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HEPATIC δ-AMINOLEVULINIC ACID SYNTHETASE: PURIFICATION, CHARACTERIZATION, AND INDUCTION BY PORPHYRINOGENIC DRUGS

by

GEORGE M. PATTON

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Mount Sinai School of Medicine of The City University of New York.

1975

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

April 23, 1975
date

Chairman of Examining Committee

April 23, 1975
date

Executive Officer

The City University of New York

Abstract

The validity of the column procedure of Ebert *et al*. (29) for the assay of δ-aminolevulinic acid (ALA) was examined. It was shown that all of [^{14}C] ALA added to the columns was recovered, and that all of the ^{14}C recovered from an incubation was ALA. It was further shown that the activity of ALA synthetase determined by the method of Ebert *et al*. (29) was in excellent agreement with the ALA synthetase activity determined by the method of Granick (33) when the enzyme was assayed under identical conditions.

The optimal conditions for the assay of δ-aminolevulinic acid were determined for several subcellular fractions, *i.e.* mitochondria, sonicated mitochondria, and microsomes. No activity could be seen in the soluble fraction. Under the conditions employed (15 minutes incubation at 30°C) the reaction was linear with time and protein concentration, and independent of succinate and glycine concentration. The dependence of the reaction on ATP, CoA, and a succinyl-CoA generating system were determined.

The location of ALA synthetase within the cell was examined. The enzyme was shown to be exclusively a mitochondrial enzyme. Further fractionation of the mitochondria revealed that the enzyme is closely associated with the inner membrane but can easily be dissociated by treatment with Lubrol or by sonication.

The activity of ALA synthetase was determined in porphyric rats. When assayed in intact mitochondria, a six to ten fold increase in ALA synthetase was observed with either

succinate, α-ketoglutarate or citrate as substrate when assayed by either the colorimetric or radiochemical assay. When assayed in sonicated mitochondria, a significant increase was observed in ALA synthetase activity with citrate or α-ketoglutarate as substrate, but no increase in activity was observed with succinate as substrate. No significant increase in the microsomal or supernatant fractions were seen in porphyric rats compared to the activity observed with control mitochondria. Cycloheximide blocked the increase in ALA synthetase activity observed in porphyric animals.

Acute ethanol intoxication caused a six fold increase in ALA synthetase activity in whole mitochondria. The increase could be blocked by pyrazole, an inhibitor of ethanol metabolism. Pyrazole also blocked the increase in ALA synthetase in intact mitochondria from rats treated with porphyrinogenic drugs. Pyrazole (3-10 mM) had no inhibitory effect on ALA synthetase when added to the incubation mixture with either intact or sonicated mitochondria.

The effect of changing the intramitochondrial redox potential and the energy charge on the activity of ALA synthetase was examined. When the mitochondrial redox state was highly reduced the enzyme was inhibited, and when the energy charge was high the enzyme was stimulated in intact mitochondria derived from both control and porphyric rats. Calcium had no effect on the activity of the enzyme.

In the course of trying to purify ALA synthetase, it was discovered that two different fractions were required for ALA synthetase activity, one fraction containing the enzyme and the other an activator. Both the enzyme and the

activator were purified from both control and porphyric rats. No significant qualitative or quantitative difference was observed in either ALA synthetase or the activator isolated from control and porphyric rats. A fraction obtained by extensive sonication of an ALA requiring mutant of _R. Spheroides_ was found to activate ALA synthetase from rat liver.

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I. Purpose

Delta-aminolevulinic acid synthetase is the first and presumably rate limiting enzyme in the biosynthesis of heme. In the mammalian liver, heme is the prosthetic group for a number of enzymes which serve several types of metabolic functions:

(1) production of energy via the mitochondrial respiratory chain,

(2) oxidative metabolism of some amino acids and other small molecules and the biosynthesis of unsaturated fatty acids,

(3) drug detoxification via the electron transport chain and mixed function oxidases of the endoplasmic reticulum.

Some heme containing enzymes (the cytochromes of the mitochondrial respiratory chain) occur in relatively constant levels throughout the life of the cell, while the level of other heme containing enzymes (the cytochromes of the endoplasmic reticulum) changes radically under certain conditions. Moreover, the activity of δ-aminolevulinic acid synthetase increases radically in certain disease states (hepatic porphyrias) and in response to the administration of a number of chemicals and drugs. This study is an attempt to gain some understanding of the mechanisms regulating the levels of δ-aminolevulinic acid synthetase activity.

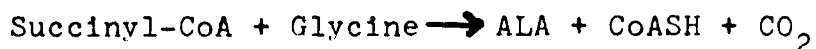
II. Introduction

A. Heme Synthesis and δ-Aminolevulinic Acid Synthetase

Biochemical studies of porphyrin synthesis were put on a firm experimental basis by the classic studies of Shemin and co-workers on the precursors of heme biosynthesis in human and duck erythrocytes (1). Shemin developed a procedure for the chemical analysis of hemes which permitted the unambiguous identification of all of the carbon and nitrogen atoms in protoporphyrin IX (2). Using ^{15}N (3) and ^{14}C (4) labeled glycine and ^{14}C labeled acetate (5), he demonstrated that all of the atoms of heme could be accounted for by these two precursors, and that the α-carboxyl group of glycine was totally lost during the early steps of heme biosynthesis. Further studies demonstrated that in addition to glycine the other immediate precursor of heme biosynthesis was "active" succinate, which was later shown to be succinyl-CoA (6). The product of the condensation of succinyl-CoA and glycine was identified as δ-aminolevulinic acid (ALA) which was subsequently shown to be an active precursor of heme (7,8). The enzyme which catalyzed the reaction, δ-aminolevulinic acid synthetase (ALA synthetase), was subsequently demonstrated in the photosynthetic bacteria Rhodopsuedomonas Spheroides and Rhodospirillum Rubrum (9,10), in chicken erythrocytes (11,12), and later in guinea pig liver (13) and rat liver (14).

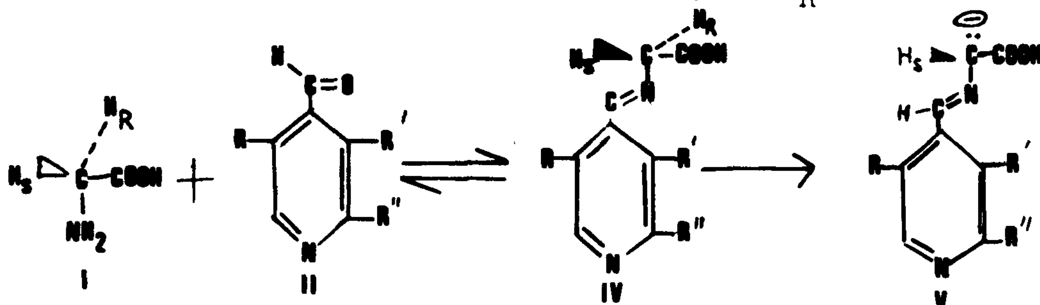
It has long been known (15,16,17) that, in addition to succinyl-CoA and glycine, pyridoxal phosphate was required for the formation of ALA in a number of organisms. The overall

reaction of ALA synthetase is:

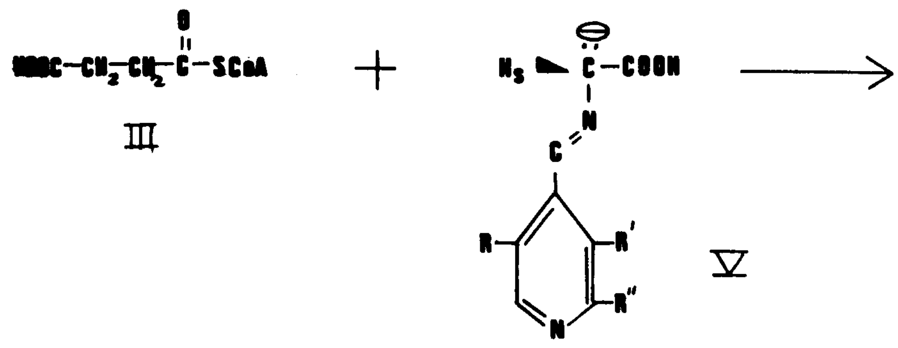


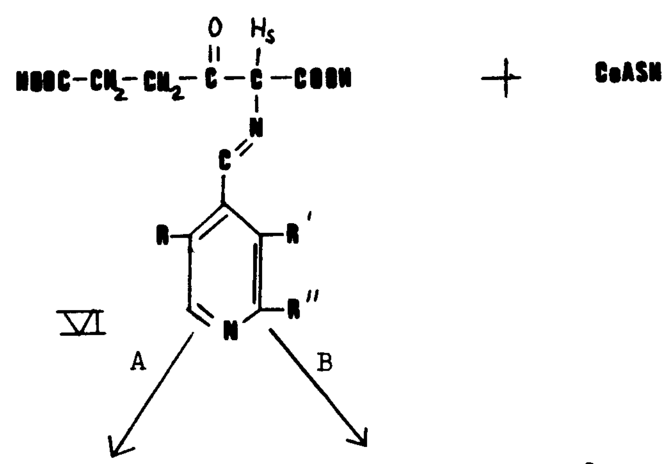
$$\text{Succinyl-CoA} + \text{Glycine} \rightarrow \text{ALA} + \text{CoASH} + CO_2$$

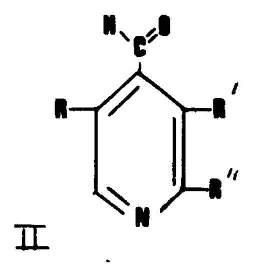
The mechanism of ALA synthetase has not been extensively studied until relatively recently, because the enzyme was not available in a pure form. Recently however, the mechanism of ALA synthetase from Rhodopseudomonas Spheroides has been studied by Zaman et al. (18) who proposed a reaction mechanism which presumably is similar to the mechanism of the enzyme from other sources. According to this mechanism, glycine (I) reacts with enzyme bound pyridoxal phosphate (II) to form a Shiff's base (IV) which loses a hydrogen (H_R^+) to yield a

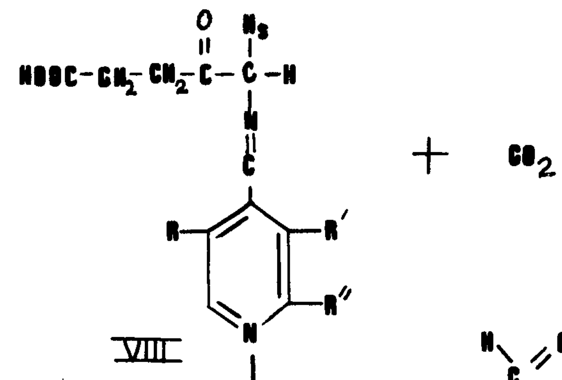


carbanion (V). Succinyl-CoA (III) is then bound to the enzyme, and reacts with the carbanion (V) to give (VI) with the release of CoASH. The pyridoxal phosphate complex (VI) either (A) decomposes to pyridoxal phosphate (II), and α-amino-β-keto adipic acid (VII) which then spontaneously decarboxylates to ALA or, alternatively, (B) (VI) decarboxylates to (VIII), which is then hydrolysed to pyridoxal phosphate (II) and ALA (IX). The work of Scholnick et al. (19) with purified rat liver ALA synthetase tends to support the first alternative pathway, i.e. (VI) hydrolyses to (VII) which then spontaneously

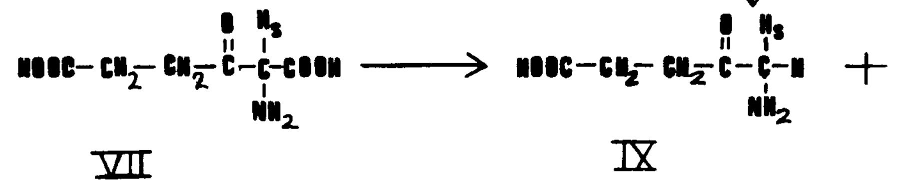

HOOC–CH2–CH2–C–SCoA
III
HS
C–COOH
V


HOOC–CH2–CH2–C–C–COOH
CoASH
VI
A
B


II


HOOC–CH2–CH2–C–C–H
CO2
VIII

+


HOOC–CH2–CH2–C–C–COOH
NH2
VII
HOOC–CH2–CH2–C–C–H
NH2
IX

decarboxylates on release from the enzyme. Their conclusions are based on the finding that ALA synthetase has no affinity for ALA. This would be predicted with pathway A, but would not be expected with pathway B, which predicts that ALA synthetase would have a high affinity for ALA. Using ^{14}C carboxyl labeled glycine, they also confirmed that the carboxyl group of glycine is not lost until after the reaction of the carbanion (V) with succinyl-CoA. Furthermore, they suggested that free pyridoxal phosphate is bound to the enzyme by a thiohemiacetal linkage to a free sulfhydral group of the enzyme.

Following the identification of ALA synthetase as the first step in the biosynthesis of porphyrins, Sano and Granick (21) determined the subsequent steps of heme biosynthesis which are outlined below.

1. Glycine + Succinyl CoA $\xrightarrow{\text{ALA Synthetase}}$ ALA + CO_2
2. 2 ALA $\xrightarrow{\text{ALA Dehyratase}}$ Porphobilinogen + H_2O
3. 4 Porphobilinogen $\xrightarrow[\text{Uroporphyrinogen Cosynthetase}]{\text{Uroporphyrinogen I Synthetase}}$ Uroporphyrinogen III + $4NH_3$
4. Uroporphyrinogen $\xrightarrow{\text{Uroporphyrinogen Decarboxylase}}$ Coproporphyrinogen III + CO_2
5. Coproporphyrinogen $\xrightarrow{\text{Coproporphyrinogen Oxidase}}$ Deuteroporphyrinogen + CO_2
6. Deuteroporphyrinogen $\xrightarrow{\text{Deuteroporphyrinogen Decarboxylase}}$ Protoporphyrinogen + CO_2
7. Protoporphyrinogen $\xrightarrow{O_2}$ Protoporphyrin IX
8. Protoporphyrin IX + Fe^{++} $\xrightarrow{\text{Ferrochelatase}}$ Heme

Many of the steps in prophyrin biosynthesis are not well understood, and it is not certain that all of the steps are determined or that all of the intermediates have been identified. Vitamin B_{12} is synthesized from uroporphyrinogen III by certain bacteria, and chlorophyll is synthesized from protoporphyrin IX by both plants and bacteria.

In the mammalian liver, the enzymes of heme biosynthesis are distributed within the cell between the cytosol and the mitochondria (21). ALA synthetase, which is the rate limiting step in the overall pathway (20), is located in the mitochondrial matrix (22,23). The next four enzymes, catalyzing the conversion of ALA to coproporphyrinogen III, are soluble enzymes of the cytoplasm. The last three enzymes are again found in the mitochondrion. Of these, the exact location within the mitochondrion has been established only for ferrochelatase, which has been shown to be a constituent of the inner membrane (23,24). The mechanism by which heme precursors are transported across the mitochondrial membrane and what relation this may have to the control of heme biosynthesis is unknown.

In higher animals (birds and mammals), ALA synthetase has been extensively studied only in the liver and erythropoetic tissues <u>i.e.</u> reticulocytes (25), red cells (26), bone marrow (27,28), and spleen (29). Recently, ALA synthetase has been observed in rat kidney (24). All of these tissues are involved either in hemoglobin synthesis, or are tissues which are known to be active in detoxification (liver and kidney), where active heme synthesis is clearly indicated. ALA synthetase has not been demonstrated in other tissues.

Barnes et al. (29) have detected ferrochelatase activity in the heart and brain of rats, however, and uroporphyrinogen III cosynthetase has been studied in the Harderian gland of the hamster (30). The presence of these enzymes of heme biosynthesis in non-erythropoetic tissues suggests that most if not all tissues are capable of heme biosynthesis at least to the extent required for the hemes of the respiratory chain.

B. ALA Synthetase and Porphyria

Much of the interest in hepatic ALA synthetase is prompted by the role of ALA synthetase in hereditary hepatic prophyrias, which are a group of hereditary conditions characterized by disturbances in heme biosynthesis. Four types of porphyria have been described clinically (31,32); (i) acute intermittent porphyria, which is characterized by the excretion of large amounts of ALA and porphobilinogen in the urine, (ii) hereditary coproporphyria, characterized by the excretion of ALA, porphobilinogen, uroporphyrinogen, and coproporphyrin in the urine and feces, (iii) variegate porphyria, characterized by the excretion of ALA, porphobilinogen, uroporphyrinogen, coproporphyrin, and protoporphyrinogen; and (iv) porphyria cutania tarda, characterized by uroporphyrinogen and coproporphyrin excretion in the feces.

A good experimental model for hepatic porphyrias has been available since the discovery by Granick (20) that a number of drugs and chemicals, when administered to rats, caused the excretion of large amounts of porphyrin precursors

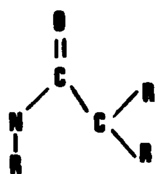
and an increase in the activity of hepatic ALA synthetase. This observation prompted Tschudy _et al_. (34) to examine the levels of ALA synthetase in patients with acute intermittent porphyria. They did indeed find increased activity of ALA synthetase in porphyric patients. Although these studies implicated increased ALA synthetase activity as the enzymatic defect causing the overproduction of porphyrin precursors, they failed to explain the pattern of excretion (ALA and porphobilinogen) found in patients with acute intermittent porphyria. This pattern suggested that there may be a block in the metabolism of porphobilinogen to uroporphyrinogen III. This possibility was explored by Strand _et al_. (35), who discovered a decreased level of hepatic uroporphyrinogen I synthetase in patients with acute intermittent porphyria. They postulated, therefore, that the primary enzymatic defect was at the level of uroporphyrinogen synthetase, and that the resulting decrease in total heme synthesis resulted in a derepression of ALA synthetase. Their suggestion received further support when it was shown that in patients with acute intermittent porphyria the level of uroporphyrinogen I synthetase is decreased in the red cell as well as the liver, but that the activity of ALA synthetase in the red cell of patients with an acquired form of porphyria is normal (36).

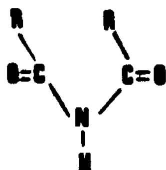
Joubert _et al_. (37) using similar reasoning, have characterized porphyria cutanea tarda as resulting from an abnormal uroporphyrinogen decarboxylase. The defective enzyme causes the formation of unusual isomers of uroporphyrinogen and coproporphyrinogen which cannot be further metabolized. The resulting block in heme synthesis then causes the increase

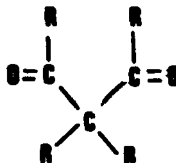
in ALA synthetase observed in these patients. Similarly, Matteis (38) was able to induce an experimental variegate porphyria by inhibiting ferrochelatase and inducing ALA synthetase. The pattern of excretion of heme precursors was identical to that observed in patients with variegate porphyria.

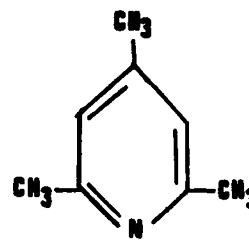
Interest in chemical porphyria as a model system for hepatic porphyrias has persisted, however, because of the remarkable similarities between chemical porphyria and the hereditary porphyrias. Induction of chemical porphyria is caused by the same drugs and chemicals which precipitate acute attacks in patients with porphyria (33). Feeding a high carbohydrate diet alleviates or prevents the symptoms of both experimental and hereditary porphyria (39). Moreover, porphyria is a post puberty condition, implying the participation of steroids. Patients with acute intermittent porphyria have high levels in their blood of the same steroids which have been shown to induce chemical porphyria in cultured chick embryo liver cells (33,40). Consequently, the regulation and mechanism of induction of ALA synthetase in experimental porphyria has been intensively studied.

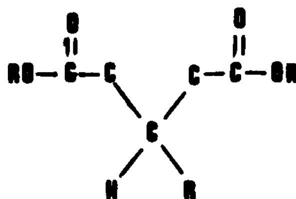
Granick (33) systematically tested a large number of chemicals and drugs for their ability to induce experimental porphyria in cultured chick embryo liver cells. As a result of that study, he classified the different inducers into four groups: steroid hormone metabolites, collidine derivatives, barbiturates, and a miscellaneous group. He further observed that the ability to induce chemical porphyria could be conferred on a molecule if it contained one or more of five chemical groupings. The barbiturates are inducers because they contain











groups A,B,C. Likewise, the collidine derivatives are inducers because they contain groups D and/or E. The miscellaneous class induce by virtue of possessing one or more of the groups, or because they are unsaturated, chlorinated hydrocarbons (mostly used as fungicides, herbicides, or insecticides). Steroids were shown to be inducers if they contained the 5-βH configuration, were fully saturated C_{19}, C_{21} or larger with an alcohol or carboxyl group at position 3,11,17, or 20. All 21 hydroxy steroids or unsaturated steroids are inactive, as are all carboxylated derivatives or the glucuronides of all steroids, even the ones which would otherwise be active inducers (41).

In his early studies on the induction of ALA synthetase by allylisoproplyacetamide (AIA) in chick embryo liver cells, Granick (33) reported that the induction of ALA synthetase began after a lag time of 6-8 hours, and then increased logarithmically for 12-22 hours. Addition of actinomycin D,

an inhibitor of RNA synthesis, or cycloheximide, an inhibitor of protein synthesis, prevented the induction. Furthermore, when cycloheximide was added during the logrithmic phase of the induction, the activity of ALA synthetase decreased with a half life of 4-6 hours. The inducer did not activate ALA synthetase directly, nor did it prolong the half life of the enzyme after administration of cycloheximide. On the basis of this data, Granick postulated a model for the regulation of ALA synthetase activity which, with certain modifications, has become the working hypothesis for most subsequent studies.

According to this model, the activity of ALA synthetase is controlled by the level of ALA synthetase in the mitochondrion. Since the half life of the enzyme is very short (4-6 hours), the enzyme level is regulated by the rate of synthesis. The rate of synthesis is regulated by a repressor consisting of an aporepressor and a corepressor (heme), which prevents the translation of the messenger RNA for ALA synthetase. Inducers act by preventing the binding of the corepressor (heme) to the aporepressor. Steroids induce by combining with a repressor on the operator gene allowing the expression of the structural gene, resulting in the production of more m-RNA for ALA synthetase. Hereditary prophyria is attributed to a faulty repressor protein.

C. Role of Steroids in Porphyria

The role of steroids in the induction of porphyria is particularly interesting because they are the only naturally occurring inducers, and because sex hormones have been shown to exacerbate acute intermittent porphyria. Granick and Kappas (41), and Kappas and Granick (42) have studied steroid induction of ALA synthetase in cultured chick embryo liver cells in considerable detail. Embryonic chicken liver is a particularly good choice for these studies in that the level of steroid metabolism in that tissue is so low that individual steroids can be studied without complications arising from their breakdown products.

None of the steroid hormones secreted by the adrenal cortex or gonads had significant inducing activity, nor did the degradation products of cholesterol. The steroids which did have activity, however, were metabolites of the hormones, especially testosterone and progesterone. These inducing metabolites are characterized by having the 5β-H configuration and alcohol or keto substituents at carbons 3,11,17 or 20. All steroids with the 5β-H configuration or a hydroxyl group at carbon 21 were inactive. The steroids which have porphyria inducing activity are generally considered physiologically inert by-products which result from the metabolism of the steroid hormones in the liver. They are normally excreted as the glucuronides, and glucuronidation of even the most potent inducers abolishes all inducing activity.

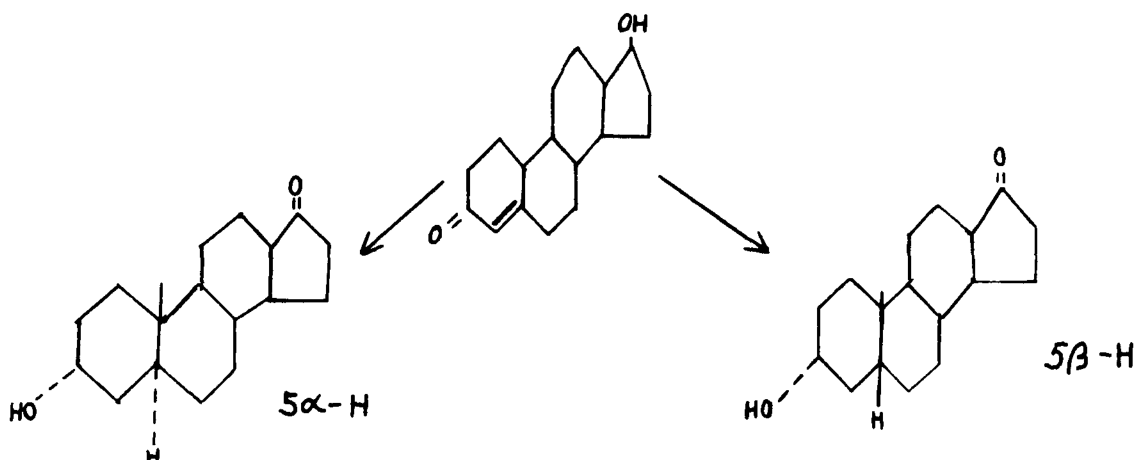
The most active steroid inducers are effective at con-

centrations below 10^{-8}M. The induction of porphyria by these compounds is prevented by inhibitors or RNA and protein synthesis, as well as by heme and protoheme, but not by glucose. The induction with suboptimal levels of steroid is additive with suboptimal doses of DDC or AIA. Unlike all other inducers of porphyria, the 5β-H steroids have been shown to stimulate heme synthesis in chick blastoderm erythroid cells (43,44), and in mouse and rat erythroid cells (45,46).

Kappas and Granick (47) also found that UDP-glucuronic acid, but none of its derivatives, was able to prevent porphyria induction. They attributed this result to an increased rate of removal of the steroid inducer by glucuronidation catalyzed by UDP-glucuronyl transferase. It has been shown by Arias et al. (47) and Hsia et al. (48) that 5-βH steroids at moderate concentrations (10^{-6}-10^{-4}M) inhibit UDP-glucuronyl transferase. This led Granick (42) to suggest that if for some reason (over production of 5β-H steroids or a decrease in UDP glucuronic acid) more 5β-H steroids were produced than could be metabolized, they would accumulate and inhibit UDP-glucuronyl transferase and start a cycle of increasing 5β-H steroid levels which could induce porphyria.

The strong implications that 5β-H steroids may be the immediate physiological inducers of porphyria prompted Kappas et al. (49) to examine steroid metabolism in patients with acute intermittent porphyria. The neutral steroid hormones have a double bond between ring A and B. In the liver, they are degraded by an enzyme or enzymes Δ^4-5α reductase and/or Δ^4-5β reductase, where the 5α metabolite is

inactive and the 5β metabolite induces porphyria. In normal



people, the ratio of the 5α to 5β metabolite is 1:1 when [^{14}C] testosterone is injected and the metabolites isolated. In patients with hereditary porphyria the ratio was 1:2 to 1:4. Patients with an acquired form of porphyria had a 5α to 5β ratio of 1:1. Similar results were obtained with [^{14}C] 11-OH, androstenedione. They attributed the change in the ratio of 5α:5β metabolites to a decrease in the activity of Δ^4-5α reductase.

Although the 5β-H steroids are potent inducers of porphyria in embryonic chicken liver and in several erythropoetic tissues as well as in man, they are apparently without effect in rat or mouse liver. It is clear, however, that steroids are involved in porphyria in rats. Stein et al. (50) and Matsuoka et al. (51) have reported that hydrocortisone potentiates induction of porphyria when administered together with DDC (50) or AIA (51). Moreover, the later the hormone was administered after the inducer, the less pronounced the induction. If the hormone was administered two hours after the inducer, no increase in induction was seen above the level without inducer. Weisman et al. (52) demonstrated

similar results using 5β-H steroids in mice. Bock *et al*. (53), using isolated perfused rat liver, were unable to demonstrate any induction of ALA synthetase by AIA unless they included dexamethasone or hydrocortisone in the perfusion medium. Likewise, Padmanaban *et al*. (54) examined porphyria induction by AIA in ovarectomized and adrenalectomized rats. They found no induction of ALA synthetase activity unless they administered cortisone along with the inducer, even though several other physiological changes produced by AIA could be demonstrated in the absence of cortisone. Matsuika *et al*. (51) observed that ALA synthetase induction in adrenalectomized rats was almost abolished unless hydrocortisone was administered along with the inducer (AIA).

More direct evidence for the involvement of steroids in the induction of prophyria in the rat resulted from the work of Moore *et al*. (55). They found that dehydroepiandrosterone, which is metabolized by P-450 and excreted as the glucuronide, induces porphyria in rats, and that patients with hereditary porphyria contained elevated levels of the steroid in their blood.

D. Role of Carbohydrate in Preventing Experimental Porphyria.

One of the first properties observed regarding the induction of ALA synthetase was that a high carbohydrate diet prevented the induction of the enzyme by drugs (14), and also alleviated the symptoms of an attack of acute intermittent porphyria (57). Marver *et al*. (39) examined glucose inhibition of prophyria

in the liver of AIA treated rats. They observed that in porphyric rats there was a 65% increase in liver weight and liver protein. The increase in liver protein was distributed equally in all cellular fractions except the mitochondria, which showed a 15% increase. ALA synthetase activity was also increased. Administration of glucose prevented the increase in ALA synthetase activity, as well as the increase in liver weight and protein content. When rats were treated with AIA for 12 hours and then injected with puromycin, an inhibitor of protein synthesis, there was an immediate drop in ALA synthetase activity, with a half life of 67-72 minutes. When the same experiment was performed with actinomycin D instead of puromycin, the level of ALA synthetase activity remained constant for two to three hours and then decreased with a half life of 72-74 minutes. When the experiment was repeated again with glucose instead of an inhibit or, the same pattern was observed as when actinomycin D was injected, indicating that glucose inhibition is primarily an effect on transcription.

In a later study, Bonkowsky <u>et al</u>. (58) examined a number of carbohydrate metabolites to see where the glucose effect was exerted . C-AMP had no effect on the induction process with AIA, nor did it effect the inhibition of induction by glucose, although theophylline did inhibit the induction. Kornek and Moses (59) have shown that the inhibition of porphyria seen with theophylline is not related to its ability to inhibit phosphodiesterase, as other more potent phosphodiesterase inhibitors are without effect. They also observed no effect of C-AMP on ALA synthetase induction. On the other hand, Pinelli and Capuano (60) have claimed a moderate inhibitory role for C-AMP.

Of a large number of sugars, glycolytic and Krebs cycle intermediates and fatty acids studied, only glucose, fructose and glycerol had a significant inhibitory effect. 2-deoxyglucose was as effective as glucose in preventing ALA synthetase induction. The further removed metabolically from glucose an intermediate is, the less effective it is in preventing the increase in activity of ALA synthetase. On the basis of their data and the known gluconeogenic capabilities of the metabolites tested, Bonkowsky _et al_. concluded that the glucose effect is indeed an effect of glucose itself (or possibly glucose-6-PO_4).

Cowger _et al_. (61), working with cultured L cells, observed the same sort of increases in cell protein and cell weight described by Marver _et al_. (39). They also observed an increase in cellular RNA and fatty acid synthesis (62), but did not see an increase in DNA or the number of cells. They further observed, that the administration of AIA resulted in a decrease in mitochondrial oxidative metabolism due to a block in NADH metabolism in the mitochondria. This resulted in a decrease in ATP concentration, and an increase in glycolysis (presumably for the synthesis of more ATP). Cowger and Labbe (63) extended their results with AIA to a number of porphyrogenic drugs. They were able to correlate potency in inducing ALA synthetase with potency in inhibiting NADH dehydrogenase from beef heart submitochondrial particles, and were further able to correlate potency in inhibiting NADH dehydrogenase with the ability to stimulate glycolysis in cultured L cells. Thus it appears that chemicals which induce porphyria also put a drain on cellular glucose reserves by stimulating

glycolysis. These results tend to support the hypothesis of Granick (33) that the glucose effect is related to the production of glucuronic acid which is used to inactivate and remove the physiological inducer by glucuronidation. In starved animals there would be little glucose availabe for metabolism to glucuronic acid. If porphyria inducing chemicals cause a further drain on glucose by stimulating glycolysis, the cell may not be able to make sufficient glucuronic acid to remove the steroid inducer, and a cycle would be set up which results in porphyria (as discussed above).

E. Studies on the Turnover of ALA Synthetase.

One of the peculiar properties of ALA synthetase, and one which is central to the model of ALA synthetase regulation proposed by Granick (33), is the short half life of the enzyme. Tschudy *et al*. (56) made a careful study of the turnover rates of ALA synthetase and the m-RNA for ALA synthetase. The results obtained with puromycin and actinomycin D indicated that ALA synthetase has a half life of 40-70 minutes. Similar results have been reported by Beattie and Stuchell (66), and by Hayashi *et al*. (65) for the half life of ALA synthetase. The results of Beattie and Stuchell also showed that when chloramphenicol, an inhibitor of mitochondrial protein synthesis, is added instead of cycloheximide, that the activity of ALA synthetase decreases with a half life of 120 minutes. As ALA synthetase is synthesized on cytoplasmic not mitochondrial ribosomes, their results indicate that some product of mitochondrial protein synthesis is required for translation of

ALA synthetase, or for the expression of ALA synthetase activity. The results of Hayashi _et al_. further complicate the calculations of the half life of ALA synthetase. They have reported that after treatment with AIA, ALA synthetase accumulates in the cytoplasm. When they measured the half life of this soluble ALA synthetase they found it had a half life of only 20 minutes. The results of these two studies, indicate that the simplifying assumptions which Marver used to calculate the half life of the mRNA may not be valid, namely that ALA synthetase is a one component system with a constant rate of decay.

Sassa and Granick (67) using cultured chick embryo liver cells examined the half life of ALA synthetase and its mRNA using cycloheximide and actinomycin D. In the course of their experiments, they discovered that enzyme induction by certain groups _e.g._, Lindane (miscellaneous class) and AIA (barbiturate class) was essentially unaffected by actinomycin D, indicating that these inducers act at the translational level. Etiocholanolone (steroid class) and DDC (collidine class), on the other hand, induce at the transcriptional level. When protein synthesis was inhibited by cycloheximide, and a transcriptional level inducer was added, it was found that mRNA for ALA synthetase accumulated in the cytoplasm. When the cycloheximide was washed out, and a translational level inducer was added, the rate of ALA synthetase formation increased drastically. By adding a translational inducer and actinomycin D (to prevent mRNA synthesis) after the cycloheximide was washed out, the half life of the mRNA could be determined

directly. Using these methods, Sassa and Granick determined the half life of ALA synthetase to be three hours and the half life of the mRNA was found to be five hours. Similar results were obtained by Strand *et al*. (68) using the same experimental system. Sassa and Granick also found that hemin inhibits the induction of ALA synthetase, and that the kinetics of inhibition closely resemble the kinetics of inhibition observed with cycloheximide, indicating that heme inhibits at the translational level.

F. Mechanism of the Enzyme Induction

Early studies on the drug induced increase in ALA synthetase activity were directed towards characterizing the kinetics of the induction process, characterizing the level of action of the drug, and examining possible effectors of the process. Little information, however, was developed relating to the mechanism of induction. Recently, several significant studies have helped explain the mechanism of induction by the two most widely used inducers AIA and DDC. One of the points developed by early researchers was that in addition to inducing ALA synthetase, porphyria inducing drugs (with the exception of DDC) also induce cytochrome P-450; however, many inducers of P-450, such as phenobarbital, do not cause a significant induction of ALA synthetase. The difference between these two classes is that prophyria inducing drugs cause a transient decrease in the level of P-450 (69). There are also strong indications that heme plays a central role in

the induction process since heme has been shown to inhibit ALA synthetase induction *in vivo* (67), and to exert feedback inhibition on ALA synthetase *in vitro* (71,72). High concentrations of tryptophan are known to cause the rapid activation of tryptophan pyrrolase by causing the insertion of free heme into the apo enzyme, resulting in a transient decrease in free heme levels. Marver *et al*. (70) reported that administration of tryptophan to rats pretreated with porphyria inducing drugs at levels insufficient to cause induction, resulted in a rapid induction of ALA synthetase activity.

Recently, the relationship between cellular heme concentration and the biochemical effects of the porphyrogenic drugs has been intensively studied. Narisawa and Kikuchi (73) demonstrated a two phase induction process in rats treated with AIA. The first phase (six to eight hours) is sensitive to inhibitors of protein synthesis, but relatively insensitive to inhibitors of RNA synthesis, while the second phase (18-24 hours) is acutely sensitive to inhibitors of both RNA and protein synthesis. Both phases of induction are reversed by the administration of heme, although the second phase is more sensitive (65). Hayashi *et al*. (65) also demonstrated that although heme prevented the AIA induced increase in ALA synthetase, it did not prevent the induction of glucose-6-PO_4 dehydrogenase, NADPH : cytochrome C reductase, or ALA dehydratase which are also induced by AIA. This indicates that heme does not provide cell wide protection against the effects of AIA, but that heme is a specific effector of the ALA synthetase system which controls the activity of ALA synthetase.

Several groups of researchers (74,75,76,77) have reported that AIA causes a rapid, transient decrease in cytochrome P-450 in the liver, as a result of the destruction of preexisting P-450 heme, and that there is a concomitant accumulation of certain unspecified green pigments in the cytoplasm (76,78). Matteis (78) has suggested that the destruction of P-450 heme is a result of a metabolite of AIA, or the result of the metabolism process. This conclusion is based on the observation that the breakdown of heme is more rapid in rats which were previously treated with compounds that induce P-450. Moreover, he observed that SKF-525-A, a drug which blocks AIA metabolism, prevents the destruction of P-450, and that addition of NADPH, a substrate for P-450 linked drug metabolism greatly stimulates the breakdown of P-450. The breakdown of P-450 heme is implicated as a step in the AIA induced increase in ALA synthetase activity because SKF-525-A which prevents the heme destruction also inhibits the increase in ALA synthetase activity (77,78), and because analogues of AIA which are weak inducers of ALA synthetase are also less effective in causing the breakdown of cytochrome P-450 heme (79). Moreover, neonatal rats (5 days) cannot metabolize AIA due to very low levels of P-450, and consequently, there is no breakdown of heme and no induction of ALA synthetase (54).

These results have led Padmanaban et al. (54,80) to postulate a mechanism for the AIA induced increase in ALA synthetase activity. They maintain that the increase in ALA synthetase activity is the result of an increase in the rate of translation of ALA synthetase mRNA which is

caused by increased levels of apo-cytochrome P-450 (or some other apo-enzyme which requires heme). AIA causes the accumulation of apo-enzyme by destroying the enzyme bound heme, and also by inducing an increase in the rate of apo-cytochrome P-450 synthesis. Heme prevents the induction by complexing with the apo-enzyme. They caution, however, that the breakdown of liver heme is not sufficient to cause an increase in ALA synthetase activity, as the induction of ALA synthetase is dependent upon protein synthesis. Their hypothesis fails to explain why glucose prevents the induction however, and also why cortisol is required for induction. Perhaps the formation of apo-cytochrome P-450 is dependent on cortisol, or perhaps a steroid metabolite is required for the stimulation.

Like AIA, DDC has been shown to cause a rapid decrease in cytochrome P-450 levels; however, it is not an inducer of P-450 (69). Sassa and Granick have also shown that DDC acts at the transcriptional level as opposed to AIA which acts at the translational level (67). But unlike AIA, DDC has also been shown to inhibit heme synthesis at the level of ferrochelatase (81,82), and thus to cause the accumulation of protoporphyrin in the liver. Matteis _et al_. (83) have argued that the induction of ALA synthetase by DDC is a consequence of the destruction of P-450 heme, combined with the inhibition of heme synthesis at the level of ferrochelatase. They observed that mice are more sensitive than rats to the porphyrogenic effects of DDC, and that rat liver ferrochelatase is correspondingly less sensitive to the inhibitory effects of DDC. Moreover, SKF-525-A, which inhibits the metabolism of drugs by P-450, prevented porphyria in the rat and prevented the inhibition

of ferrochelatase. It was less effective in preventing both porphyria and the inhibition of ferrochelatase in mice. These results led Matteis to conclude that the inhibition of ferrochelatase is caused by a metabolite of DDC, and that induction of porphyria is directly related to the degree of inhibition of ferrochelatase. They obtained similar results (unpublished) with griseofulvin, another porphyrogenic drug which inhibits ferrochelatase.

Support for the hypothesis that a crucial step in the induction of porphyria is a block in heme synthesis comes from the work of Strand _et al_. (68). They observed that a number of compounds which inhibit various steps in heme synthesis cause an increase in ALA synthetase activity and the accumulation of heme precursors before the site of inhibition. Addition of the precursors of heme synthesis which occur after the site of inhibition decrease the activity of ALA synthetase. For example, lead acetate, which inhibits the conversion of ALA to porphobilinogen, causes an increase in ALA synthetase activity which is completely inhibited by cycloheximide but unaffected by actinomycin D. This indicates that a block in heme synthesis increases ALA synthetase by a translational control mechanism. Moreover, administration of lead acetate reversed the actinomycin D inhibition of AIA induced porphyria.

Since DDC has been shown to be an inducer of ALA synthetase at the transcriptional level (67), Nawata and Kato (84) undertook a study of the effects of DDC on RNA metabolism in rats. They observed that in the time between the administration of DDC and the increase in ALA synthetase activity that there

was a rapid rise in the rate of RNA synthesis which returned to the control level before ALA synthetase induction. They noted that the rapidly labeled [^{32}P] RNA accumulated in the nucleus and remained there for some time. A similar increase of labeled RNA in the cytoplasm was not observed at any time. Moreover, after analysis of labeled polyribosomal RNA on sucrose density gradients, no difference in the amount or distribution of the labeled RNA was observed when comparing control and porphyric rats.

In an attempt to clarify some of the contradictory information on the induction of ALA synthetase, Kikuchi <u>et al</u>. (85) undertook a comparative study of the induction process in rat liver with AIA and DDC as inducers. In previous studies with AIA they showed that as much as 35% of the ALA synthetase accumulates in the cytoplasm during induction (65); that the soluble enzyme had a half life of 20 minutes, and the mitochondrial form had a half life of 68 minutes; that heme, when administered along with AIA, prevented the induction of ALA synthetase; and that when heme was administered two to four hours after the inducer it had no effect on the total cellular ALA synthetase activity. Closer examination revealed that the soluble ALA synthetase was accumulating in the cytoplasm as if no heme were present, while the mitochondrial ALA synthetase was declining rapidly with a half life of 20 minutes (86). Kikuchi <u>et al</u>. interpreted this to mean that heme prevented the conversion of the soluble form (M.W. = 250,000) to the mitochondrial form (M.W. = 115,000). This same group, working with cock liver, observed a time dependent increase in the molecular weight of the soluble ALA synthetase from 170,000 to 250,000. This change in molecular weight was

prevented by heme (88). The addition of heme also changed the apparent half life of the soluble form from 20 minutes to 120 minutes. C-AMP exhibited a similar but less pronounced effect when administered several hours after AIA (87). When DDC was the inducer, however, there was only a slight increase in the soluble form of ALA synthetase, and neither heme nor C-AMP caused any further accumulation in the cytoplasm. While the mitochondrial form of ALA synthetase has a half life of 60 minutes when AIA is the inducer, it has a half life of 120 minutes when DDC is the inducer. Likewise, with AIA the half life of soluble ALA synthetase was 20 minutes, but with DDC it was 60 minutes.

When they performed the same experiments in liver cell culture instead of in vivo, they found that there was no accumulation of ALA in the soluble fraction with either inducer whether or not heme was present. They also observed that the half life of mitochondrial ALA synthetase was about twice that obtained in vivo i. e., two hours with AIA and four hours with DDC. Moreover, the molecular weight of the mitochondrial enzyme in cultured liver cells was 250,000 as compared to a molecular weight of 115,000 observed in rat liver or cock liver (88). It is, therefore, apparent that there are not only significant differences in the mechanism of induction by DDC or AIA, but also differences in the mechanism of induction in vivo and in cell culture.

Any real understanding of the regulation of ALA synthetase or the regulation of the induction of the enzyme by porphyrogenic drugs will ultimately depend on the isolation and characterization of the components involved. Progress along

this line has been made in several laboratories. As noted above, Kikuchi first observed the existence of two forms of ALA synthetase, a soluble form (M.W. 178-600,000) and a mitochondrial form (M.W. = 115,000). Subsequently Scholnick *et al*. (71) partially purified the soluble form of the enzyme from rat liver. They found the partially purified enzyme has a molecular weight of 500,000, but that it dissociates in 0.8 M NaCl to a complex with a molecular weight of 300,000 (89). The enzyme had a K_m of 1 X 10^{-2} M for glycine, 7 X 10^{-5} M for succinyl-CoA, and 3 X 10^{-6} M for pyridoxal phosphate, and a K_I for inhibition by heme of 2 X 10^{-5} M (89). The inhibition by heme was competitive with pyridoxal phosphate (19), but was unaffected by glycine or succinyl-CoA. The enzyme is maximally active in 0.3 M NaCl. Soluble ALA synthetase was further purified by Whiting and Elliot (72) and shown to have a molecular weight of 178,000. They also purified the enzyme from the mitochondria of DDC treated rats. The mitochondrial enzyme was shown to have a molecular weight of 77,000 after solubilization of a large aggregate by 10^{-4} M DTE and 0.8 M NaCl. They obtained a Km of 1.9 X 10^{-2} M for glycine, 2 X 10^{-4} M for succinyl-CoA, and 10^{-6}-10^{-5} M for pyridoxal phosphate. The K_i for heme inhibition was 1 X 10^{-5} M. Whiting and Elliot obtained antibodies to the purified mitochondrial enzyme and demonstrated that it cross reacted with the purified soluble ALA synthetase, although it required four to five times as much antibody. They were unable to demonstrate any immunologically active precursor of ALA synthetase in the cytoplasm of non-induced

animals, although the mitochondrial ALA synthetase of non-induced animals reacted with the antibody to the induced mitochondrial enzyme.

Although there is considerable confusion regarding many details of the properties of ALA synthetase, and the mechanism of its induction, there does seem to be general agreement on several points:

(1) steroids in some form are a necessary prerequisite for the induction of ALA synthetase,

(2) heme, at least in vivo, is able to block the induction of ALA synthetase.

(3) the activity of ALA synthetase is in some way coupled to the induction of cytochrome P-450.

(4) protein synthesis, and probably RNA synthesis is necessary for the induction of ALA synthetase.

(5) ALA synthetase exists as part of a stable high molecular weight complex.

There would probably also be general agreement that there is as yet no comprehensive theory capable of explaining the regulation of ALA synthetase.

III. Materials and Methods

A. Treatment of Animals.

Male Sprague-Dawley rats weighing between 75g and 125g were used throughout. Chemical porphyria was induced by injecting 8 hour or 24 hour fasted rats with either DDC or AIA. When porphyria was induced with AIA, the drug was dissolved in 0.9% NaCl at a concentration of 15 mg/ml and administered by subcutaneous injection at a dose of 40 mg per 100g body weight. Controls received an equal volume of 0.9% NaCl. When DDC was the inducer, the drug was suspended in corn oil at a concentration of 25 mg/ml and administered by intraperitoneal injection at a dose of 25 mg of DDC per 100 g body weight. Controls recieved an equal volume of corn oil. The rats were continued fasting until sacrifice after 16-17 hours or as indicated. When indicated, cycloheximide (5 mg/ml in 0.9% saline) was administered by intraperitoneal injection at 5mg/100g body weight just prior to the administration of the porphyria inducing agent.

Ethanol (50%) was administered by gastric intubation at a dose of 600 mg/100 g body weight. Control rats recieved isocaloric glucose. Pyrazole (30 mg/ml in 0.9% NaCl) was administered by intraperitoneal injection at a dose of 30mg/100g body weight one hour before ethanol administration. The rats were continued fasting until sacrifice after 4 hours.

B. Preparation of Tissue

Rats were sacrificed by decapitation, and the livers were removed. Mitochondria were prepared by the method of Beattie *et al.* (90) in a medium containing 0.25 M sucrose, 0.001 M EDTA and 0.01 M Tris-HCl (pH 7.6). The postmitochondrial supernatant was centrifuged at 30,000 X g for 10 minutes, and the resulting supernatant was further centrifuged at 108,000 X g for 60 minutes. The pellet was resuspended in the isolation medium and used as the microsomal fraction. The supernatant was used directly or, alternatively, was treated with 2 volumes of saturated $(NH_4)_2SO_4$ after the method of Hayashi *et al.* (65). The resulting precipitate was dissolved in 0.01 M Tris-HCl (pH 7.5) and used as the soluble fraction.

C. Fractionation of Mitochondria

Mitochondria, washed four times, were resuspended in the isolation medium to a concentration of 1 mg/ml and sonicated for 10 seconds at full power with a Bronson Sonifier (model W 1850). Alternately, mitochondria were resuspended to a concentration of 10 mg/ml and frozen and thawed three times in a dry ice-acetone bath. Sonicated or frozen mitochondria were centrifuged at 30,000 X g for 10 minutes and the pellet was resuspended in the isolation medium.

Inner and outer membranes and matrix fractions were prepared by the digitonin-Lubrol method of Schnaitman and Greenawalt (91). Mitochondria at 70 mg/ml were treated with digitonin (20 mg/ml

+ 2 mg BSA) in the ratio 1.1 mg digitonin per 10 mg mitochondrial protein. After 15 minutes the mitochondria were diluted with four volumes of isolation medium and centrifugated at 9500 X g for 15 minutes. The supernatant was recentrifuged at 104,000 X g for 60 minutes to sediment the outer membranes. The 9500 X g pellet (inner membrane-matrix fraction) was suspended in 10 mM Tris-HCl (pH 7.5) 1 mM EDTA, 0.1 mM DTE to a concentration of 10 mg/ml, and 1 mg of Lubrol was added for 10 mg protein. After gently stirring for 15 minutes, the suspension was centrifuged at 165,000 X g for 60 minutes. The resulting pellet (inner membrane) was resuspended in isolation medium, while the supernatant was used directly as the matrix fraction.

D. Preparation of Succinyl CoA-Synthetase

Succinyl-CoA synthetase was prepared from an ALA requiring mutant of Rhodopsuedomonas Spheroides. The bacteria were grown aerobically in the light at 30°C after the method of Lascelles (96). The culture was centrifuged at 9000 X g for 10 minutes and washed in one tenth the culture volume of 0.05 M Tris-HCl (pH 6.8). The cells were resuspended in one twentieth the culture volume of 0.05 M Tris-HCl (pH 6.8) and sonicated three times for 20 seconds at full power with a Bronson Sonifer (model W 1850), and centrifuged at 165,000 X g for 60 minutes. The supernatant was used as the succinyl-CoA synthetase preparation.

E. Purification of ALA Synthetase

The 165,000 X g supernatant from the Lubrol extracted inner membrane was used for the further purification of ALA synthetase. All purification procedures were performed at 0-5° C. Solid $(NH_4)_2SO_4$ was added until 40% saturation and the precipitate (FRACTION I) was dissolved in Buffer A containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTE, to a concentration of 5-10 mg/ml. Solid $(NH_4)_2SO_4$ was added to the supernatant until 60% saturation and the precipitate was removed by centrifugation and discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant until saturation. The precipitate (FRACTION III) was collected by centrifugation and dissolved in Buffer A to a concentration of 3-5 mg/ml. FRACTION I and FRACTION III were dialyzed against fresh Buffer A to remove $(NH_4)_2SO_4$.

FRACTION I was diluted five fold with Buffer A and applied to a 0.5 cm X 5 cm DEAE cellulose column previously equilibrated with Buffer A. The column was washed with one half the sample volume of Buffer A and eluted with twice the void volume of 0.25 M NaCl in Buffer A. The eluate was reprecipitated with $(NH_4)_2SO_4$, dialysed, diluted and applied to the DEAE cellulose column as described above. The column was washed with 3 times the void volume of 0.1 M NaCl in Buffer A. The column was then eluted with 3 times the void volume of 0.15 M NaCl in Buffer A.

The eluate was concentrated with carbowax (polyethylene glycol 30-50,000 m.w.), and applied to a sephadex G-200 column (1 X 50 cm) previously equilibrated with Buffer A.

The ALA synthetase elutes at the void volume with an apparent molecular weight greater than 200,000. If the enzyme is not equally distributed in the leading protein peak, the active fractions are reconcentrated and applied to the G-200 column again. FRACTION III was dissolved in 2 ml of Buffer A and chromatographed on a sephadex G-200 column (1 X 50 cm), previously equilibrated with Buffer A. The active fractions were pooled and applied to a DEAE cellulose column (0.5 X 5 cm). The material which passed through the column was applied to a carboxylmethyl cellulose column (0.5 X 5 cm) and the material which passed through the column was saved and frozen. Upon thawing, a precipitate formed which was collected by centrifugation in a clinical centrifuge, and prepared for SDS polyacrylamide electrophoresis as described below.

FRACTION III used for assays was dissolved in Buffer A and frozen until use.

F. Enzyme Assays

1. ALA Synthetase.

ALA synthetase was assayed either by the colorimetric method of Granick (33) or the radiochemical method of Ebert *et al.* (29). For the colorimetric assay, unless otherwise noted, 10 mg of mitochondria were incubated for 30 minutes at 30° C in an incubation mixture containing 100 mM glycine, 10 mM Tris-HCl, 20 mM $MgCl_2$, 10 mM EDTA, 0.1 mM pyridoxal phosphate, 1 mM ATP and 50 mM citrate, succinate, or α-keto glutarate in a final volume of 4.0 ml at pH 7.3. The reaction

was stopped with 2 ml of 15% TCA and centrifuged in a clinical centrifuge. To 5 ml of the supernatant were added 1 ml of 2 M sodium acetate (pH 4.6) and 0.1 ml of acetyl acetone. After boiling for 10 minutes a 1.5 ml aliquot was reserved, and a 2.0 ml aliquot was extracted with 5 ml of ether previously equilibrated with a solution containing 7.7 mM trichloroacetic acid (TCA), 15 mM sodium acetate, 0.68 mM NaOH, and 1.1 mM potassium phosphate. 1.5 ml of modified Ehrlich's reagent (92) was added to both the reserved aliquot and to 1.5 ml of the ether extracted aliquot. After 15 minutes the optical density was read at 552 nm. The moles of ALA were determined by the following equation:

$$\text{Moles of ALA} = 1.7 \times 10^{-7} \; (z - 0.045\, w)$$

where z = optical density of the ether extracted aliquot

w = optical density of the original aliquot.

For the radiochemical assay, the tissue sample was incubated for 15 minutes at 30° C in an incubation medium containing 100 mM glycine, 50 mM Tris-HCl, 20 mM $MgCl_2$, 10 mM EDTA, 0.1 mM pyridoxal phosphate and 1 µCi of either [^{14}C] succinate, [^{14}C] citrate, or [^{14}C] α-ketoglutarate in a final volume of 2 ml at pH 7.3. When the microsomal fraction, supernatant fraction, sonicated mitochondria, or submitochondrial fractions were assayed, the incubation medium contained, in addition to the above, 1 mM ATP, 0.1 mM reduced coenzyme A, and 250 µg of a succinyl coenzyme A synthetase preparation. Assays of the microsomal fraction also contained 5 µg of pyruvate kinase and 10 mM phosphoenolpyruvate.

The incubation was stopped by the addition of 0.5 ml

of 25% TCA and centrifuged in a clinical centrifuge, the precipitate was washed with 5 ml of 1% TCA. The TCA extracts were pooled and 3 ml of 1 M sodium acetate (pH 4.8) was added, the volume was taken to 20 ml and the pH was adjusted to pH 3.9 ± 0.2. The samples were applied to a 2-3 ml column of Dowex AG 50- X 8 (100 - 200 mesh) and washed successively with 20 ml of 0.1 M sodium acetate (pH 3.9), 20 ml 0.1 M acetate : methanol (1 : 2 v/v, pH 3.9), and 10 ml 0.01 M HCl. The ALA was eluted with 4 ml of 1 M NH_4OH and a 0.5 ml aliquot was counted in a Packard liquid scintillation counter.

2. Succinate Dehydrogenase

Succinate dehydrogenase was assayed at room temperature by the method of Bachman _et al._ (93) in a reaction mixture containing 10 mM potassium phosphate (pH 7.6), 10 mM succinate, 1 mM KCN, and 20 µg of dichloroindolphenol in a volume of 1 ml. The reaction was initiated by the addition of succinate and the reaction was followed by the decrease in optical density at 600 nm.

3. Isocitrate Dehydrogenase

Isocitrate dehydrogenase was assayed at room temperature by the method of Bachmann _et al._ (94) in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.3 mM $MgCl_2$, 0.4 mM $NADP^+$, 5 µg of antimycin A, and 0.5 mM isocitrate in a volume of 1 ml. The reaction was initiated by the addition of $NADP^+$, and the reaction was followed by the increase in optical density at 340 nm.

4. Glutamic Dehydrogenase

Glutamic dehydrogenase was assayed by the method of Beaufay *et al.*(94) in a reaction mixture containing 20 mM potassium phosphate (pH 7.7), 0.4 mM KCN, 1% Triton X - 100, 1 mM EDTA, 50 mM NH_4Cl, 10 mM α-ketoglutarate and 0.28 mM NADH. The incubation mixture minus α-ketoglutarate was incubated for 15 minutes at room temperature after which time the reaction was initiated by the addition of α-ketoglutarate and the progress of the reaction was followed by the increase in optical density at 340 nm.

5. Succinyl-CoA Synthetase

Succinyl-CoA synthetase was assayed by the method of Kaufman (95) in a reaction mixture containing 5 mM ATP, 50 mM succinate, 0.13 mM reduced CoA, 2.5 mM reduced glutathione, 50 mM Tris-HCL (pH 7.4), 5 mM $MgCl_2$, and 0.5 M hydroxylamine in a volume of 2 ml. The reaction mixture was incubated for 30 minutes at 37° C, after which 2 ml of a 1 : 1 : 1 mixture of 5% $FeCl_3$: 12% TCA : 3N HCL was added. After 15 minutes the reaction mixture was centrifuged in a clinical centrifuge and the optical density was determined at 540 nm.

G. Chromatography of ALA

Samples were prepared for chromatography by lyophilization of the Dowex AG-50 eluate (4 ml). The dried sample was

redissolved in 0.5 ml of 1 M NH_4OH. Descending paper chromatography was performed on 50 cm strips of Whatman No. 1 paper with 1-butanol : acetic acid : water (25 : 4 : 10) as solvent. The chromatograms were cut into 1 cm strips and counted in a Packard liquid scintillation counter.

Thin layer chromatography was performed on Eastman Silica Gel G plates with 1-butanol:acetic acid:water (4 : 1 : 5) as solvent. The chromatograms were either cut into 0.5 cm slices and counted, or the spots were scraped directly from the plate into a vial and counted. All chromatograms were developed both with ninhydrin spray and exposure to iodine vapor.

Standard δ-aminolevulinic acid and δ-aminolevulinic acid isolated from the Dowex columns were converted to the pyrrole by the method of Mauzerall and Granick (97) and chromatographed on Eastman Silica Gel G plates with 1-butanol : acetic acid : water (4 : 5 : 1). The chromatograms were developed with Ehrlich's reagent and counted as described above.

H. Gel Electrophoresis

Samples for electrophoresis were dissolved in a solvent system containing 10% glycerol (w/v), 1% SDS, 1% mercaptoethanol, 0.01 M phosphate buffer (pH 7.1), and .002% bromphenol blue at a protein concentration of 1-2 mg/ml (98). The samples were then heated at 70° C for 20 minutes. 50-100 µg of protein were applied to 7 cm, 10% acrylamide gels and electrophoresis was performed at room temperature according to the method of Weber and Osborn (99) at 3 mA per gel for 17-24 hours.

Gels were stained overnight with 1% Coomassie Blue and 1% Napthol Blue Black in methanol : acetic acid : water (5 : 1 : 5) and were destained electrophoretically with 7.5% acetic acid in 5% methanol.

I. Liquid Scintillation Counting

Radioactivity was determined in a Packard liquid scintillation counter with an efficiency of 68% when counting ^{14}C samples containing 0.5 ml of 1 M NH_4OH in 10 ml of Bray's counting solution (100), or 73% efficiency with 0.5 ml of 1 M NH_4OH in 5 ml of toluene-triton X 100 counting solution (101).

J. Protein Determination

Protein concentrations were determined by the Folin method of Lowry _et al_.(102), or by the Biuret method of Gornall _et al_. (103) with bovine serum albumin as standard.

K. Materials

δ-amino [4-^{14}C] levulinic acid (11.4 mCi per m mole) and [2,3-^{14}C] succinate (10 mCi per m mole) were obtained from New England Nuclear; [1-^{14}C] citrate (11.4 mCi/m mole) was obtained from Schwartz-Mann; uniformly labeled [^{14}C] l-glutamate (10 mCi/m mole), [5-^{14}C] α-ketoglutarate (10 mCi/m mole), and [2,3-^{14}C] succinate (10 mCi/m mole) were obtained from Amersham-Searle. Nucleotides, pyrazole, Coenzyme A, ALA, phosphoenolpyruvate, pyrdoxal phosphate, pyruvate kinase, oligomycin, antimycin A, cycloheximide were obtained from

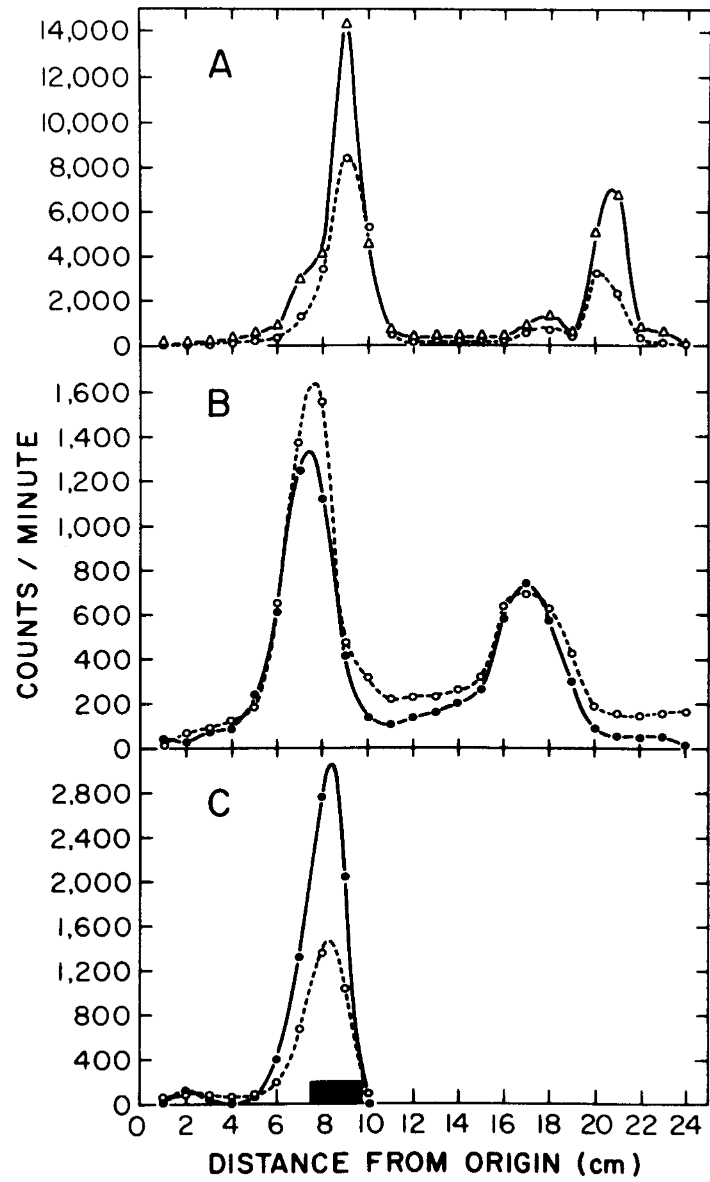
Sigma. Valinomycin was obtained from Pfizer; acrylamide, bisacrylamide, tetramethylethylenediamine, and DDC from Eastman; allylisopropylacetamide from Hoffman-La Roche; Triton X 100 from Rohm and Haas. Digitonin, obtained from Sigma, was recrystallized from ethanol. Lubrol was a gift of ICI American, Inc. The δ-aminolevulinic acid requiring mutant of Rhodopsuedomonas Spheroides was a gift from Dr. June Lascelles, University of California, Los Angeles.

IV. Results

A Verification of the Column Assay for ALA

Studies on ALA synthetase in this laboratory and others have been hindered by the lack of a specific and sensitive assay for ALA. Previous assays (33,104,73) have relied on the condensation of ALA with acetyl acetone to form a pyrrole which subsequently forms a colored complex with Ehrlich's reagent. The condensation with acetyl acetone is not specific for ALA, nor is Ehrlich's reagent specific for the pyrrole formed from ALA. The radiochemical assay of Ebert _et al_. (105), which is used throughout, overcomes these objectionable aspects of the previous assays, by isolating ALA specifically on a Dowex AG-50 resin. Moreover, the radiochemical assay, using [^{14}C] succinate detects only the ALA formed during the assay incubation, since ALA present in the tissue preparation or ALA formed by a pathway not dependent on succinyl-CoA would not be radioactive.

The greatest potential source of error in the measurement of ALA synthetase by the method of Ebert _et al_. (29) is the specificity of the ALA isolation. On analysis by descending paper chromatography, it was found that standard [^{14}C] ALA incubated with complete reaction mixture minus [^{14}C] succinate (Figure 1A), and the product formed from [^{14}C] succinate in the complete reaction mixture (Figure 1B) chromatographed exactly as standard [^{14}C] ALA. Recoveries in all cases were greater than 90%. No contamination was seen when a ten fold excess of ^{14}C was added to the column as radioactive succinate, leucine,



A
B
C
COUNTS / MINUTE
DISTANCE FROM ORIGIN (cm)
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
1,600
1,400
1,200
1,000
800
600
400
200
0
2,800
2,400
2,000
1,600
1,200
800
400
0
0 2 4 6 8 10 12 14 16 18 20 22 24

Figure 1: Recovery of δ-amino [^{14}C] levulinic acid ([^{14}C] ALA) from Dowex columns. A, 2 ml of an incubation mixture containing 100 mM glycine, 50 mM Tris-HCl, pH 7.3, 20 mM $MgCl_2$, 10 mM EDTA, 0.1 mM pyridoxal phosphate, 1 mM ATP, 10 mg of mitochondrial protein plus 1 μCi of [^{14}C] ALA were incubated for 30 min at 30°. The reaction was terminated by precipitation with trichloroacetic acid and separated on Dowex columns (16). The column eluate (Δ——Δ) plus standard [^{14}C] ALA (0---0) were spotted on Whatman No. 1 paper and developed by descending chromatography with butanol-1-acetic acid-water (25:4:10). The chromatograms were cut into 1-cm strips and counted. B, 2 ml of the incubation mixture (A) were incubated with 1 μCi of [^{14}C] succinate instead of [^{14}C] ALA and chromatographed as described above (●——●). Standard [^{14}C] ALA (0---0) was also chromatographed. C, 2 ml of the incubation medium described in A were incubated as described in B. The product of the incubation mixture was converted to the pyrrole, chromatographed on silica gel, developed with Ehrlich's reagent, and counted (●——●). Standard [^{14}C] ALA (0---0) was also chromatographed. The shaded bar represents that part of the chromatogram which formed a pink color with Ehrlich's reagent.

histidine, glutamic acid, aspartic acid, adenine, or uridine. That the ALA runs as two spots (Figures 1,A and B) is most likely due to decomposition after the isolation procedure.

The product of the incubation mixture was also converted to the pyrrole by condensation with acetyl acetone (33), chromatographed on silica gel G plates, developed with Ehrlich's reagent, and counted. The radioactivity was observed to co-chromatograph with the material giving a color with Ehrlich's reagent (Figure 1C). Furthermore, the color and the radioactivity of the pyrrole formed from the incubation mixture had the same R_f as the color and radioactivity of the pyrrole formed from standard [^{14}C] ALA.

In order to further verify the accuracy of the column procedure, mitochondria were incubated in a complete reaction mixture with citrate as substrate. Half of the product was assayed by the method of Ebert _et al_. (29) and the other half by the method of Granick (33). Table 1 shows that identical results were obtained when the ALA was isolated form the columns, converted to the pyrrole and detected by reaction with Ehrlich's reagent, as when it was assayed by the colorimetric method of Granick. It should be emphasized that the column procedure and the colorimetric method yield identical results when the enzyme is assayed under identical conditions. The main advantage of the column procedure is that it is more sensitive when coupled to radiochemical analysis.

TABLE 1

Comparison of δ-aminolevulinic acid synthetase activity as determined by colorimetric and chromatographic procedures

Assay procedure	Control			Porphyric		
	Zero time	30 min	30 min - zero time	Zero time	30 min	30 min - zero time
	nmoles ALA			nmoles ALA		
Colorimetric method.	29.4	91.4	62.0	35.6	171	135
Column procedure....	19.1	88.5	69.4	24.5	160	135

Mitochondria (10 mg, either control or porphyric) were incubated for 30 min at 30° in 4 ml of a mixture containing 100 mM glycine, 50 Tris-HCl, 20 mM $MgCl_2$, 10 mM EDTA, 0.1 mM pyridoxal phosphate, 1 mM ATP, 50 mM citrate at a final pH of 7.3. The reaction was stopped by addition of 1 ml of 30% trichloroacetic acid. Half of the supernatant was assayed by the method of Granick (33); the other half was assayed by the column procedure of Ebert et al.(29). The δ-aminolevulinic acid (ALA) isolated from the column was neutralized to pH 4.5 with acetic acid and assayed (33).

B. Determination of Optimal Conditions for Assaying ALA Synthetase

Once the accuracy of the assay for ALA had been verified, the experimental conditions for the assay of ALA synthetase were examined. As can be seen in Figure 2, the amount of ALA formed was linearly dependent on the amount of protein in both the mitochondrial and microsomal fractions over a wide range of protein concentration. The activity in the supernatant fraction was too low to allow for accurate evaluation.

Figure 3 shows that the synthesis of ALA is linear for at least 15 minutes in all fractions. It should be noted that a linear time relationship could be obtained only at temperatures below 33°C and at a pH of approximately 7.0. It was also observed that with the microsomal fraction, the reaction was linear with time only if an ATP-generating system consisting of phosphoenol-pyruvate and pyruvate kinase was employed. This requirement may reflect the extremely active ATPase activity present in the microsomal fraction. Attempts to inhibit the ATPase activity with ouabain resulted in nearly total inhibition of ALA synthetase activity.

At sufficiently low levels of tissue (100 µg of sonicated mitochondria, 1 mg of intact mitochondria, 1 mg of microsomes), the rate of ALA formation was appreciably independent of [^{14}C] succinate concentration (Figure 4). An apparent dependence of ALA formation on succinate concentration was observed with sonicated mitochondria: *i.e.* a two fold increase in activity with an eight fold increase in succinate concentration. Several ob-

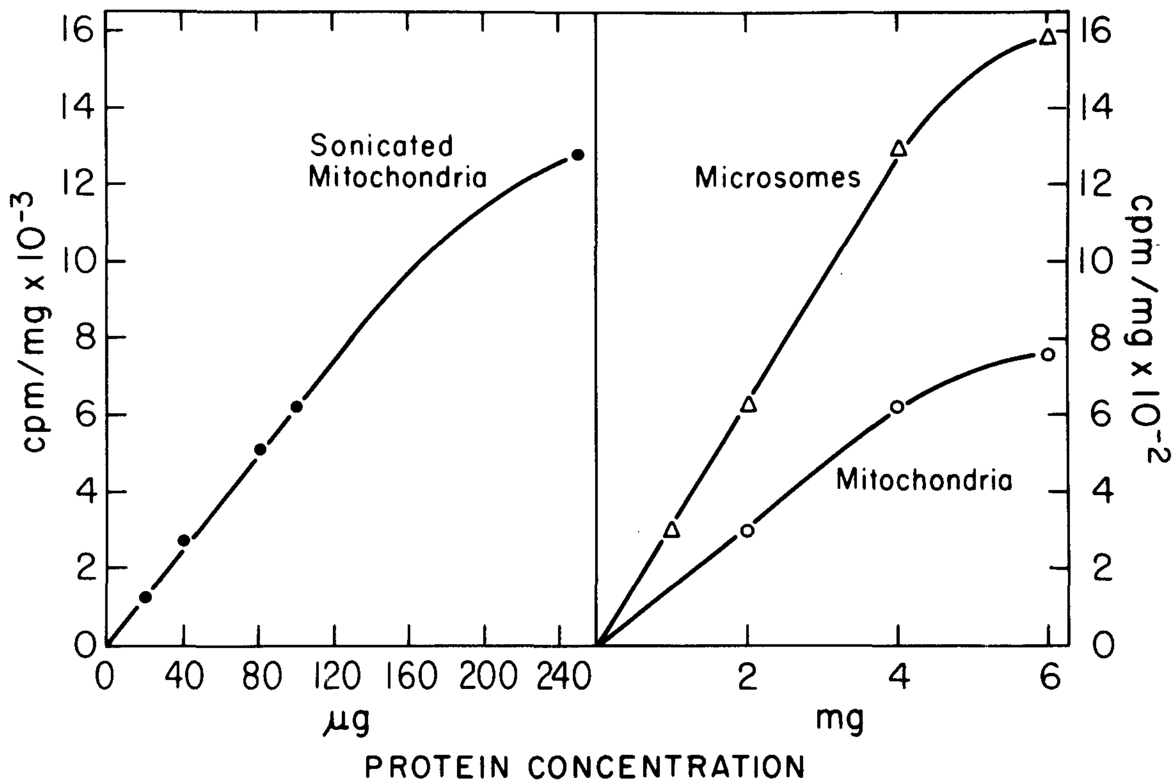


Figure 2: Effect of increasing protein concentration on rate of δ-aminolevulinic acid (ALA) synthetase in sonicated mitochondria (●——●), intact mitochondria (O——O), and microsomes (Δ——Δ). ALA synthetase was assayed in 2 ml of incubation mixture containing 100 mM glycine, 50 mM Tris-HCl, pH 7.3, 20 mM $MgCl_2$, 10 mM EDTA, 0.1 mM pyridoxal phosphate, 1 µCi of 2, 3 -[^{14}C] succinate (10 mCi per mmole), 1 mM ATP, and 100 µM CoA. The reaction was stopped by addition of trichloroacetic acid as described in legend to Table I. The succinyl-CoA synthetase preparation (250 µg) was added to both the sonicated mitochondria and the microsomes. Phosphoenolpyruvate (10 mM) and pyruvate kinase (5 µg) were also added to the microsomes.

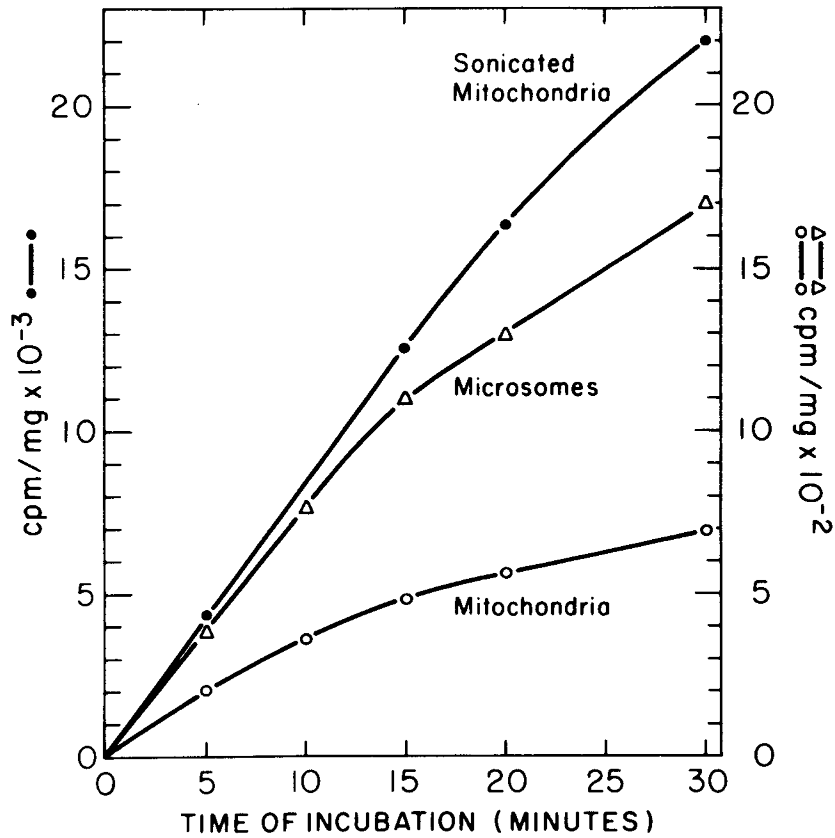


Figure 3: Time course of δ-aminolevulinic acid (ALA) synthetase activity in sonicated mitochondria (●——●), intact mitochondria (O——O), and microsomes (Δ——Δ). ALA synthetase was assayed as described in legend to Figure 2 with 100 μg of sonicated mitochondria, 1 mg of intact mitochondria, and 3 mg of microsomes.

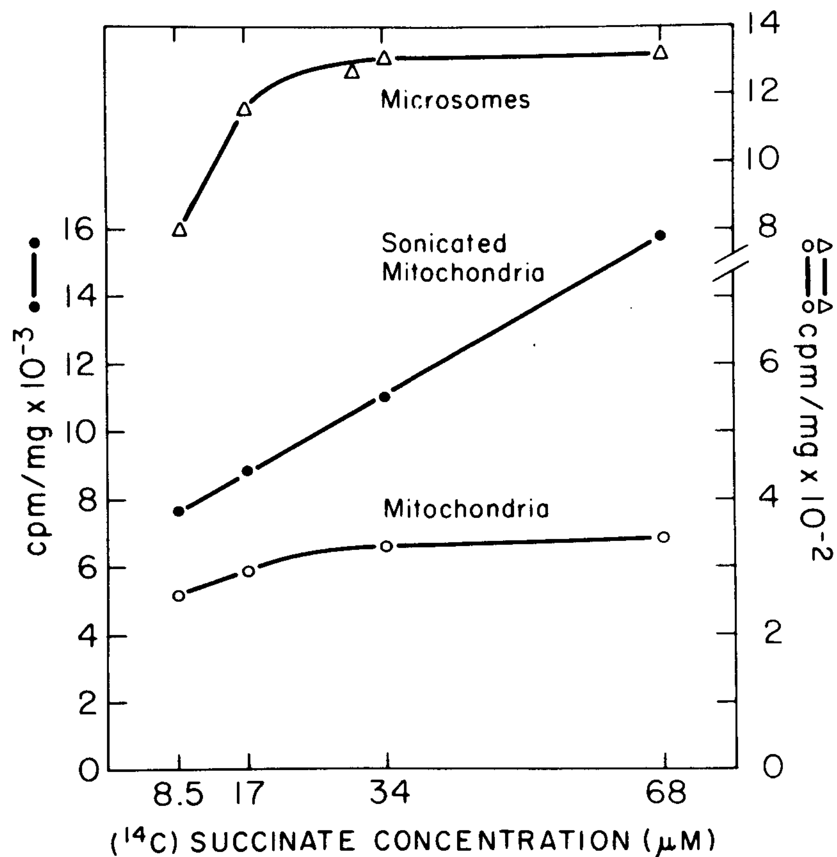


Figure 4: Effect of [^{14}C] succinate concentration on δ-aminolevulinic acid (ALA) synthetase activity. Symbols and assay of ALA synthetase are as described in legends to Figures 2 and3.

servations suggest that the effect is not a simple substrate concentration dependence. First, the amount of ALA formed was linear with both time and protein concentration, which would not be the case if succinate was limiting. In addition, the succinyl-CoA generating system (consisting of succinyl-CoA synthetase, CoA, and ATP) was added in excess which should drive the reaction toward succinyl-CoA, scavenging even low levels of succinate while generating high levels of succinyl-CoA.

In order to clarify the succinate dependence with sonicated mitochondria, the [^{14}C] succinate was diluted with varying amounts of non-radioactive succinate over a wide range and assayed at two different protein concentrations, (100 µg and 500 µg). As seen in Table 2 the specific activity of ALA synthetase remained constant over the entire range of succinate concentrations tested and had the same activity on a "per mg protein" basis with either 100 µg or 500 µg of sonicated mitochondria. The values obtained with 5 mM succinate are subject to considerable error as very low counts were obtained when 1 µCi of [^{14}C] succinate was diluted with such a large amount of unlabeled succinate.

When sonicated mitochondria were used as the source of enzyme, there was a marked dependence on the addition of exogenous coenzyme A (Figure 5). In addition, as can be seen in Table 3, both ATP and a complete succinyl-CoA gererating system were required for activity. An ATP-generating system cannot substitute for ATP, and in fact, addition of the ATP-generating system caused a slight inhibition of ALA synthetase activity when ATP was present.

The addition of glycine was necessary for the formation of ALA

TABLE 2

Effect of succinate concentration on δ-aminolevulinic acid synthetase activity in sonicated mitochondria.

Succinate Concentration	Control		Porphyric	
	Protein			
	100μg	500μg	100μg	500μg
	ALA nmoles/mg protein			
5 mM	1.2	1.51	3.3	1.4
1 mM	0.92	0.95	0.94	0.83
0.25 mM	0.85	0.77	1.03	0.65
0.05 mM	1.0	0.62	1.05	0.57

Rats were treated with 3, 5-dicarbethoxy-1, 4-dihydrocollidine as described in "Materials and Methods." δ-aminolevulinic acid synthetase activity was assayed as described in legend to Figure 2 with additional succinate added as indicated.

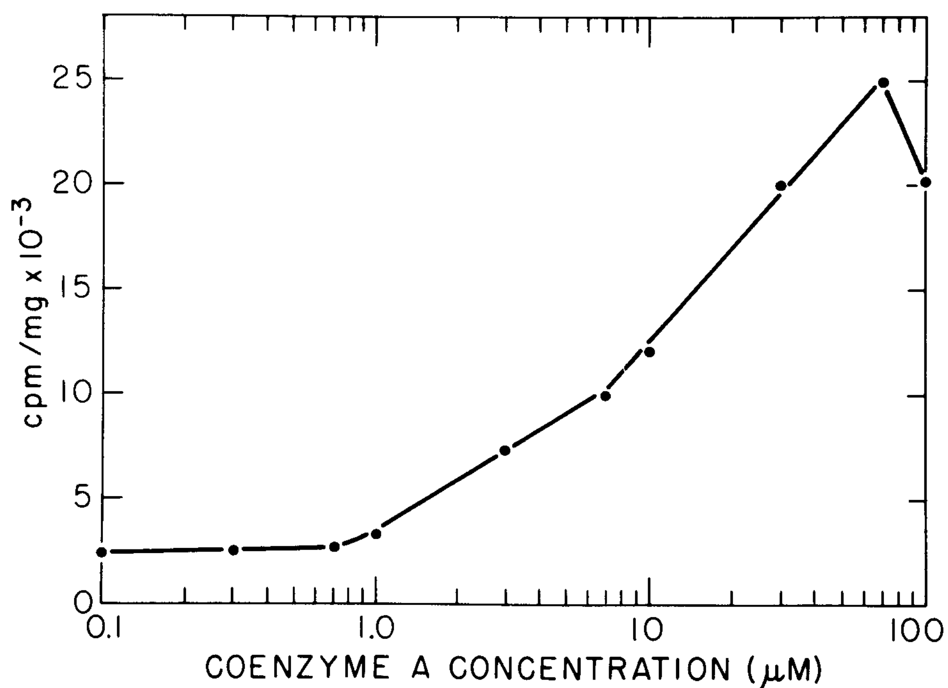


Figure 5: Effect of CoA concentration on the activity of δ-aminolevulinic acid (ALA) synthetase in sonicated mitochondria. ALA synthetase was assayed as described in the legend to Figure 2 with 100 μg of sonicated mitochondria.

TABLE 3

Effects of ATP and succinyl-CoA-generating systems on δ-aminolevulinic acid synthetase activity

Additions and omissions	cpm/mg
Complete incubation mixture...........................	18,000
+PEP[a] + pyruvate kinase.................................	12,800
-ATP + PEP + pyruvate kinase...........................	5,200
-Succinyl-CoA-generating system......................	4,570
-ATP...	3,260
ATP + succinyl-CoA-generating system................	3,200
-CoA + Succinyl-CoA-generating system................	2,950

[a]PEP, phosphoenolpyruvate

δ-Aminolevulinic acid synthetase was assayed as described in the legend to Fig. 2 with 100 μg of sonicated mitochondria.

as determined by either the colorimetric or radiochemical assay (Table 4). Moreover, at each concentration of glycine tested the amount of ALA formed was identical as measured by both assay procedures. To calculate the amount of ALA formed by the radiochemical assay, it was assumed that endogenous substrates caused negligible dilution of the $[^{14}C]$ succinate added to the incubation mixture. Attempts to show radioactive glycine incorporation into ALA were frustrated by the high concentration of glycine required for maximal activity, and because glycine is incompletely separated from ALA by the column procedure. Hence, the few counts incorporated into the ALA were masked by the high and variable background.

C. Intracellular Localization of ALA Synthetase.

Once the optimal conditions for the assay of ALA synthetase were determined, the distribution of the enzyme within the cell was examined. Table 5 shows the intracellular distribution of ALA synthetase as compared to the specific marker enzymes succinate dehydrogenase, a marker for the mitochondrial inner membrane, and glutamic dehydrogenase, a marker for the mitochondrial matrix (93). Only 40% of the total ALA synthetase activity in the homogenate was recovered in either the mitochondrial, microsomal, or supernatant fractions. The majority of the missing ALA synthetase was lost during the four mitochondrial washes. Again the lack of significant activity in the supernatant fraction was observed. It has been suggested (65) that the supernatant fraction contains an inhibitor of ALA synthetase which can be removed by ammonium sulphate precipitation of the supernatant fraction.

TABLE 4

Dependence of δ-aminolevulinic acid synthetase on glycine concentration

Glycine concentration	Control		Porphyric	
	Radio-chemical	Colori-metric	Radio-chemical	Colori-metric
	nmoles ALA/mg			
30 mM	13.1	0	90.7	98.6
6 mM	11.9	0	73.1	83.0
0	4.8	0	3.1	0

Rats were treated with 3,5-dicarbethoxy-1, 4-dihydrocollidine as described under "Materials and Methods." δ-Aminolevulinic acid (ALA) synthetase activity was assayed as described in Table I with 10 mg of intact mitochondria. ALA was determined either colorimetrically by the method of Granick (33) or radiochemically by the method of Ebert <u>et al</u> (29).

TABLE 5

Subcellular distribution of δ-aminolevulinic acid synthetase

Fraction	Protein	ALA synthetase	Succinic dehydrogenase	Glutamic dehydrogenase
	mg	total cpm	total nmoles/min	total nmoles/min
Homogenate[a]	702	1,610,000	279	678
Mitochondria	69	631,000	198	389
Supernatant	503	0	0	156
Microsomes	114	86,000	68	84
Percentage of recovery	98	44	95	92

[a]Homogenate refers to the 600 X g supernatant.

δ-Aminolevulinic acid (ALA) synthetase was assayed as described in the legend to Fig. 2 with 1 mg of homogenate, 1 mg of microsomes, 3 mg of supernatant, and 100 μg of sonicated mitochondria. Units are total counts per min per fraction. Succinic and glutamic dehydrogenases were assayed as described under "Materials and Methods." Units are total nanomoles per min per fraction.

Even after this treatment, however, there is no detectable ALA synthetase activity in the supernatant fraction with the radiochemical assay. The activity of ALA synthetase in the microsomal fraction is extremely low and probably represents contamination of the microsomes with mitochondrial fragments, as indicated by the activity of succinate and glutamic dehydrogenases in this fraction.

As ALA synthetase appears to be an exclusively mitochondrial enzyme, the submitochondrial distribution of the enzyme was examined. The mitochondria were fractionated into inner and outer membrane and matrix fractions, and compared with the inner membrane marker succinate dehydrogenase and the matrix marker isocitrate dehydrogenase. As can be seen in Table 6, the distribution of ALA synthetase closely resembles the distribution of the matrix enzyme isocitrate dehydrogenase, especially with respect to the solubilization of the inner membrane fraction with Lubrol. The inital treatment of the mitochondria with digitonin, however, releases significantly more isocitrate dehydrogenase activity than ALA synthetase activity into the 9500 X g supernatant. In an attempt to further elucidate the partition of ALA synthetase between the inner membrane and matrix, mitochondria were disrupted either by sonication or freezing and thawing three times, and the distribution of the enzyme was compared to the marker enzyme succinate and isocitrate dehydrogenase (Table 7). After freezing and thawing, a gentle procedure for disrupting the mitochondria, ALA synthetase segregated as the inner membrane marker succinate dehydrogenase; while after sonication, a more vigorous procedure, ALA synthetase segregated as the matrix marker isocitrate dehydrogenase. These results indicate

TABLE 6

Submitochondrial distribution of δ-aminolevulinic acid synthetase

Fraction	Protein		ALA synthetase		Succinic dehydrogenase		Isocitric dehydrogenase	
	mg	%	total cpm	%		%		%
Mitcohondria........	215	100	1,820,000	100	121	100	220	100
9,500 X g supernatant............	72		74,300		3		78	
140,000 X g supernatant...........	54	25	2,300	0.1		0	81	37
140,000 X g pellet	23	11	34,900	2	3	2	1.5	0.6
9,500 X g pellet....	139	65	1,730,000	95	76	63	142	64
9,500 X g pellet with Lubrol..........	134	100	2,760,000	100	119	100	240	100
Supernatant after Lubrol.........	49	37	1,640,000	59	1	0.8	125	52
Pellet after Lubrol	85	63	900,000	33	150	126	88	37

Mitochondria were fractionated by the digitonin-Lubrol method of Schnaitman and Greenawalt (91). The 9,500 X g pellet contains the inner membrane-matrix fraction. After treatment with Lubrol and centrifugation, the Lubrol pellet contains the inner membrane while the supernatant fraction contains the matrix. The 9,500 X g supernatant was centrifuged at 100,000 X g for 1 hour to yield a pellet containing the outer membrane. Enzymes were assayed as described in the legend to Table 5 and under "Materials and Methods." ALA, δ-aminolevulinic acid. Units of succinic and isocitric dehydrogenase are total nanomoles per min.

TABLE 7

Distribution of δ-aminolevulinic acid synthetase activity after sonication or freezing and thawing

Fraction	Protein	ALA synthetase	Succinic de-hydro-genase	Iso-citric de-hydro-genase
	mg	total cpm		
Mitochondria............	60	400,000	56.3	9.9
Frozen mitochondria......	60	469,000	22.3	13.4
30,000 X g supernatant..	9.3	67,000	0.1	25.2
30,000 X g pellet.......	45.5	390,000	23.7	16.5
Sonicated mitochondria...	60	563,000	21.2	25.1
30,000 X g supernatant..	17.8	566,000	1.2	35.0
30,000 X g pellet.......	39.2	360,000	22.0	8.4

δ-Aminolevulinic acid (ALA) synthetase, succinic, and isocitric dehydrogenases were assayed as described in the legends to Tables 5 and 6. Units of succinic and isocitric dehydrogenases are total nanomoles per min.

that the enzyme is closely associated with the inner membrane but is easily dissociated from it.

D. ALA Synthetase Activity in Chemical Porphyria.

Having characterized ALA synthetase in normal rats, an attempt was made to study the mechanism by which ALA synthetase activity is increased during chemical porphyria. Table 8 shows a comparison of the activity of ALA synthetase in the livers obtained from control and AIA-treated rats. A five fold increase in enzymatic activity was observed in mitochondria obtained from AIA-treated rats when ALA was determined by the colorimetric method of Granick (33). In contrast, no significant increase in enzymatic activity was observed in either the microsomal or supernatant fractions obtained from rats treated with AIA using the colorimetric assay. Rats treated with cycloheximide immediately before AIA administration showed a slight decrease in ALA synthetase activity in all fractions compared to control rats. When ALA was determined by the radiochemical assay, different results were obtained. ALA synthetase activity was increased better than four fold in both intact mitochondria and the supernatant fraction from porphyric animals; whereas no increase in activity was observed with sonicated mitochondria or in the microsomal fraction. Cycloheximide blocked the increase in activity in all fractions.

Since the colorimetric assay was performed with citrate as substrate and the radiochemical assay with [^{14}C] succinate as substrate, it was decided to reinvestigate the ALA synthetase activity in the mitochondrial fraction by both analytical methods

TABLE 8

Effects of Allylisopropylacetamide on hepatic δ-aminolevulinic acid synthetase activity

A. Colorimetric assay			
Fraction	Control	Porphyric	Porphyric plus cycloheximide
	nmoles ALA/min		
Mitochondria...........	1.25	6.65	0.84
Microsomes.............	0.64	0.49	0.09
Supernatant............	0.51	0.09	0.03
B. Radiochemical assay			
Fraction	Control	Porphyric	Porphyric plus cycloheximide
	cpm/mg		
Mitochondria...........	926	4,020	437
Sonicated mitochondria.	27,400	29,300	19,000
Microsomes.............	3,730	3,830	1,890
Supernatant............	221	1,030	383

Rats fasted for 24 hours were injected with saline (control), allylisopropylacetamide, or cycloheximede and then allylisopropylacetamide and sacrificed after 4 hours. The colorimetric assays were performed as described in the legend to Table I with 10 mg of protein. The radiochemical assay was performed as described in the legends to Fig. 2 and 3. ALA, δ-aminolevulinic acid.

using citrate, α-ketoglutarate and succinate as substrates for both assays (**Table** 9). Regardless of the substrate used, ALA synthetase activity assayed by the colorimetric procedure was increased nearly six-fold in animals treated with AIA. When ALA was determined by the radiochemical method after incubation with sonicated mitochondria, a two-fold increase in activity was observed with [^{14}C] citrate as substrate, a six-fold increase with [^{14}C] α-ketoglutarate as substrate and no increase with [^{14}C] succinate as substrate. When ALA synthetase was assayed with intact mitochondria, there was a small increase (almost two-fold) with [^{14}C] citrate as substrate, a six-fold increase with [^{14}C] α-ketoglutarate as substrate, and a nine-fold increase with [^{14}C] succinate as substrate.

It has been suggested by Sassa and Granick (105) that there are at least two different classes of inducers for ALA synthetase with different mechanisms of activation. It was possible that the effects observed in AIA-treated animals were only valid for one class of inducer. Therefore, the experiments in Table 9 were repeated with DDC as the inducer. As can be seen in Table 10, identical results were obtained with both inducers.

It should be noted, that under optimal conditions for the assay of ALA synthetase (dilute, sonicated mitochondria with [^{14}C] succinate and a succinyl-CoA generating system) there is no increase in the activity of ALA synthetase in rats treated with either of the porphyria inducing drugs tested. Attempts to confirm this observation with the colorimetric assay were frustrated because the large amount of tissue required for the colorimetric assay did not permit the enzyme to be assayed under the same conditions (dilute sonicated mitochondria) as was used for the more sensitive radiochemical assay.

TABLE 9

Effect of various substrates on apparent "induction" of δ-aminolevulinic acid synthetase in allylisopropylacetamide-treated rats

Substrate	Radiochemical assay				Colorimetric assay	
	Sonicated mitochondria		Mitochondria		Mitochondria	
	Control	Porphyric	Control	Porphyric	Control	Porphyric
	cpm/mg				nmoles ALA/mg	
Succinate...........	20,800	17,800	242	2,190	0.82	5.02
α-Ketoglutarate.....	565	3,190	614	3,730	0.69	4.23
Citrate.............	2,880	4,140	522	1,050	0.80	3.69

Twenty-four hour fasted rats were injected with either saline (control) or allylisopropylacetamide and sacrificed after 16 hours. The colorimetric determination of δ-aminolevulinic acid (ALA) synthetase was performed as in Table I except that where indicated 50 mM succinate or 50 mM α-ketoglutarate was substituted for citrate (50 mM). Likewise, the radiochemical assay was performed as in Fig. 2 with 1 μCi of α-keto[5-^{14}C] glutarate, (10 mCi per mmole) or 1 μCi of [1-^{14}C]citrate (21.7 mCi per mmole) substituted for 1 μCi of [2,3-^{14}C]succinate (10 mCi per mmole) and either 1 mg of intact mitochondria or 100 μg of sonicated mitochondria.

TABLE 10

Effect of various substrates on apparent "induction" of δ-aminolevulinic acid synthetase in 3, 5-dicarbethoxy-1, 4-dihydrocollidine-treated rats

Substrate	Radiochemical assay				Colorimetric assay	
	Sonicated mitochondria		Mitochondria		Mitochondria	
	Control	Porphyric	Control	Porphyric	Control	Porphyric
	cpm/mg				nmoles ALA/mg	
Succinate.........	13,000	17,500	600	2,780	1.47	15.3
α-Ketoglutarate...	1,230	3,230	630	3,700	1.70	16.9
Citrate...........	570	2,200	500	1,350	1.52	13.1

All procedures were the same as in Table 9 except the rats were injected with either corn oil (control) or 3, 5-dicarbethoxy-1, 4-dihydrocollidine and sacrificed 4 hours later. ALA, δ-aminolevulinic acid.

E. Effects of Acute Ethanol Intoxication and Pyrazole on the Activity of ALA Synthetase.

Since there was no increase in ALA synthetase activity in mitochondria from porphyric rats when the enzyme was assayed under optimal conditions with sonicated mitochondria, and since there was an increase in activity when the enzyme was assayed in whole mitochondria from porphyric rats, it appeared that the increase in ALA synthetase activity observed in chemical porphyria resulted from an activation of the enzyme already present within the mitochondria rather than from an increase in the amount of enzyme in the mitochondria. Rubin et al. (106) had previously reported that acute or chronic ethanol intoxication also resulted in increased ALA synthetase activity in rat liver homogenates. In an attempt to gain some understanding of the control of ALA synthetase activity in intact mitochondria the effect of acute ethanol intoxication on the activity of the enzyme was investigated. Studies with ethanol have the advantage that the metabolic consequences of ethanol intoxication have been extensively studied, while little is known about the metabolism or metabolic consequences of either AIA or DDC. As can be seen in Table 11, ALA synthetase activity was increased six-fold in intact liver mitochondria four hours after ethanol administration. Administration of pyrazole, an inhibitor of ethanol metabolism in vivo (67) which acts by inhibiting alcohol dehydrogenase (107), blocked the ethanol-induced increase in ALA synthetase activity in intact mitochondria by 75%. Moreover, the administration of pyrazole caused a similar inhibition of the DDC-stimulated increase in ALA synthetase activity. The effects of pyrazole do not result

TABLE 11

Effect of pyrazole on the apparent 'induction' of ALA synthetase in DDC or ethanol-treated rats

Treatment	ALA synthetase activity cpm/mg
Control	970
Pyrazole	1,090
Ethanol	6,700
Ethanol and pyrazole	2,370
DDC	6,300
DDC and pyrazole	2,130

δ-aminolevulinic acid synthetase was assayed as described in the legend to Figure 2 with 1 mg of intact mitochondria. Animals were treated as described in "Materials and "Methods. Each value is the average of six different animals.

from an inhibition of ALA synthetase. Addition of 3-10 mM pyrazole to the incubation mixture did not result in any change in ALA synthetase activity in either intact or sonicated mitochondria obtained from control, pyrazole treated, ethanol treated, or DDC treated rats.

F. Effect of Intramitochondrial Redox Potential and Engery Charge on the Activity of ALA Synthetase.

Since ethanol, which causes a six fold increase in the activity of ALA synthetase in intact mitochondria, is known to alter the intracellular redox state (108) and thereby affect the regulation of energy metabolism, the effects of various respiratory inhibitors were examined to see if the redox state [$(NAD^+)/(NADH)$ ratio] or the phosphate potential [(ATP)/(ADP)(Pi)] had any effect on the activity of ALA synthetase in intact mitochondria. As can be seen in Table 12, rotenone, a respiratory inhibitor which causes an accumulation of NADH by blocking its oxidation *via* the respiratory chain, severely inhibited ALA synthetase activity. Both valinomycin, a potassium specific ionophore which allows potassium to equilibrate across the mitochondrial membrane in response to a charge gradient, and carbonyl cyanide m-chlorophenyl hydrazone (CCP) or 2, 4 dinitrophenol (DNP), classical uncouplers of oxidative phosphorylation, significantly stimulated ALA synthetase activity. The action of all of these compounds results in lower levels of both ATP and NADH. Oligomycin, which blocks ATP synthesis without uncoupling respiration, thereby leading to lower ATP and higher NADH levels had no effect

TABLE 12

Effect of respiratory inhibitors and uncouplers on ALA synthetase activity in intact mitochondria

Additions	Control	Porphyric
	% Control [Range]	% Control
+ Rotenone (6) (10 ug)	-61% [46% - 77%]	-63%
+ Valinomycin (5) (10 ug)	+51% [20% - 110%]	+64%
+ Dinitrophenol (5) (20 ug)	+70% [40% - 110%]	+90%
+ Oligomycin (4)	-10% [0 - 20%]	No effect
	Control value = 720 cpm/mg	Porphyric value = 3800 cpm/mg

δ-amin olevulinic acid synthetase was assayed as described in the legend to Table 11. Rats were treated with 3, 5-dicarb-ethoxy -1, 4-dihydrocollidine as described in "Materials and Methods." Numbers in parenthesis indicate number of experiments.

on the activity of ALA synthetase. Mitochondria from both control and porphyric rats responded identically to the inhibitors. Similar experiments using α-ketoglutarate or glutamate as substrate yielded appreciably identical results. It should be noted that none of the inhibitors tested had any effect on the activity of ALA synthetase with sonicated mitochondria, indicating that they do not affect the enzyme directly.

Since valinomycin causes the equilibration of potassium across the mitochondrial membrane, it causes the loss of potassium from mitochondria suspended in a potassium free medium. An experiment was performed to determine whether the stimulation of ALA synthetase activity observed with valinomycin was due to the uncoupler effect of valinomycin or due to the decrease in intramitochondrial potassium concentration. As can be seen in Table 13, maximal stimulation of ALA synthetase by valinomycin was observed in the absence of potassium in the external medium. Increasing concentrations of potassium caused increasing inhibition of the activity of ALA synthetase activity with [^{14}C] succinate or [^{14}C] α-ketoglutarate as substrate in mitochondria from control rats, and with [^{14}C] succinate in mitochondria from porphyric rats. No stimulation in ALA synthetase activity was observed with [^{14}C] glutamate as substrate in mitochondria from either control or porphyric rats, although increasing concentrations of potassium showed increasing inhibition of ALA synthetase.

An attempt was made to regulate the NAD^+/ NADH and the ATP/ADP ratios independently to determine what effect these ratios have on ALA synthetase activity (Table 14). Oligomycin

TABLE 13

Effect of K^+ concentration on δ-aminolevulinic acid synthetase activity from valinomycin treated mitochondria.

Additions	Control			Porphyric	
	Succinate	α-Ketoglutarate	Glutamate	Succinate	Glutamate
	cpm/mg protein				
No additions	615	335	290	5,425	1,450
Valinomycin	890	440	275	6,500	1,320
Valinomycin + 25 mM K^+	515	215	110	3,550	1,040
Valinomycin + 50 mM K^+	415	145	110	3,250	800
Valinomycin + 100 mM K^+	300	135	70	3,360	-

Mitochondria were incubated in a K^+ free medium as described in the legend to Figure 2 with 10 μg of valinomycin and K^+ added to the incubation mixture as indicated. Rats were treated with 3, 5-dicarbethoxy -1, 4-dihydrocollidine as described in "Materials and Methods."

TABLE 14

Effect of $NADH/NAD^+$ and ATP/ADP ratios on ALA synthetase activity in intact mitochondria

	SUCCINATE		GLUTAMATE	
Additions	Control	**Porphyric**	Control	Porphyric
		cpm/mg protein		
None	610	3900	886	1870
Oligo, DNP, ADP	540	3800	1886	1980
Oligo, DNP, ATP	610	5280	1090	3090
Oligo, rot, ADP	90	430	795	745
Oligo, rot, ATP	180	760	840	1050
Oligo = oligomycin, Rot = rotenone, DNP = dinitrophenol				

δ-aminolevulinic acid synthetase was assayed as described in the legend to Figure 2. ATP and ADP (10 mM), oligomycin (20 µg), rotenone (10 µg), and dinitrophenol (20 µg) were added as indicated. Rats were treated with 3, 5-dicarbethoxy-1, 4-dihydrocollidine as described in "Materials and Methods."

was added so that the ATP/ADP ratio could be controlled independently of the NAD^+/NADH ratio. Since the mitochondrial membrane is impermeable to pyridine nucleotides, the NAD^+/NADH ratio can be adjusted by adding either rotenone which raises the NADH levels (thus lowering the NAD^+ level), or an uncoupler which lowers the NADH levels by stimulating respiration, thereby raising the NAD^+ levels. Since adenine nucleotides are transported across the mitochondrial membrane by an exchange carrier mechanism (109), the intramitochondrial levels of ATP or ADP can be altered by adding either ATP (10 mM) or ADP (10 mM) to the external medium. As can be seen in Table 14, ALA synthetase activity in mitochondria from both control and porphyric rats is increased by high energy charge [(ATP)/(ADP)] and decreased by high redox potential [(NADH)/(NAD^+)] when either [^{14}C] succinate or [^{14}C] glutamate was the substrate. The addition of calcium, an ion which is actively accumulated by mitochondria at high energy charge, had no effect on ALA synthetase activity in intact mitochondria.

G. Partial Purification of ALA Synthetase

Several groups have observed that the increase in ALA synthetase activity during experimental porphyria is prevented by inhibitors of cytoplasmic RNA and protein synthesis (65,105,110). However, the results obtained in this study have suggested that the actual amount of ALA synthetase does not differ in control and porphyric rats. These observations imply that some specific product or products of cytoplasmic protein synthesis are capable

of altering the activity of ALA synthetase in the mitochondria. Attempts to demonstrate such an activation by incubating mitochondria obtained from control rats with various cell fractions obtained from porphyric rats or to demonstrate the reverse process using mitochondria from porphyric rats and various fractions from control rats have been singularly unsuccessful. Therefore, an attempt was made to partially purify ALA synthetase from both control and porphyric rats to determine whether the activation of ALA synthetase causes any gross changes in the physical properties of the enzyme. The results in Table 15 revealed that when mitochondria from both control and porphyric rats were fractionated into inner and outer membrane fractions, all the detectable ALA synthetase activity is present in the inner membrane fraction. After treatment of the inner membrane fraction with Lubrol (1 mg/10 mg protein) all the detectable ALA synthetase activity is solubilized. Approximately 80% of the ALA synthetase activity remains in solution when the Lubrol treated inner membrane is centrifuged for two hours at 165,000 X g. All further attempts to purify the enzyme from the Lubrol supernatant resulted in an almost total loss of ALA synthetase activity. This obstacle was overcome when it was discovered that an activator, also present in the Lubrol supernatant, was required to measure ALA synthetase activity. Table 16 shows how this effect was manifest when the enzyme was precipitated from the Lubrol supernatant with $(NH_4)_2SO_4$. Although the 0 - 40% $(NH_4)_2SO_4$ precipitate and the 60 - 100% $(NH_4)_2SO_4$ precipitate had very low activity by themselves, the combination of the two had appreciably the same specific activity as the

TABLE 15

Partial purification of ALA synthetase

	Control		Porphyric	
	Specific Activity cpm/mg	Total Activity cpm	Specific Activity cpm/mg	Total Activity cpm
Intact Mitochondria	1,370	0.6 X 10^6	16,400	7.8 X 10^6
Sonicated Mitochondria	20,700	9.3 X 10^6	17,100	8.1 X 10^6
Inner Membrane	23,800	8.2 X 10^6	27,000	9.7 X 10^6
Outer Membrane	6,800	0.5 X 10^6	850	0.1 X 10^6
Lubrol Supernatant	40,100	9.3 X 10^6	43,300	9.0 X 10^6
Lubrol Pellet	5,700	0.6 X 10^6	5,200	.6 X 10^6

Mitochondria were fractionated according to the digitonin/Lubrol method of Schnaitman and Greenawalt (91) as described in "Materials and Methods." δ-aminolevulinic acid synthetase was assayed as described in the legend to Figure 2.

TABLE 16

δ-aminolevulinic acid synthetase activity after fractionation of Lubrol supernatant with $(NH_4)_2SO_4$

Fraction(s) assayed		Activity cpm/mg
Lubrol supernatant		50,000
#1	(0 - 40% ppt)	5,600
#2	(40 - 60% ppt)	11,900
#3	(60 - 100% ppt)	2,600
#1 + #2		25,000
#1 + #3		46,500
#2 + #3		15,100

Lubrol supernatant was fractionated with $(NH_4)_2SO_4$ as described in "Materials and Methods." ALA synthetase was assayed as described in the legend to Figure 2.

Lubrol supernatant. Although the 40 - 60% $(NH_4)_2SO_4$ precipitate had appreciable activity by itself and was active in reconstitution with both of the other fractions, it could be refractionated by repeating the $(NH_4)_2SO_4$ fractionation to yield a pattern similar to that obtained when the Lubrol supernatant was fractionated. Similar results were obtained when the Lubrol supernatant was fractionated on a Sephadex G-200 column (Figure 6). None of the fractions had significant activity by itself, but the total ALA synthetase applied to the column was recovered when the fractions were assayed in the presence of either the 0 - 40% $(NH_4)_2SO_4$ precipitate or the 60 - 100% $(NH_4)_2SO_4$ precipitate.

The 0 - 40% $(NH_4)_2SO_4$ precipitate (Fraction 1) from both control and porphyric rats, was further purified by DEAE Sephadex chromatography, $(NH_4)_2SO_4$ precipitation, and Sephadex G-200 chromatography as described in Materials and Methods. The results presented in Table 17 show that there is no detectable difference in the behavior of ALA synthetase isolated from control or porphyric rats by the purification procedure employed. Furthermore, SDS disk electrophoresis on 10% polyacrylamide gels of the more purified fractions failed to show any significant difference between the enzyme as purified from control or porphyric rats (Figure 7).

As can be seen from the gels of the most purified fraction (Figure 7), the enzyme isolated by the procedure employed is far from pure. The enzyme appears to be part of a very stable complex which cannot be dissociated without a total loss of activity. Treatment with phospolipase A, a number of ionic and nonionic detergents, or salts did not appear to yield any further purification.

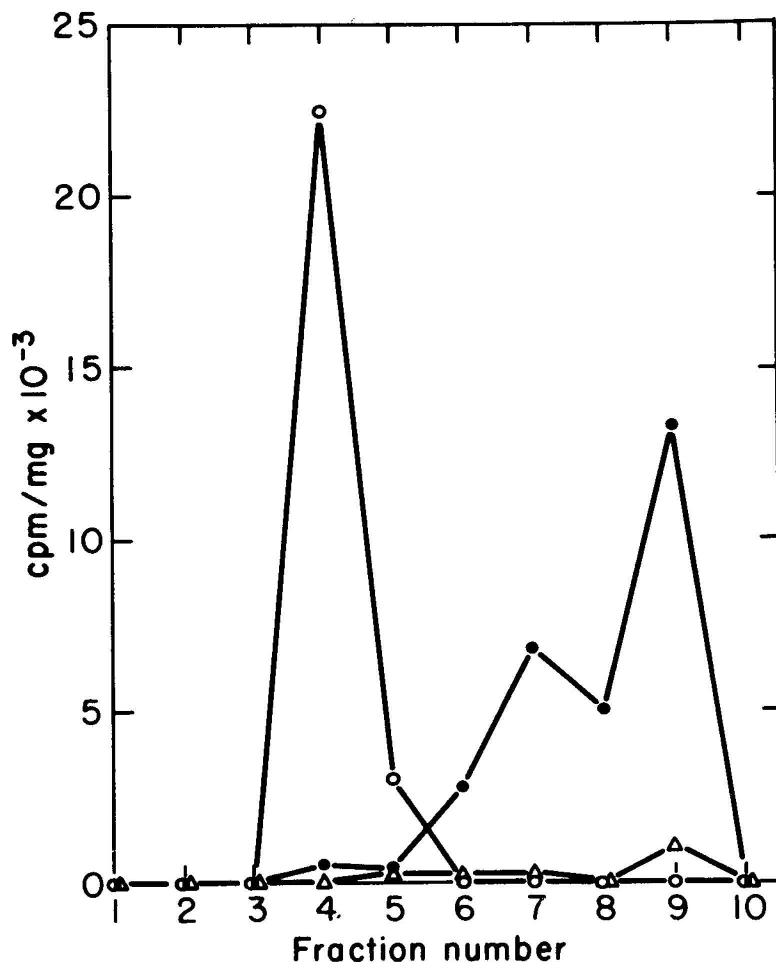


Figure 6: Sephadex G-200 fractionation of Lubrol supernatant. Sufficient Lubrol supernatant to give 25,000 cpm when assayed for δ-aminolevulinic acid synthetase was applied to a Sephadex G-200 column as described in "Materials and Methods." δ-aminolevulinic acid synthetase was assayed as described in the legend to Figure 2 with 500 ug of either Fraction 1 (●——●) or Fraction 3 (O——O) as defined in Table 16, were added to the incubation mixture. Fraction 1 and Fraction 3 when assayed together had an activity of 27,000 cpm. (Δ——Δ) assay performed without either Fraction 1 or Fraction 3.

TABLE 17

Purification of δ-aminolevulinic acid synthetase from control and porphyric rats

Fraction	Control			Porphyric		
$(NH_4)_2SO_4$ Fractionation	Specific Activity cpm/mg	Total Activity cpm	per cent recovery	Specific Activity cpm/mg	Total Activity cpm	per cent recovery
Lubrol Supernatant	40,100	20×10^5	-	43,300	20×10^5	-
#1	20,300	16×10^5	78%	27,200	17×10^5	87%
#2	4,400	2.7×10^5	14%	2,700	1.7×10^5	8.7%
#3	0	0	0	0	0	0
DEAE Column I Void	4,400	1.0×10^5	5.2%	21,500	4.6×10^5	23%
Eluate	27,700	15×10^5	73%	47,400	9.0×10^5	45%
DEAE Column II Void	2,400	0.4×10^5	2.2%	18,500	0.8×10^5	3.8%
0.1 M NaCl Eluate	92,220	5.2×10^5	26%	70,700	6.4×10^5	32%
0.15 M NaCl Eluate	24,800	1.1×10^5	5.3%	28,600	1.5×10^5	7.3%
0.25 M NaCl Eluate	23,000	0.6×10^5	3.1 %	19,500	0.9×10^5	4.3%
Pooled Sephadex G-200	107,000	1.4×10^5	7.2%	85,300	1.5×10^5	7.7%

Lubrol supernatant was fractionated as described in "Materials and Methods." δ-aminolevulinic acid synthetase was assayed as described in the legend to Figure 6.

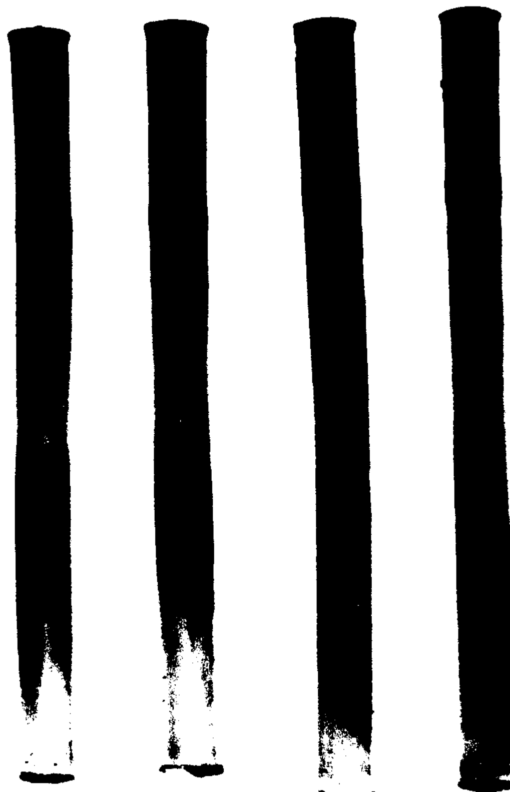


Figure 7: SDS polyacrylamide-gel electrophoresis of δ-aminolevulinic acid synthetase isolated from control and 3, 5-dicarbethoxy-1, 4-dihydrocollidine treated rats. The samples are derived from the purification shown in Table 17. Electrophoresis was performed as indicated in "Materials and Methods."

The most purified ALA synthetase preparations from both control and porphyric rats show two major bands (MW = 175,000 and 48,000) and two minor bands (MW = 78,000 and 68,000). The band with a molecular weight of 175,000 is presumably an undissociated complex containing ALA synthetase and a number of other components. It should be noted that the value of 175,000 MW agrees reasonable well with the minimum molecular weight complex reported by several workers (72,86,115). The major band at M.W. = 48,000 is assumed to be ALA synthetase as it shows enrichment during the purification of the enzymatic activity, although it is possible that one of the minor bands represents the enzyme. Whiting and Elliott (72) have reported a molecular weight of purified ALA synthetase of M.W. = 77,000.

The factor in the 60 - 100% $(NH_4)_2SO_4$ precipitate (Fraction 3) which is responsible for the activation of ALA synthetase was also further purified, as described in the materials and methods, from control and prophyric rats, and the most purified fractions were analyzed by SDS polyacrylamide gel electrophoresis (Figure 8). As was the case with ALA synthetase itself, there were no significant differences observed between the activator isolated from control rats and that isolated from porphyric rats.

It was observed during the purification of the activator from the 60 - 100% $(NH_4)_2SO_4$ precipitate, that the activator had several peculiar properties, namely:

(1) it does not precipitate in 60% $(NH_4)_2SO_4$;

(2) when chromatographed on Sephadex G-200 the activtiy appears in many fractions;

(3) it does not bind to either DEAE or carboxymethyl cellulose;

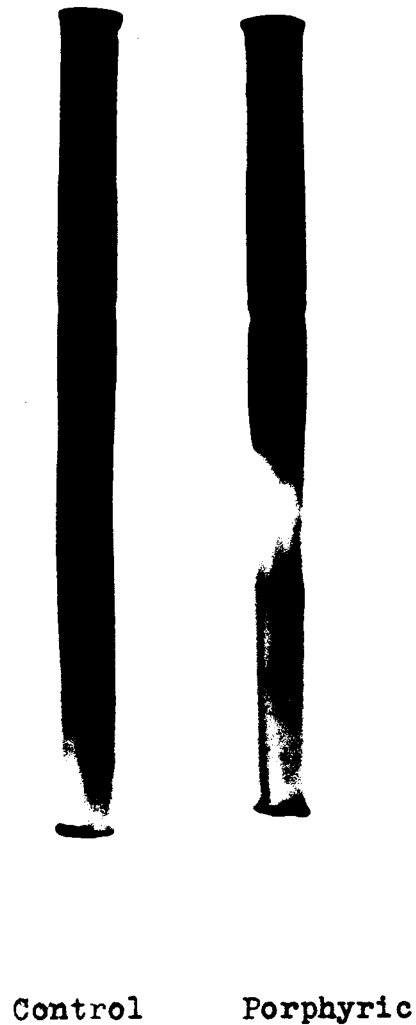


Figure 8: SDS polyacrylamide gel electrophoresis of the "Activator" of δ-aminolevulinic acid synthetase isolated from control and 3, 5-dicarbethoxy-1 4-dihydrocollidine treated rats. The "Activator" was purified from Fraction 3 as described in "Materials and Methods." Electrophoresis was performed as indicated in "Materials and Methods."

(4) it readily precipitates when any of the fractions obtained beyond the initial $(NH_4)_2SO_4$ fraction are frozen;

(5) once precipitated by freezing it forms a spongy precipitate which is nearly impossible to re-dissolve.

These properties suggested that the activator may not be a protein. However, the activator is non-dialysable, is heat labile, and phospholipase resistant. Table 18 shows that a sonicated membrane preparation from an ALA requiring mutant of Rhodopseudomonas Spheroides can serve as an alternative source of the activator.

TABLE 18

Effect of a sonicated membrane fraction of R. Spheroides on the activity of δ-aminolevulinic acid synthetase

Additions	ALA synthetase activity	
	cpm	cpm corrected
+ ALA synthetase	600	-
+ ALA synthetase, Activator	6,500	5,900
+ ALA synthetase Sonicated R. Spheroides	8,100	6,500
+ Activator	0	-
+ Sonicated R. Spheroides	1,000	-

δ-aminolevulinic acid synthetase was assayed as described in the legend to Table 16. R. Spheroides was extract was prepared as described in "Materials and Methods" except that sonication was performed for three minutes.

V. Discussion

Prior to the publication of the radiochemical assays of Irving and Elliot (111) and Ebert et al. (29), accurate quantitative determination of ALA synthetase could be achieved only with large amounts of tissue (3-10 mg depending upon the fraction being assayed). As a consequence, several potentially interesting lines of experimentation were difficult to pursue, notably purification of the enzyme. Of the two published radiochemical procedures available, the method of Ebert et al. (29) appeared more reliable for routine assays as it was more specific even though more time consuming. Before using the radiochemical assay of Ebert et al., however, it was necessary to establish that the analytical method was specific for ALA. All of the radioactivity recovered from the Dowex column after an incubation with [^{14}C] succinate chromatographed as standard ALA on paper chromatography. Likewise when the ALA isolated from the column was converted to the pyrrole and chromatographed on silica gel, the radioactivity had the same R_f as the pyrrole made from standard ALA. When standard [^{14}C] ALA was applied to the column after incubation under the assay conditions, more than 90% of the ALA was recovered from the columns. Furthermore, our results and those of others (24,29,111,112) indicated that the radiochemical and colorimetric assays yielded identical results when identical samples of ALA synthetase are assayed by both methods.

Since the sensitivity of the radiochemical assay for ALA synthetase is largely determined by the specific activity

of the substrate used for the assay, relatively low concentrations of [^{14}C] succinate were used so as not to dilute the radioactivity. It was therefore necessary to reexamine the conditions under which the enzyme was assayed to be certain that it was being assayed under optimal conditions with the level of substrate employed. In order to insure that the assay was linear with both time and enzyme concentration, it was necessary to use a relatively short incubation period (15 min), and relatively low protein concentrations (1-3 mg of protein for mitochondria and microsomes, and 100-250 μg of protein for sonicated mitochondria). Although intact mitochondria are capable of generating succinyl-CoA via the α-ketoglutarate dehydrogenase complex or the reversal of succinyl thiokinase, sonicated mitochondria and the other subcellular fractions were dependent upon an exogenous succinyl-CoA generating system (bacterial succinyl-CoA synthetase, CoA and ATP) for maximal activity. Both succinate and glycine were present in excess so that the assay was not substrate limited during the incubation period.

There has been some controversy regarding the distribution of ALA synthetase within the cell. Hayashi et al. (65), Scholnick et al. (71), and Beattie and Stuchell (66) using the colorimetric assay of Granick, have demonstrated considerable ALA synthetase activity in the post mitochondrial supernatant as well as in the mitochondria. On the other hand, Barnes et al. (24) using both the colorimetric assay of Granick and ~~the~~ radiochemical method of Irving and Elliot (111), were able to demonstrate appreciable enzymatic activity only in the

mitochondrial fraction of control animals. The present results likewise indicate that ALA synthetase is exclusively a mitochondrial enzyme. Although the total activity of ALA synthetase in the microsomal and supernatant fractions of porphyric animals is comparable to that observed in intact mitochondria, this activity represents only a few per cent of the total activity observed in sonicated mitochondria. The activity observed in the microsomal and supernatant fractions is undoubtedly due to contamination of these fractions by mitochondria damaged during preparation as judged by the amount of the mitochondrial marker enzymes succinate and glutamic dehydrogenase in these fractions. Discrepancies in the reported ratios of ALA synthetase activity in the supernatant, microsomal, and mitochondrial fractions may be due to differences in the amount of damage to the mitochondria caused by the different tissue fractionation procedures used in the respective laboratories.

McKay *et al*. (22), and Zuyderhoudt *et al*. (23) using porphyric animals, attempted to determine the intramitochondrial localization of ALA synthetase. Although they concluded that it was difficult to assign the enzyme exclusively to either the inner membrane or the matrix fraction, they tentatively decided that the enzyme was localized in the matrix. The present study corroborates their findings with control animals. The results obtained by either sonication or freezing of the mitochondria indicate that ALA synthetase is loosely bound to the inner membrane, but that it is easily dissociated by sonication or treatment with detergents.

Numerous studies, as reviewed by Granick and Sassa (113) have demonstrated an increase in porphyrin synthesis and in

ALA synthetase activity in rats with experimental porphyria. In addition, the increase in ALA synthetase activity can be blocked by inhibitors of cytoplasmic RNA or protein synthesis. On the basis of such data, it has been concluded by most workers that the increase in porphyrin synthesis is due to an increased synthesis of ALA synthetase. The results of the present study also show an increase in ALA synthetase activity when assayed in whole mitochondria. The increase in ALA synthetase activity assayed in the intact mitochondria is indeed blocked by cycloheximide, a potent inhibitor of cytoplasmic protein synthesis. When ALA synthetase is assayed in sonicated mitochondria, however, no increase in ALA synthetase was observed in porphyric rats when [^{14}C] succinate was the substrate, although moderate increases in ALA synthetase activity were observed in porphyric rats when [^{14}C] citrate or [^{14}C] α-ketoglutarate were used as substrate. Since the maximum activity of ALA synthetase is obtained in sonicated mitochondria with succinate as substrate, the results of the present study imply that the increase in ALA synthetase activity observed in chemical porphyria is a result of enzyme activation rather than <u>de novo</u> synthesis of enzyme. It must be emphasized that there is no significant difference in ALA synthetase activity between control and porphyric rats when the enzyme is assayed under optimal conditions (i. e. with dilute sonicated mitochondria using succinate and a succinyl-CoA generating system), and furthermore, that the levels of maximally induced enzyme measured in whole mitochondria from porphyric rats is always less than or equal to but never greater than the activity observed with sonicated mitochondria from either control or porphyric rats.

On the other hand, when α-ketoglutarate or citrate was used as substrate, there was a demonstrable increase in ALA synthetase activity with sonicated mitochondria from porphyric rats. Both of these substrates, however, must first be metabolized by the α-ketoglutarate dehydrogenase complex, which is under stringent metabolic regulation, and in the case of citrate by several enzymes which are also tightly regulated *i. e.* isocitrate dehydrogenase. Since both isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are regulated by the intramitochondrial redox potential [ratio of (NAD^+)/(NADH)] and by the phosphate potential [(ATP)/(ADP) + (AMP) + (Pi)], these parameters were considered likely control mechanisms for ALA synthetase. Further support for this concept came from the observation that acute ethanol intoxication, which drastically alters the cellular redox potential, also activated ALA synthetase (106). Furthermore, pyrazole, which blocks ethanol metabolism, and as a consequence the change in the cellular redox potential, not only prevented the increase in ALA synthetase activity caused by ethanol, but also prevented the increase in ALA synthetase activity caused by DDC, a potent porphyria inducing drug.

On the basis of these observations, a series of experiments were performed to determine the effects the redox potential and the phosphate potential on the activity of ALA synthetase. Although the activity of ALA synthetase varied over a four to five fold range with changes in the intramitochondrial redox and phosphate potentials, the maximum activity obtained was at most twice the control values, which is well below the

ten fold or more increase in ALA synthetase activity observed in porphyric rats. Moreover, the activity of ALA synthetase in mitochondria from both porphyric and control rats responded to changes in the redox and phosphate potentials in an identical manner, indicating that these changes are not responsible for the increased activity observed in experimental porphyria.

In a further attempt to elucidate the mechanism by which ALA synthetase is activated during chemical porphyria, the enzyme was partially purified from both DDC treated and control rats. In its most purified form, the enzyme exists as part of a large aggregate with a molecular weight of over 200,000. The complex shows at least six bands on SDS polyacrylamide gel electrophoresis indicating that it is not a high molecular weight aggregate of smaller ALA synthetase monomers.

Several workers (114,115,116,117) have reported the partial purification of ALA synthetase from porphyric rats. Scholnick _et al_. (117), Kaplan (115), and Whiting and Elliot (116) have reported the purification of a high molecular weight aggregate with ALA synthetase activity which dissociates into smaller units in high concentrations of NaCl. Whiting and Elliot were able to further purify the enzyme after salt dissociation of the aggregate and obtained a molecular weight of 77,000 for the enzyme. Hayashi _et al_. obtained a molecular weight of 115,000 for ALA synthetase isolated from a salt extract of an acetone powder of mitochondria. Attempts to dissociate the complex isolated in this study with high concentrations of NaCl and other salts have been unsuccessful.

No significant quantitative or qualitative difference was observed between the enzyme during purification from control

and porphyric rats. Moreover, no difference was observed in the banding patterns on SDS gel electrophoresis. These results strongly suggest not only that there is no increase in the amount of enzyme during experimental porphyria, but also, that there is no gross alteration in the structure of the enzyme as a result of the activation process.

These studies have indicated, however, that there is an activator present in the mitochondria which is necessary for maximal expression of ALA synthetase activity. Again, no significant differences in either the amount or physical properties of the activator could be seen, when it was purified from the mitochondria of both control and porphyric rats. The behavior of the activator during purification, however, suggested that it may not be a protein. Further support for this came from the observation that a sonicated membrane preparation from an ALA requiring mutant of *R. Spheroides* could also activate ALA synthetase purified from rat liver.

Irving and Elliot (111) have observed a non-protein effector of ALA synthetase in a membrane fraction of mitochondria in control rats which inhibits ALA synthetase activity when added to mitochondria from porphyric rats. Their results, together with the results of this study suggest the possibility that the activity of ALA synthetase is controlled by its association with one of at least two different "factors" in the mitochondrial membrane. According to this scheme, in control rats the mitochondrial membrane exists in a form which binds ALA synthetase such that the enzyme has low activity. In control mitochondria, the activator is buried in the membrane such that ALA synthetase does not have access to the activator,

while during experimental porphyria, the conformation of the mitochondrial membrane is altered in such a way that the activator is unmasked, and free to interact with ALA synthetase. Normal regulation of ALA synthetase could be achieved by subtle changes in membrane conformation in response to salts, redox and phosphate potential or hemin, which expose more or less activator as required. ALA synthetase is activated by sonication or detergents because both ALA synthetase and the activator, presumably a normal component of the mitochondrial inner membrane, are both released into solution where they are free to interact. Freezing and thawing the mitochondria, which ruptures the mitochondria but causes only slight damage to the membrane, causes only a slight activation of ALA synthetase.

The increase in ALA synthetase activity observed in experimental porphyria, is assumed to be a consequence of the cellular adaption to the toxic effects of the inducing chemical. Although it is clear that the inducing chemicals are not the immediate cause of the alteration of the membrane (as addition of the drugs to whole homogenates or mitochondria _in vitro_ does not result in an increase in ALA synthetase activity), it should be noted that porphyria inducing chemicals are uniformly lipophilic (104) and presumably interact directly with biological membranes. It may be that it is the attempt by the cell to correct dysfunctions in the properties of the membrane which are caused by the chemicals that prompts the cell to alter the composition of the membrane which then results in the increase in ALA synthetase activity.

If the cell is specifically altering the composition of the membrane it must, presumably, make specific modifications in the protein composition of the cell *i. e.* changes in certain enzyme levels, regulatory proteins or membrane proteins. If these specific protein adaptions are prevented by inhibiting cytoplasmic protein synthesis, then the specific alterations in the membrane would not occur, and there would be no increase in ALA synthetase activity. Moreover, since the membrane in question is the mitochondrial membrane, which synthesizes some of its own membrane proteins, inhibiting mitochondrial protein synthesis could also prevent the increase in ALA synthetase activity as was shown by Beattie and Stuchell (66).

ABBREVIATIONS

ADP - adenosine dephosphate

AIA - allylisoproplyacetamide

ALA - δ-aminolevulinic acid

ATP - adenosine triphosphate

C-AMP - 3',5' cyclic adenosine monophosphate

CCP - carbonyl cyanide m-chlorophenyl hydrazone

CoA - coenzyme A

Copro - coproporphyrinogen

cpm - counts per minute

DDC - 3,5-dicarbethoxy-1, 4-dihydrocollidine

Deutero- deuteroporphyrinogen

DNP - 2,4-dinitrophenol

DTE - dithioerythrotol

EDTA - ethylenediamine tetraacetic acid

gm - gram

hr - hours

I.P. - intraperitoneal

K_I - concentration of inhibitor which causes 50% inhibition

K_M - Michealis binding constant i.e. concentration at which 50% is bound

M - moler

mg - milligram

min - minutes

ml - milliliter

mM - millimoler

m-RNA - Messenger Ribonucleic acid

M.W. - molecular weight

n - normal

NAD^+ - nicotine adenine dinucleotide

NADH - reduced nicotinamide adenine dinucleotide

$NADP^+$ - nicotinamide adinine dinuceotide phosphate

nm - nanometer

Oligo - oligomycin

PBG - porphobilinogen

Proto - protoporphyrinogen

R_f - retardation factor

RNA - Ribonucleic acid

Rot - rotenone

s - seconds

SDS - sodium sosecyl sulfate

SKF-525-A 2-diethylaminoethyl 3, 3-diphenylpropylacetate

TCA - trichloroacetic acid

TRIS - tris(hydroxymethyl)aminomethane

UDP - uridine diphosphate

URO - uropophyrinogen

Val - valinomycin

°C - degrees centigrade

μCi - microcurie

μg - microgram

X g - times gravity

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