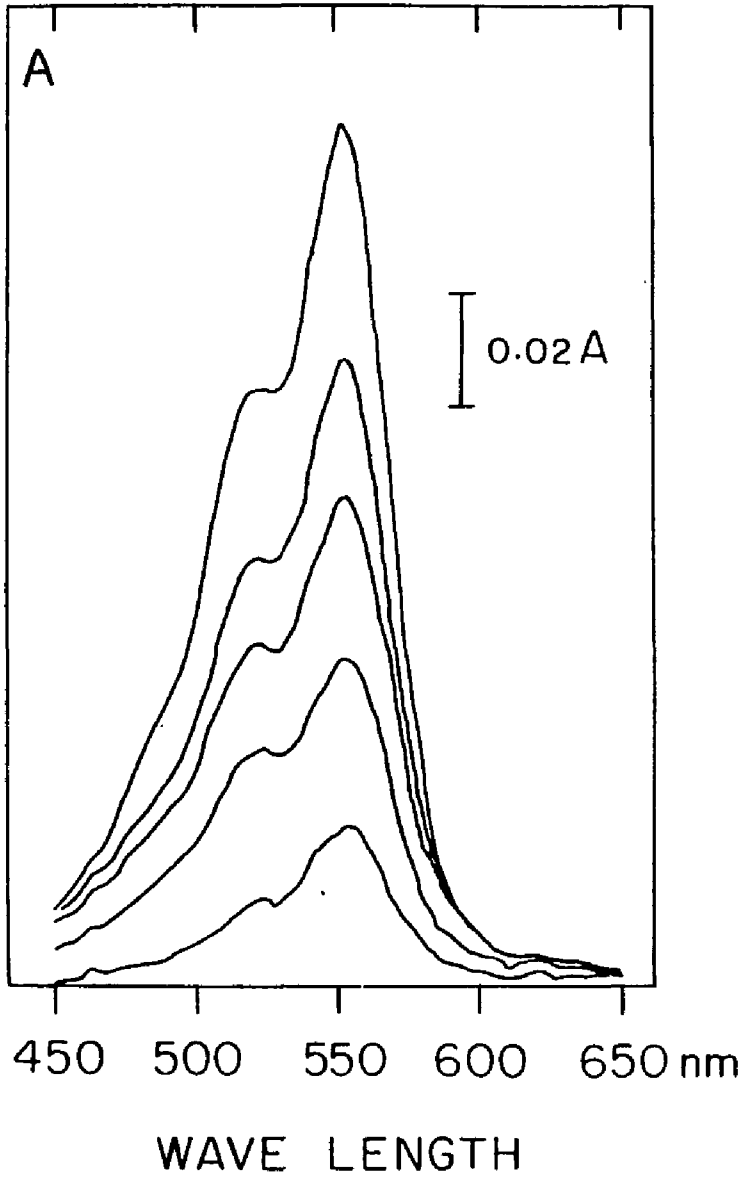

0.3
0.2
0.1
0
$A_{552\,nm}$
MITOCHONDRIA
HOMOGENATE
0
10
20
30
40
50
60
TIME (minutes)

Figure 4. Difference Spectrum of the Colored Salt of ALA-Pyrrole with Modified Ehrlich's Reagent

A. Liver mitochondria obtained from rats induced with one injection of allylisopropylacetamide were incubated for various periods of time in assay medium. The ALA-pyrrole was formed and purified by extraction with ether. The zero time assay mixture was used as a reference and the spectrum scanned from 650 to 450 nm. The spectra, in order of increasing absorption, were obtained at 5, 10, 15, 20 and 35 minutes of incubation.

B. Authentic ALA was converted to the pyrrole in water. The Spectra were obtained by scanning from 650 to 450 nm and represent, in order of increasing absorption, the final concentrations of ALA in the cuvette: 2.56, 5.13, 7.69, 10.25 and 15.4 μm.


A
0.02 A
450 500 550 600 650 nm
WAVE LENGTH

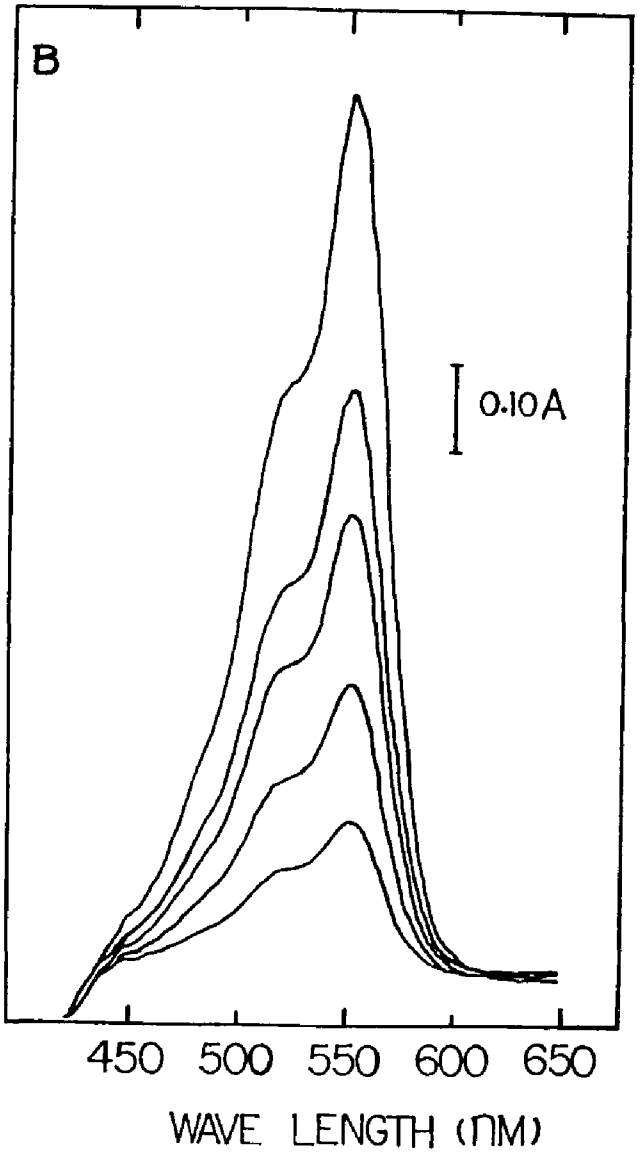

B
0.10A
450 500 550 600 650
WAVE LENGTH (nM)

Figure 5. Protein Concentration Curve by Using Colorimetric Assay

ALAS activity of control mitochondria was assayed as described in Methods and the ALA-pyrrole purified by dichloromethane extraction. The indicated amounts of mitochondrial protein are per 1 ml of incubation mixture.

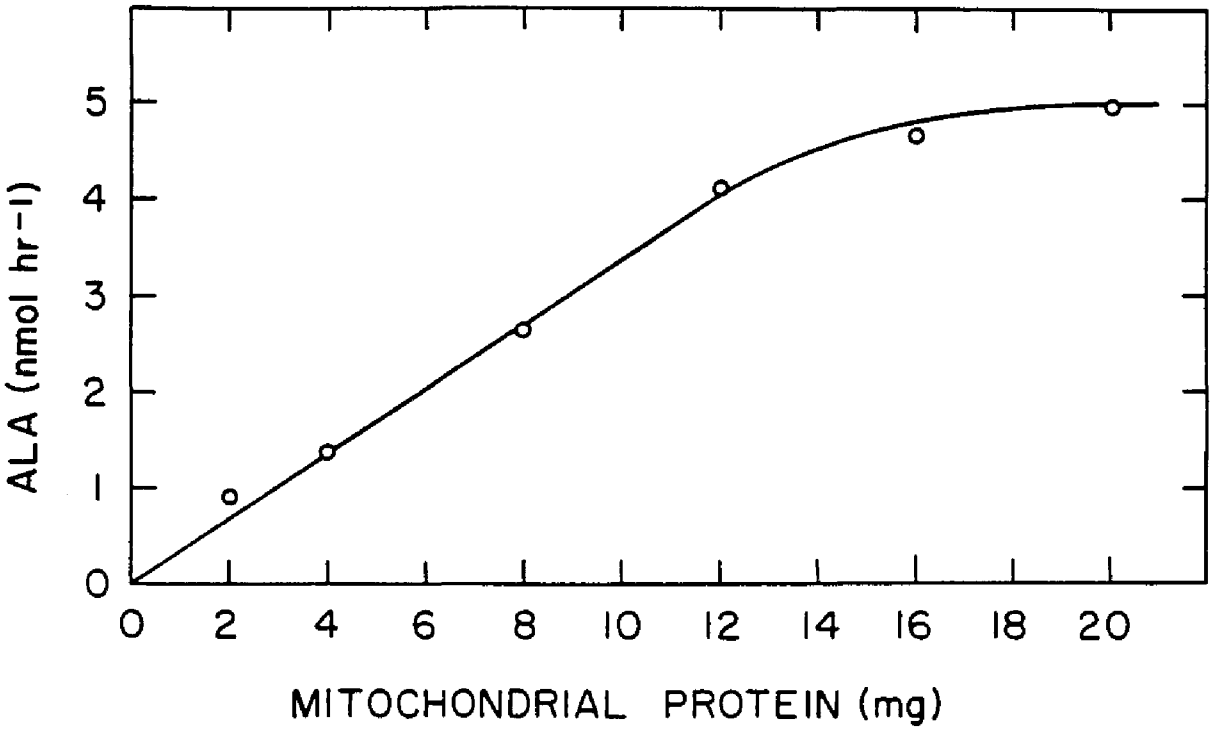

ALA (nmol hr-1)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16
18
20
MITOCHONDRIAL PROTEIN (mg)

Table 4. Comparison of Different Methods to Isolate ALA-Pyrrole

Pyrrole purification method	ALAS Activity[1]	
	Homogenate u/g liver	Mitochondria u/mg protein
Dichloromethane extraction (7)	-	0.35 ± 0.20
Ether extraction (4)	105 ± 18	0.41 ± 0.08
Ion-exchange chromatography (3)	-	0.27 ± 0.18

[1]Liver homogenates (10%) or mitochondria were prepared and ALAS assayed as described under Materials and Methods. Either 500 µl of a 10% homogenate or 10 mg of mitochondrial protein were incubated at 37°C for 30 minutes. The ALA produced was converted to the pyrrole with acetylacetone and the ALA-pyrrole isolated by extraction with dichloromethane or ether. Alternately, the ALA pyrrole was purified by chromatography on Dowex 1. The absorbance of the pyrrole was measured at A552-A650. The different apparent extinction coefficients calculated from Figure 1 were used for determination of the specific activity. The numbers in parenthesis indicate the groups of animals used. Each group contained livers pooled from 2 animals.

per mg of protein. These values can be compared to those previously reported: 0.23 units per mg (63), 0.12 units per mg (149), 0.038 units per mg (127) and 0.112 units per mg (50).

2. Discussion

Among three methods which were used for isolation of the pyrrole, extraction with ether was the preferred method. When this method applied to either homogenates or mitochondria from rat liver yielded values of 105 units per g liver and 0.41 units per mg mitochondrial protein. The method is rapid, simple and inexpensive; however, its use is restricted to activities of 0.56 units per ml of incubation mixture or greater. The colorimetric assay has been widely used to assay ALAS in mammalian tissues, avian cells and bacteria such as Rhodopseudomonas spheroides. The assay is both accurate and reproducible.

B. The Characterization of ALAS and an Associated Amidase in Rat Liver Mitochondria Using an Improved Assay for Both Enzymes

As described in the previous chapter, the activity of ALAS has generally been assayed in tissues with high levels of the enzyme by a colorimetric assay. In order to improve the sensitivity, the radiochemical assay was developed in which radioactive ALA was separated from the radioactive substrate, succinate, by ionexchange chromatography on Dowex 50.

In recent years, several groups have reported the presence of radioactive substances other than ALA in the assay mixtures of ALAS with liver homogenates or mitochondria. To solve this problem, I have confirmed the presence of a contaminant which co-elutes with ALA in the radiochemical assay for ALAS and tentatively confirmed its structure as the cysteamine thioester of succinate. Based on the knowledge of the formation of this contaminant, two new, rapid, assay procedures to measure ALA and the product of the amidase have been derived. Using these new assays, optimal conditions to measure ALAS and amidase which degrades succinyl-CoA have been developed and these two enzymes have been characterized in rat liver mitochondria.

1. Results

a. Modification of the Single Column Dowex 50 Procedure for the Isolation of ALA

Several investigators (61,60,62) in the past few years have reported the presence of radioactive contaminants other than ALA in the eluates from the single column method of Ebert et al. (38) for assay of ALAS activity. Consequently, the accurate determination of enzyme activity in tissues with insufficient ALAS activity for colorimetric determination (43) has necessitated the formation of the pyrrole of the radioactive ALA and its subsequent isolation on a second column as described by Strand et al. (63). A comparison of the Ebert's (38) and the modified Strand (63) procedure in our laboratory revealed no significant difference in the estimation of ALAS activity with the intact rat liver mitochondria as the source of enzyme (Table 5). By contrast, large differences in the two procedures were observed when soluble matrix protein was assayed in a similar manner with an external succinyl CoA-generating system and the reaction stopped by the addition of trichloracetic acid, a procedure used in all published radiochemical assays for ALAS. As

Table 5. Comparison of Trichloracetic Acid and SDS As Stopping Solutions for the Assay of ALAS

Intact liver mitochondria or a Lubrol extract of mitoplasts were incubated in the reaction medium described in Experimental Procedures. The reaction was stopped by the addition of either 0.125 ml of trichloracetic acid (TCA) or 1 ml of 10% SDS. In some experiments glycine was omitted. The single cation exchange column procedure (43) was used to isolate the radioactive products. These products were converted to the pyrrole and isolated on an anion exchange column (63). The specific activity is expressed as nmol product formed per mg mitochondrial protein per hour. Each value is the mean and the standard deviation of triplicate determinations corrected for recovery by both internal and external ALA standards.

	TCA-STOP		SDS-STOP	
Mitochondria	ALA	ALA Pyrrole	ALA	ALA Pyrrole
+ glycine	1.03 ± 0.10	1.02 ± 0.07	1.10 ± 0.05	1.06 ± 0.04
- glycine	0.07 ± 0.04	0.04 ± 0.02	0.08 ± 0.05	0.02 ± 0.01
Matrix Fraction				
+ glycine	4.82 ± 0.96	1.79 ± 0.05	1.95 ± 0.12	1.89 ± 0.06
- glycine	10.1 ± 5.9	0.03 ± 0.02	0.57 ± 0.09	0.02 ± 0.01

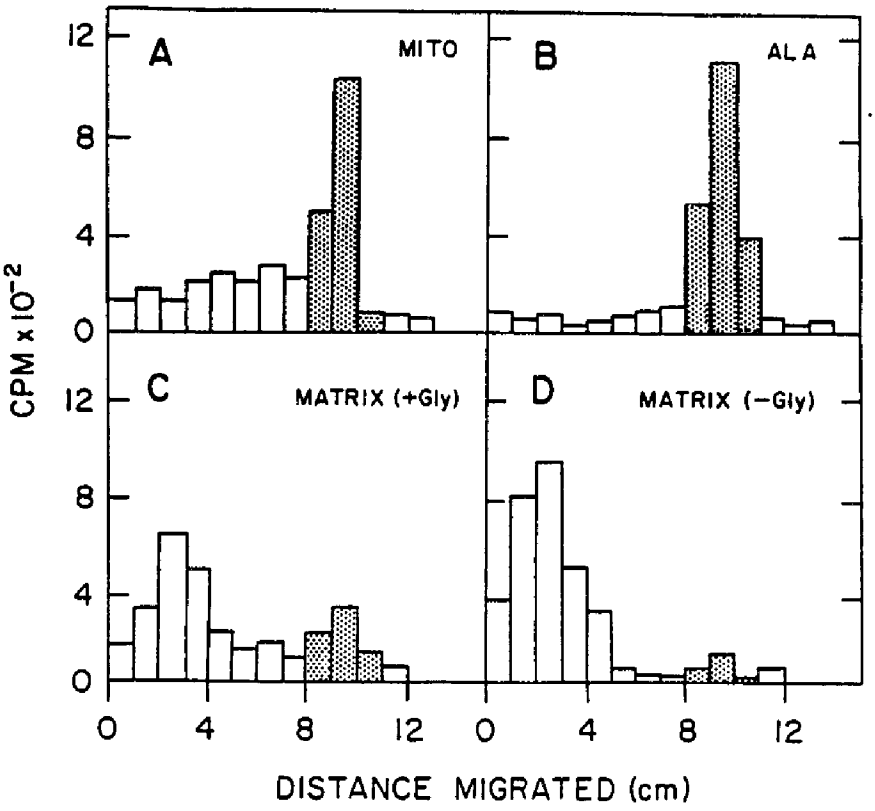
seen in Table 5 (left panel), only 37% of the counts eluted from Dowex 50 column under these conditions was ALA as indicated by the conversion to the ALA-pyrrole and its subsequent isolation on a Dowex 1 column. Furthermore, in the absence of glycine in the assay mixture, a two-fold increase in the amount of the non-ALA radioactive material was eluted from the column suggesting that glycine may interfere with its production.

The presence of a radioactive product other than ALA in the reaction mixture was confirmed when the eluate from the single Dowex 50 column was reacted with acetylacetone to form the pyrrole of ALA and the mixture separated by thin layer chromatography (63). As seen in Figure 6A, intact mitochondria produced predominately genuine ALA, while the soluble fraction of the mitochondrial matrix produced a prominent contaminant in addition to ALA when assayed in the presence of glycine (Figure 6C). It is interesting to note that when the soluble matrix fraction from AIA or DDC treated animals was assayed for ALAS activity, the proportion of contaminants compared to ALA shifts in favor of ALA synthesis such that ALA now represents 85% of the total counts.

The presence of these contaminating radioactive compounds in the ALAS assay mixture was prevented by stopping the reaction with SDS rather than with trichloracetic acid (Table 5, right panel). Genuine ALA was selectively eluted

Figure 6. Thin-Layer Chromatography of the Radioactive Products Produced During Assays of ALAS

Mitochondria and Lubrol extracts of mitochondria were incubated as indicated in Table a and the reaction stopped by addition of trichloracetic acid. After elution from the Dowex 50 column, the radioactive products were converted to the pyrrole and separated by thin-layer chromatography as described in Experimental Procedures. The shaded area in each panel indicates the position to which the authentic ALA-pyrrole migrated, determined colorimetrically by spraying with Ehrlich's reagent. A. Radioactive products synthesized during an incubation of intact mitochondria. B. Standard [^{14}C]ALA. C. Radioactive products synthesized by a Lubrol extract of mitochondria. D. Radioactive products synthesized by a Lubrol extract incubated in the absence of glycine.


A
MITO
B
ALA
C
MATRIX (+Gly)
D
MATRIX (−Gly)
CPM x 10⁻²
0
4
8
12
DISTANCE MIGRATED (cm)

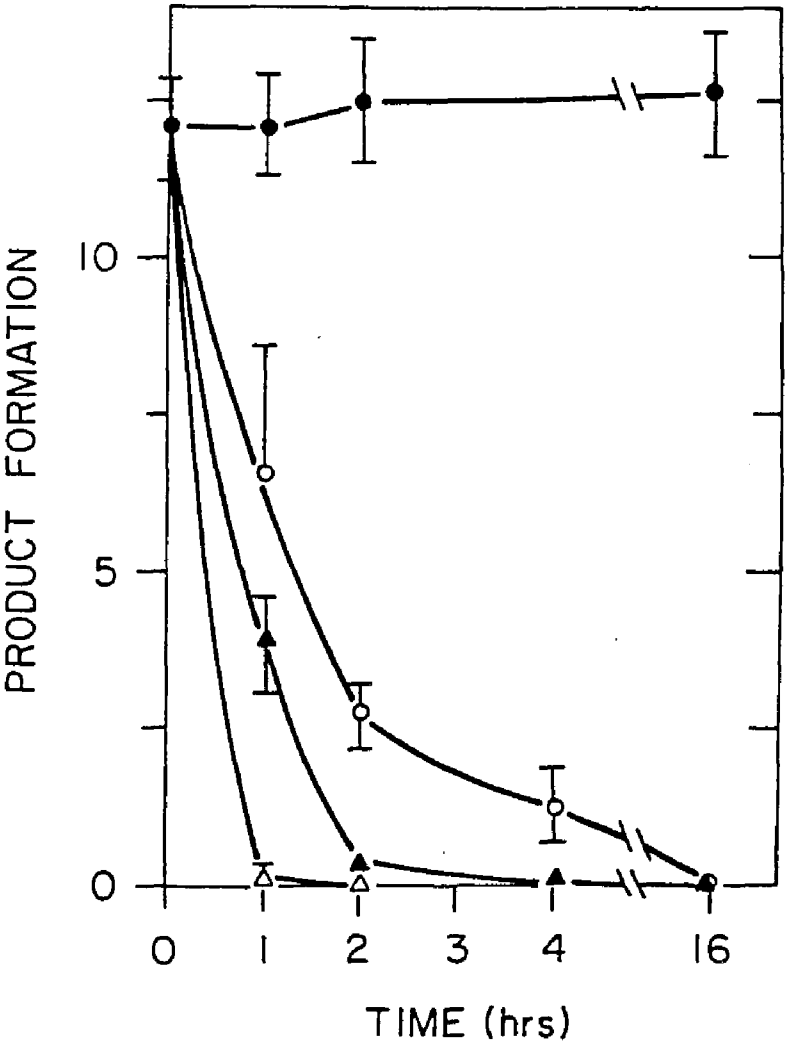
from the Dowex 50 column in the presence of SDS when either intact mitochondria or soluble matrix protein was assayed, suggesting that the contaminating compounds in the assay mixture either no longer were bound to the Dowex 50 column or were eluted prior to the ALA. This change in the chromatographic behavior of the non-ALA radioactive compounds in the presence of SDS occurred in a time and temperature dependent manner (Figure 7). The effect of SDS was also concentration dependent. Addition of less than one ml of 10% SDS to each 0.250 ml assay did not yield complete elimination of the contaminant. Addition of greater than one ml of 15% SDS per assay tended to increase the variability of the recovery of genuine ALA. It should also be noted that using SDS for stopping the reaction simplifies the procedure by allowing the direct application of the sample to the ion exchange resin, thus avoiding the tedious acid precipitation, centrifugation and subsequent neutralization with the resultant loss of quantitation.

b. Characterization of the Contaminant in the ALAS Assay

The behavior of the contaminant in the ALAS assay in the presence of SDS suggests that this compound may be the degradation product of succinyl CoA originally proposed by Minaga *et al.* (60). This group suggested that degradation

Figure 7. The Effect of Time and Temperature on the Elution of the Non-ALA Contaminating Compounds

Lubrol extracts of mitochondria were incubated in the reaction mixture for ALAS described in Experimental Procedures in the absence of glycine. The reaction was stopped with SDS and incubated at various temperatures for the times indicated. The amount of contaminating product present in the Dowex 50 column eluate was expressed as nmol per mg per h. o——o, 22°; ▲——▲, 37° and Δ——Δ, 65°. A control reaction was stopped with trichloroacetic acid and incubated at 4° or 22° for the times indicated (●——●).


PRODUCT FORMATION
10
5
0
0
1
2
3
4
16
TIME (hrs)

of succinyl CoA by an amidase or a peptidase could produce two distinct products: 1. A thioester of succinate and cysteamine or 2. A thioester of succinate and β-alanyl-cysteamine. The cysteamine thioester of succinate, the proposed major contaminant, could initially bind to the Dowex 50 resin at pH 3.9. Subsequently, during the alkaline elution of the column, this product would undergo an internal nucleophilic attack by the terminal amino group resulting in the formation of N-succinyl-cysteamine, which would be oxidized to the stable disulfide dimer. This putative product of succinyl CoA degradation was characterized by treating the thin layer plates described in Figure 6 with ninhydrin to indicate the presence of a free amino group (Table 6). Alternatively, the products eluted from the Dowex 50 column after treatment with acetylacetone were separated by descending paper chromatography and the chromatograms treated with nitroprusside to indicate the presence of a free SH group. Both reactions were negative, indicating that the radioactive contaminant, which was eluted from the Dowex 50 column by NH_4OH, has neither a free amino nor a free SH group. Furthermore, the contaminating product initially eluted from the Dowex 50 no longer binds to this cation exchange resin, but instead will bind at pH 7.0 to the anion exchange resin, Dowex 1. All of these results are consistent with the proposal of Minaga et al.

Table 6. Identification of Amidase Product

Test System	Results	Conclusions
Silica Gel TLC plate	1) Ninhydrin negative 2) Rf. of succinate = 0.85 Rf. of X cpd = 0.1 ~ 0.3 3) Rf. of succinimide = 0.517	1) No amino group 2) Does not comigrate with succinate 3) Does not comigrate with succinimide
Fixion TLC ion exchange plate	1) Rf. of succinimide = 0.46 ~ 0.52 Rf. of X cpd = 0.47 ~ 0.53	1) Comigrates with succinimide
Paper descending chromatography	1) Nitroprusside negative 2) Rf. of β-mercaptoethylamine = 0.47 Rf. of X cpd = 0.48	1) No -SH group 2) Comigrates with β-mercaptoethylamine
Recrystallization	1) No recrystallization with succinimide	1) The X cpd is not succinimide
Binding to Anion Exchange Column	1) Binds to Dowex 1 resin	1) Contains -COOH group

(60) that the final elution product from the Dowex 50 is the disulfide dimer of the cysteamine thioester of succinate. The latter compound which does not contain a free amino group could no longer bind to a Dowex 50 column in a similar way as does ALA.

An alternative proposal to the disulfide dimer as the final elution product from the Dowex 50 column is that the cysteamine thioester of succinate breaks down through a nucleophilic attack on the carbonyl group by the 1 N NH_4OH, used to elute from Dowex 50, and thus forms succinimide. To assess the possibility that succinimide is the final contaminating product of succinyl CoA degradation eluted by the Ebert's procedure, the radioactive product(s) formed in the absence of glycine were collected and recrystallized repetitively with genuine succinimide. After 3 recrystallizations of the succinimide, the specific radioactivity decreased from 193 cpm mg^{-1} to 9 cpm mg^{-1}, indicating that the radioactive contaminant is not succinimide.

The proposed pH dependent molecular rearrangement and dimerization of the cysteamine thioester of succinate explains our success in eliminating this contaminant when the assay is stopped with a 10% SDS solution which has a basic pH, 8.8. We propose that the rearrangement of the cysteamine thioester of succinate now occurs during the incubation in the SDS stopping solution rather than on the column, resulting

in the elution of the non-ALA [^{14}C] compound prior to elution of ALA. As indicated in Figure 7, incubating the reaction mixture with SDS for 1 hour at 65°C or overnight at 22°C effectively removes all the non-ALA radioactive contaminants from the assay mixture. To test the suggestion that the alkaline pH of the SDS used for stopping the reaction is critical for rearrangement of the putative contaminant, the cysteamine thioester of succinate, the reaction was stopped with acidic SDS. As seen in Table 7, the specific activity of ALAS was 2.33 nmol hr^{-1} mg^{-1} when SDS, pH 8.8, was used for stopping. This value corresponds to genuine ALA as determined by ALA-pyrrole formation (Table 5). In the presence of acidic SDS, pH 3.9 or pH 4.8, however, a much higher apparent specific activity (19.4 nmol hr^{-1} mg^{-1}) was observed in the column eluates. By comparison with the values observed at pH 8.8, approximately 80% of these counts probably represent the cysteamine thioester of succinate which bound to the Dowex 50 column at pH 3.9. In this manner, stopping the reaction with acidic SDS duplicates the difficulties observed when stopping with trichloracetic acid as the co-elution of ALA and the contaminating products occurs. In the absence of glycine in the reaction mixture, even greater amounts of the contaminating product were formed when the reaction was stopped with acidic SDS. In addition, high activity was observed in the blanks incubated in the

Table 7. Effect of pH on SDS Stopping of ALAS Assay

Enzyme assays were performed as described in Table 1, except that the "blank" refers to the omission of the Lubrol extract (or matrix fraction) during the incubation. The pH of the 10% SDS was adjusted with Na acetate buffer as necessary.

	pH 3.6	pH 4.8	pH 8.8
Matrix Fraction			
Plus Glycine	19.4 ± 3.1	19.3 ± 0.9	2.33 ± 0.12
Blank	0.35± 0.05	0.43± 0.02	0.036± 0.000
Minus Glycine	24.3 ± 1.1	23.9 ± 3.4	0.95 ± 0.10
Blank	6.6 ± 1.1	7.9 ± 0.7	0.52 ± 0.05

absence of mitochondrial protein. This value may represent an amidase or peptidase activity present in the porcine succinyl CoA synthetase. The activity of this enzyme may be suppressed by glycine leading to the low values of product present in the blanks containing glycine (Table 7).

c. Optimal Assay Conditions for Soluble ALAS

Before attempting to purify ALAS and the amidase-like enzyme which cleaves succinyl CoA, it was necessary to establish optimal conditions to assay both these enzymes. The soluble ALAS liberated from the interior of the mitoplast was saturated with concentrations of succinate as low as 25 μM. Previous studies from our laboratory (133) and others (43) have reported a substrate level of 25-50 μM succinate as optimal for the soluble enzyme. Maximum activities were observed with 0.1 mM CoA and 1 mM GTP. As seen in Figure 8C, the optimal concentration of glycine for the soluble ALAS was 75 mM, a similar value to that noted for intact mitochondria (150,151). The activity of soluble ALAS increased linearly with protein from 0.1 to 0.5 mg per 250 μl assay (Figure 8A). Furthermore, the assay was linear with time for 10 minutes (Figure 8B). The optimal pH for the reaction was 7.4 (Figure 9). The reaction was temperature dependent, doubling when the reaction was performed at 37°C. When the enzyme was assayed with either Tris or Hepes buffer using

Figure 8. Optimal Conditions for the Assay of ALAS in a Lubrol Extract of Mitochondria

The standard 0.25 ml incubation medium contained, except for the component varied: 50 mM Tris-HCl, pH 7.4, 100 mM glycine, 10 mM EDTA, 0.1 mM pyridoxal-5'-phosphate, 20 mM $MgCl_2$, 1.0 mM coenzyme A, 1 mM GTP, 0.25 μCi of [^{14}C]succinate, 50 μM succinate, 0.006 U of porcine succinyl CoA and 0.2 mg of a Lubrol extract of mitoplasts. After incubation for 20 minutes, except where indicated in B, the reaction was stopped by addition of 1.0 ml of 10% SDS and ALA isolated as described in Experimental Procedures. Units are A and B, pmol ALA; C, nmol ALA per mg per hour.

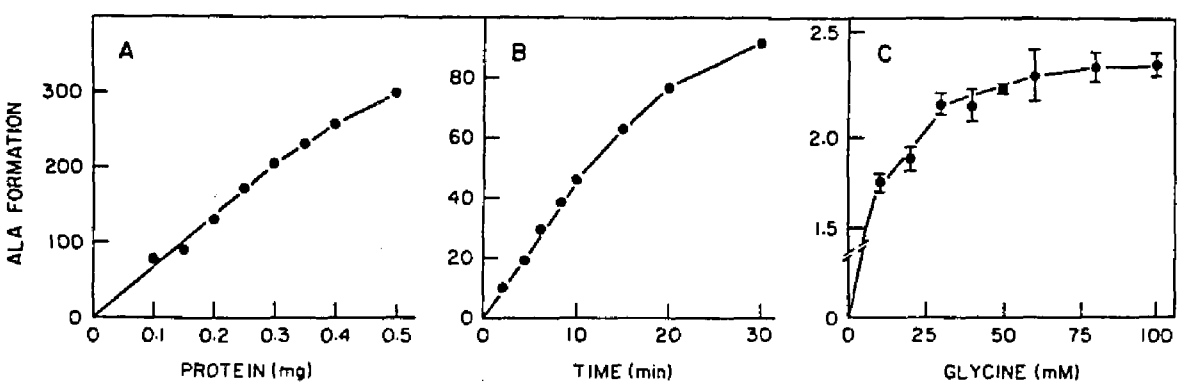
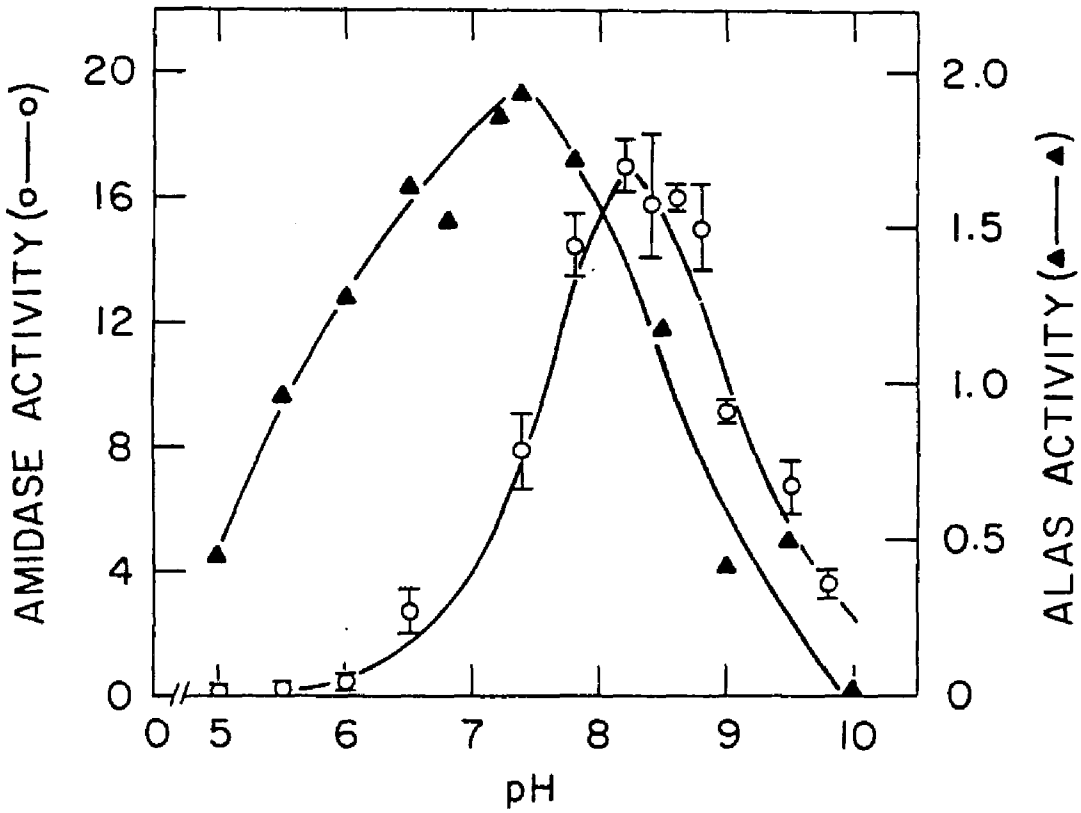

A
B
C
ALA FORMATION
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
PROTEIN (mg)
80
60
40
20
0
0
10
20
30
TIME (min)
2.5
2.0
1.5
0
0
25
50
75
100
GLYCINE (mM)

Figure 9. Optimal pH for ALAS and Amidase Activities

The assay medium for both enzymes described in Experimental Procedures and in the legends to Figures 8 and 10 were preincubated at pH 7.4 with succinyl CoA synthase in the absence of mitochondrial proteins for 5 minutes at 22°C. The pH of the reaction mixture was then adjusted as desired and the reaction started by the addition of the Lubrol extract of mitochondria. The assay conditions and isolation of products were as described in Experimental Procedures and in the text. Activity of both enzymes is expressed as nmol per mg per hour.


AMIDASE ACTIVITY (o——o)
ALAS ACTIVITY (▲——▲)
pH
0
4
8
12
16
20
0.5
1.0
1.5
2.0
5
6
7
8
9
10

the modified SDS stopping method, an excellent estimate of genuine ALA was obtained by Dowex 50 isolation when compared to the pyrrole conversion protocol; however, in the presence of Hepes buffer, the ALAS-activity was only 60% of that observed with Tris buffer (Table 8). The substitution of Hepes for Tris buffer in the ALA-assay was originally proposed by Bishop and Wood (61), who attributed the major contaminant observed during the Ebert procedure to the presence of Tris buffer. In addition, Hepes is a commonly used buffer for many mitochondrial isolation procedures.

However, a major contaminant which did not undergo a molecular rearrangement with the basic SDS stopping solution appeared in the presence of Hepes buffer and could not be separated from ALA on the single ion exchange column procedure as shown in Table 8.

d. Optimal Assay Conditions for Soluble Amidase (Succinyl CoA Degrading Enzyme)

The optimal conditions and substrate concentration for the putative amidase were determined in a Lubrol extract of rat liver mitoplasts. As shown in Figure 10A and B, the optimal [^{14}C]succinate and coenzyme A concentrations were 50 μM and 0.5 mM, respectively. The enzymatic reaction proceeded linearly with time for 35 minutes and was also linear with protein from 0.02 mg to 1.5 mg in the 1 ml assay

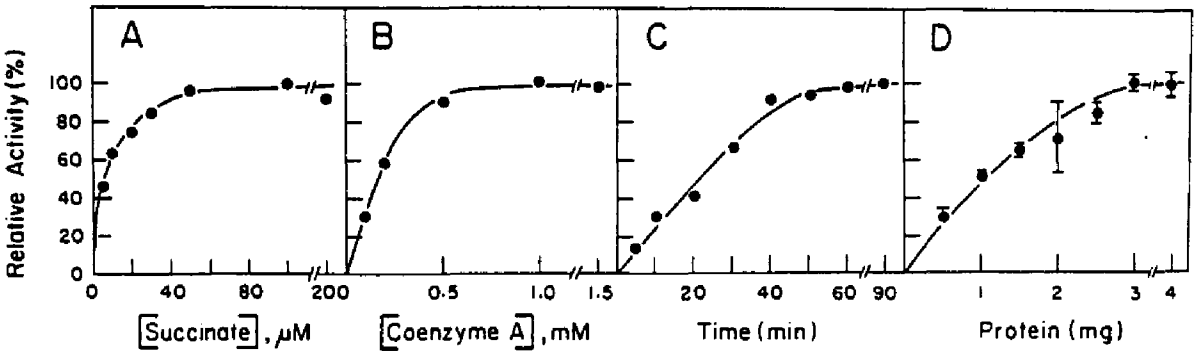
Table 8. Effect of Different Buffers on ALAS Activity

Enzyme assays with the Lubrol extract of mitoplast were performed as described in Table 1 except that Hepes buffer, pH 7.4, was substituted for Tris-HCl throughout the isolation procedure for mitochondria and the Lubrol extract, as well as in the enzyme reaction mixture. Activities are nmol per mg per hour.

	Tris		Hepes	
Matrix Fraction	ALA	ALA Pyrrole	ALA	ALA Pyrrole
Plus Glycine	1.73 ± 0.02	1.60 ± 0.05	1.10 ± 0.03	0.94 ± 0.02
Minus Glycine	0.18 ± 0.003	0.03 ± 0.03	0.68 ± 0.02	0.03 ± 0.01

Figure 10. Optimal Conditions for the Assay of the Amidase

The standard 1.0 ml reaction mixture contained, except where indicated, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 20 mM $MgCl_2$, 1.0 mM coenzyme A, 1 mM GTP, 1 μCi of [^{14}C] succinate, 50 μM succinate, sufficient porcine succinyl CoA synthase to produce 1 μmol of succinyl CoA per 15 minutes and 0.5 mg of Lubrol extract. After a 30 minute incubation in a shaking water bath at 37°C, the radioactive product was isolated as described in Experimental Procedures. Activity is expressed as the percentage of the maximum specific activity obtained, 17.8 $\pm$ 3.6 nmol labeled product formed per mg of protein per hour.


A
B
C
D
Relative Activity (%)
100
80
60
40
20
0
0
40
80
200
0·5
1·0
1·5
20
40
60
90
1
2
3
4
[Succinate], μM
[Coenzyme A], mM
Time (min)
Protein (mg)

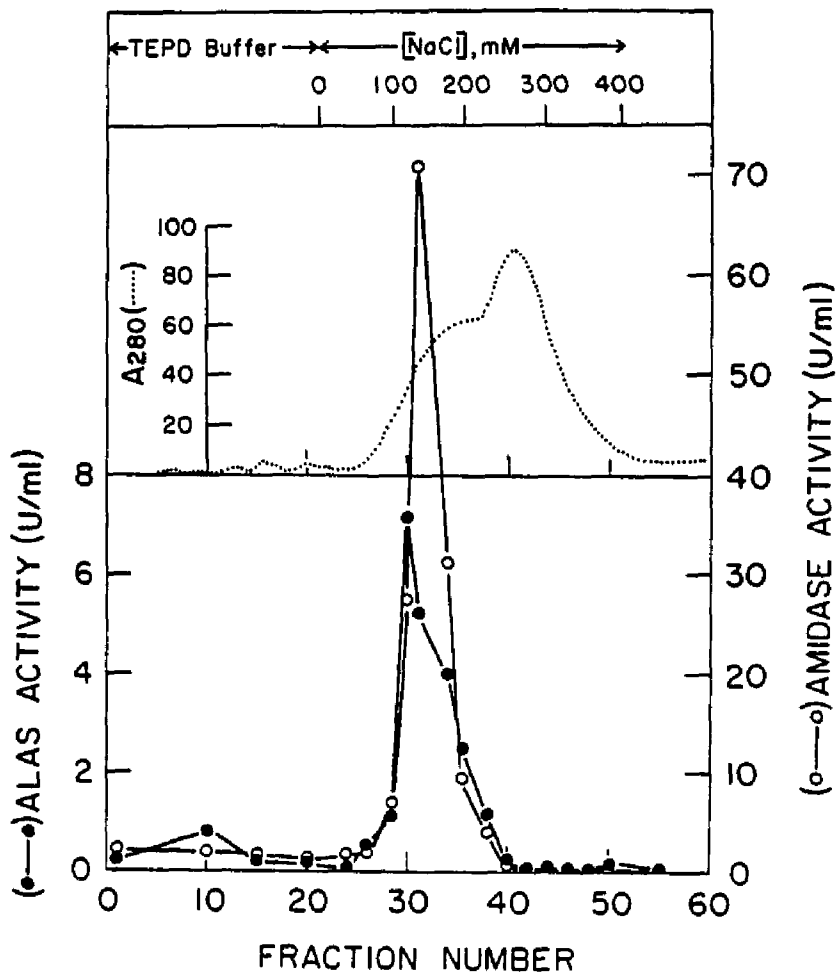
mixture (Figure 10C and D). The formation of product by the amidase was independent of both pyridoxal 5'-phosphate and glycine. A biphasic pattern of stimulation and inhibition was obtained when the concentration of EDTA in the assay mixture was varied. At a 5 mM concentration of EDTA, the rate of product formation approached the maximum rate observed; however, the reaction was completely inhibited by 15 mM EDTA. A similar finding was reported by Wolfson et al. (62), who showed that 10 mM EDTA blocked succinyl CoA degradation. The optimal pH for amidase activity was found to be approximately 8.2 (Figure 9).

e. Relationship of the Amidase to ALAS

The possible association of the proposed amidase, the succinyl CoA degrading enzyme, and ALAS was examined by monitoring the behavior of both enzymes on DEAE column chromatography. The Lubrol extract of mitoplasts was loaded onto a DE52 column and eluted with a continuous NaCl gradient. As shown in Figure 11, both enzyme activities were eluted at the same salt concentration, 125-150 mM NaCl, suggesting a possible aggregation (or association) between these two enzymes. The possibility that both activities were catalyzed by the same protein but different subunits or different active sites was examined by studying the heat lability of both enzymatic activities. As shown

Figure 11. DEAE-Cellulose Elution Profile of ALAS and the Amidase

The Lubrol extract of mitoplast was applied to a 3 x 20 cm column of DEAE equilibrated with 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM pyridoxal 5'-phosphate and 0.1 mM dithiothreitol, pH 7.5. Elution of the protein with a linear NaCl gradient (0-600 mM) at 3 ml/minute. Both enzymes were assayed as previously described in the text and in Experimental Procedures. •——•, ALAS; o——o, amidase; ..., absorption at 280 nm. Units are nmol per hour.


←TEPD Buffer→
←[NaCl], mM→
0 100 200 300 400
A280(----)
100
80
60
40
20
(•—•)ALAS ACTIVITY (U/ml)
(o—o)AMIDASE ACTIVITY (U/ml)
0 2 4 6 8
0 10 20 30 40 50 60 70
0 10 20 30 40 50 60
FRACTION NUMBER

in Figure 12, ALAS was much less sensitive to heat than was the amidase. A 20 minute preincubation at 38°C resulted in the loss of 50% of the amidase activity; however, this preincubation caused a slight activation of ALAS. When the DEAE column eluates were preincubated at 45°C, 60% of the amidase activity was destroyed in 5 minutes while only 25% of ALAS activity was lost. These results clearly indicated that these two enzyme activities are catalyzed by different proteins.

The porphyrinogenic agent, AIA, has been well established as a potent inducer of ALAS. As shown in Table 9, treatment with AIA resulted in a 10-fold increase of ALAS activity as described by many other groups (58,137,56). The amidase activity, however, was not significantly increased by AIA treatment. During AIA-induced porphyria, it has been shown that the synthesis of ALAS occurs de novo (152). The lack of induction of amidase activity by AIA provides further evidence for the suggestion that these reactions are catalyzed by different proteins and are regulated by different mechanisms.

Figure 12. Effect of Heat Treatment on ALAS and Amidase

A portion of the pooled peak fraction obtained with DEAE-cellulose was preincubated at 38°C and 45°C for the times indicated prior to addition to the incubation medium, maintained in ice. ALAS and the amidase were assayed as described in the legends to Figures 8 and 10 and the radioactive products isolated as described in Experimental Procedures. ●——●, Amidase at 45°C; o——o, amidase at 38°C; ▲——▲, ALAS at 45°C; △——△, ALAS at 38°C.

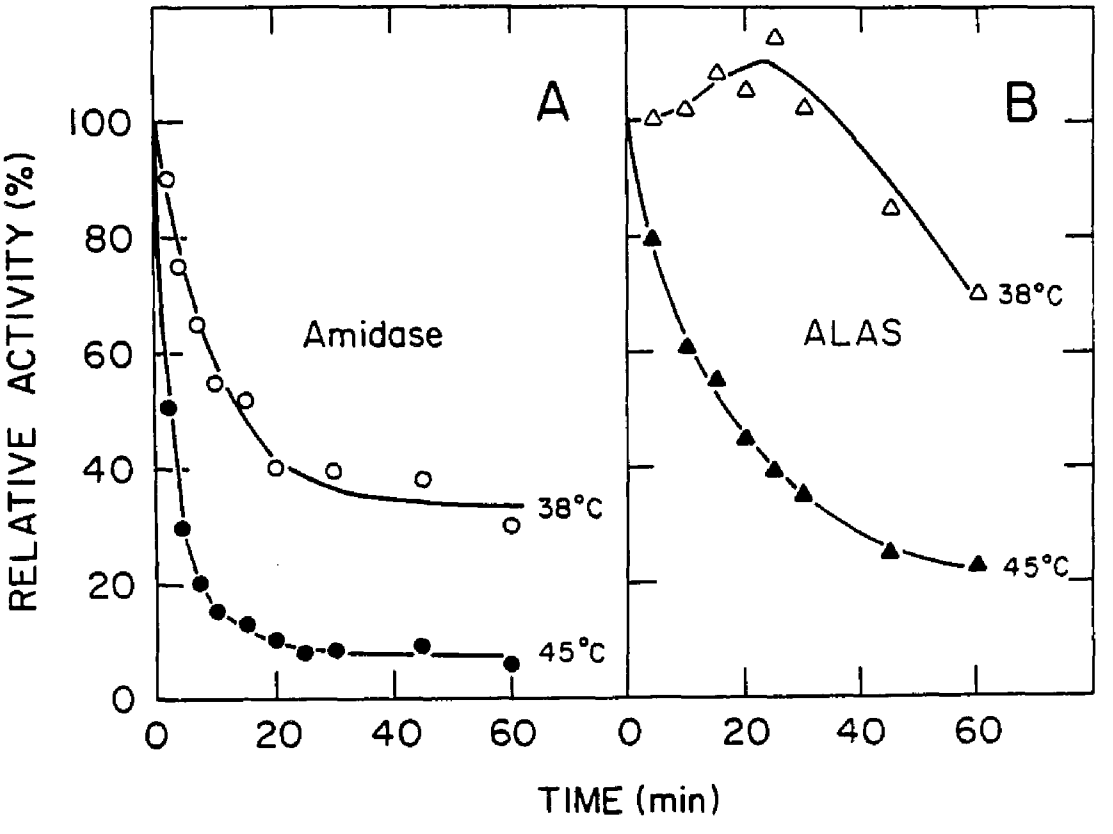

A
B
Amidase
ALAS
38°C
45°C
38°C
45°C
RELATIVE ACTIVITY (%)
TIME (min)
0
20
40
60
80
100

Table 9. Effect of AIA Induction on ALAS and the Amidase Activity

All animals were fasted 24 hours prior to treatment with AIA. AIA (dissolved in 0.9% NaCl) was administered subcutaneously at a dose of 50 mg/100 g body weight, 16 hours prior to sacrifice. ALAS and the amidase were assayed as described in the legends to Figures 8 and 10.

	Control	AIA
	(nmol mg^{-1} h^{-1})	
ALAS	1.98 ± 0.16	19.3 ± 5.0
Amidase	14.2 ± 3.1	18.7 ± 4.3

2. Discussion

In the present chapter, a rapid and convenient assay for ALAS is presented in which SDS is used for stopping the reaction. The major advantage of this new procedure is that it eliminates the co-elution of degradative products of succinyl CoA with ALA from the Dowex 50 columns. The presence of these contaminating products in the eluates of the single column procedure for the isolation of ALA (61,60,62) had necessitated, for the accurate measure of ALAS activity, the use of a time-consuming multi-column procedure in which the ALA-pyrrole is finally isolated (63). Variable recoveries from the different columns, however, had made this procedure difficult to quantitate on an individual assay basis. The new assay presented here avoids these difficulties while retaining the potential sensitivity of the radiochemical assay (38,63) with the rapidity of the single column procedure.

An additional benefit of stopping the assay with SDS is that the entire reaction mixture can be applied directly to the Dowex 50 columns. The need to acid-precipitate proteins and subsequently remove them by centrifugation is thus avoided. The SDS-denatured proteins which have a net negative charge pass through the Dowex 50 columns with the succinate

and other anions and do not interfere with the binding of the positively charged ALA to the column. Furthermore, the column does not apparently become clogged due to the presence of SDS-protein micelles. The SDS stopping procedure for the assay of ALAS has also been used successfully in the multi-column procedure of Strand et al. (63), in which ALA is converted to the pyrrole and isolated. In this case, a 4 cm x 1 cm resin bed of the initial Dowex 1 column was found to have a bind capacity which is not exceeded by 1 ml of 10% SDS and the anions present in the assay mixture. It should be noted that in our laboratory we have found that a Dowex 50 column, followed by a Dowex 1 column after conversion of ALA to the pyrrole, is adequate for the quantitative isolation of the ALA pyrrole.

Several disadvantages of the SDS stopping method should be mentioned. First, Hepes buffer, commonly present in isolation media for mitochondria, cannot be used. A contaminating compound which does not rearrange during incubation procedure with SDS is produced and co-eluted with ALA from the Dowex 50 columns. In addition, significantly lower activities of ALAS are obtained. Secondly, elution of the columns with 1 N NaOH rather than NH_4OH, as reported by Minaga et al. (60), reduces the [^{3}H]ALA/[^{14}C]ALA ratio in the final eluate, suggesting that NaOH causes a degradation of the [^{3}H]ALA, resulting in a decreased and variable

recovery of the internal standard.

The attempts at characterization of the contaminating product in the eluates by the use of different chromatographic procedures have indicated that it most probably is the disulfide dimer of the cysteamine thioester of succinate, as originally proposed by Minaga, et al. (60). This compound would be formed by cleavage of succinyl CoA at the amide bond between the β-alanine and the cysteamine residues by the action of an amidase or endopeptidase in the solubilized mitochondria. This product would then undergo a rearrangement during elution from the Dowex 50 columns by the strong base. It was found that an identical rearrangement of the cysteamine thioester of succinate occurs during incubation in the basic SDS and hence the contaminant no longer binds to the cation exchange column, as does ALA, but is eliminated.

The initial interest in further studies of the proposed amidase was to elucidate its possible relationship to ALAS, both structurally and functionally. Initially, the optimal condition for measuring the amidase activity was established. The omission of glycine from the usual ALAS reaction mixture resulted in significantly higher rates of product formation by the amidase and also eliminated the formation of any ALA as a contaminant. The reaction was then stopped with acidic SDS, a condition which does not permit rearrangement of the

cysteamine thioester of succinate (Table 7). Finally, the usual elution scheme from the Dowex 50 columns was modified in order to obtain maximum recovery of the amidase product as discussed in the results section.

Using these newly established assays for ALAS and the putative amidase, it was shown that the two enzymes co-purify during the initial steps in the purification procedure for ALAS developed in the laboratory of Dr. D.S. Beattie (153). Preliminary studies in this laboratory also indicate that the two enzymic activities co-purify during column chromatography on both Sephacryl S-200 and S-300 as well as on hydoxyapatite. The two enzymes, however, exhibit different heat denaturation profiles and respond differently to the porphyrinogenic drug, AIA, an established inducer of ALAS (159,56,175,152). This suggests that the two enzymatic activities, ALAS and the amidase, are indeed catalyzed by two different proteins.

C. The Submitochondrial Localization of ALAS and Metal Requiring Amidase in Rat Liver

In the preceding chapter the structure of the contaminant originally proposed by Minaga et al (60) was confirmed, and a new rapid assay procedure for the separation of ALA and the amidase product was reposted. In the present chapter the intramitochondrial localization of the amidase compared to both ALAS and established marker enzymes was further explored. Treating rat liver mitoplasts, defined as an intact inner membranes containing the matrix (154) with varying concentrations of the non-ionic detergent Lubrol or with either trypsin or chymotrypsin suggested that the amidase is localized on the outer surface of the inner membrane. This intramitochondrial localization is similar to that reported for cytochrome c (155); however, treatment of mitoplasts with dilute salt did not remove the amidase as it did cytochrome C (156). The amidase activity was inhibited by ortho-phenanthroline and reconstituted by addition of either Co^{2+} or Mn^{2+} suggesting the need for a metal ion at the active site of the enzyme. A possible functional role for the amidase is suggested based on the intramitochondrial localization of the enzyme and the metal requirement for activity.

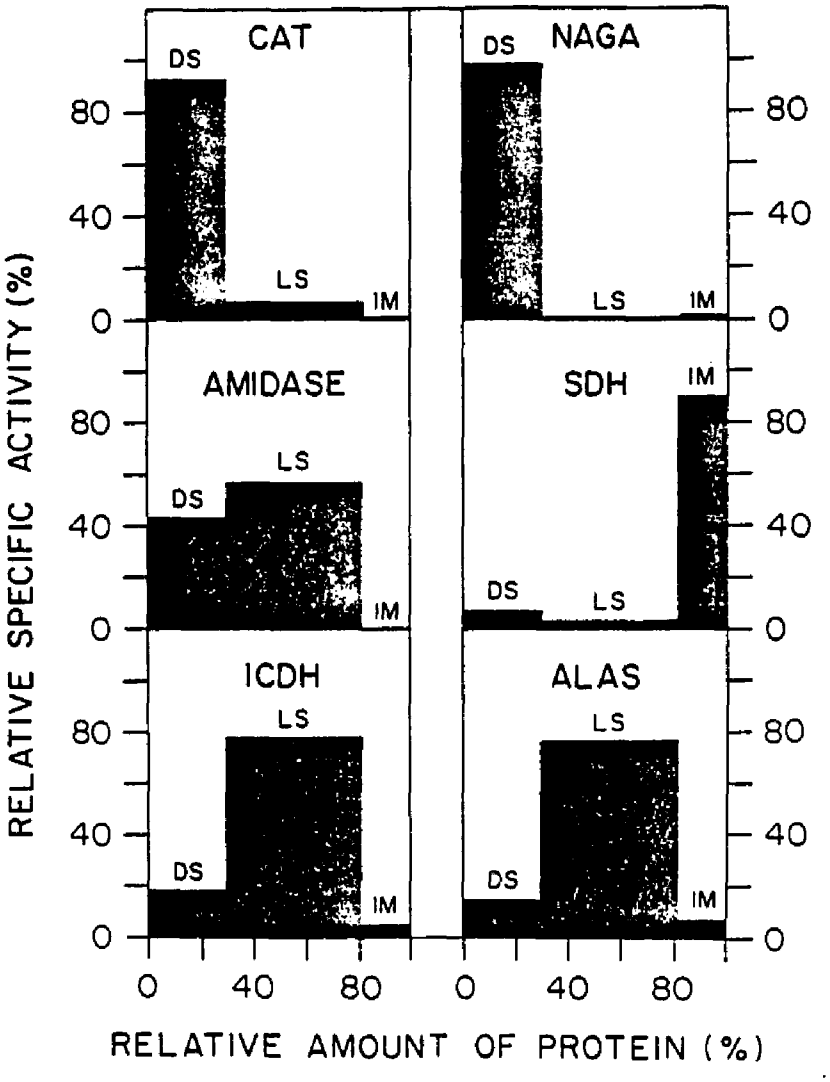
1. Results

a. The Localization of ALAS and the Amidase

In the preceding chapter (V.B), the presence of an amidase-like activity in Lubrol-solubilized rat liver mitochondria was demonstrated and optimal conditions for its assay were determined. Before further characterizing the enzyme, it was necessary to establish its true mitochondrial localization. The distribution of the lysosomal marker enzyme, N-acetyl-glucosaminidase and the peroxisomal marker enzyme, catalase, was compared to the amidase and ALAS during the sub-fractionation of mitochondria with digitonin and Lubrol. Both contaminating lysosomes and peroxisomes were lysed by treatment of the mitochondrial fraction with digitonin, as the marker enzymes were limited to the digitonin-soluble fraction (Figure13). These results indicate that the mitoplast fraction (digitonin insoluble) used in all our studies does not contain any other contaminating subcellular organelles. Figure 13 also indicates that during the digitonin and Lubrol fractionations, both the amidase and ALAS activities distribute in a manner similar to the matrix enzyme, isocitric dehydrogenase. A greater percentage of amidase activity (30%) is released from the mitoplasts by digitonin than ALAS or isocitric

Figure 13. The Distribution of Marker Enzymes in Submitochondrial Fractions

Rat liver mitochondria were fractionated with digitonin and Lubrol as described under Experimental Procedures. Each of the fractions was assayed for the following enzymes: CAT (catalase), NAGA (N-acetylglucosominidase), the amidase; SDH (succinate dehydrogenase); ICDH (isocitrate dehydrogenase) and ALAS. The fractions assayed were the digitonin-soluble (DS) containing both the outer membrane and the intermembrane space, the Lubrol-soluble (LS) containing the matrix and the inner membrane (IM).


CAT
NAGA
AMIDASE
SDH
ICDH
ALAS
DS
LS
IM
0
40
80
RELATIVE SPECIFIC ACTIVITY (%)
RELATIVE AMOUNT OF PROTEIN (%)

dehydrogenase activity (15-20%). However, the majority of activity remains in the Lubrol-soluble fraction. These results make it extremely unlikely that the amidase activity observed in our mitochondrial preparations are due to contaminating lysosomes or peroxisomes.

The latency of both ALAS and the amidase was further investigated by treating either intact mitochondria or mitoplasts with Lubrol and studying the release of enzymatic activity. No significant amidase activity was observed in intact mitochondria with the presence or absence of an exogenous succinyl CoA generating system (Table 10). These results indicate that the amidase is localized within the mitochondria in such a manner that the active site of the enzyme is inaccessible to either exogenous succinyl CoA or to that generated by enzymes within the matrix. Addition of the succinyl CoA generating system to intact mitochondria, however, decreased the apparent rate of ALAS. An explanation might be that the exogenous succinyl CoA synthase converts a significant amount of the labeled succinate to succinyl CoA which cannot cross the inner membrane and thus is unavailable to the ALAS within the matrix. Lubrol treatment of the mitochondria resulted in an increase in the total amidase activity but resulted in an apparent decrease of ALAS activity. After centrifugation of the Lubrol-treated mitochondria to remove membrane fragments, the activities of

TABLE 10

The Effect of Lubrol Treatment on Amidase and ALAS Activity in Mitochondria and Mitoplasts

Mitochondria were treated with Lubrol at a concentration of 1.5mg Lubrol/10mg mitochondrial protein for 15 min at 0°. This fraction, the Lubrol-treated mitochondria, was subsequently centrifuged at 100,000 xg for 45 min to yield a Lubrol extract and Lubrol pellet. Mitoplasts were prepared from digitonin-treated mitochondria, solubilized with Lubrol (1.05mg/10mg mitoplast protein) and centrifuged at 100,000 xg for 45 min to yield the Lubrol soluble and Lubrol pellet fractions. The amidase, ALAS, isocitrate dehydrogenase (ICDH) and succinate dehydrogenase (SDH) were assayed as described under Experimental Procedures. Results are reported as mean and standard deviation of triplicate determinations.

		Amidase		ALAS		ICDH		SDH	
Enzyme Preparation	Protein (mg)	SpAct nmol $hr^{-1}mg^{-1}$	Total Act	SpAct nmol $hr^{-1}mg^{-1}$	Total Act	SpAct nmol $min^{-1}mg^{-1}$	Total Act	SpAct nmol $min^{-1}mg^{-1}$	Total Act
Mitochondria (-SCS)	172	-	-	0.35 ± .12	60.2	0	0	1.0 ± .4	173.7
(+SCS)		0.21 ± .03	36.1	0.1 ± .04	17.2	-	-	-	-
Mitochondria treated with Lubrol WX	172	0.53 ± .08	91.2	0.13 ± .07	22.4	42.6 ± 7.8	7327.2	2.6 ± 4.3	450.6
Lubrol Extract	95	14.5 ± 1.4	769.5	0.78 ± .15	74.9	70.7 ± 7.2	6716.5	.4 ± .02	4.1
Lubrol Pellet	61	0.30 ± .05	1.2	0.06 ± .02	3.7	21.0 ± 1.7	179.4	6.9 ± .2	425.9
Mitoplast	108	0.23 ± .08	24.8	0.21 ± .03	22.7	6.9 ± .7	745.2	4.12 ± 1.5	444.1
Lubrol Extract	86	17.6 ± .24	1513.6	1.6 ± .3	136.5	85.4 ± 14.1	7378.6	.3 ± .02	25.1
Lubrol Pellet (IM)	20	0.17 ± .09	3.3	0.04 ± .02	0.8	9.2 ± .8	179.4	20.9 ± .7	408.4

A- The intact mitochondria were assayed either with or without exogenous succinyl CoA synthetase. All other fractions were assayed with the exogenous succinyl CoA synthetase.

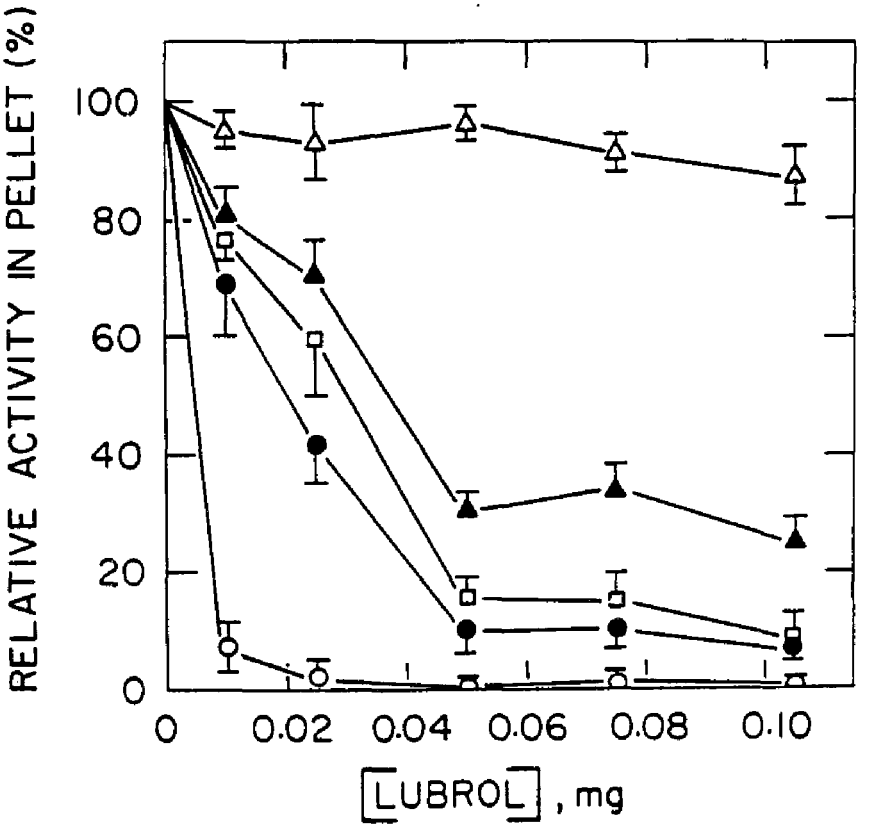
both ALAS and the amidase were increased. The most likely explanation for this result is that the succinate dehydrogenase present in an activated form in the membrane fragments obtained after Lubrol treatment rather than the succinyl CoA synthase may preferentially use the radioactive succinate. Similarly, the amidase activity is very low in isolated mitoplasts but released by Lubrol treatment (Table 10).

For a more precise intramitochondrial localization of both the amidase and ALAS, the effects of varying both the Lubrol and digitonin concentrations during fractionation were examined. Washed mitoplasts were treated with different amounts of Lubrol prior to centrifugation to obtain a pellet and Lubrol-soluble fraction. ALAS activity was released from the mitoplasts in a Lubrol-concentration dependent manner analogous to the bulk of protein and isocitrate dehydrogenase activity (Figure 14). No succinate dehydrogenase activity, an inner membrane-bound enzyme, was released by these concentrations of Lubrol. By contrast, almost all of the amidase activity was released from the mitoplasts by addition of as little as 10 μg of Lubrol per mg of mitochondrial protein.

Similarly, studies involving treatment of mitochondria with various concentrations of digitonin also indicated a unique intramitochondrial localization for the amidase. At

Figure 14. The Effect of Lubrol Concentration on the Release of the Amidase from Mitoplasts

Rat liver mitoplasts prepared as described under Experimental Procedures. Mitoplasts (1 mg) were treated with Lubrol in the indicated concentrations for 15 minutes at 0°C and then centrifuged at 100,000 xg for 45 minutes. The pellet was resuspended in TEPD buffer. Both the supernatant and pellet were assayed for protein (▲——▲) and the following enzymes: △——△ succinate dehydrogenase; □——□ isocitrate dehydrogenase; ●——●, ALAS and ○——○ the amidase.


RELATIVE ACTIVITY IN PELLET (%)
100
80
60
40
20
0
0
0.02
0.04
0.06
0.08
0.10
[LUBROL], mg

a ratio of 50 μg digitonin per 6 mg of mitochondrial protein, a concentration of digitonin reported to lyse lysosomes within the mitochondrial fraction (157), only 12% of amidase activity was released (Figure 15). At a ratio of 160 μg of digitonin per mg of mitochondrial protein, approximately 50% of the amidase activity was released. Interestingly, when the 9500 xg pellet obtained from the digitonin-treated mitochondria was resuspended in TEPD buffer and incubated at 4°C for 24 hours, significant amounts of amidase activity were released into the supernatant (Figure 15). In some experiments, nearly 80% of the amidase was solubilized by this treatment.

The effects of both Lubrol and digitonin upon the release of amidase suggested that this protein may be localized on the outside of the inner membrane. To test this possibility, the effect of proteolytic digestion of mitoplasts with trypsin or chymotrypsin was studied (Table 11). Mild treatment with both proteases resulted in the disappearance of significant amounts of the amidase activity but had no effect on the activities of the two matrix enzymes, isocitric dehydrogenase or ALAS, suggesting that the amidase is indeed on the outer surface of the inner membrane as is cytochrome c. Interestingly, negligible amounts of cytochrome c are released from the inner membrane with concentrations of Lubrol, which completely release both the amid-

Figure 15. Extraction with Digitonin and the Effect of Aging Mitoplasts on the Release of the Amidase

Mitochondria (1 mg) were treated with varying amounts of digitonin for 15 minutes at 0°C and then centrifuged at 9500 xg for 10 minutes. The mitoplasts resuspended in TSE were incubated for 24 hours at 4°C and recentrifuged at 9500 xg for 10 minutes. The following enzymes were assayed in the 9500 xg supernatant. (●——●) ALAS at zero time; (□——□) protein concentration; (●----●) ALAS in 9500 xg supernatant in mitoplasts aged for 24 hours; (o....o) amidase in supernatant in mitoplasts aged for 24 hours; (o——o) amidase activity at zero time.

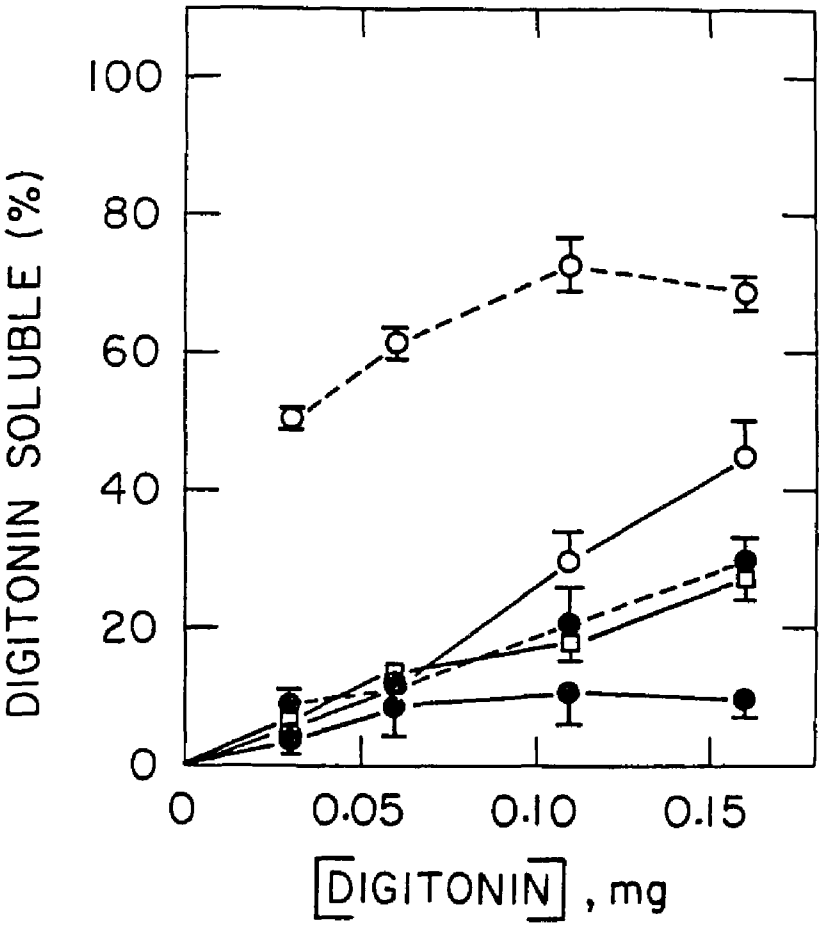

DIGITONIN SOLUBLE (%)
100
80
60
40
20
0
0
0.05
0.10
0.15
[DIGITONIN] , mg

Table 11. Effect of Mild Proteolytic Digestion of Mitoplasts

Mitoplasts were resuspended in TEPD Buffer to 20 mg/ml. Trypsin (15 μg/mg protein) was added and incubated for 30 minutes at 4°C. The reaction was stopped with a 4-fold excess of trypsin inhibitor. The same amount of trypsin inhibitor plus tryspin were added to the controls which were incubated for the same time. Chymotrypsin (20 μg/mg protein) digestion was performed in the same way and the reaction stopped with a 2-fold excess of PMSF. The PMSF and chymotrypsin were added to the controls at the beginning of the incubation. The mitoplasts were then solubilized with Lubrol and centrifuged as described under Experimental Procedures to yield a Lubrol-soluble and an inner membrane fraction. The enzymes were assayed in the Lubrol-soluble fraction as described under Experimental Procedures.

Treatment	Amidase		ALAS		ICDH	Cyt c[a]	
	nmol $h^{-1}mg^{-1}$	% Decrease	nmol $h^{-1}mg^{-1}$	% Decrease	nmol $min^{-1}mg^{-1}$	nmol mg^{-1}	% Decrease
Trypsin	13.1	35.7	2.2	none	62.4	.54	8.4
Control	20.4	-	2.1	-	60.5	.59	-
Chymotrypsin	16.0	28.5	1.9	-13.6%	N.D.	.40	26.0
Control	22.4	-	2.2	-	N.D.	.54	-

a. Cytochrome c was determined in the inner membrane fraction as insignificant amounts were released into the Lubrol-soluble fraction.

ase and the matrix enzymes. In addition, cytochrome c is insensitive to trypsin digestion but sensitive to chymotrypsin (Table 11). Increasing the amount of trypsin and the time of incubation resulted in the disappearance of 85-89% of the amidase activity but had a minimal effect on either ALAS or isocitric dehydrogenase activities (Table 12).

The possibility was next investigated that the amidase was bound to the outside of the inner membrane by ionic bonds as is cytochrome c (156). Washing the mitoplasts three times with 0.154 M NaCl released almost all of the cytochrome c from the membrane. However, less than 10% of the amidase activity was released by the salt, suggesting that this protein is not bound to the membrane by electrostatic forces (Table 13). Similarly, addition of chaotropic agents, shown to weaken the hydrophobic interactions of extrinsic membrane proteins (159), did not release amidase activity from the membrane.

2. Substrates and Inhibitors of the Amidase

In order to establish the possible endopeptidase activity of this amidase, several synthetic peptide substrates were tested. Benzoyl-4'-tyrosine-p-nitroanilide (BTNA) and Succ-Ala-Ala-Pro-Phe-pNA, specific substrates for chymotrypsin-like endopeptidases, were not cleaved by

Table 12. Effect of Trypsin Digestion on Enzyme Activities in Mitoplasts

Mitoplasts resuspended in TEPD buffer at 20 mg/ml were incubated with trypsin (40 μg/mg) protein or trypsin plus trypsin inhibitor (controls) at 4°C for the times indicated. To stop the reaction, a 4-fold excess of trypsin inhibitor was added. The mitoplasts were then solubilized with Lubrol as described under Experimental Procedures and centrifuged to yield a Lubrol-soluble fraction. ALAS, the amidase and isocitrate dehydrogenase were assayed in this fraction.

	ALAS			AMIDASE			ISOCITRATE DEHYDROGENASE		
TIME OF INCUBATION	TRYPSIN TREATED	CONTROL	% DECREASE	TRYPSIN TREATED	CONTROL	% DECREASE	TRYPSIN TREATED	CONTROL	% DECREASE
Zero	-	1.7	-	-	16.8	-	-	61.6	-
30 min	1.7	1.9	11	2.7	18.2	85	68.6	68.6	Zero
60 min	1.3	2.0	35	2.2	13.0	83	65.3	69.4	6%
105 min (1 hr. 45)	1.4	1.6	13	1.6	14.4	89	51.9	65.6	20%

Table 13. Effect of Salt Extraction of Mitoplasts on Amidase Activity and Cytochrome c

The initial mitoplast fraction obtained after treatment with digitonin followed by centrifugation at 9500 xg for 10 minutes was resuspended in 0.154 M NaCl and gently stirred for 15 minutes at 0°C. The suspension was then centrifuged at 9500 xg for 10 minutes. The supernatant was the first salt extract. The procedure was repeated two more times to yield the second and third salt extract. After the third salt extraction, the pellet was resuspended in TEPD buffer and extracted with Lubrol as described under Experimental Procedures.

Fraction	Protein (mg)	Cyt c			Amidase		
		Specific Content	Total	% Mito Total	Specific Activity	Total Activity	% Mito Total
First Salt Extract	2.8	13.2	36.3	74.4	10.2	28.1	1.6
Second Salt Extract	2.8	2.0	5.6	11.5	9.2	25.3	1.4
Third Salt Extract	1.3	1.3	1.6	3.3	8.5	10.6	0.6
Lubrol Extract	87.4	0	0	0	17.5	1529.5	85.1

a partially purified amidase obtained from the DEAE chromatography step (153). Benzoyl-arginine-p-anilide (BAPA), specific for trypsin-like endopeptidase, and Ac-Ala-Ala-Ala-p-Nitroanilide, a substrate for elastase, were also not cleaved by the purified amidase. Interestingly, the Lubrol extract of mitochondria did contain detectable amounts of both BTNA and BAPA hydrolyzing activity which did not fractionate with ALAS and the amidase during DEAE chromatography.

The amidase activity in the Lubrol extract of mitoplasts was not affected by the protease inhibitors PMSF, TLCK or TPCK; however, it was sensitive to ortho-phenanthroline (Figure 16). A concentration of 2.3 mM o-phenanthroline caused a complete inhibition of amidase activity after a 10 minute preincubation, suggesting that the enzyme contains a transition-state metal at the active site. In order to test which metals might reconstitute the amidase activity, the enzyme preparation was preincubated with sufficient o-phenanthroline to inhibit 70% of the enzyme activity. Subsequently, various metals were added for an additional 20 minutes preincubation prior to addition of the enzyme to the assay mixture. As seen in Table 14, only Co^{2+} and Mn^{2+} of all the metals tested had the ability to restore activity to the inhibited enzyme. In addition, both these metals stimulated the amidase activity in the absence of inhibitors. The amount of o-phenanthroline present in

Figure 16. Effect of Protease Inhibitors on the Amidase Activity

One ml aliquots of partially purified amidase obtained by DEAE chromatography of Lubrol-solubilized mitoplasts (1) were preincubated with the indicated concentrations of PMSF (o——o), TLCK (▲——▲), TPCK (△——△) and o-phenanthroline (●——●) for 10 minutes. With shaking at room temperature a 125 μl aliquot was then added to the assay mixture to determine the amidase activity.

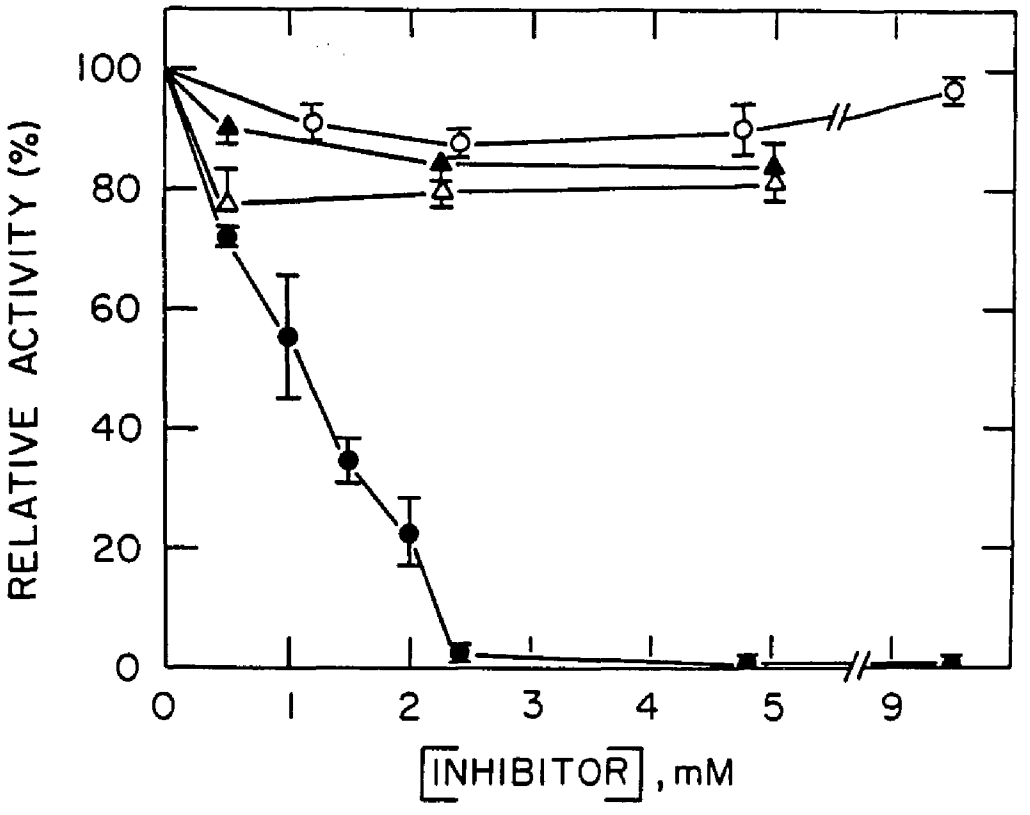

RELATIVE ACTIVITY (%)
100
80
60
40
20
0
0
1
2
3
4
5
9
[INHIBITOR], mM

Table 14. Reconstitution of Amidase Activity with Metal Ions After Inhibition with Ortho-phenanthroline

Into each 100 µl aliquot of partially purified amidase obtained by DEAE chromatography of Lubrol-solubilized mitoplasts (153) was added 1.7 mM o-phenanthroline and preincubated for 10 minutes at room temperature with shaking. Then the indicated metals were added and the enzyme preincubated for an additional 20 minutes at room temperature. Suitable aliquots were then added to the assay mixture to determine amidase activity as described under Experimental Procedures.

Table 14. Reconstitution of Amidase Activity with Metal Ions After Inhibition with Ortho-phenanthroline

Preincubated with 1.75 nM o-phenanthroline	Preincubated with Metals (mM)	Amidase nmol $h^{-1}mg^{-1}$	% of Control
-	-	62.0	-
-	Co^{++} (2.5)	74.4	120.0
-	Mn^{++} (2.5)	70.1	113.0
+	-	16.7	26.9
+	Co^{++} (1.25)	38.1	61.5
+	Co^{++} (2.5)	67.9	109.5
+	Mn^{++} (1.25)	34.7	56.0
+	Mn^{++} (2.5)	48.5	78.2
+	Fe^{++} (1.25)	28.5	45.9
+	Fe^{++} (2.5)	22.5	36.3
+	Zn^{++} (1.25)	28.8	46.4
+	Zn^{++} (2.5)	22.5	36.3
+	Mo (1.25)	16.8	27.1
+	Mo (2.5)	16.7	27.0
+	Di (1.25)	16.7	27.0
+	Di (2.5)	11.5	18.6
+	Cd (1.25)	9.8	15.8
+	Cd (2.5)	11.8	19.0
+	Cu^{++} (1.25)	13.0	21.1
+	Cu^{++} (2.5)	13.0	9.0

of inhibitors. The amount of o-phenanthroline present in the final incubation mixture was shown to have no effect on the succinyl CoA synthase coupling enzyme in the presence of the 10 mM $MgCl_2$ in the assay mixture. The possibility that iron was at the active site of the enzyme was also eliminated by the observed lack of inhibition by desferoxamine, a specific iron inhibitor (159).

The role of sulfhydryl groups in the amidase was investigated. Preincubation with p-chloromercuribenzoate was without effect on the amidase activity, suggesting that a sulfhydryl group is not necessary for activity. By contrast, high concentrations of dithiothreitol in the incubation mixture inhibited 50% of the amidase activity.

2. Discussion

The further characterization of the putative amidase or endopeptidase present in rat liver mitochondria was prompted by its apparent association with ALAS. Attempts to separate these two activities by chromatography of Lubrol-solubilized mitochondria on DEAE-cellulose, Sephacryl or hydroxyapatite were unsuccessful. In addition, succinyl CoA is a substrate for both enzymes. This suggests a possible functional association in the mitochondria. As discussed in the preceding chapter, however, the different sensitivities of the two enzymatic activities to heat plus

the lack of response of the amidase to AIA, a well-characterized inducer of ALAS (148), had indicated that the two activities were indeed catalyzed by two different enzymes. The possibility existed that the two might be closely associated in the mitochondria and hence be co-purified.

The results of the present study indicate that the amidase and ALAS have a completely different intramitochondrial localization. ALAS is present exclusively in the mitochondrial matrix as it responds to Lubrol and digitonin extractions and sonication exactly as does isocitric dehydrogenase, a matrix marker enzyme. By contrast, the amidase appears to be localized on the outer surface of the inner membrane. This conclusion is based on the observation that the amidase was released by concentrations of Lubrol-Wx, which had little or no effect on the release of ALAS. In addition, digitonin treatment did not release significant amidase activity; however, leaving the mitoplasts, obtained by digitonin treatment, for 24 hours at $0^{o}C$ resulted in the solubilization of almost 80% of the amidase activity. This suggests that the enzyme was gradually released from the membrane. The most compelling evidence for the localization of the amidase on the surface of the inner membrane was its complete sensitivity to trypsin or chymotrypsin digestion. These proteases had no effect on the matrix enzymes ALAS or isocitric dehydrogenase (160). This localization is similar to that proposed for cytochrome c, but more probably is held to the membrane by mild hydrophobic bonds

weakened by the mild detergents employed in subfractionations.

A major unanswered question is the chemical nature of the actual substrate for the amidase in the mitochondria. Succinyl CoA, the substrate used in the studies, does not appear to be the actual substrate. Neither radioactive succinyl CoA, generated by an external succinyl CoA synthase and hence, external to the mitoplasts, nor succinyl CoA generated within the matrix (and used by ALAS), can serve as substrate. Both mitochondria and mitoplasts must be disrupted with either detergents or sonication for detection of amidase activity. Furthermore, slight amidase activity can be assayed in some mitochondrial preparations which are damaged during preparation. Likewise, other coenzyme A derivatives, such as acetyl CoA or malonyl CoA, might serve as substrates for the amidase, but these compounds are also compartmentalized within the cell in such a manner as to be inaccessible to the activated amidase.

The possibility that coenzyme A itself is the natural substrate for the amidase also appears unlikely. Concentrations of coenzyme A greatly above that needed for optimal amidase activity (Figure 10B) do not cause a decrease in the activity of cleaving the [^{14}C]succinyl CoA. Furthermore, the established pathway for coenzyme A degradation in rat liver involves the action of a lysosomal phosphatase followed by that of a pyrophosphatase localized on the plasma mem-

brane (161,162). In the final step, pantetheine is cleaved at the same bond between the cysteamine and pantethenic acid, the identical bond in succinyl CoA which we and Minaga et al. (60) have suggested is cleaved by the amidase in solubilized mitochondria. The pantetheinase purified from horse kidney cortex (162,163) had an optimal pH of 4-5.5 and was specific for pantetheine or pantetheine-4-phosphate. By contrast, the amidase described in this study has an optimal pH of 8.2 and cleaves succinyl CoA.

The possibility that the amidase is a simple peptidase also does not appear likely despite the similarity of the amide bond cleaved in succinyl CoA and a peptide bond. Several synthetic substrates specific for trypsin, chymotrypsin and elastase were not cleaved by the partially purified amidase. Furthermore, the amidase activity in Lubrol-solubilized mitochondria was not inhibited by PMSF, TLCK or TPCK established inhibitors of some proteases (164). By contrast, the amidase was inhibited by o-phenanthroline, a metal chelator and potent inhibitor of metal-containing proteases (164).

Based on the intramitochondrial localization of the amidase and its sensitivity of o-phenanthroline, it is suggested that the amidase may be involved in the processing of mitochondrial proteins synthesized as larger precursors in the cytoplasm. Currently, evidence has accumulated

indicating that these precursors are post-translationally cleaved to the mature form of the protein by mitochondrial proteases (165,166). Recent studies with rat liver mitochondria have suggested that the processing to ornithine transcarbamylase occurs deep in the mitochondria (most probably in mitoplasts) but not completely in the matrix space (148). The protease which processed subunits of F_1 of the mitochondrial ATPase, however, was assigned a matrix localization (167). As noted in this dissertation, the "classical" methods for subfractionating mitochondria and mitoplasts can be misleading for assigning localizations of enzymes, such as the amidase, which are easily released by detergent treatment. Even more interesting are the reports that the yeast mitochondrial processing enzyme is sensitive to o-phenanthroline (167). The suggestion that the amidase described in this study is a peptidase is attractive, albeit tentative, and is currently under investigation in the laboratory of Dr. D.S. Beattie.

D. Determination and Characterization of Skeletal Muscle ALAS

The presence of the heme biosynthetic pathway in skeletal muscle was anticipated, since the synthesis of myoglobin the oxygen-binding hemoprotein has been demonstrated in this tissue (168). In this study, the existence of ALAS in skeletal muscle mitochondria was shown and optimal conditions for its assay determined by using the modified radiochemical assay. An investigation of the factors which regulate skeletal muscle ALAS such as fasting, diabetes, insulin and dexamethasone administration and porphyrinogenic drugs, indicates that the ALAS of skeletal muscle is controlled in a unique manner which differs from the control of ALAS in other mammalian organs.

The effect of diabetes on skeletal muscle heme biosynthesis was particularly interested. Since it has been reported in Dr. D.S. Beattie's laboratory (169) that the rate of skeletal muscle mitochondria protein synthesis is decreased 50-60% in experimental diabetic rats. Also phenotypic changes observed in diabetic muscle mitochondria included a 36% decrease in the content of cytochromes $\underline{aa}_3$ and a 27% decrease in cytochrome $\underline{b}$, both established as mitochondrial translated products in lower eukaryotes (169).

Those studies have prompted us to study heme biosynthesis in skeletal muscle of diabetics. The knowledge of the regula-

tion of heme biosynthesis in skeletal muscle would seem to be essential to an understanding of the control of mitochondrial cytochrome levels and ultimately energy utilization in skeletal muscle.

1. Results

a. Determination of Optimal Conditions to Assay Skeletal Muscle ALAS

Our initial studies involved determining the optimal substance concentrations to assay ALAS in skeletal muscle mitochondria. As seen in Figure 17, maximum activity of the enzyme was obtained at a glycine concentration of 30 mM, a value somewhat lower than that reported as optimal for ALAS determination in liver homogenates (137,133), adrenal mitochondria (37), heart mitochondria (40) or testicular homogenates (42), but similar to the optimal concentration reported for brain homogenates (138). The succinate concentration needed for maximal activity was 0.5 mM, a value identical to that reported for adrenal (37) or testicular (42) homogenates but lower than that for brain (138) or heart (40) homogenates (Figure 17).

No requirement for exogenous PLP was evident suggesting that sufficient endogenous PLp is present in skeletal muscle mitochondria (Table 15). It is interesting to note that previous studies of ALAS in adrenal (37) or brain (138) have

Figure 17. Effects of Substrate Concentration on Skeletal Muscle Mitochondrial ALAS Activity

Except as noted in the figure, the assay system contained (final concentrations): 75 mM Hepes, pH 7.4, 10 mM EDTA (disodium salt), 0.1 mM PLP, 0.5 mM succinate containing 2 μCi of [^{14}C]succinate, 75 mM glycine and 2 mg of skeletal muscle mitochondrial protein in a final volume of 1.0 ml. The reaction was carried out at 37°C for 30 minutes and terminated with 3 ml of 10% SDS. The product [^{14}C]ALA was determined by the single column procedure as described under Materials and Methods.

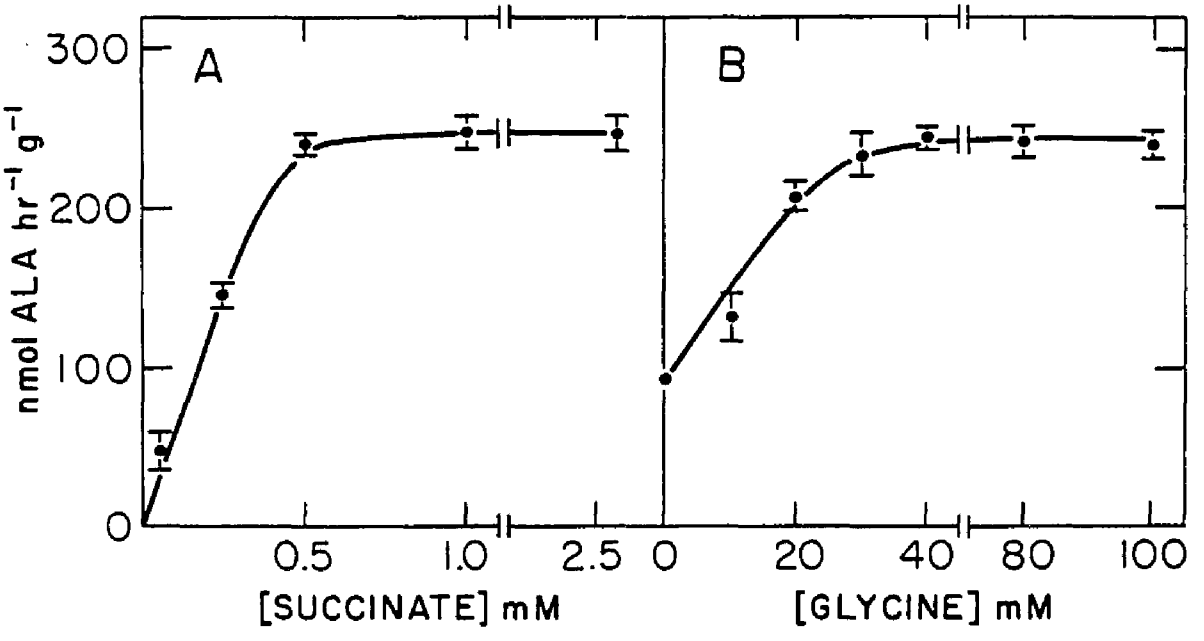

300
200
100
0
nmol ALA hr⁻¹ g⁻¹
A
B
0.5
1.0
2.5
0
20
40
80
100
[SUCCINATE] mM
[GLYCINE] mM

Table 15. The Effect of Cofactors on Skeletal Muscle ALAS Activity

Experimental Conditions	ALAS Activity nmol ALA formed/h/g protein
Complete System	234 ± 34
minus EDTA	264 ± 17
minus PLP	255 ± 40
plus Succinyl CoA generating system	198 ± 16

The complete system for assay of ALAS was as described in the legends to Figure 1 with 0.5 mM succinate and 75 mM glycine. The succinyl CoA generating system contained 1 mM GTP, 0.1 mM coenzyme A, 20 mM $MgCl_2$ and 0.05 units of succinyl CoA synthetase.

indicated a need for exogenous PLP in the assay. Addition of low concentrations of EDTA had no effect on skeletal muscle ALAS activity, although 15 mM EDTA resulted in a slight decrease in the rate of ALAS. EDTA is routinely added to assay mixtures for the liver enzyme as it prevents the conversion of ALA to porphobilinogen by inhibiting ALA dehydratase (170). In this way, the production of ALA is reduced. In extrahepatic tissues, however, EDTA appears to have little effect on ALAS activity (138,42). Including a succinyl CoA generating system consisting of coenzyme A, GTP, $MgCl_2$ and succinyl CoA synthetase decreased that rate of ALA formation in skeletal muscle mitochondria (Table 15). Since the inner mitochondrial membrane is impermeable to succinyl CoA synthetase and succinyl CoA, the presence of this enzyme in the incubation medium may result in a lowered availability of radioactive succinate to the intramitochondrial succinic thiokinase. Hence, a lowered amount of succinyl CoA would be available for ALAS.

The time course of ALA formation in skeletal muscle mitochondria was linear for 45 minutes at 37°C (Figure 18). The reaction was also linear with mitochondrial protein concentration up to 4 mg per ml (Figure 19).

The rate of ALA production by skeletal muscle mitochondria under optimal conditions was compared when the product [^{14}C] ALA was eluted by either the single column procedure or after conversion to the pyrrole and separation on the second

Figure 18. Time Course of ALA Formation in Skeletal Muscle Mitochondria

The rate of [^{14}C]ALA production was measured by either the single column procedure (●——●) or after conversion to the pyrrole and separation on a second column (○——○) as described in the legend to Figure 1.

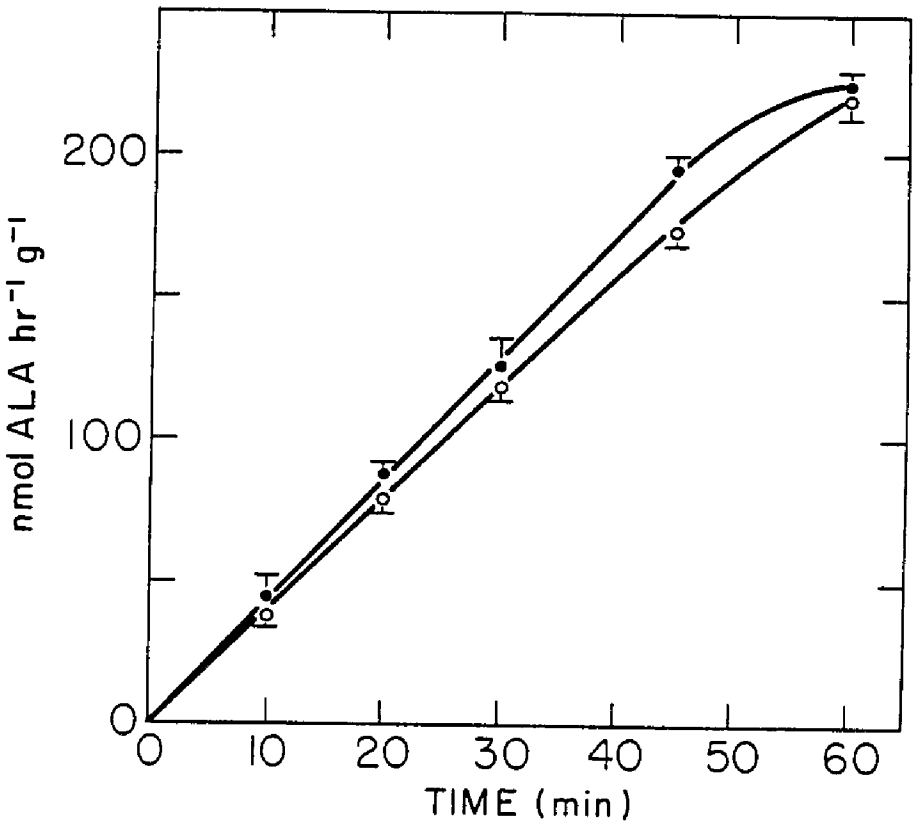

200
100
0
nmol ALA hr⁻¹ g⁻¹
0
10
20
30
40
50
60
TIME (min)

Figure 19. Effect of Protein Concentration on ALAS Activity in Skeletal Muscle Mitochondria

ALAS activity was assayed as described in the legend to Figure 1.

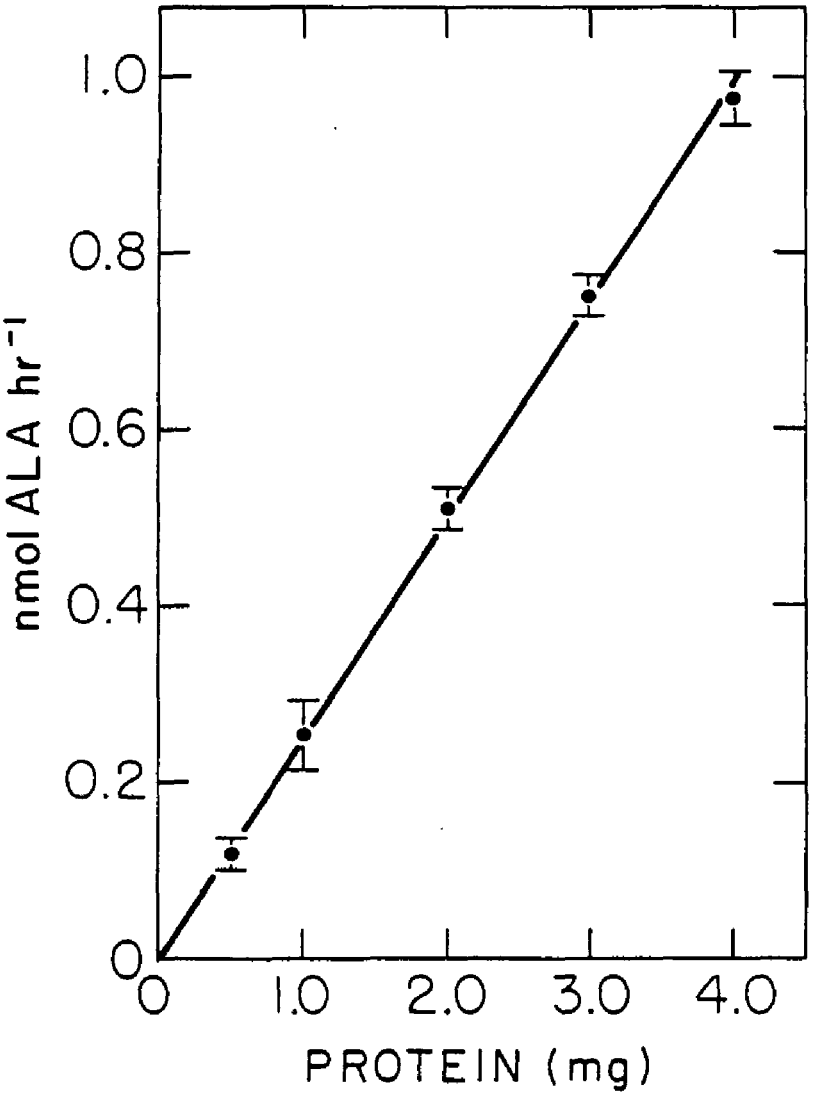

1.0
0.8
0.6
0.4
0.2
0
nmol ALA hr⁻¹
0
1.0
2.0
3.0
4.0
PROTEIN (mg)

Table 16. Comparison of ALA Production by Different Assays

ALA Determination	ALA Activity nmol ALA formed /g protein 30 min	60 min
Radiochemical Assay		
Single Column	138 ± 6	225 ± 13
Double Column	121 ± 15	219 ± 9
Colorimetric Assay	-	203

For the radiochemical assay, 2 mg of mitochondrial protein was added to the assay mixture described in the legend in Table 1 and incubated for 30 and 60 minutes. The product [^{14}C]ALA was isolated directly or after conversion to the pyrrole as described under Materials and Methods.

For the colorimetric assay, 7 mg of mitochondrial protein was incubated in the same assay mixture containing 10 mM succinate for 1 hour. The reaction was stopped with trichloracetic acid. The ALA-pyrrole was formed and reacted with Ehrlich's reagent as described under Materials and Methods.

column, as described in Materials and Methods. The results obtained by both procedures are identical and compare favorably to those obtained with the colorimetric assay (Table 16) (43). As discussed previously (43), the colorimetric assay does not have the required sensitivity for accurate determination of ALAS in tissues with low activity of the enzyme.

b. Effects of Porphyrinogenic Drugs on ALAS of Skeletal Muscle and Liver

AIA and DDC are potent inducers of hepatic ALAS resulting in an experimental porphyria (171,46,149,172); however, administration of these chemicals to rats had no effect on skeletal muscle ALAS while increasing the activity of hepatic ALAS more than 10-fold (Table 17). Previously, it was reported that the ALAS activity of rat adrenal (37), heart (40), brain (138) or testes (42) also was not increased by AIA or DDC.

c. Effect of Fasting and Streptozotocin-Induced Diabetes on ALAS of Skeletal Muscle

The activity of mammalian ALAS in different organs has been shown to be highly responsive to both the nutritional and hormonal status of the animal. Similarly, the activity of skeletal muscle ALAS decreased during a 48 hour fast reaching a value only 50% of the feed controls (Table 18). Administration of dexamethasone to the fasting animals did

Table 17. Effects of Porphyrinogenic Drugs on Skeletal Muscle and Liver ALAS Activity

Tissue	Treatment	ALAS Activity nmol ALA formed /h/g protein
Skeletal Muscle Mitochondria	Control	208 ± 9
	AIA	241 ± 17
	DDC	200 ± 15
Liver Mitochondria	Control	850
	AIA	7,200
	DDC	5,600

All animals were fasted 24 hours prior to sacrifice. Test substances were prepared and administered as described under Materials and Methods. ALAS was assayed as described in Table 1 with 2 mg of mitochondrial protein.

Table 18. Effects of Fasting on Skeletal Muscle ALAS Activity

Treatment	ALAS Activity nmol ALA formed /h/g protein	p Values With:	
		Fed	Fasted 48 hrs.
Fed (4)	223 ± 6.8	1.0	-
Fasted 48 hours (7)	99.9 ± 5.8	0.0091*	1.0
Fed + Dexamethasone (5)	208 ± 7.1	0.797	-
Fasted (48 hours) + Dexamethasone (7)	129 ± 7.3	0.0063*	0.443

* Significant at the 99% confidence level.

Fed animals were permitted food ad libitum prior to sacrifice. All animals were given water. Dexamethasone was administered as described under Materials and Methods. ALAS was assayed as described in Table 1. Each value is the mean plus or minus the standard error of the mean. The numbers in parenthesis indicate the groups of animals used. Each group contained muscles pooled from 2-3 different animals on a given day. The p values were calculated by Student's t test.

not reverse the effects on skeletal muscle ALAS activity as was reported for the enzyme in either heart (40) or adrenal (37). In addition, administration of dexamethasone to control fed animals had no effect on the activity of skeletal muscle ALAS (Table 18).

The effects of diabetes on skeletal muscle ALAS was investigated in animals which had received streptozotocin five days prior to sacrifice, at which time blood glucose levels were >400 mg per dl. As seen in Table 19, the activity of skeletal muscle ALAS was decreased 55% in the diabetic animals, although the activity of hepatic ALAS was unaffected by the experimental diabetes. Administration of insulin to the diabetic animals resulted in a lowering of blood glucose levels to 70-150 mg per dl and an increase in ALAS activity. Interestingly, ALAS activity of insulin-treated diabetic animals was significantly higher than that of the diabetics but remained significantly lower than that of the control animals. Insulin administration to control animals also lowered blood glucose levels to below 100 mg per dl and resulted in a significant 21% decrease in ALAS activity. The activity of ALAS observed in both groups of insulin-treated rats (controls and diabetics) did not differ significantly (Table 19). By contrast, the effectivity of hepatic ALAS was not changed by either diabetes or insulin administration.

Table 19. Effect of Diabetes on Skeletal Muscle and Liver ALAS

Tissue	Treatment	ALAS Activity nmole ALA /h/g prot.	p Values with Control	p Values with Control & Insulin	p Values with Diabetes
Skeletal Muscle Mitochondria	Control (14)	244 ± 7.0	1.0	-	-
	Control & Insulin (10)	193 ± 8.2	0.036*	1.0	-
	Diabetic (12)	107 ± 5.8	0.009**	-	1.0
	Diabetic & Insulin (15)	175 ± 8.4	0.003**	0.43^{NS}	0.010*
Liver Mitochondria	Control (5)	1101 ± 18.2	1.0		
	Control & Insulin (4)	1163 ± 26.4	0.86^{NS}		
	Diabetic (4)	1156 ± 14.3	0.81^{NS}		

* Significant at the 95% confidence level

** Significant at the 99% confidence level

Diabetes was induced and insulin administered as described under Materials and Methods. The numbers in parenthesis indicate the groups of animals used. Each group contained muscles pooled from 2-3 different animals but was 1 liver from 1 animal. The p values were calculated by Student's t test.

2. Discussion

The presence of ALAS in isolated mitochondria of rat skeletal muscle mitochondria has been investigated. The activity of ALAS observed is similar to that previously reposrted for mitochondria from cardiac muscle (40). These results indicate that skeletal muscle has the ability to synthesize the heme necessary for formation of its own hemoproteins including both myoglobin and mitochondrial cytochromes. All mammalian tissues investigated to date appear to contain sufficient ALAS for heme biosynthesis, the activity correlating with the overall hemoprotein content of the tissue. For example, drug metabolizing tissues, such as liver or kidney which contain high levels of cytochrome P450 as well as steroidogenic tissues, such as the adrenal with several cytochrome P450's involved in hydroxylations, appear to contain a much higher activity of ALAS than other tissues examined.

The development of a sensitive method for ALAS determination and the definition of optimal conditions to assay ALAS activity permitted an investigation of the hormonal and nutritional factors which regulate the skeletal muscle enzyme. A need for insulin to maintain skeletal muscle ALAS is evidenced by the 50% decrease in activity observed in either diabetic or fasted animals. During a 48 hour fast, plasma insulin levels have been reported to decrease to 50%

of control levels (172). Many of the pathophysiological effects observed in skeletal muscles of diabetic animals are also seen after a fast of this duration. Although the underlying mechanism for the decrease in ALAS activity under these conditions has not been elucidated, the well-documented decrease in overall rates of skeletal muscle protein synthesis during starvation (173,174,175) and diabetes (135) may be responsible. The half-life of hepatic ALAS has been reported to be 60-70 minutes (137). Assuming that the skeletal muscle enzyme has a similar half-life as the hepatic enzyme, then a profound decrease in cellular protein synthesis would be reflected in a lowered amount and hence, in the activity of ALAS and other proteins with a rapid turnover. A similar decrease in ALAS activity during a 48 hour fast was previously reported for cardiac muscle, another tissue in which the overall rate of protein synthesis decreases during fasting or diabetes (135,173). To date, no report on the effect of diabetes on ALAS in cardiac muscle mitochondria has appeared.

Insulin administration partially reversed the decrease in ALAS activity observed in the diabetic animal. The inability of insulin administration to cause ALAS activity to reach control levels may reflect the effect of the profound hypoglycemia induced by insulin on ALAS. For example, insulin administration to control animals also resulted in a significant decrease in ALAS activity accompanied as well

by a lowering of the plasma glucose levels to less than 100 mg per dl. These high doses of insulin may inhibit the actual synthesis of ALAS in skeletal muscle or block translocation of newly-synthesized ALAS from its site of synthesis on cytosolic ribosomes to the mitochondria (175,176,177). Alternatively, the increased rate of uptake of glucose into skeletal muscle in the presence of high plasma levels of insulin may cause a decrease in ALAS activity. Previous studies had indicated that the activity of hepatic ALAS was decreased in animals given large amounts of glucose (44), the so-called glucose effect. Despite much speculation, no satisfactory explanation for the glucose repression of mammalian enzymes has been advanced.

The possibility was considered that during diabetes or starvation the amounts of substrates or cofactors necessary for optimum ALAS activity might become limiting so that the maximum activity of the enzyme could not be determined. Increasing the concentrations of succinate, glycine or PLP present in the incubation medium did not increase the activity of ALAS in mitochondria from fasted or diabetic rats. Likewise, addition of an exogenous succinyl CoA generating system to either intact or cholate-solubilized mitochondria did not increase ALAS activity to that of controls. This suggests that under our assay conditions some change in ALAS itself is responsible for thelowered rate of ALA production

in these animals. Either a change in the amount of the enzyme or a change in its catalytic efficiency would explain these observed decreases.

The results of the present study also extend the concept that ALAS activity is regulated in a tissue-specific manner. Differences reported here in the response of the skeletal muscle and liver enzyme to assay conditions, hormonal changes, fasting or inducers are complemented by the findings of altered regulation of the heart (40), adrenal (37) and testicular (42) enzyme by starvation and hormonal effects. Viewed as a whole, the studies demonstrate that the regulation of ALAS differs in each tissue studied. The biochemical basis for the observed differences in regulation of the various ALAS activities may reflect differences in the physiology of each tissue. One pertinent example of this tissue specificity is the lack of response of ALAS in skeletal muscle in administration of the porphyrinogenic drugs, AIA and DDC, which increase the activity of hepatic ALAS several-fold (25,46,149,172). Both inducers apparently act by binding to the cytochrome P450 of the drug detoxifying system of the endoplasmic reticulum, causing the breakdown of the heme (178,179). Thus, only the ALAS present in tissues with an active drug detoxification system, such as liver or kidney, is induced by porphyrinogenic drugs. The activity of ALAS in tissues, such as adrenal, brain, heart,

testis or skeletal muscle, which lack the drug detoxification system, is not affected by the porphyrinogenic drugs.

Similarly, the response of ALAS to glucocorticoids differs in a tissue-specific manner. For example, administration of dexamethasone to fasted animals restored the rate of ALAS in cardiac mitochondria to control levels but was without effect in reversing the decreased ALAS activity of skeletal muscle during fasting. In non-muscle tissue, glucocorticoids, such as hydrocortisone, have been shown to potentiate the induction of hepatic ALAS by porphyrinogenic drugs (26,27). Other hormones reported to regulate ALAS are human chorionic gonadotrophin, which regulates the testicular enzyme (42) and ACTH, which is involved in maintaining the activity of the adrenal enzyme (40). In general, the tissue-specific regulation of mammalian ALAS by hormones attest to the importance of the organism controlling the overall rate of heme biosynthesis.

VI. CONCLUDING REMARKS

The ability to measure ALAS activity specifically and rapidly has been demonstrated. The characterization and regulation of this enzyme in both rat liver and rat skeletal muscle have been investigated. The effects of porphyrinogenic drugs and hormones on ALAS in both tissues have revealed the nature of the tissue-specific regulation of the overall rate of heme biosynthesis. Particularly, the studies of ALAS in normal animals and streptozotocin induced diabetic animals have indicated the molecular pathology of diabetes.

The procedures for ALAS determination would enable screening the enzymatic activity efficiently on each hybridoma cell line for monoclonal antibody development. The antibody will become a tool for the study of the molecular nature of glucose effect, hormonal effect and aging effect on ALAS. Thus, the metabolic abnormality occurring in aging, diabetes and porphyria would be elucidated elegantly.

The finding of an amidase which co-purified with ALAS has become very interesting and important. The unique submitochondrial localization of this amidase and its sensitivity toward o-phenanthroline has suggested that the amidase may be involved in the processing of mitochondrial proteins synthesized as larger precursors in the cytoplasm. However, there are still many questions remaining unanswered

such as: (1) Does this amidase have a peptidase nature and what is the native substrate in the mitochondria for this amidase? Interestingly enough, it was shown that ALAS is synthesized as a larger precursor on free polysomes, and yet, it has to be imported to its functional compartment, mitochondrial matrix, as well as to be converted to its mature size. Therefore, the development of ALAS monoclonal antibody and the rapid, specific assay method for ALAS would provide a good tool to study the possible role of the amidase on the processing of mitochondrial proteins. Another approach will be finding other appropriate substrate(s) for demonstrating its signal peptidase activity.

(2) If the amidase does participate in the import or processing of ALAS, then is the import or processing energy-dependent, and does it need ATP itself or a membrane potential? This question could be approached by using uncoupler and ionophore for dissipating membrane potential or by using atractyloside for inhibiting the ATP/ADP translocator.

(3) What is the major regulatory factor for the amidase? Does it respond to hormone, infection, aging or other physiological conditions?

(4) How do the mitochondria regulate the import process of ALAS? Does the heme interact with the precursor of ALAS and block the import of ALAS?

Those investigations would contribute enormously to the

knowledge of mitochondrial biogenesis. Several elaborate investigations of mitochondrial biogenesis have been done and are currently under way in the laboratory of Dr. D.S. Beattie. The research detailed in this dissertation hopes to provide a springboard for further studies. Hopefully, these ongoing investigations of this progress will be productive and enlightening.

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VITA

Li-Fen L. Chang, the daughter of Mr. and Mrs. Wan-Fa Lien, was born in Taiwan, R.O.C., on November 7, 1956. She received her Bachelor of Science degree in Chemistry from Chung Yuan University, Taiwan, in 1978. She has attended Mt. Saini School of Medicine for her Doctor of Philosophy degree in Biomedical Sciences since 1978.

Mrs. Chang is the author of papers which have appeared in ABB and Enzeme (monograph, ed. D. Bishop). She is currently a post Doctor fellow in the Biochemistry and Nutrition department of Virginia Polytechnic Institute and State University.

She is married to Tien-Chien Chang and has no children.