SIMPSON, DENYSE MARIE

STUDIES ON THE REGULATION OF 5-AMINOLEVULINIC ACID SYNTHASE FROM RAT LIVER MITOCHONDRIA: PURIFICATION OF A SPECIFIC ACTIVATOR AND IMMUNO-CHEMICAL QUANITITATION OF THE ENZYME DURING DRUG INDUCTION

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STUDIES ON THE REGULATION OF 5-AMINOLEVULINIC ACID SYNTHASE FROM RAT LIVER MITOCHONDRIA: PURIFICATION OF A SPECIFIC ACTIVATOR AND IMMUNOCHEMICAL QUANITITATION OF THE ENZYME DURING DRUG INDUCTION.

by

DENYSE M. SIMPSON

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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The City University of New York

ABSTRACT

A protein capable of activating 5-aminolevulinic acid synthetase (ALA-s) has been purified from rat liver mitochondria. By treatment of the mitochondrial inner membrane-matrix fraction with Lubrol a soluble and active preparation of ALA-s has been obtained. Ammonium sulfate fractionation yielded a 60-90% saturated fraction containing an activity capable of stimulating the catalytic ALA-s activity 3-fold. The fraction which activates ALA-s contains no catalytic ALA-s activity, is non-dialyzable, heat-stable and sensitive to pronase digestion. The activator activity has been purified 6,250 fold by means of Carboxymethyl sephadex chromatography, Sephacryl S-200 Gel Exclusion chromatography and Hydroxylapatite chromatography. The purified activator was judged to be homogenious by its migration as a single band on sodium dodecyl sulfate containing polyacrylamide gels, non-denaturing polyacrylamide gels and isoelectric focusing gels. The activator displayed a native molecular weight of 57,000 as determined by gel exclusion chromatography and a molecular weight of 54,000 as determined by sodium dodecyl sulfate gel electrophoresis. The isoelectric point was determined to be 7.5. The activator does not diminish or enhance the inhibition of hemin on catalytic ALA-s. A non-specific protein phosphatase from rabbit liver did not activate or inhibit ALA-s suggesting that the mechanism of the activator was not via a phosphatase or kinase reaction. Reconstitution of purified ALA-s and

purified activator resulted in the formation of a tetramer and a high molecular weight aggregate of ALA-s which exists as a monomer and dimer in its purified form. The tetramer and high molecular weight aggregate contained both ALA-s catalytic units and activator units. It is possible that the formation of high molecular weight aggregates of ALA-s catalytic monomers is the mode of action of the activator.

ALA-s was purified and antisera against it was obtained from goat. The anti-ALA-s antibodies were judged to be monospecific by diffusion analysis on an Ouchterlony plate. Precipitin lines between wells containing antigen and the antisera intersected without the formation of spurs. ALA-s was stimulated by treatment of rats with allylisopropylacetamide (AIA), 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) and ethanol. The drug treatment stimulated ALA-s activity by 11.6, 11 and 3 fold respectively. The relative quantities of mitochondrial ALA-s in drug treated and control rats was compared by single electroimmunophoresis. The results indicated that AIA increased the quantity of ALA-s protein 2-fold and that this increase is blocked by cycloheximide. DDC and ethanol do not increase the quantity of ALA-s enzymes.

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DEDICATION

This Thesis is Dedicated to my Family for their Love and Support

Donald B.

Donna Mae

Dawn Ellen

Donetta Lynn

Daria Ann

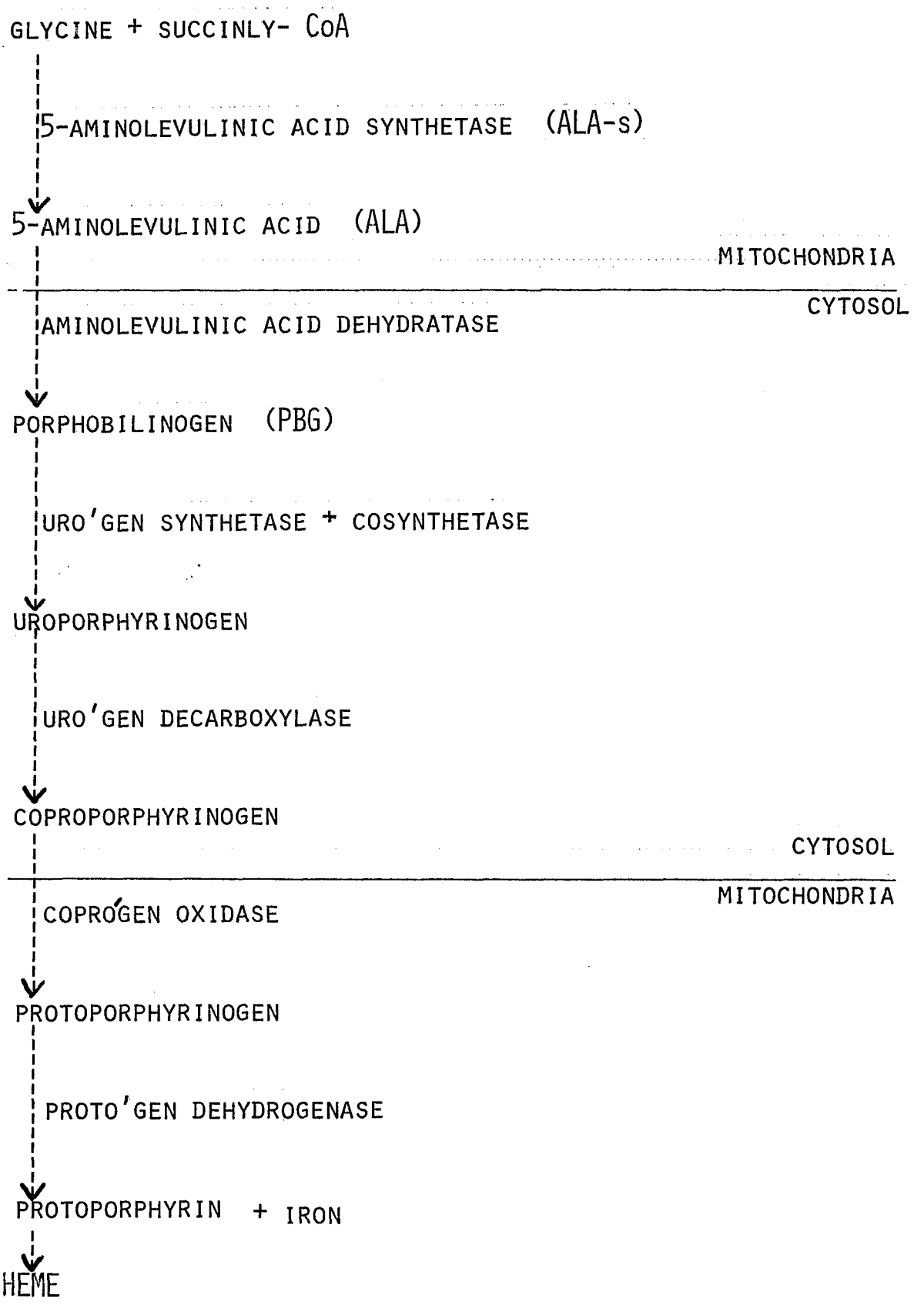
Darla Jean

SIMPSON

and also

Sebastian
and
Lancelot

FIGURE A


GLYCINE + SUCCINLY- CoA
5-AMINOLEVULINIC ACID SYNTHETASE (ALA-S)
5-AMINOLEVULINIC ACID (ALA)
MITOCHONDRIA
CYTOSOL
AMINOLEVULINIC ACID DEHYDRATASE
PORPHOBILINOGEN (PBG)
URO'GEN SYNTHETASE + COSYNTHETASE
UROPORPHYRINOGEN
URO'GEN DECARBOXYLASE
COPROPORPHYRINOGEN
CYTOSOL
MITOCHONDRIA
COPROGEN OXIDASE
PROTOPORPHYRINOGEN
PROTO'GEN DEHYDROGENASE
PROTOPORPHYRIN + IRON
HEME

INTRODUCTION

A. Heme Biosynthesis : The Control

The sequence of reactions by which heme is synthesized was originally elucidated by Shemin and his coworkers in a series of elegant experiments in which isotopically labelled protoporphyrin IX of hemoglobin was achieved by the administration of labelled glycine to animals (1-6).The series of enzymatic steps from the condensation of glycine and succinyl-CoA to form 5-aminolevulinic acid to the insertion of iron into protoporphyrin IX to yield heme was determined by Sano and Granick and is shown in Figure A (7). Unequivocal evidence is available for all steps although details of some of these reactions remain uncertain; namely, the precise manner of oxidative decarboxylation of coproporphyrinogen III to protoporpyrinogen IX, the mechanism of dehydrogenation of protoporphyrinogen IX to protoporphyrin IX and themanner of insertion of iron into the porphyrin nucleus to form heme.

The heme biosynthetic pathway is compartmentalized, with enzymes distributed between the mitochondria and the cytosol (7). ALA-s is found loosely associated with the inner mitochondrial membrane(8) and also in the matrix(9,10). ALA-dehydratase, Uro'gen-synthetase, Uro'gen-cosynthetase and Uro'gen decarboxylase are soluble enzymes located exclusively in the cytosol. The precursor copro'gen III enters the mitochondrion where the last three enzymes of the pathway,

Copro'gen oxidase, Proto'gen dehydrogenase and Ferrochelatase are located. Proto'gen dehydrogenase and Ferrochelatase are known to be constituents of the inner membrane(10,11,12). It is unknown whether or not the transport of heme precursors across the mitochondrial membrane is under regulatory control.

In mammals, the principal site of heme biosynthesis is the liver,where 70% of the heme formed serves as the prosthetic group for mitochondrial and microsomal cytochromes, and the erythropoietic tissues. These tissues are involved in either drug detoxification via the microsomal oxygenase system which requires heme for cytochromes P-450 and b_5 or in hemoglobin synthesis. Other than the liver, ALA-s has been detected and studied in reticulocytes(13), red blood cells(14), bone marrow(15,16), spleen (17), heart and adrenal(18), kidney (12) and recently in the brain(19). This suggests that all tissues are capable of carrying out heme biosynthesis to meet their needs.

The rate of formation of heme in both procaryotes and eucaryotes is considered to be controlled by the first enzyme of the pathway, 5-aminolevulinic acid synthetase (ALA-s)(20-23). ALA-s is present in the liver mitochondria under normal conditions in very low levels, apparently just sufficient to meet the heme requirements of the cell. A wide range of chemicals and steroids can upset this delicate balance leading to increases in the activity of the enzyme and to pophyrin and porphyrin precursor accumulation and excretion (20,21,24-27). This condition, the loss of regulation of ALA-s and heme

biosynthesis has been entitled chemically induced porphyria and has attracted much attention because of the biochemical similarities to the genetically determined porphyric diseases in man.

B. PORPHYRIA : THE LOSS OF CONTROL

The porphyrias are a group of diseases in which heme metabolism is disturbed. They are either hereditary or aquired in origin. Enzymic lesions with subsequent loss of regulation of heme biosynthesis have been postulated as possible causes for some types of porphyria. The role of ALA-s in certain porphyrias has sparked interest in this enzyme and its regulation.

The porphyrias are distinguished biochemically based on the pattern of heme precursors which are accumulated and excreted. The clinical descriptions of hepatic porphyrias are as follows (28,29): Acute Intermitant Porphyria, characterized by ALA and PBG in the urine, an increase in the activity of ALA-s is observed; Hereditary Coproporphyria, characterized by ALA, PBG, uro'gen and coproporphyrin in the feces. There are also erythropoietic porphyrias such as Protoporphyria which is characterized by accumulation of protoporphyrin in the bone marrow, skin and teeth leading to acute photo sensitivity and increased levels of protoporphyrin in the blood. The activity of ferrochelatase is markedly decreased in all tissues studied.

All inherited hepatic porphyrias are characterized by increased levels of ALA-s (29). There are two reasons why ALA-s is considered to regulate heme biosynthesis. The first reason is that increasing the supply of ALA <u>in vivo</u> and <u>in vitro</u> leads to increased production of liver heme but an increase in the precursors of ALA does not (22, 30-33). So the rate of

heme synthesis is determined by the supply of ALA. Also, a comparison of the in vitro activities of the enzymes of the pathway shows ALA-s to be rate limiting, Figure B. Secondly, ALA-s is the site where heme, the end product, exerts its feedback control over its own synthesis. It is not clear whether heme exersizes end-product repression, affecting the quantity of ALA-s, but it is known for certain that heme inhibits the activity of the enzyme in purified preparations (34-36). Possibly both mechanisms are in effect. It has been suggested that heme controls the migration within the cytosol, the assembly and uptake into the mitochondria of the ALA-s complex (37).

The activity of Uroporphyrinogen synthetase is also very low and could become rate limiting under conditions where ALA-s activity is increased. Indeed, PBG is accumulated in various porphyrias. Strand et al (38) discovered a decreased level of hepatic Uro'gen I synthetase in patients with Acute Intermitant Porphyria and postulated that this was the primary defect and that ALA-s was stimulated due to the decreased total heme output. Though ALA-s is increased in porphyrias the pattern of heme precursors which accumulate suggests that other enzymic defects may be the primary cause of the various porphyrias.

Experimentally induced hepatic porphyrias have been in use since the early sixties when Granick and Urata described a number of drugs which cause the excretion of heme precursors and also increase the activity of hepatic ALA-s. Two drugs

Which induce porphyria in animals are 2-allyl-2-isopropyl-acetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Studies indicate that the liver is the only site of precursor accumulation (39). The total amount of precursors excreted in 24 h is 40-fold in excess of the heme required for liver hemoproteins, indicating that the rate of production has been increased. These two drugs induce different types of experimental porphyria as witnessed by the difference in intemediates which are excreted. DDC treatment leads to the accumulation and excretion of protoporphyrin whereas AIA results in PBG and ALA accumulation. The reason for the difference can be explained by the mode of action of the drug.

Granick (22) suggested that these drugs increased the activity of ALA-s by interfering with the feedback control of heme. In the case of DDC a rapid inhibition of ferrochelatase activity is apparent well before the increase in ALA-s activity. This inhibition of heme synthesis could be responsible for the stimulation of ALA-s (32). In the case of AIA the loss of liver heme is due to increased destruction and conversion to a green pigment which has been identified as an adduct formed by the covalent association of AIA and the heme moiety of cytochrome P-450 (40-42). Thus, the loss of liver heme and probable loss of feedback inhibition are responsible for AIA induced porphyria as well.

Chemically induced porphyria can be caused by the same drugs which precipitate acute attacks in patients with porphyria (22). A high carbohydrate diet alleviates the symptoms of both experimental and hereditary porphyria and is

known as the "glucose effect" (43). Porphyria is a post puberty disease suggesting the participation of steroids. Indeed, glucocorticoid hormones are essential for the induction of ALA-s by AIA both in whole animals and in isolated perfused liver and glucose in large doses inhibits the induction of the enzyme (21, 44-45). Patients with AIP have high blood levels of the same steroids which induce experimental porphyria in cultured chick embryo liver cells (22, 46).

None of the steroid hormones secreted by the gonads had a significant inducing ability. Metabolites of testosterone and progesterone have the inducing ability and are generally considered physiologically inert by-products which result from metabolism of steroid hormones in the liver (47-48). They are normally excreted as the glucuronides. Glucuronidation of most potent inducers abolishes all inducing activity. The induction with suboptimal levels of steroid is additive with suboptimal dosed of AIA or DDC. Kappas and Granick (49) also found that UDP-glucuronic acid was able to prevent porphyria induction. This suggests that the steroid inducer is removed by glucuronidation via UDP-glucuronyl transferase. Moore et al (50) found that dihydroepiandrosterone, which is excreted as the glucuronide, and metabolized via P-450, induces porphyria in rats. The found that patients with hereditary porphyria contained elevated blood levels of this steroid. Glucocorticoid hormones are known to induce certain liver enzymes and to stimulate the liver formation of RNA andproteins. Protein synthesis has been found to play a key role in ALA-s stimulation during experimental porphyria.

Marver et al (43) observed that in porphyric rats there was a 65% increase in liver weight and liver proein, distributed equally in all cellular fractions except the mitochondria, which showed a 15% increase. ALA-s activity also increased. Glucose prevents the increase in liver weight and protein content as well as the increase in ALA-s activity. Treatment of rats with puromycin, an inhibitor of protein synthesis, after induction with AIA caused an immediate drop in ALA-s activity. Treatmemt with actinomycin D instead of puromycin resulted in a lag period of 2-3 h before the decline of ALA-s activity, suggesting a transcriptional mechanism of control. Glucose mimics the effect of Antimycin D.

A number of carbo hydrate metabolites were tested by Bonkowsky et al (51) and only glucose, fructose and glycerol had significant inhibitory effects.

The stimulation of ALA-s by either AIA or DDC can be prevented by the prior administration of cycloheximide. The same effect is seen with SKF-525-A, an inhibitor of drug metabolizing enzymes. It also prevents the destruction of liver heme and the inhibition of ferrochelatase. These results suggest a two stage mechanism in the induction process. The first stage, inhibited by cycloheximide, leads to increased activity of ALA-s and porphyria, and involves protein synthesis (52). The dependance of ALA-s induction on protein synthesis has been known for some time. Granick (22) proposed that the drugs induce de novo synthesis of ALA-s. However, protein synthesis could be related to the activity of the enzyme in some other way. If ALA-s is subject to regulation

by free cellular heme, cycloheximide, by inhibiting the synthesis of apoproteins of the cytochromes, might increase the cellular concentration of heme and lead to the inhibition of ALA-s.

FIGURE B.

ACTIVITIES OF THE HEME BIOSYNTHETIC ENZYMES

Comparison of the In Vitro activites of the enzymes of the heme biosynthetic pathway

ENZYME	SPECIFIC ACTIVITY
5-ALA SYNTHETASE	0.70
5-ALA DEHYDRATASE	60.0
URO'GEN SYNTHETASE	0.7
URO'GEN DECARBOXYLASE	0.8
COPRO'GEN OXIDASE	11.5
FERROCHELATASE	67

Specific activities are expressed as nmol of ALA equivalents produced or utilized per min by 1 g wet liver.
Values are from fasted animals.

C. CYTOCHROME P-450 and the FREE HEME POOL : CLIENTS OF ALA-SYNTHETASE

According to Marver and Schmid (53), who measured the turnover rate of various hemoproteins and their concentrations, the amount of heme needed to be synthesized to keep a steady state of hemoproteins is of the same order as that expected from the activity of ALA-s. They also calculated that cytochrome P-450 accounts for more than half of the total heme requirement of the liver cell. This becomes significant when considering drugs which result in a large and rapid change in the concentration of cytochrome P-450.

The formation of apoproteins and the synthesis of heme must be coordinated in some way. In the case of hemoglobin, heme is necessary for the synthesis of globin chains at the ribosomal site (54-55). Marver (56) and Baron and Tephly (57) have reported that the activity of ALA-s and the rate of liver heme synthesis precedes the increase in cytochrome P-450 after phenobarbitone treatment. Baron and Tephly (58) have also reported that the accumulation of P-450 caused by phenobarbitone can be prevented by the administration of 3-amino-1,2,4-triazole, an inhibitor of hepatic heme synthesis. They interpreted their findings to mean that an increased supply of heme leads to the accumulation of P-450. This conclusion should be viewed with caution. The results may simply indicate that heme, as a necessary constituent of P-450, is essential for its formation and under the circumstances where heme synthesis is inhibited, the heme supply may become rate limiting.

Bock et al (45) and DE Matteis (59) have shown that after repeated doses of drugs, P-450 accumulated with no observable change in ALA-s or the rate of heme synthesis. Also, Song et al (60) and Druyan and Kelly (61) have shown that the administration of exogenous ALA to rats does not result in the accumulation of P-450 even though it causes increased formation of heme and bile pigments. This shows that the an increased supply of ALA and heme is not sufficient to stimulate the formation of P-450. It is unlikely that heme is the main controlling factor in stimulating the synthesis of liver hemoproteins since this would not account for the selectivity of the response. Drug administration can cause accumulation of only one or of a few liver hemoproteins. More likely, the controlling factor is the synthesis of the specific apoproteins, which utilize heme and increase heme synthesis. This is consistent with the findings of free apoprotein pools which have been reported for cytochrome c by Kadenbach (62), for cytochrome b_5 by Bock and Siekevitz (63) and for catalase by Lazarow and De Duve (64).

The interesting question exists as to whether heme is utilized irreversibly in hemoprotein formation or to what extent an exchange between free heme and hemoprotein-bound heme occurs. Druyan and Kelly (61) have obtained evidence that an exchange may occur in vivo. Radiolabbled heme from radioactive ALA was able to exchange with pre-existing hemoproteins even whenprotein synthesis was completely inhibited by cycloheximide. This indicates a rapid exchange between

newly synthesized free heme and the heme of pre-existing hemoproteins.

Heme is degraded by microsomal heme oxygenase to bile pigments and other degradation products which are eliminated from the liver. Excessive amounts of heme, due to the inhibition of protein synthesis by cycloheximide or the administration of large amounts of ALA, is degraded to bile pigments (60). Thus an overflow pathway exists which ensures that the hepatic concentration of heme will not become too large. One can visualize a regulatory heme pool of relatively small size and rapid turnover into which newly synthesized heme is fed and out of which heme is drawn for either the synthesis of hemoproteins or for degradation (Figure C). A decrease in the concentration of regulatory heme and a consequent stimulation of ALA-s can be brought about by any of the following mechanisms: 1. inhibition of synthesis of heme, 2. an increase in the utilization of heme or 3. an increase in the rate of degradation of heme.

This scheme explains the observation that the activity of ALA-s is increased after treatment with drugs which stimulate the formation of P-450. These drugs may act by increasing the amount of apoprotein of P-450 and thereby stimulating the rate of heme utilization (32). In labelling experiments with radioactive glycine and with radioactive ALA, an increase in labelled heme was found in P-450 after treatment with phenobarbitone (60). Since ALA by-passes the rate-limiting step of the pathway and since the heme of cytochrome b_5 was not

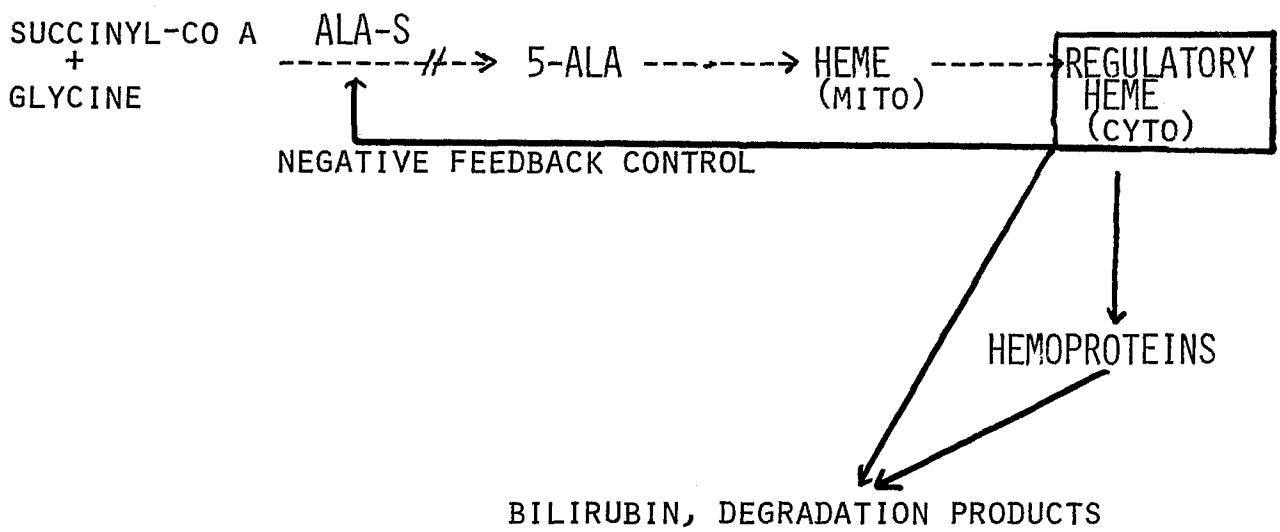
labelled, the inference was that phenobarbitone specifically stimulates the utilization of heme for cytochrome P-450. An increased utilization of heme will result in lowering of the concentration of the regulatory heme pool and therefore in the stimulation of ALA-s activity. The extent of the stimulation of ALA-s depends on the balance between utilization of heme and the other two pathways of heme metabolism which control the level of the heme pool, namely, heme synthesis and heme degradation.

Under normal conditions phenobarbitone does not result in an apprecialble elevation in the activity of ALA-s(59). This suggests that hepatic levels of ALA and heme are sufficient to meet the increased demand without upsetting the level of regulatory heme. If heme synthesis is inhibited by DDC, phenobarbitone treatment results in a marked stimulation of ALA-s activity (32), greater than seen with DDC alone. Under these conditions the two subsequent enzymes in the pathway, ALA-Dehydratase and Uro'gen Synthetase become rate limiting and ALA and PBG accumulate in excess. IF liver heme distruction is enhanced by treatment with AIA, phenobarbitone will markedly stimulate ALA-s activity (65). As descussed above, the interpretation of the findings is that drugs lead to a decrease in regulatory heme and to a stimulation of ALA-s presumably by lifting the feedback inhibition mechanism.

FIGURE C.

EQUILIBRIUM OF THE CELLULAR HEME POOL

POSTULATED MECHANISM FROM DE MATTEIS, 1973



D. ALA-SYNTHETASE : THE SIZE AND SHAPE OF THE ENZYME

Earlier attempts to purify ALA-s from bacterial, avian and mammaliam sources were unsuccessful due to the formation of large aggregates by the enzyme and the ambiguity surrounding the molecular size. Whiting and Elliot (66), the first to report a pure enzyme, made this statement concerning ALA-s,

> "ALA-s, as extracted from mitochondria by conventional methods, exists in an aggregate form, and treatments with salt, detergents, lipolytic enzymes and sonication, while possibly reducing the particle size of the aggregate, do not achieve total release of the enzyme molecule from other components."

Molecular weights found for mammalian ALA-s, from partially purified preparations, vary over a wide range. This could be an outcome of the ease with which the enzyme forms aggregates with other proteins (66, 67). Whiting and Elliot (66) obtained molecular weight values of 77,000 for the mitochondrial enzyme and 178,000 for the cytoplasmic enzyme from rat liver. Scholnick et al (68), who were unable to disassociate the aggregate, gave a molecular weight value of 300,000 from rat liver cytosol. More recently, Ohashi and Kikuchi (69) have defined the enzyme in greater detail. They report a cytosolic enzyme of 650,000-700,000 which is a dimer of two 320,000 complexes. Each of the complexes contains the ALA-s catalytic enzyme and two non-identical binding proteins. The catalytically active portion of the complex has a molecular weight of 110,000.

Whiting and Granick (70) purified the enzyme from chick embryo liver mitochondria and reported a molecular weight of

87,000, composed of two polypeptide chains of 49,000 each. Paterniti and Beattie (71) recently purified the rat liver enzyme to homogeneity. It has a dimer molecular weight of 118,000 composed of two monomers of 58,000 each. All of the avian and mammalian ALA-s enzymes from adult animals reported thus far are dimers of two identical monomers. Woods and Murphy(72), working with fetal rat liver mitochondria observe a monomer enzyme of 47,000 which is not inhibited by hemin.

The ALA-s from R. spheroides (73) and M. denitrificans (74) were both reported to be monomers. Their molecular weights are 57, and 68,000 respectively. Nandi and Shemin (75) disputed that information and described a dimer enzyme of 80,000 composed of two non-identical monomers of 45,000 and 41,000 from R. spheroides. A high activity form of ALA-s from that microorganism has been observed by Davies and Neuberger (76). The enzyme can be converted to the highly active form by air or cysteine trisulfide. They report the enzyme as being mainly monomeric. The high activity form and three other active isozymes in the mixture do not differ by molecular weight but by isoelectric point. The most basic enzyme, pI 5.5, as compared to pI 5.45, 5.35 and 5.2, is the supposed active configuration. They suggest an interchange of disulfide bonds as being responsible for the conversion.

Several reasons have been proposed for the association of ALA-s with other proteins. Ohashi and Kikuchi (69) consider the proteins associated with ALA-s in the cytosol to be carriers, necessary for assembly of ALA-s in the cytosol and transport to and into the mitochondrion. They believe hemin

interferes with the uptake of the cytosolic enzyme into the mitochondria by causing the condensation of two 300,000 d molecular weight complexes to a 650 ,000 aggregate which can not be converted to the mitochondrial enzyme. Ohashi and Sinohara (77) prepared antibodies against the cytosolic enzyme and observed its uptake into mitochondria and mitoplasts. If the enzyme was stripped of the non-catalytic binding proteins it could not be taken up by the mitochondria.

It has been suggested (66) that solubilization by detergents and sonication release ALA-s from the mitochondrial inner membrane along with other proteins that bind it to the membrane or regulate its activity in some way. Thus the activity of the enzyme is dependant on the solubilization technique employed.

Elucidation of the native structure of ALA-s awaits an understanding of the proteins associated with it and the function and organization of the ALA-s complex.

METHODS

PREPARATION OF ALA-SYNTHETASE-

Mitochondria were prepared from the livers of male Sprague-Dawley rats according to the method of Bustamante et al (78). ALA-s was purified according to Paterniti and Beattie (79), which utilized Diethylaminoethyl cellulose column chromatography, Sephacryl S-200 chromatography and preparative gel electrophoresis.

ASSAY OF ALA-SYNTHETASE-

Enzyme preparations were assayed by a modication of the method of Ebert et al (80). One tenth of a ml of enzyme preparation (20 ug of protein) was added to an incubation mixture which contained 50 mM Tris- HCL pH 7.5, 100 mM glycine, 10 mM EDTA, 0.1 mM pyridoxal 5'-phosphate, 20 mM magnesium chloride, 0.1 mM coenzyme A, 1.0 mM ATP, 2.0 µCi of (^{14}C)-succinic acid and sufficient succinyl-CoA synthetase to produce 1 µmol of succinyl CoA in 15 min in a final volume of 2.2ml. The reation mixture was incubated in a shaking water bath at 125 RPM at 35^{o}C for 30 min. The reation was terminated by the addition of 0.5 ml 25% trichloroacetic acid. The quantitation of ALA formed proceeded according to Ebert et al (80). One unit of ALA-s activity is defined as that quantity which catalyzes the formation of 1 nmol of ALA at 35^{o}C in 1 hour.

ASSAY OF ALA-SYNTHETASE ACTIVATOR-

Activator preparations were assayed by incubating 50 µl of ALA-s enzyme preparation with 50 µl of activator preparation

and proceding according to the ALA-s assay outlined above. The activity of ALA-s in the presence of activator fractions was compared to a control which contained no activator but contained an equivalent amount of protein. One unit of activator activity is defined as that quantity which stimulates ALA-s activity by 100% in 30 min at 35°C. Specific activity is expressed as units per mg protein.

BUFFERING SYSTEMS-

Buffer A consists of 10 mM Tris-HCL pH 7.5 containing 1.0 mM EDTA, 0.1 mM pyridoxal phosphate and 0.1 mM dithiothreitol. Buffer B consists of 10 mM Tris-HCL pH 7.0 containing 1.0 mM EDTA, 10% glycerol and 0.1 mM dithiothreitol. Buffer A is used for ALA-s containing preparations and Buffer B is used for Activator containing preparations.

PREPARATIONS OF $(NH_4)_2SO_4$ FRACTION CONTAINING ALA-S AND ALA-S ACTIVATING ACTIVITIES-

A Lubrol extract of liver mitochondria was prepared from mitoplasts as follows: The inner membrane-matrix fraction was prepared from washed mitochondria by treatment with digitonin after the method of Schnaitman and Greenawalt (81). The mitoplasts were resuspended in Buffer A at a protein concentration of 10 mg/ml. The suspension was treated with a solution of the non-ionic detergent Lubrol-WX (20 mg/ml in Buffer A) at a concentration of 1.05 mg Lubrol/10 mg protein, with gentle stirring for 15 min. Over 80% of the ALA-s activity present in the Lubrol treated preparation was recovered in the supernatant fraction after centrifugation for 1 h at 100,000 g. The Lubrol extract was brought to 33% sat

uration by the slow addition of solid ammonium sulfate. After 30 min stirring on ice the precipitated protein was collected by centrifugation. The supernatant was brought to 40% saturation with ammonium sulfate as above. The protein was collected in the same manner and resuspended in Buffer A. The supernatant was brought to 60% saturation and the protein collected and resuspended in Buffer A. In the final step, the supernatant from the 60% saturated fraction was brought to 90% saturation with ammonium sulfate, the protein collected and resuspended in Buffer B. The resuspended protein pellets were dialyzed 20 h against their respective buffers and diluted to a final protein concentration of 2 mg/ml.

POLYACRYLAMIDE GEL ELECTROPHORESIS-

Sodium Dodecyl Sulfate containing polyacrylamide gel electrophoresis was performed in 10% or 12.5% gels by the method of Weber and Osborn (82). Non-denaturing gel electrophoresis was performed according to Davis (83) with the exception that the gels contained 10% glycerol. Isoelectric focusing gel electrophoresis was performed in a pH gradient of 3.5-10 using 5% gel containing 8 M urea by the method of Danno (84). A 100 µl sample of the activator was extracted in 2% ampholytes (3.5-10), 8 M urea and 5% sucrose by shaking the mixture at room temperature. The sample was applied to the IEF gel and electrophoresed for 16 h at 150 volts. A gel without sample was sliced into 0.5 cm sections which were extracted for two h in distilled water at 35^{o}C. A gel which contained sample was soaked overnight in 12.5% TCA to remove ampho-

lytes and then stained with coomassie blue.

CARBOXYMETHYL SEPHADEX CHROMATOGRAPHY-

The activator fraction was dialyzed for 20 h against 2 X 4 liters of buffer B and then diluted 2-fold with the same buffer. A 250 ml sample was applied to a 2.5 X 40 cm column of CM-Sephadex. The column had previously been washed with buffer B at 4^{o}C. The column was washed with two bed volumes of buffer and the proteins were eluted with a 320 ml linear salt gradient of 0.01-0.60 M NaCl in the same buffer. Fractions of 5 ml were collected at a flow-rate of 24 ml/h. The fractions were assayed and those that contained the single peak of ALA-s activating activity which eluted at 0.37 M NaCl were pooled. This constitutes the CM-sephadex fraction.

SEPHACRYL S-200 GEL FILTRATION-

A 4 ml sample of the CM-sephadex fraction was applied to a 2.5 X 100 cm column of Sephacryl S-200. The column had previously been equilibrated with buffer B at 4^{o}C. The proteins were eluted with the same buffer and 3.7 ml fractions were collected. The fractions that constituted the major peak of ALA-s activating activity were pooled and comprise the S-200 fraction.

HYDROXYLAPATITE CHROMATOGRAPHY-

The S-200 fraction was diluted to three times its volume with buffer B and adsorbed onto a 1.5 X 30 cm column of hydroxylapatite which had been equilibrated at 4^{o}C with buffer. The activator was eluted with a linear gradient of 300 ml of 0.0-0.3 M Nacl in buffer B. Fractions of four ml were collected. The pure activator eluted at 0.1 M Nacl. This fraction

was concentrated against 90% glycerol containing 10 mM Tris-HCl, pH 7.0, 1.0 mM EDTA and 0.01 mM DTT and is refered to as the pure activator.

IMMUNODIFFUSION ANALYSIS-

Immunodiffusion analysis was carried out on plates prepared with 1% agarose on 38 mM Tris-glycine, pH 8.6. The center well contained 75 µl of antigen. The plates were developed 3-5 days at room temperature. The preparation of Lubrol extract of mitochondria and the ammonium sulfate fractions which contain the antigen were described above.

SINGLE ELECTROIMMUNOPHORESIS-

Solubilized mitochondria for immunoelectrophoresis were prepared by treating mitochondria with 1% sodium cholate in buffer A, and incubating for 30 min on ice. Immunophoresis was carried out as described by Nelson and Mendel-Hastvig (85). A 30 ml slab of 1% agarose in 38 mM Tris-glycine, pH 8.6, contqaining either 3 or 2.5 ml of antiserum was poured. A constant voltage of 100 v was used for the immunophoresis. Gels were washed three days in a buffer of 38 mM Tris-glycine pH 8.6, containing 150 mM NaCl and 0.1% sodium azide. Gels were stained with coomassie blue.

DRUG TREATMENT OF ANIMALS-

Male Sprague-Dawley rats, 170-200 g were fasted 24 h prior to drug treatment. Allylisopropylacetamide was dissolved in 0.9% saline at a concentration of 30 mg/ml and injected subcutaneously at a dosage of 400 mg/kg body weight. Control rats recieved the equivalent volume of saline. 3,5-diethoxycarbonyl-1,4-dihydrocollidine was suspended in corn oil by sonication

at a concentration of 25 mg/ml and injected intraperitoneally at a dosage of 25 mg/kg body weight. Controls recieved corn oil. Ethanol was diluted to 30% with 0.9% saline and administered intraperitoneally at a dosage of 300 mg/100 g body weight. Cycloheximide was disolved in saline at 10 mg/ml and injected intraperitoneally at a dosage of 15 mg/kg body weight at the same time as AIA. The drug treated rats were sacrificed 16 h after injection, ethanol and cycloheximide treated rats were sacrificed 4 h after injection.

PROTEIN DETERMINATION-

Protein content was measured by the method of Lowry et al (86) using crystalline serum albumin (Frac V) as standard.

RESULTS

A. THE PURIFICATION OF ALA-SYNTHETASE ACTIVATOR

THE STIMULATION OF ALA-SYNTHETASE- Lubrol supernatant and ammonium sulfate fractions were prepared as described under methods. Samples of the proteins precipitating at 33-40%, 40-60% and 60-90% saturated ammonium sulfate were assayed for ALA-s activity. All assays contained 200 µg protein. Table 1 shows the activity of ALA-s in each fraction and the effect of mixing the fractions. The 60-90% fraction is seen to stimulate the 33-40% fraction 3-fold. The 60-90% fraction is refered to as the activator fraction. Activator activity was linear with time under the conditions used.

THE PURIFICATION TABLE- The activator was purified to homogeneity by a combination of CM-sephadex, Sephacryl S-200 and hydroxylapatite column chromatographies. The column profiles are shown in Figures 1,2 and 3 respectively. Chromatography procedures are described in detail under methods. A summary of the purification scheme is shown in Table 2. The final yield of activator was 5% with a 6,250 fold increase in specific activity over that of the 60-90% saturated ammonium sulfate fraction.

CRITERIA OF PURITY AND MOLECULAR WEIGHT DETERMINATIONS- A 100 µl sample of the purified activator which had been concentrated was applied to a Davis non-denaturing gel and subto electrophoresis. The gel was scanned at 280 nm in a Gilford gel scanner. Figure 4 shows the ultraviolet absorbance trace which exhibits one major peak. The gel was then sliced into

TABLE 1

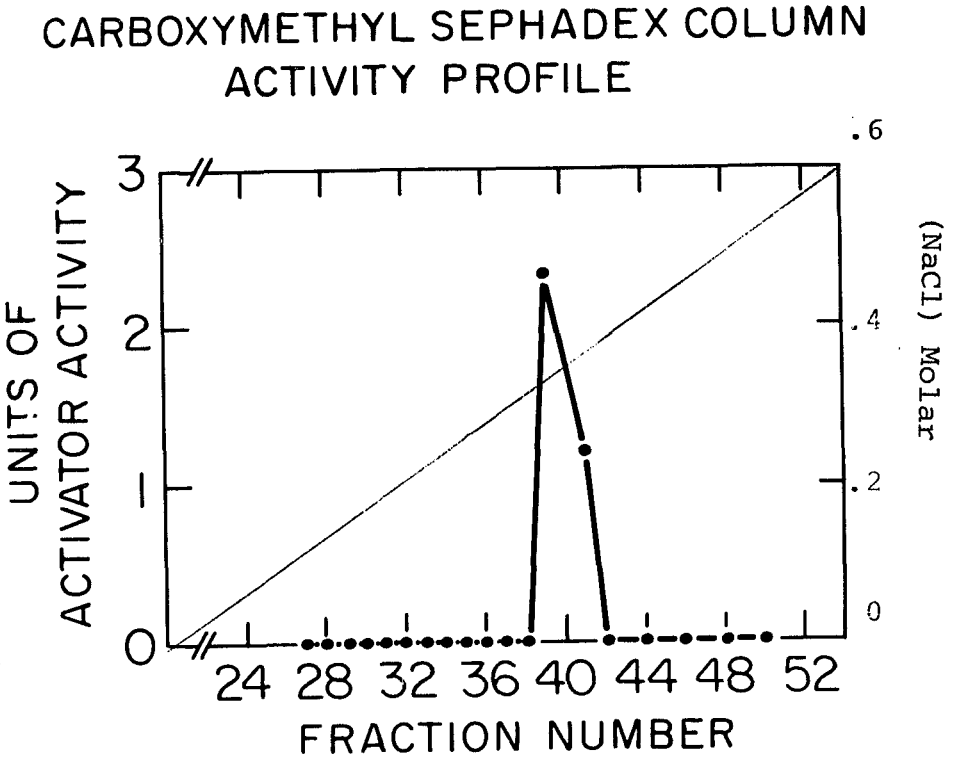
RECONSTITUTION OF AMMONIUM SULFATE FRACTIONS

FRACTION	SPECIFIC ACTIVITY UNITS/MG PROTEIN	ACTIVATION FOLD OF STIMULATION
33-40% I	2.87	
40-60% II	0.71	
60-90% III	0.078	
I + II	3.80	
I + III	8.72	3.0
II + III	0.95	

Fractions are percent saturation with ammonium sulfate. 100 ul of fractions I,II and III and 50 ul of each in the combined fractions (200 ug protein total)were assayed for ALA-s activity. Units are nmol/h.

FIGURE 1

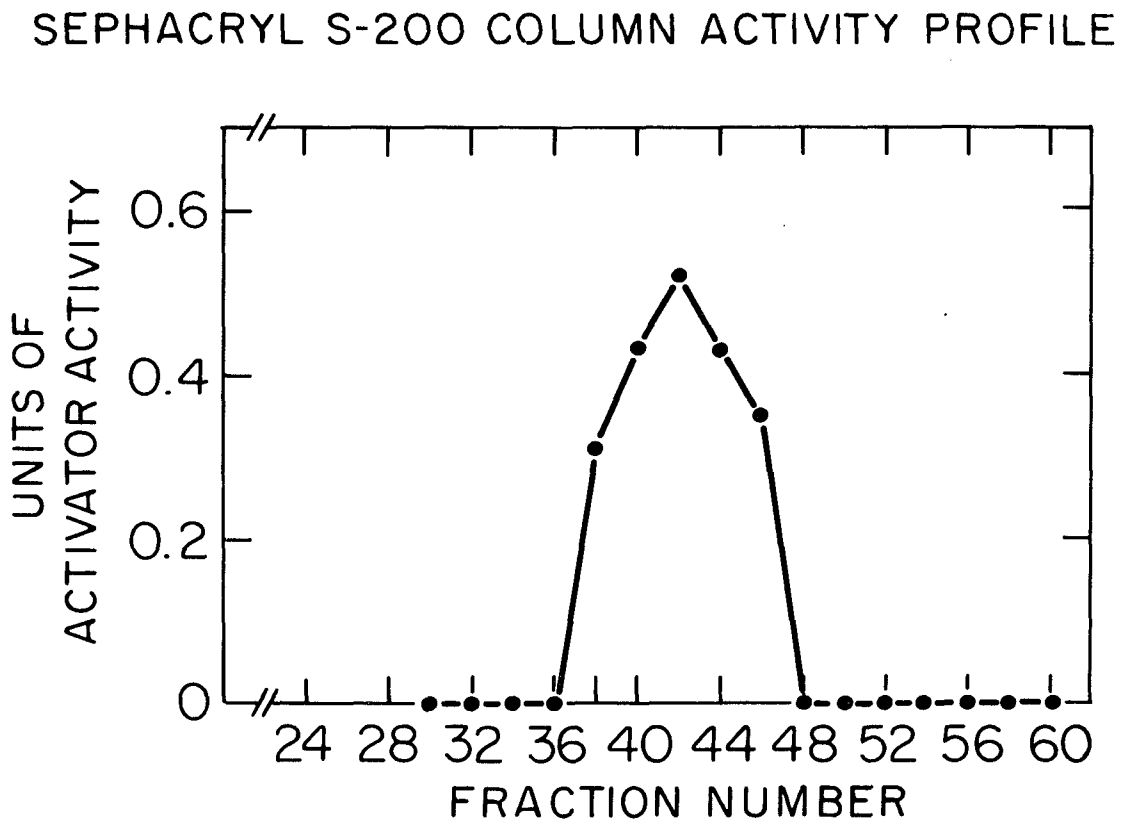
CARBOXYMETHYL SEPHADEX COLUMN ACTIVITY PROFILE



A 200 mg sample of 90% ammonium sulfate fraction was subjected to CM-Sephadex column chromatography. Assays were conducted on 50 ul of each column fraction combined with 50 ul of 40% fraction as described in methods. One unit of activator activity stimulates ALA-s 100%.

FIGURE 2

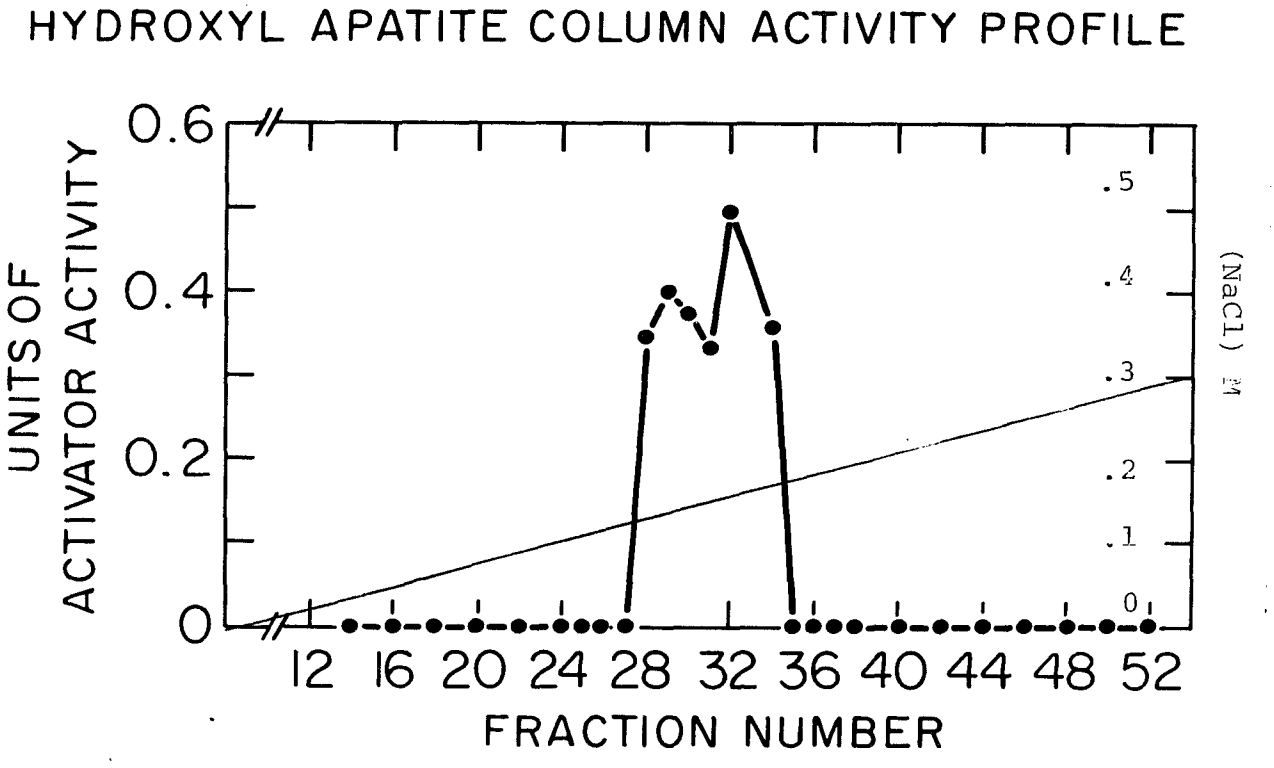
SEPHACRYL S-200 COLUMN ACTIVITY PROFILE



A 4 ml sample of the pooled CM-sephadex fractions, those that eluted at 0.37 Molar NaCl, was subjected to chromatography on a sephacryl S-200 column. A 50 ul aliquot of each column fraction was combined with 50 ul of 40% ammonium sulfate fraction and the activator activity was assayed as described under Methods. One unit stimulates ALA-s 100%.

FIGURE 3

HYDROXYLAPATITE COLUMN ACTIVITY PROFILE



The pooled S-200 fractions amounting to 18.5 ml were subjected to hydroxylapatite column chromatography. A 50ul aliquot of each column fraction was combined with 50 ul of 40% ammonium sulfate fraction and the activator activity was assayed as described under Methods. One unit stimulates ALA-s 100%.

TABLE 2

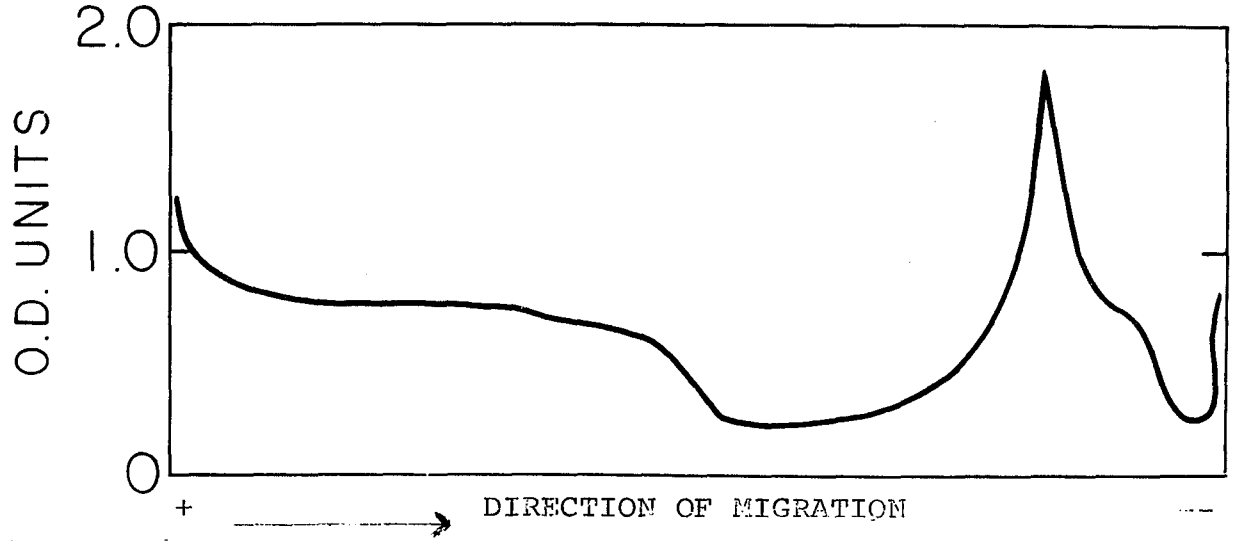
PURIFICATION OF ALA-SYNTHETASE ACTIVATING PROTEIN

PURIFICATION STEP	PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	YIELD	FOLD PURIFICATION
	MG	UNITS	UNITS/MG PROTEIN	%	
60-90% FRACTION	250	4000	16	100	
CM-SEPHADEX	0.105	966	9.2×10^3	24	575
SEPHACRYL S-200	2.22×10^{-2}	222	1.0×10^4	5.5	625
HYDROXYLAPATITE	2.00×10^{-3}	200	1.0×10^5	5.0	6250

Fractions were assayed as described in Methods. One unit of activator activity stimulates ALA-s activity 100%. Each fraction contained 100 ug protein.

FIGURE 4

ULTRAVIOLET SCAN OF A DAVIS NON-DENATURING GEL CONTAINING THE ACTIVATOR



A .1 ug sample of the hydroxylapatite fraction was applied to a Davis non-denaturing gel and subjected to electrophoresis for 5h. The gel was scanned at 280 nm and the trace is shown above.

2 mm sections and assayed for ALA-s activating activity. The gel slices containing the activator activity corresponded to the peak of the U.V. absorbance and the activity profile is shown in Figure 5.

A 200 µl fraction of the purified activator was extracted overnight at 60°C with 2% SDS and 1% 2-mercaptoethanol. The sample was applied to a 12.5% polyacrylamide gel containing SDS and subjected to electrophoresis. After staining, no bands were visible. The gel was scanned at 280 nm and the absorbance peak observed is shown in Figure 6. The molecular weight of the denatured activator was estimated with phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase as standards which are indicated by arrows. The estimated molecular weight is 54,000.

The molecular weight of the native activator was estimated by gel filtration on Sephacryl S-200 with bovine serum albumin, ovalbumin, trypsin and cytochrome c as markers. The estimated molecular weight is shown in Figure 7 to be 57,000. The isoelectric point was determined to be 7.5 as shown in Figure 8.

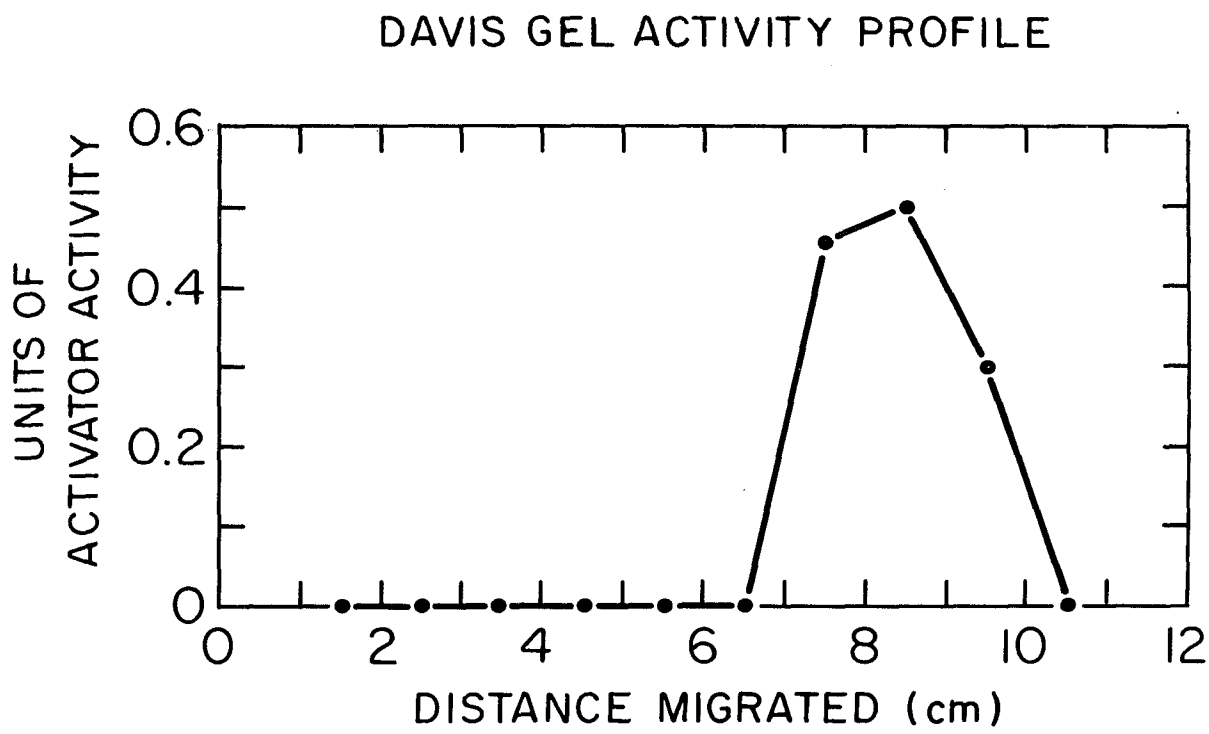
B. CHARACTERIZATION OF THE ACTIVATOR

ALA-s in a 40% ammonium sulfate fraction was assayed in the presence of varying quantities of 90% activator fraction. The amount of activation was determined and a linear relationship between quantity and activation was seen up to a protein concentration of 67 µg. Saturating amounts of activator fraction were used in all experiments. See Figure 9.

The actiator did not lose activity after 20 h dialysis

FIGURE 5

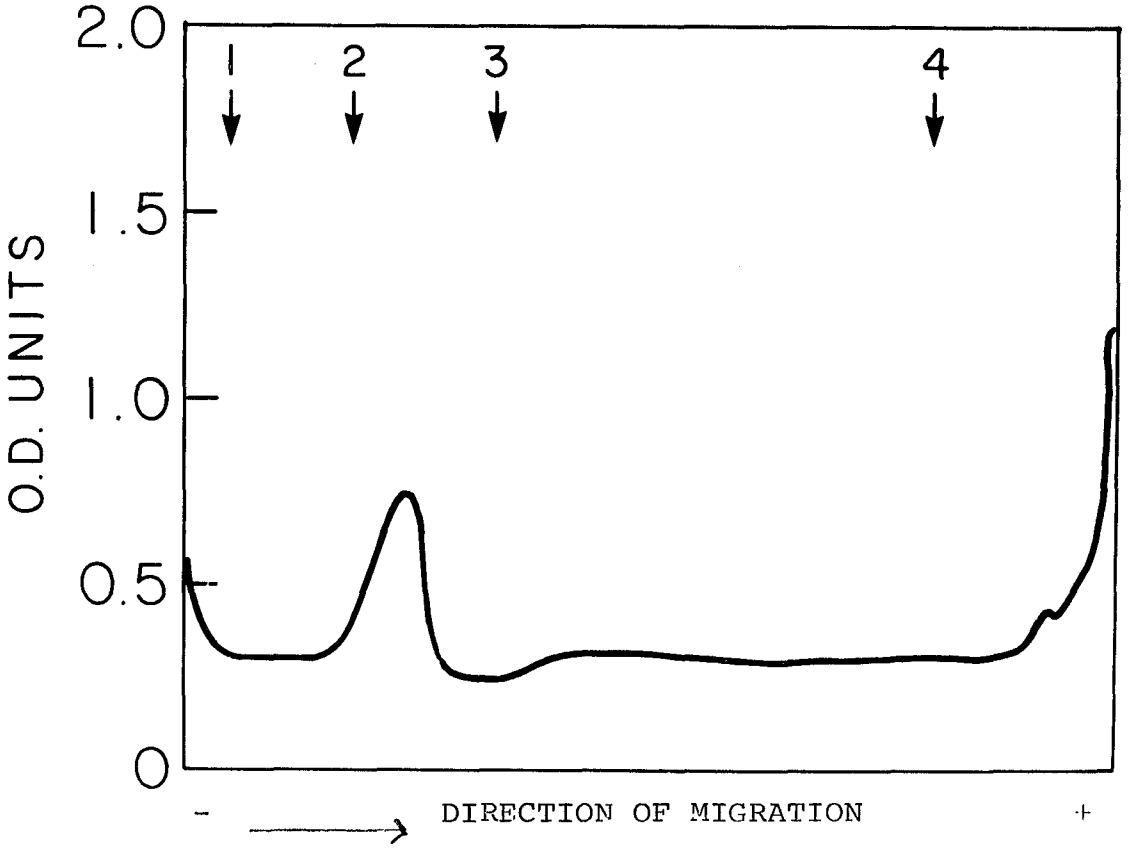
DAVIS GEL ACTIVITY PROFILE



A 0.2 ug sample of hydroxylapatite fraction was applied to a Davis non-denaturing gel and subjected to electrophoresis for 5 h. Gel slices of 0.5 cm were assayed for activator activity as described in Methods. One unit of activator activity stimulates ALA-s activity 100%. One centimeter on the gel activity profile is equivalent to 1.04 cm on the previous gel scan.

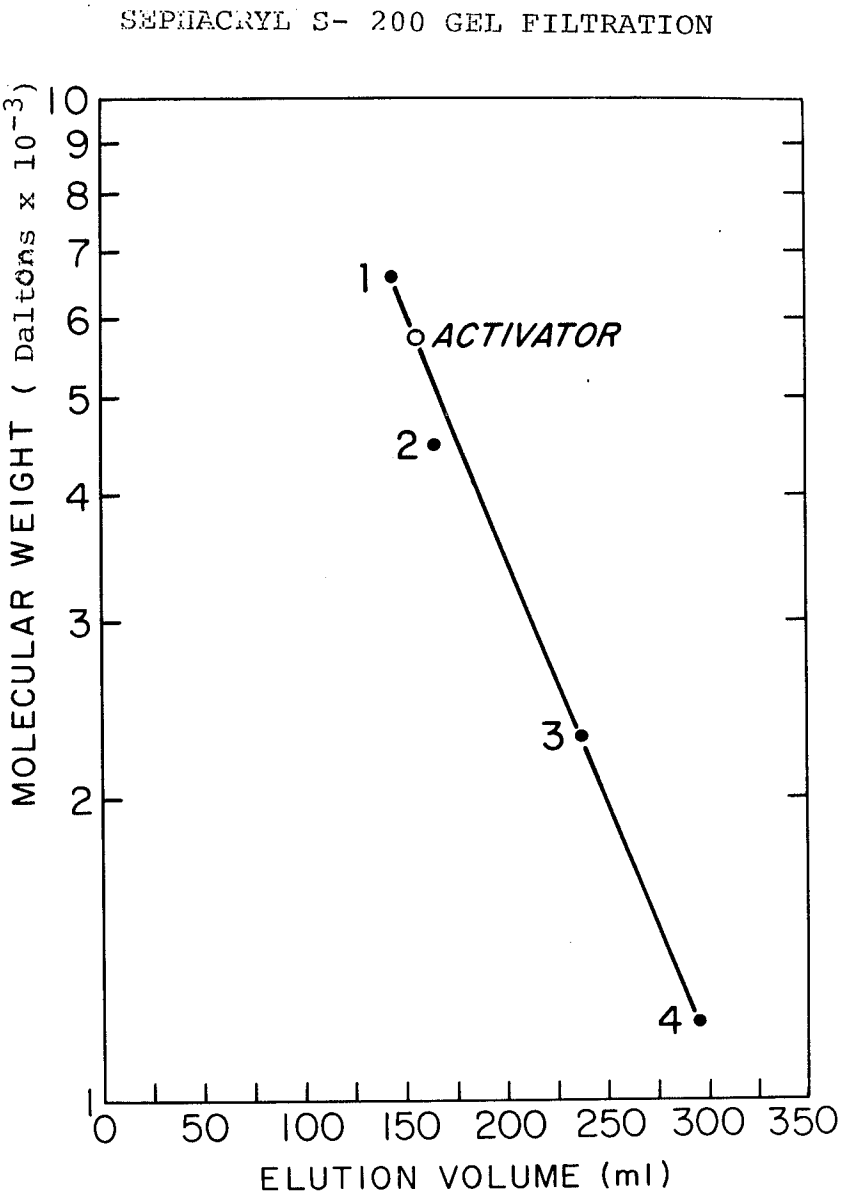
FIGURE 6

ULTRAVIOLET SCAN AT 280 NM OF AN SDS CONTAINING GEL CONTAINING THE ACTIVATOR



A 0.2 ug sample of hydroxylapatite fraction was extracted overnight in SDS and 2-mercaptoethanol and subjected to SDS gel electrophoresis for 3 h at 150 volts. The gel was scanned at 280 nm and the trace is shown above. The molecular weight was estimated to be 54,000 daltons with the following molecular weight standards; 1; phosphorylase b; 2, BSA; 3, ovalbumin; 4, carbonic anhydrase.

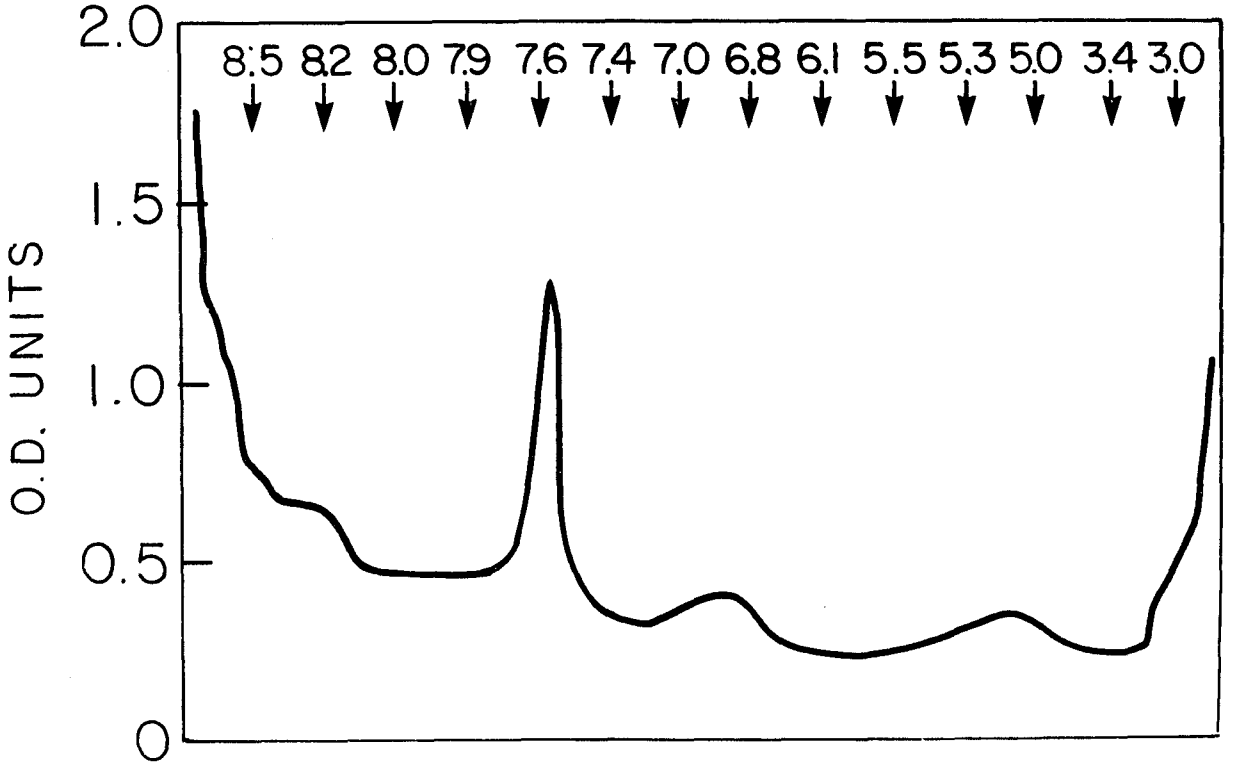
FIGURE 7
MOLECULAR WEIGHT ESTIMATION OF THE ACTIVATOR



The volume of Carboxymethyl-Sephadex applied to the Sephadex S-200 column was 4 ml. The column had previously been calibrated with the following molecular weight standards: 1. BSA; 2, ovalbumin; 3, trypsin; 4, cytochrome c. The native molecular weight of the activator was estimated to be 57,000 daltons.

FIGURE 8

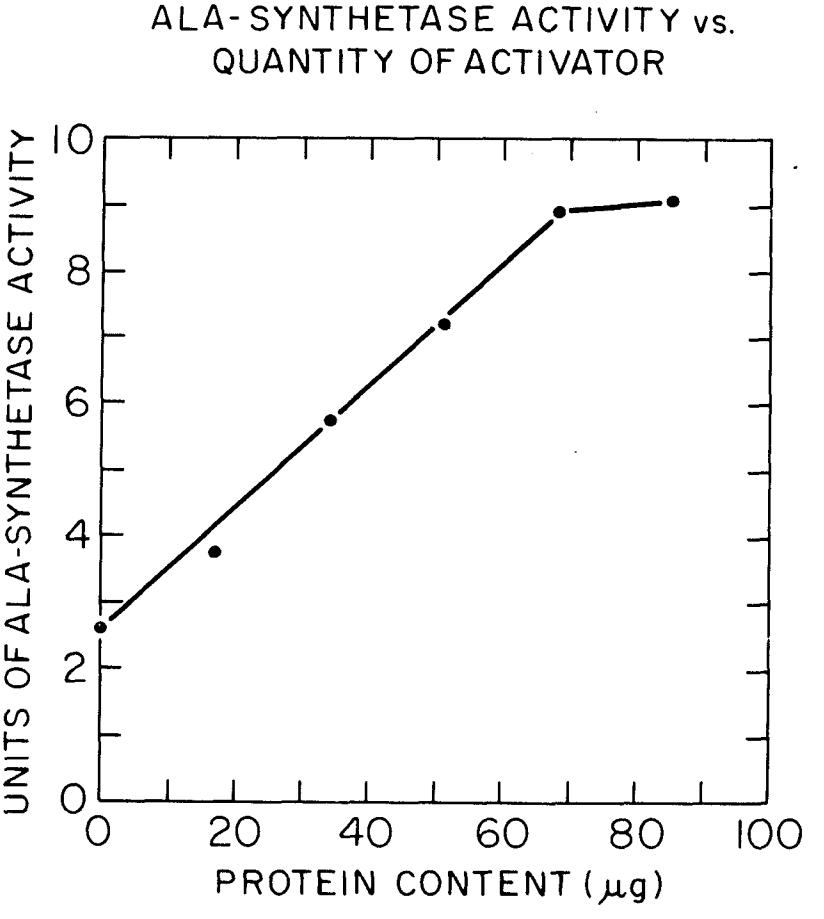
VISIBLE SCAN AT 560 NM OF AN ISOELECTRIC FOCUSING GEL CONTAINING THE ACTIVATOR



The isoelectric point was determined by extracting 0.5 ug of hydroxylapatite fraction in ampholytes and urea as described in Methods. The sample was applied to an isoelectric focusing gel and subjected to electrophoresis for 16 h at 150 volts. Ampholytes were extracted with trichloroacetic acid and the gel was stained with coomasie blue and scanned at 560 nm. The pH gradient was determined on a gel which did not contain sample by extracting 0.5 cm sections in distilled water and determining the pH.

FIGURE 9

ACTIVITY CURVE OF THE ACTIVATOR AT DIFFERENT CONCENTRATIONS



Aliquots of 90% ammonium sulfate fraction containing the indecated protein concentrations were combined with 50 ul of 40 % ammonium sulfate fraction and ALA-s was assayed as described in Methods. One unit of activity catalyzes the formation of one nmol of ALA per hour.

against 12 l buffer B in dialysis tubing with an exclusion limit of 20,000 d. Incubating the activator fraction at 4^{o} for 3 days, at room temperature for two hour or at $60^{o}C$ for two min did not cause loss of activity. Activity was lost after storing the fraction at $^{-}60^{o}C$ for two weeks or at $^{-}20^{o}C$ for one month, even in the presence of glycerol. The pH optima were determined to be 7.0 and 7.8 and the activity was seen in the pH range of 6.0-8.0 as shown in Figure 10.

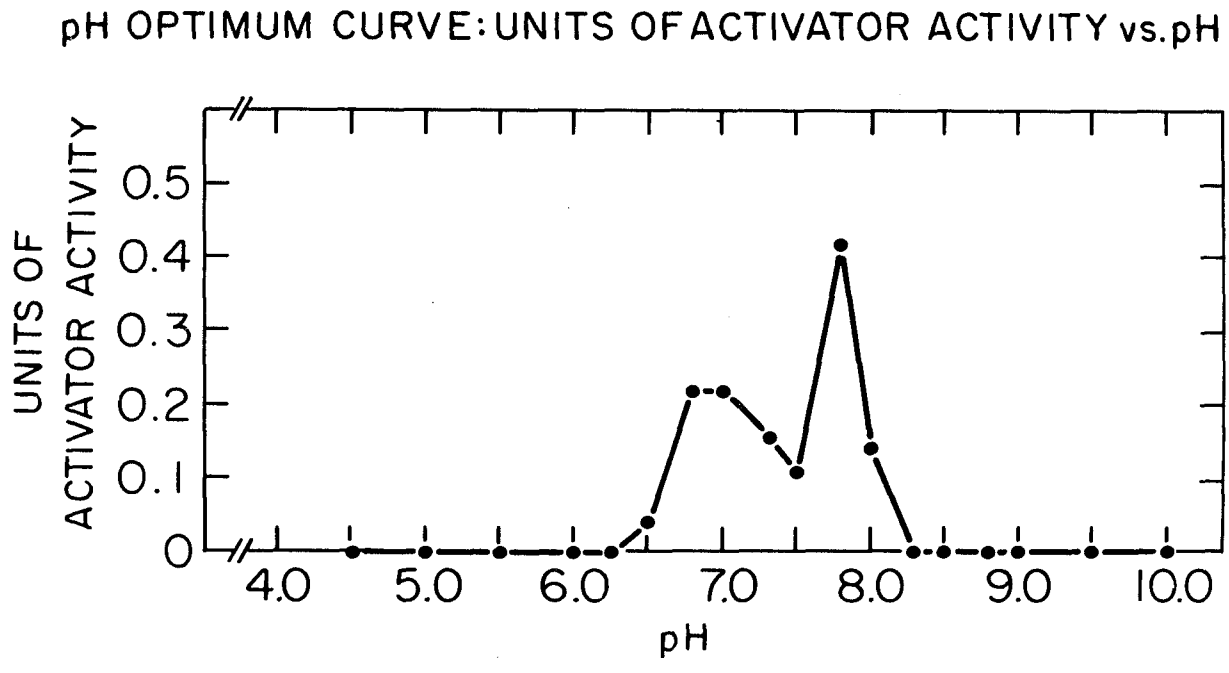
During purification of ALA-s, the elution of ALA-s activity from a DEAE-cellulose column occurs at a NaCl concentration of 0.1 M, as shown in Figure 11, when the activator has not been separated from ALA-s catalytic activity during ammonium sulfate fractionation. However, when the catalytic ALA-s and the activator have been separated, the ALA-s activity elutes from DEAE-cellulose at a NaCl concentration of 0.3 M. Thus the Activator changes the charge characteristics of the ALA-s complex in such a fashion as to make it less strongly anionic, binding less tightly to DEAE-cellulose when complexed with the activator.

C. THE MODE OF ACTION OF ALA-SYNTHETASE ACTIVATOR

One mg of activator fraction was incubated for 15 min at $35^{o}C$ with 75 µg of pronase. Aliquots containing 100 µg of activator fraction were then assayed for activator activity. No activator activity was observed after treatment with pronase as seen in Table 3. It was concluded that the activator is a protein. The activator was not sensitive to trypsin.

FIGURE 10

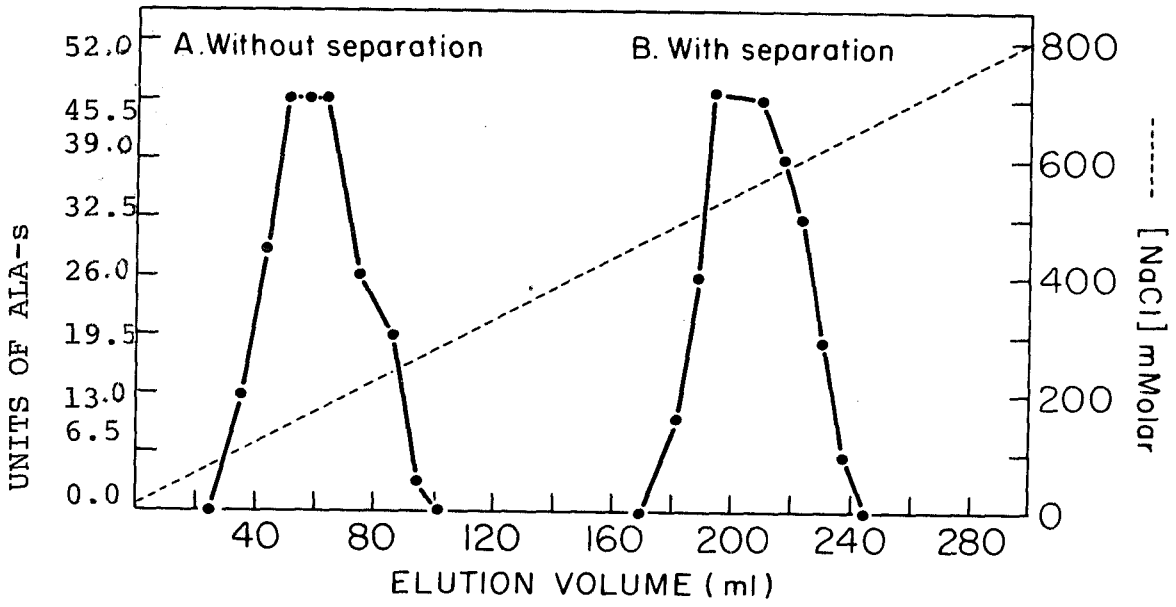
THE pH OPTIMUM OF THE ACTIVATOR



A 50 ul sample of 90% fraction and 50 ul of 40% fraction were combined and incubated in an assay medium at the appropriate pH as described in Methods.. One unit of activator activity stimulates ALA-s activity 100%.

FIGURE 11

DEAE-CELLULOSE COLUMN PROFILES OF
ALA-S ACTIVITY WITH AND WITHOUT SEPARATION OF THE ACTIVATOR



This graph shows relative elution volumes of ALA-s with and without the removal of the activator protein. The columns were run during different experiments and are not comparable with regards to activity. 100 ul of each fraction was assayed for ALA-s activity as described in Methods. One unit of activity catalyzes the formation of one nmol of ALA per hour.

TABLE 3

PROTEASE DIGESTION OF ACTIVATOR FRACTION

PROTEASE	ACTIVITY (UNITS)	
	0 MIN	15 MIN
BSA CONTROL	2.4	2.9
TRYPSIN	2.0	2.6
PRONASE	2.1	0

ALA-s activator activity was assayed as described in Methods. One unit is the quantity which stimulates ALA-s activity 100%. The BSA control consists of 50 µl of 40% fraction incubated with 50 µl of 90% fraction which had been treated with BSA. The protease treated fractions consist of 50 µl of 40% fraction incubated with 50 µl of 90% fraction which had been treated with protease. The activation was determined by assaying 40% fraction with a protease treated BSA stock and comparing that to the activity determined by assaying 40% fraction with a protease treated 90% fraction. In this way any degradation of ALA-s by proteolysis during the assay period was corrected for.

ALA-s in the 33-40% ammonium sulfate fraction and enzyme preparations partially purified by DEAE-cellulose and Sephacryl S-200 were assayed in the presence of 30 µg hemin.ALA-s ALA-s was inhibited 85% in the S-200 fraction as shown in Table 4.The presence of the activator fraction did not reverse or enhance the inhibition of ALA-s by hemin.

A sample of partially purified ALA-s containing 0.4 mg of protein was incubated with 6.4 µg of pure rabbit liver phosphatase. The incubation was for 20 min at 37°C. The ALA-s was then assayed for activity and found to possess identical activity as a control incubated with 6.4 µg of BSA. This is shown in Table 5. This suggests that the mechanism of action of the activator is not via a phosphatase or kinase activity because the phosphatase neither inhibited nor enhanced the ALA-s activity. The activation of ALA-s does not appear to have the same function as the R. spheroides enzyme described by Hayasaka and Tuboi (110) Which activates ALA-s by an intramolecular thiol-disulfide conversion because the activator does not require cysteine or other disulfide compounds.

These results suggest that the mode of action of the activator might not be via chemical modifications of ALA-s or by interference with the feedback inhibition exerted by heme on ALA-s.

An 800 µl aliquot of pure ALA-s and a 200 µl ALiquot of pure activator were combined and incubated at room temperature for 20 min. They were then applied to to 1 X 20 cm column of Sephacryl S-200 which had been equilibrated with buffer A at 4°C. The proteins were eluted with the same buffer and fractions

TABLE 5

EFFECT OF PROTEIN PHOSPHATASE ON ALA-SYNTHETASE

FRACTION	TREATMENT	SPECIFIC ACTIVITY
LUBROL SUPT	0 TIME CON	5.2
LUBROL SUPT	TREATED	6.0
LUBROL SUPT	UNTREATED	5.2
DEAE FRAC	0 TIME CON	7.5
DEAE FRAC	TREATED	6.2
DEAE FRAC	UNTREATED	6.0
S-200 FRAC	0 TIME CON	3.5
S-200 FRAC	TREATED	2.0
S-200 FRAC	UNTREATED	2.2

Samples of 200 ul were incubated with 6.4 ug of pure Rabbit liver protein phosphatase for 20 min at 37^{o}. The ALA-s activity was assayed as described in Methods. Specific activity was 1 nmol ALA formed/h/mg protein.

TABLE 4

Hemin Inhibition Table Of ALA-s In The Presence Of Activator

Fraction	Specific Activity (nmole/mg 1hr)	% Inhibition
ALA-s	5.33	
ALA-s + Activator	12.89	
ALA-s + Hemin	1.35	75%
ALA-s + Activator + Hemin	1.69	87%

ALA-s was assayed as described in Methods. Percent inhibition refers to untreated ALA-s.

Either 50 ul of DEAE-Cellulose fraction or 50 ul of DEAE-Cellulose plus 50 ul 90% fraction were assayed for ALA-s activity in the presence and absence of 30 uM Hemin. Specific activity was nmol of ALA formed /h/mg protein.

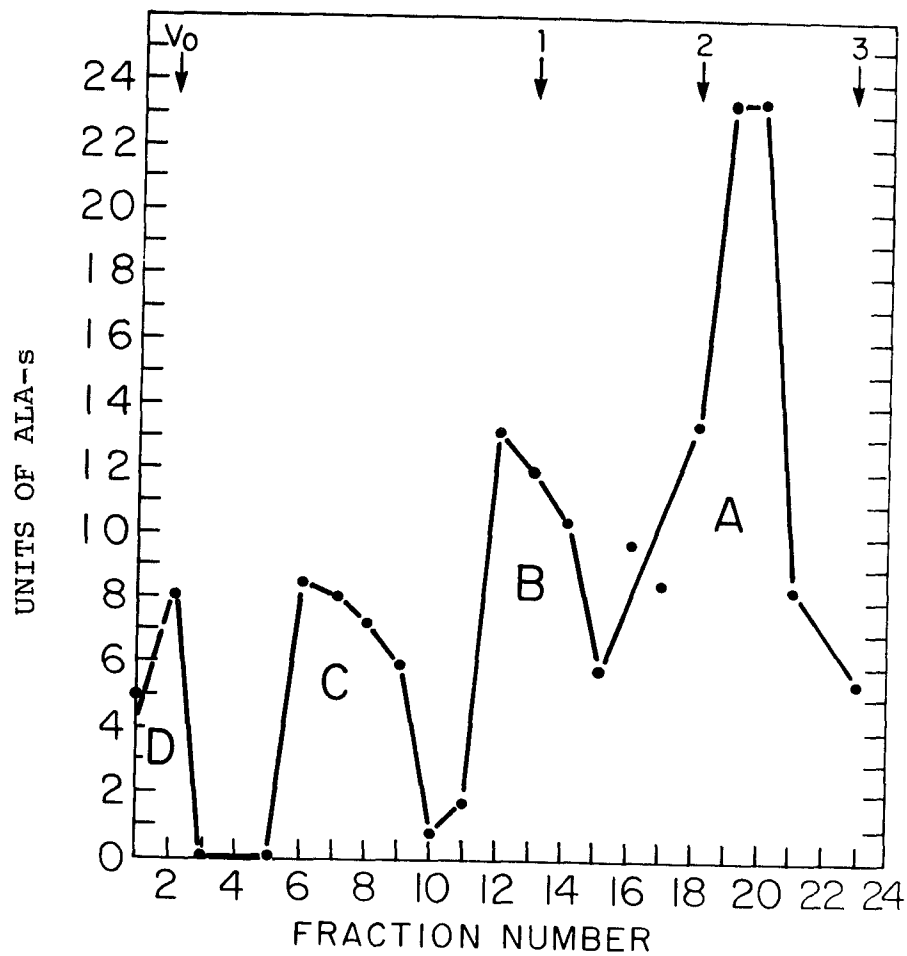
of 0.84 ml were collected at a flow rate of 30 ml per h. The fractions were assayed for ALA-s activity. The activity profile is shown in Figure 12. The protein standards indicated by arrows are yeast alcohol dehydrogenase, BSA, and ovalbumin. Peak A, corresponding to a molecular weight of of 58,000 is the ALA-s monomer. The second peak, B, of 118,000 d is the ALA-s dimer. These two peaks were observed previously by Paterniti and Beattie (71) who consider that the the dimer may be the native configuration. In the present experiment a third peak, Peak C, of approximately 218,000 d was observed. Peak C is a tetramer composed of both ALA-s and activator molecules. This was determined by pooling the fractions comprising this peak and subjecting them to SDS gel electrophoresis. Two bands were observed. Peak D eluting at the void volume is an aggregate of high molecular weight, greater than 250,000 and contains both ALA-s and activator protein. These results suggest that the activator may be responsible for the aggregation of ALA-s into the high molecular weight complexes which characterize it and that these large complexes are the most active forms of the enzyme.

D. THE ANTI-ALA-SYNTHETASE ANTIBODY

ALA-s purified from 210 rats yielded 67 ug of protein. This was sent to Dr. Ursula Muller-Eberhard who raised anti-bodies against the enzyme in a goat. Antisera prepared against ALA-s was cross-reacted with Lubrol solubilized mitochondria, the 33-40% ammonium sulfate fraction, the 60-90% ammonium sulfate

FIGURE 12

SEPHACRYL S-200 COLUMN PROFILE OF ALA-s ACTIVITY IN THE PRESENCE OF THE ACTIVATOR



An 800 ul aliquot of pure ALA-s and 200 ul of pure activator were incubated together at room temperature for 20 min. They were then chromatographed on Sephacryl S-200. 100 ul of each fraction was assayed for ALA-s activity. The protein standards were: yeast alcohol dehydrogenase; 2, BSA; 3, ovalbumin; 4, myoglobin (not shown). V_o was determined with Blue Dextran.

fraction, a sonicated extract of R. spheroides and a BSA standard. Only the Lubrol supernatant and the 33-40% fraction reacted with the antisera and formed a precipitin line (Fig 13). The precipitin lines formed between the wells containing the antigen and antisera intersect without showing spurs, indicating that the antibody is specific for one antigen, ALA-s from rat liver mitochondria. The antibody was used to quantitate ALA-s by immunophoresis as described under methods.

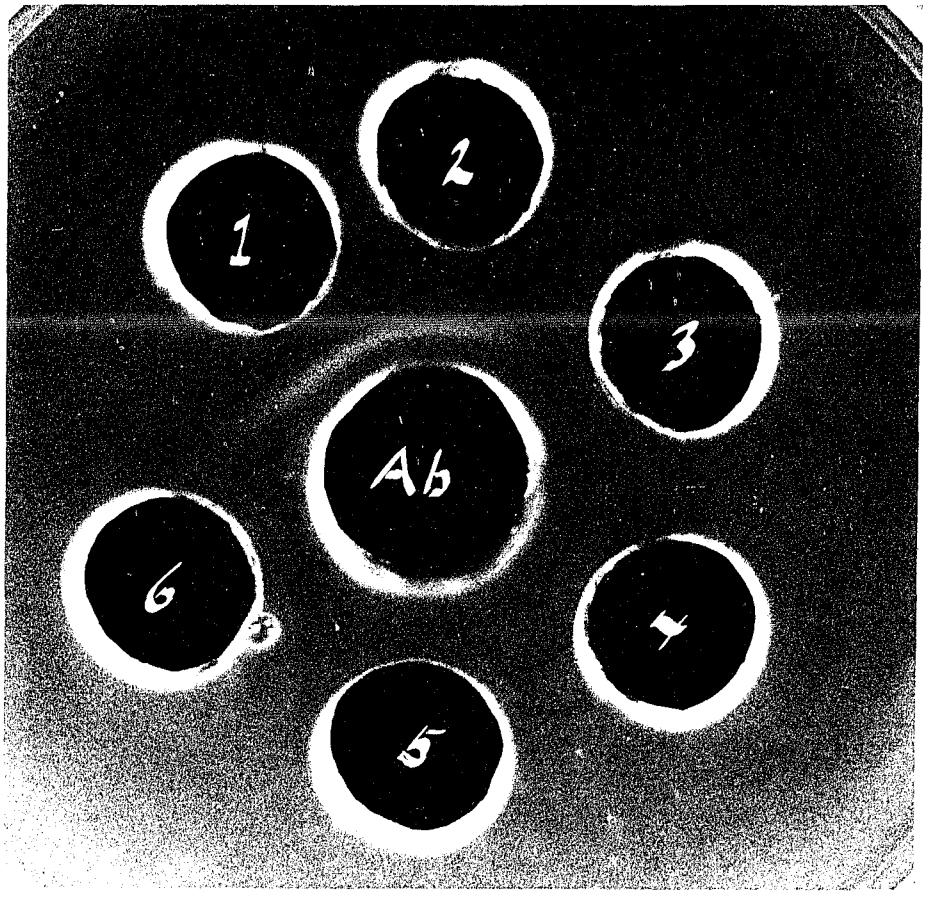
E. THE QUANTITATION OF ALA-SYNTHETASE

ALA-s activity in rat liver mitochondria was induced with the drugs AIA, DDC and ethanol as described in methods. The effect of cycloheximide on AIA induction was also tested. The ALA-s was assayed after solubilizing the mitochondria with 1% sodium cholate, a treatment which results in recovery of all the activity of intact mitochondria. As seen in Table 5 the induction ratio for AIA, DDC and ethanol (11.6,10.9 and 3.3 respectively) are similar to those reported previously. In addition, the stimulation of ALA-s activity was blocked by cycloheximide.

Immunophoresis was used to quantitate the amount of ALA-s during drug induced states relative to uninduced controls. Figure 14 shows a typical immunophoresis gel with rockets from DDC treated and control animals. The mitochondria were diluted to 2 mg/ml of protein and solubilized with 1% cholate in buffer A. The heights of the rockets from drug treated and control animals were plotted against the quantity of solubil-

FIGURE 13

OUCHTERLONY IMMUNODIFFUSION ANALYSIS



Wells contain: Ab, 75 ul antiserum; 1, Lubrol solubilized mitoplasts; 2, 33-40% saturated ammoniun sulfate fraction; 3, 60-90% saturated ammoniun sulfate fraction; 4, sonicated R. spheroides; 5, BSA. Each peripheral well contains 40 ul of sample at a protein concentration of 2 mg/ml.

TABLE 6

ACTIVITY OF ALA-SYNTHETASE AFTER TREATMENT WITH DRUGS AND CYCLOHEXIMIDE

FRACTION	SPECIFIC ACTIVITY	INDUCTION RATIO
	NMOL PRODUCT/H/MG PROTEIN	
CONTROL (8)	0.82	-
AIA (6)	9.50	11.6
AIA + CYCLOHEXIMIDE (2)	0.75	0
DDC (4)	8.94	10.9
ETOH (4)	2.71	3.3

ALA-s activity was assayed as described in Methods. The induction ratio is the ratio of the drug treated activity to the control activity. Samples were cholate solubilized mitochondria containing 2 mg/ml, 100 ul of each sample was assayed. Numbers in parenthesis are the number of animals in each experimental group.

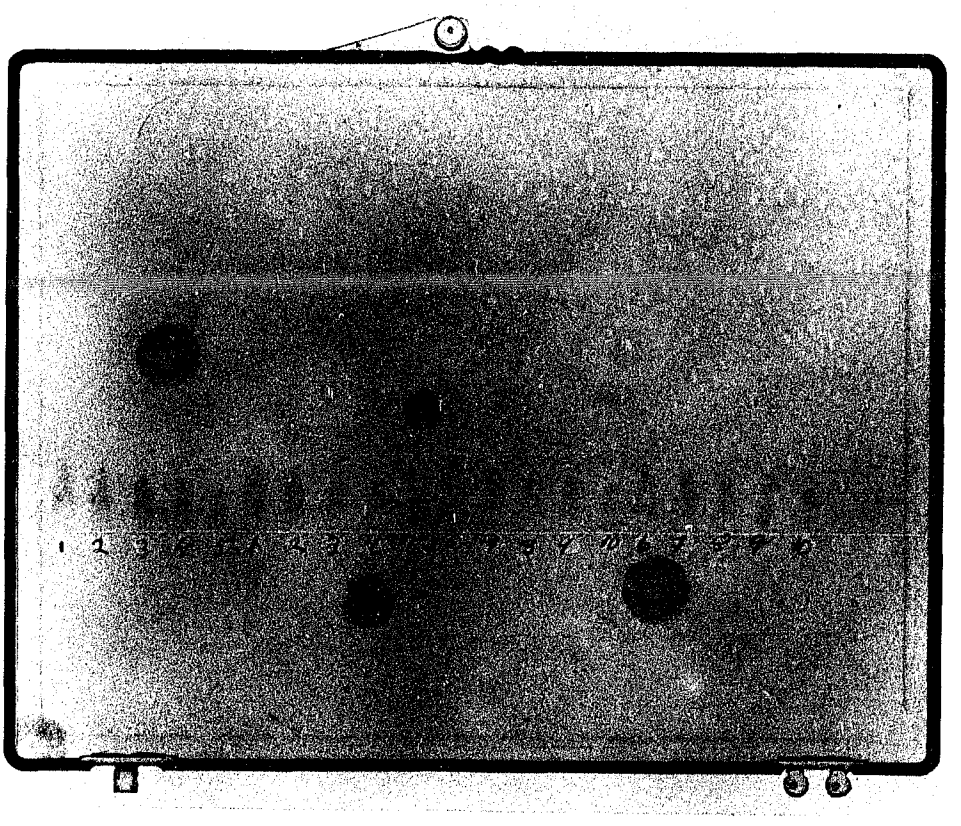
FIGURE 14

PHOTOGRAPH OF AN IMMUNOPHORESIS GEL OF CONTROL AND DDC TREATED RATS

Wells contain varying volumes of cholate solubilized mitochondria(2 mg/ml). Wells 1-5 are 13,12,10,8 and 5 ul of control mitochondria respectively. Wells 6-10 contain 13,12,10,8, and 5 ul of DDC treated mitochondria respectivly.

FIGURE 15

PHOTOGRAPH OF AN IMMUNOPHORESIS GEL OF CONTROL AND ETHANOL TREATED RATS

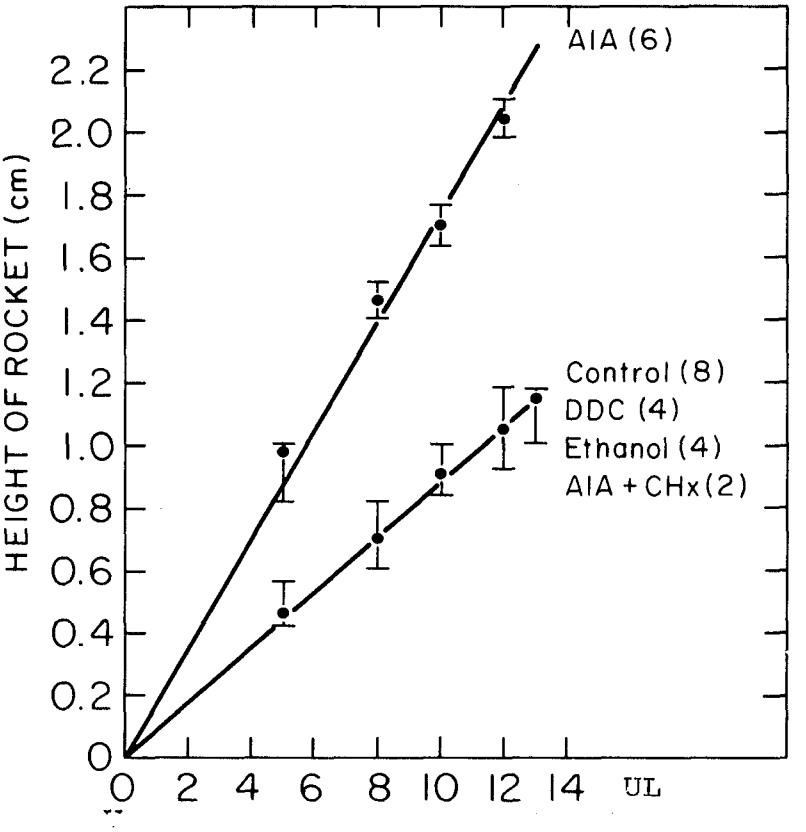


Wells contain varying concentrations of cholate solubilized mitochondria (2mg/ml) as described in the text. Wells 1-5 are 13,12,10,8 and 5 ul of control mitochondria respectively. Wells 6-10 are 13,12,10,8 and 5 ul of DDC treated mitochondria respectively.

ized mitochondria, Figure 16. Treatment of rats with AIA caused a two-fold increase in the content of ALA-s protein. Treatment of rats with either DDC or ethanol did not result in any increase in ALA-s protein despite the large increases in activity observed. The AIA induced increase in protein was blocked by cycloheximide.

FIGURE 16

GRAPH OF ROCKET HEIGHTS VERSUS VOLUME OF CHOLATE SOLUBILIZED MITOCHONDRIA DURING DRUG INDUCTION OF ALA-SYNTHETASE



Approprate volumes of cholate solubilized mitochondria were applied to the wells of a 1% agarose gel containing 10% antiserum. Immunophoresis was carried out at 100 volts for 16h. Rocket heights were measured with a micrometer. Bars represent deviation within experimental groups and points are average values. Numbers in parenthesis are the number of animals in each experimental group.

DISCUSSION

The regulation of heme biosynthesis and the key regulatory enzyme of the pathway, ALA-s, is a complex problem. In this thesis two aspects of this regulation have been attacked. The induction and feedback inhibition of the enzyme and another protein which is fundamental to its activity.

The finding of a stimulatory activity necessary for the optimum activity of ALA-s was first reported in this lab by George Patton in his Ph.D. thesis (87). He described an activity which was soluble in 60% saturated ammonium sulfate, which was heat labile, non-dialyzable and insensitive to phospholipase. My work has confirmed these findings, however, the separation of the activator from the catalytic fraction is difficult to reproduce in an identical manner each time it is purified. The evidence indicates that the association between ALA-s and the activator is ionic in nature but ammonium sulfate fractionation may not be the most reliable and reproducible method of disassociating the two. Different levels of ALA-s activity are found in the 40% saturated ammonium sulfate cut after fractionation. The lower the ALA-s activity the more it is stimulated by the activator. The greatest stimulation that I achieved was 3.2-fold, while George Patton reported a 40% cut which was devoid of activity until combined with the activator.

The pattern of fractionation and activation was identical in fed and fasted rats, thus the physical association of ALA-s

and the activator is not dependant on the state of the liver.

The pronase sensitivity established the protein nature of the activator. Because it is present in such low quantities it is not visible by staining after it has been purified to homogeneity. This had raised the question of its chemical nature. All biological and histological stains tested, for example, those for glycoproteins, lipoproteins and nucleo-proteins, failed to stain the activator. Ultaviolet scans were used to detect the activator in gels and a micro-assay based on coomassie blue was used to make a rough estimate of the protein concentration. Staphalococcus aureus protein A is a protein of 40,000 d molecular weight which contains only two tryptophan or tyrosine residues, which are necessary for most protein determinations. It is possible that the activator is similar to this protein and thus is not stainable at low quantities.

The isoelectric point of the activator was estimated to be pH 7.5-7.6. The isoelectric point of ALA-s is known to be 4.5. Thus at neutral pH the two have opposite charges. The pH optimum curve of the activator displays two peaks, one at 7.0 and one at 7.8..Assays are carried out between pH 7.0 and 7.3 when the activator is positively charged and catalytic ALA-s is negatively charged. This explains why ALA-s sticks more tightly to DEAE-cellulose after the activator has been removed. If it has lost a positively charged subunit, the overall charge is more negative. It is difficult to explain the pH optimum at 7.8.

Based on these observations regarding the charge characteristics of ALA-s and its activator, it is easy to imagine an aggregation taking place. The most likely explanation based on the supporting data is the following: ALA-s is more active in a large aggregate which serves either to orient the subunits of ALA-s properly for substrate interaction and perhaps for regulation or for attachment to the inner membrane. Perhaps it performs both functions. The activator is neither catalytic nor regulatory in function but serves as a sort of glue or orientor. Rather than orbital stearing this would be molecular stearing. Data from this laboratory which supports this is the finding by Paterniti and Beattie (79) that high salt concentrations cause the inactivation of ALA-s and that after purification of ALA-s to the monomer by preparative gel electrophoresis it is much less active.

The other major aim of this research involved the regulation of ALA-s via induction. Antisera against ALA-s was used to study this proposed induction of ALA-s by porphyrinogenic drugs. Immunodiffusion studies with the antiserum and various ALA-s containing antigen preparations suggested that the antiserum contained a monospecific antibody directed against rat liver mitochondrial ALA-s. The antibody did not react with the activator.

Immunotitration experiments were performed to see if the antibody would inhibit the enzyme activity. There appeared to be some inhibition at low anti seum concentrations but a strong stimulation of ALA-s activity by both the antiserum

and the non-immune serum was observed at higher concentrations. Whatever was responsible for the stimulation, it was found to be non-dialyzable..The inhibition studies were discontinued.

The sensitive immunophoresis technique was used to quantitate ALA-s during drug induction. The three drugs tested are known to act differently in the cell and their induction effects on ALA-s also differ. Ethanol affects ALA-s in an unknown way. It stimulates ALA-s activity 3-6-fold and this stimulation is dependant on its metabolism via Alcohol dehydrogenase(88). DDC inhibits ferrochelatase leading to decreased cellular heme concentrations as described in the introduction and AIA destroys cytochrome P-450 heme. In these studies ethanol, DDC and AIA lead to a 3.3, 10.9 and 11.6-fold stimulation of ALA-s activity respectively. Only AIA causes an increase in ALA-s protein and that induction is only two-fold. This induction is sensitive to cycloheximide demonstrating its dependance upon protein synthesis. The interesting thing is that the entire 11.6-fold increase in ALA-s activity by AIA is also blocked by cycloheximide.

The data suggest that all the drug treatments increase ALA-s specific activity in the mitochondria by reducing feedback inhibition of the enzyme by cellular heme. The cellular heme pool is reduced in the case of DDC by inhibiting heme synthesis and in the case if AIA by increasing heme utilization. The function of ethanol is not understood at this point, but ethanol may work by changing the redox state of the cell. Cycloheximide was tested on AIA induction only and was found to block this induction. Several explanations for the effect of

cycloheximide on the increase of ALA-s activity suggest themselves yet none is particularly satisfying or experimentally verified. Feedback regulation by heme might require ongoing protein synthesis, or the stimulation of ALA-s activity may require the synthesis of other proteins in the mitochondria, prehaps the activator. The half-lives of ALA-s and its mRNA are very short, 70 min, (90-92) and sufficitient degradation could take place in 4 h that the activity is observed to decline drastically. As stated in the introduction, cycloheximide could also inhibit the apoprotein pool for hemoproteins thus causing the cellular heme to accumulate shifting the steady state to inhibit ALA-s.

AIA is reported to result in enlargement of liver size. The increased protein content could be responsible for the increase of ALA-s protein which is only two-fold. This could be a nonspecific effect of the drug. During drug metabolism, 30% of the total oxygen uptake of the organ can be due to cytochrome P-450 dependant monooxygenases (104). Oxygen might become limiting leading to a competition between mitochondria and microsomes for molecular oxygen. An interdependance between the organelles is suggested by the observation that during drug metabolism the mitochondria are surrounded by E.R. membranes (105).

Phenobarbitol, an inducer of P-450 and heme synthesis leads to proliferation of the endoplasmic reticulum (106) and to an increase in liver mass (107). It also leads to an increase in the number of mitochondria and peroxisomes (108) and an increase in the amount of mitochondrial and peroxisomal

enzymes corresponds to the increase in liver weight (109).

These results tend to support the suggestion (24) that ALA-s is not induced de novo specifically by AIA or other drugs but is increased in activity.

CONCLUSION

Hemoproteins occupy key positions in the function of hepatocyte organelles. Thus the regulation of hemoprotein levels can be linked to the general regulation of these organelles. The regulation of hemoprotein synthesis requires coordinating the synthesis of heme and the apoprotein, the attachment of the cofactor to the apoprotein and the localization of the holoenzyme at its site of function.

The term induction was originally defined by microbiologists for the bacterial system, where protein degradation plays a minor role in determining enzyme levels and the protein level implicates the mRNA level. In eukaryotes a steady state situation exists and proteins are regulated by their synthesis and degradation (93). The term induction is used operationally, here, to describe an increase in the quantity of a protein without mechanistic implications. Induction is thus an increase in the ratio of the rate of protein synthesis over the rate of degradation.

Cytochrome P-450 is part of a microsomal electron transport chain consisting of two components, cytochrome P-450 is the terminal oxidase and binds a lipid-soluble substrate to molecular oxygen. The other component is a NADPH-dependant flavoprotein called cytochrome P-450 Reductase. The complex catalyzes the oxidation of lipid soluble chemicals such as aliphatic and aromatic hydrocarbons as well as physiological compounds like steroids, cholesterol, bile acids and fatty acids. The substrate specificity of the system resides in P-450

which is a family of related cytochromes with different substrate specificities.

The protein components of the monooxygenase system can be induced by a great variety of chemicals. The induction can be prevented by inhibition of protein synthesis (94). Treatment with phenobarbitol results in increased in vivo incorporation of amino acids into P-450 (95) and increased incorporation of heme precursors into a preparation which contained P-450 as the only hemoprotein (96). As stated in the introduction, most of the heme synthesized in the hepatocyte is needed for P-450 and treatment of patients who have defects in heme metabolism with inducers of P-450 leads to an acute porphyric attack(97). The level of ALA-s activity is increased by phenobarbitol treatment (96), and returns to normal after P-450 has reached the induced steady state.

Evidence for a pool of free P-450 apoprotein has been reported (98). Phenobarbitol administration leads to the synthesis of apoprotein which accumulates if heme synthesis is inhibited. Inhibition of heme synthesis leads to a decrease in P-450 and prevents its induction. However, an increased rate of heme synthesis does not increase P-450, just the heme pool in the microsomes (99). The amount of cytochrome b_5 also increases after treatment with phenobarbitol and there is some evidence for a cytochrome b_5 apoprotein pool in the microsomes (100).

Tryptophan pyrrolase, the rate-limiting enzyme in tryptophan degradation, was the first enzyme whose concentration was shown to be dependant on a hormone (101). The enzyme

is present usually in equal proportions as a holoenzyme and as an apoenzyme. Feigelson and Grengard (102) showed that corticoids and tryptophan increased the concentration of tryptophan pyrrolase in the liver. Tryptophan converts the inactive apoenzyme to the active holoenzyme by saturation with heme. Increased activity of tryptophan pyrrolase is seen after administration of phenobarbitol and AIA (103).
In fact, conditions leading to destruction, inhibition of synthesis, increased utilization and enhanced synthesis of liver heme will modify the saturation of apotryptophan pyrrolase with heme. Thus the increase of the enzyme is not due to induction but to an increase of heme saturation of the enzyme.

In light of all this data on hemoprotein induction and that on heme synthesis regulation described in the introduction, the following scheme is adduced to explain the experimental observations described in this thesis.

The normal dynamic situation in the cell involves an unassigned heme pool also refered to as regulatory heme. This pool is regulated in size by a balance between its utilization, degradation and its syrthesis which it in turn regulates by feedback inhibition via its size. Any stimulus which diminishes its size results in immediate lift of inhibition and concommitant replenishment of the pool to the steady state level. The drug DDC results in an inhibition of ferrochelatase and thus a decrease in the size of the heme pool. ALA-s is derepressed to replenish the pool, but until the drug is removed, only precursors will accumulate because heme can

not be formed. This results in experimental porphyria. AIA causes destruction of P-450 heme. Either the P-450 protein is recycled by combination with a new heme moiety or else an equilibrium between P-450 holoenzyme and apoenzyme is shifted to cause the formation of more holoenzyme. This is accomplished by an association of apoenzyme with heme of the unassigned pool, thus diminishing its volume and derepressing ALA-s. Other manifold reactions associated with drug metabolism and microsomal oxygenase induction may lead to increased liver size, mitochondrial and organelle proliferation and enhanced protein synthesis. This will be manifested in an increased quantity of ALA-s as a secondary or tertiary response but not a specific induction. Alcohol may act in a similar manner due to its induction of the microsomal oxygenase system during cronic alcohol intake. As far as the single dose response is concerned, it is probably not due to induction of the microsomal oxygenase system but to a more immediate response which is not clear at this time. Chemicals which stimulate drug metabolism and induce P-450 do not lead to porphyria as a rule because no blockage in the heme biosynthetic pathway is effected, the steady state shifts to accomidate the increased demand for cellular heme and ALA-s is stimulated as a result. The regulation of heme formation is constantly responsive to the needs of the cell and is not wasteful.

ALA-s, the enzyme responsible for this delicate and efficient regulation, is very complex requiring the optimum

placement of subunits and positioning in the mitochondrial membrane. This is accomplished by at least one non-catalytic protein which is involved in ionic binding of the catalytic subunits. There may be other aspects of the regulation of ALA-s but this picture is consistent with the data.

ABBREVIATIONS

Ab	Antibody
AIA	Allylisopropylacetamide
ALA	5-aminolevulinic acid
ALA-s	5-aminolevulinic acid synthetase
ATP	Adenosine 5'-triphosphate
CM-Sephadex	Carboxymethyl-Sephadex
CoA	Coenzyme A
Copro'gen	Coproporphyrinogen
DDC	1,4-diethoxycarbonyl-2,5-dihydrocollidine
DEAE-Cellulose	Diethylaminoethyl-Cellulose
DTT	Dithiothreitol
EDTA	Ethlyenediamine tetraacetate disodium salt
IEF	Isoelectric Focusing
mRNA	messenger Ribonucleic acid
PBG	Porphobilinogen
Proto'gen	Protoporphyrinogen
R.E.R.	Rough Endoplasmic Reticulum
S.E.R.	Smooth Endoplasmic Reticulum
SDS	Sodium Dodecyl Sulfate
TCA	Trichloroacetic acid
Uro'gen	Uroporphyrinogen

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