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ABSTRACT

DIFFERENTIAL PROPERTIES AND CONTROL OF δ-AMINOLEVULINIC ACID SYNTHETASE ISOZYMES FROM ERYTHROID AND NON-ERYTHROID TISSUE

By

David Franklin Bishop

Fetal animals exhibit a transient overproduction of heme precursors, suggesting that heme synthesis in the fetus is controlled differently than it is in the adult. In this dissertation research, δ-aminolevulinic acid synthetase, the rate limiting enzyme of heme biosynthesis, has been studied in tissue from fetal and adult guinea pigs.

Mitochondrial ALA synthetase activity in fetal liver increased during mid-gestation in the guinea pig from 25 U/gm liver to a maximum of 60 U/gm liver at 44 days of gestation. It steadily declined in activity until at parturition (68th day) it was at the low adult level of 3.0 U/gm liver. Erythropoietic activity in the liver also followed this pattern suggesting that the fetal enzyme is an erythroid cell product, in contrast to the enzyme from the adult, non-erythroid liver.

ALA synthetase from the erythroid fetal liver had more properties in common with the adult erythroid enzyme of bone marrow than with the 3,5-dicarbethoxy-1,4-dihyro-

collidine induced enzyme of adult liver, a non-erythroid tissue. The mitochondrial ALA synthetase of fetal liver (45th day of gestation) and the synthetase of adult bone marrow did not bind to either AMP (C_6-amino)- or CoA (sulfhydryl)-carboxyhexyl Sepharose. In contrast, the form induced in adult liver was tightly bound and was eluted from both columns by 50 mM 5'-AMP. Furthermore, when chromatographed in the presence of the substrate glycine, both enzymes from erythroid tissue were partially retarded on AMP-Sepharose and more completely retarded on CoA-Sepharose affinity columns. Glycine had no effect on the behavior of the adult liver enzyme. The Km for glycine for the non-erythroid enzyme was four-fold lower than that for the erythroid enzymes. The non-erythroid enzyme displayed negative cooperativity while the other enzymes did not. The erythroid forms are strongly inhibited by high KCl or NaCl concentrations whereas the adult liver isozyme is not. The molecular weights of all three enzymes are the same. Different heat denaturation rates were determined for all three isozymes.

Evidence is presented suggesting that the isozyme from adult liver binds nucleotides at some other site than that of succinyl-CoA binding.

A new assay procedure was developed in which δ-aminolevulinic acid synthetase activities in the range of 0.1 to 100 nmoles ALA/hr/ml enzyme were routinely assayed using a modified Beckman 121 amino acid analyzer. This method reproducibly and specifically quantitates

^{14}C-aminolevulinate from a mixture of ^{14}C-succinate metabolites shown to interfere in other methods.

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By

David Franklin Bishop

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1976

DEDICATION

To C. F. Bishop,

father and biologist

extraordinaire

ACKNOWLEDGEMENTS

The author thanks Dr. W. A. Wood for his generous support and encouragement throughout this endeavor. His unfailing friendship did much to lighten the task. I have appreciated his wisdom in his dealings with me and recognize its positive influence on my development. Thanks also to my committee for being helpful in so many ways, and especially to Dr. Hyram Kitchen for his encouraging me and for talking Dr. Wood into taking on this project. Special thanks to Jim Kurtz for his friendship and help, particularly for reading and commenting on the text. I am indebted to Ron and Debora Troyer and Ana for typing the dissertation, and to the MSU Mennonite Fellowship for being a community of friendship and support. Finally, a note of thanks to Dr. R. S. Bandurski for starting me on this course and urging me to ask nature a question.

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LIST OF ABBREVIATIONS

ALA	δ-Aminolevulinic acid
ALA synthetase	δ-Aminolevulinic acid synthetase
AIA	Allylisopropylacetamide
Copro'gen III	Coproporphyrinogen III
DDC	3,5-dicarbethoxy-1,4-dihydrocollidine
DTT	Dithiothreitol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
PLP	Pyridoxal-5'-phosphate
Proto'gen III	Protoporphyrinogen III
TCA	Trichloroacetic acid
Uro'gen I	Uroporphyrinogen I
Uro'gen III	Uroporphyrinogen III

INTRODUCTION

Life on our planet is supported by the utilization of the sun's radiant energy to produce glucose. The magnesium porphyrin of green plants, chlorophyll, traps this energy and uses it to split water to yield oxygen and the energy to synthesize glucose from carbon dioxide. In the process of recovering this energy, animals utilize the iron porphyrin, heme, to bind oxygen and enable it to regain the electrons it lost to glucose.

In both plants and animals, heme functions in the cytochromes to facilitate the transfer of small packages of energy from the excited electrons to form ATP for general use in the energy requiring chemistry of the cell.

The intense green and red colors of these porphyrins have long interested scientists. Their studies have led to our current understanding of these pigments' central role in supporting life.

While the general strategies of porphyrin synthesis and control are similar in plants, microorganisms and animals, they do differ in detail. This dissertation will deal only with experiments on the control of heme synthesis in animals.

The importance of heme in animal life is emphasized by the pathologies resulting from changes in normal heme

metabolism and control. Obviously, a mutation resulting in a complete block in the heme pathway would be lethal. Situations do occur, however, in which heme synthesis is reduced, leading to various anemias. Hereditary and environmental factors can also cause overproduction of heme and related porphyrins leading to chronic metabolic disorders. These porphyrias may cause skin irritations, neurological symptoms, abdominal pains and, occasionally, death. Thus, strict controls are needed to maintain sufficient levels of heme while preventing toxic overproduction.

In animals, heme biosynthesis is controlled in part by regulation of the activity or synthesis of δ-aminolevulinic acid synthetase (ALA synthetase), the rate limiting enzyme of the pathway. Though it is well established that regulation of synthesis of ALA synthetase is an important controlling factor under some conditions, the focus of this investigation is on some of the structural and functional factors determining the activity of the enzyme.

The discovery that ALA synthetase and porphyrin levels are elevated during fetal life implies a change in control of the pathway during development. One hypothesis is that this transient fetal porphyria may be caused by a fetal isozyme with different control characteristics than the adult enzyme. This hypothesis would be supported if functional or structural differences could be found between ALA synthetase of fetal and adult origins.

The purposes of this research are two-fold. One is to compare the properties of ALA synthetase from fetal and

adult liver to determine if they are isozymes. The other is to investigate their possible relationship to the dissimilarity in fetal and adult control of heme synthesis. This research problem was approached in three separate but related stages.

First, the developmental pattern of ALA synthetase activity in relation to other fetal events was studied in the guinea pig to determine possible factors controlling its activity. Knowledge of this pattern also permits one to define the gestational age of maximal fetal ALA synthetase activity. Enzyme isolated at this stage of development should be most representative of the fetal enzyme whereas at a later stage there might be a mixture of fetal and adult types.

Secondly, a study of the functional and structural differences between ALA synthetase of fetal and adult origin was made to investigate the possibilities of regulation via isozymes and allosteric ligands.

Finally, the success of all of these inquiries depended on the existence of a sensitive, specific and reproducible assay for ALA synthetase. The currently available methodology being inadequate, an assay was developed to overcome the problems of contaminating substances and competing reactions and of the low levels of enzyme activity in the various preparations.

Following a general literature review, these three stages are presented as three separate sections. Each section contains its own introduction, materials and

methods section, results and discussion. The final section is being submitted for publication and therefore differs slightly in style from the preceding sections.

I. GENERAL LITERATURE REVIEW

A. Historical

Hoppe-Seyler coined the term "hematoporphyrin" to describe the red pigment he isolated from hemoglobin in 1871 (1). In 1880 he suggested its relation to chlorophyll based on the latter's conversion to a red pigment by alkali. Subsequent research culminated in the elucidation of the structure of heme in 1928 and that of chlorophyll in 1934 (2). It was not until 1945 that glycine was implicated as a building block of porphyrins. Between 1945 and 1955, the pioneering work in the elucidation of the heme synthetic pathway was carried out by David Shemin and co-workers (3). Using labeled precursors they were able to show that all the carbon atoms of heme are derived from the α-carbon of glycine and an asymmetric succinate derivative (4,5). This derivative was shortly identified as succinyl CoA which with glycine formed δ-aminolevulinic acid (ALA) in the presence of sonicated mitochondria from chicken erythrocytes (6,7). Several excellent reviews contain additional historical information concerning porphyrins and their synthesis (2,3,8,9).

B. The heme pathway

The intermediates and corresponding enzymes of the heme pathway in animals are summarized in Figure 1.

The condensation of succinyl-CoA and glycine to form δ-ALA with the release of CO_2 and CoA is catalyzed by the mitochondrial enzyme, δ-aminolevulinic acid synthetase[1] (10-12). The enzyme requires pyridoxal-5'-phosphate and is solubilized and stabilized by dithiothreitol and high salt or detergent (13,14). Its lipophilic nature is suggested by these properties and its probable association with the mitochondrial inner membrane (15). Its activity is apparently rate limiting for the pathway and its synthesis is negatively controlled by the final product, heme (16,17).

The aminoketone δ-ALA passes through the mitochondrial membrane to the cytoplasm where two molecules undergo aldol condensation to yield porphobilinogen and water. ALA dehydratase,[2] a zinc metalloenzyme, catalyzes this reaction (18). Sulfhydryl compounds like glutathione activate the enzyme while lead inhibits it (19). This latter characteristic makes excessive urinary ALA excretion diagnostic for lead poisoning (20). The oligomeric dehydratase has been extensively purified from various sources and is the best characterized enzyme in the pathway.

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[1]Succinyl-CoA: glycine succinyl transferase, E.C. 2.3.1.37.

[2]5-Aminolevulinate hydrolase, E.C. 4.2.1.24.

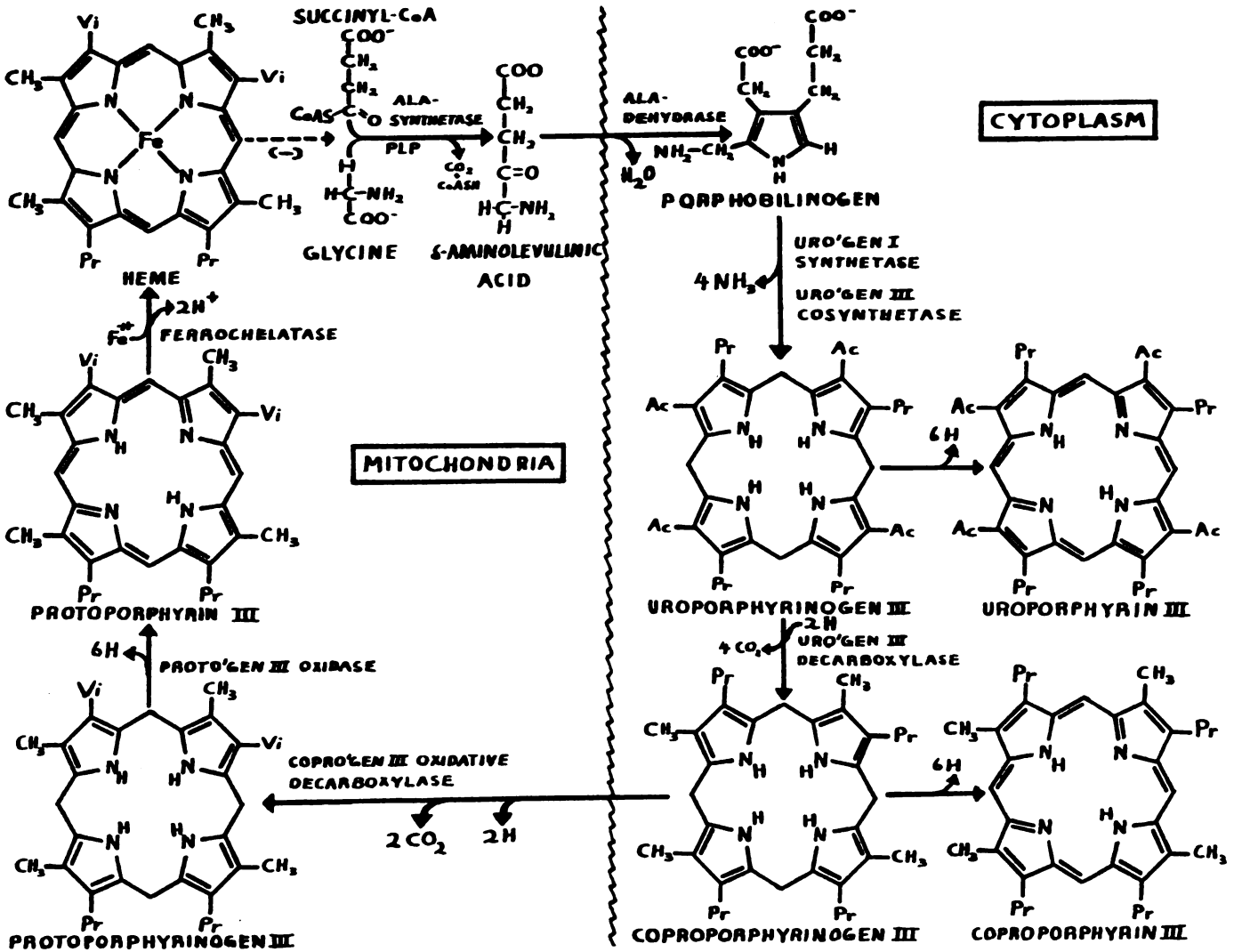

Figure 1. The heme biosynthetic pathway.

The concerted action of two enzymes, uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase, is required for formation of the tetrapyrrole, uroporphyrinogen III (Uro'gen III). Uro'gen I synthetase acting alone forms only uroporphyrinogen I (21,22). The mechanism of the interconversion has remained an enigma for more than a decade. Resolution is in sight with the recent discovery of a crucial intermediate (23).

Uroporphyrinogen III decarboxylase catalyzes the stepwise decarboxylation of uro'gen III to copro'gen III[3] (24). The partially purified enzyme is specific for the fully reduced uro'gen III but will also decarboxylate uro'gen I at reduced rates (25).

Copro'gen III must first pass through the mitochondrial membrane before being converted to proto'gen III[3] by copro-porphyrinogen III oxidative decarboxylase. The enzyme has been partially purified and requires molecular oxygen for the sequential oxidation of two propionate groups (26,27).

Proto'gen III auto-oxidizes easily, but the evidence supports its enzymatic converstion to protoporphyrin III (28).

Ferrous iron is incorporated into protoporphyrin III by ferrochelatase[4] in the terminal step of heme biosynthesis. Like ALA-synthetase, it is lipophylic and solubilized from mitochondrial cristae by detergent or salts (29).

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[3]See abbreviation list, p. x.

[4]Protoheme ferrolyase, E.C. 4.99.1.1.

In pathological states, porphyrinogens can also be found in urine, feces and bone. In the presence of light and oxygen they are photo-oxidized to the corresponding porphyrins (30).

The initial step and final steps of the pathway occur in the mitochondrion while the remaining are cytoplasmic. One explanation for this segregation is the need to prevent oxidation of the porphyrinogens, a situation which would be more likely in proximity to the electron transport chain of mitochondria (10).

Additional details concerning heme biosynthesis may be found in several comprehensive reviews (2,25,31).

C. The uses of heme

Unmodified heme is the prosthetic group for numerous hemoproteins. In vertebrates they include the oxygen carriers hemoglobin and myoglobin, tryptophan oxygenase, the hydroperoxidases, catalase and peroxidase, and the class B redox carrier cytochromes, b, b_5 and P_{450}. Cytochromes a, a_3, c and c_1 contain heme with modified side chains (32).

The regulatory role of heme is highlighted by its stimulation of globin synthesis and repression of the synthesis of ALA synthetase (17,33).

Heme is degraded in the liver by a microsomal heme oxygenase to yield bilirubin (34). Heme also binds strongly to its specific carrier, hemopexin. The latter may

function in transporting heme from senescent red blood cells to the oxygenase (35).

D. ALA synthetase as the rate controlling step

ALA synthetase is considered to be the key regulatory enzyme in the pathway of heme biosynthesis. This is based on its activity being rate-limiting under normal conditions. Two lines of evidence support this conclusion. First, incubation of chick blastoderms with ALA led to intense porphyrin fluorescence assumed to be due to heme precursors. No fluorescence was noted after incubation with succinate and glycine (36). Second, in vitro assays of other enzymes in the pathway demonstrate specific activities greater than that of ALA synthetase (37,38).

E. Regulation of ALA synthetase

1. Control of enzyme amount

a. Chemical induction. Theories on the control of ALA synthetase had their origin in studies of the porphyrias, the diseases of porphyrin overproduction. It was soon realized that certain chemicals precipitated porphyric attacks or made existing cases worse (30). An experimental porphyria was produced in rabbits by the drug sedormid (Figure 2) (39).

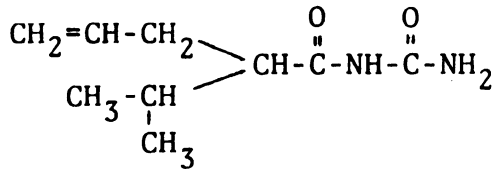

Figure 2. Structure of Sedormid (allylisopropyl-acetyl urea).

Allylisopropylacetamide (AIA), the soluble derivative of Sedormid lacking the carbamyl group, is a potent inducer of porphyria and ALA synthetase in rats and rabbits. Guinea pigs, however, are too sensitive to its hypnotic effects to withstand doses large enough to induce porphyria (30).

While being used as a blue fluorescent marker for the forestomach of mice, 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) (Figure 3) was found to cause bright red fluorescence of the gallbladder and intestines (40).

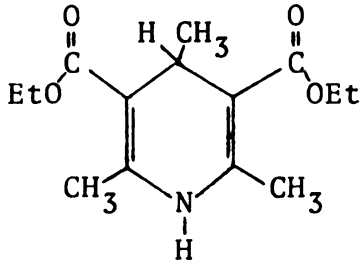

Figure 3. Structure of DDC.

DDC was subsequently identified as a powerful inducer of ALA synthetase in guinea pigs, rats, rabbits and other animals (37).

Granick has divided the chemical inducers of porphyria into four classes: barbiturates, e.g., AIA; collidine derivatives, e.g., DDC; steroids, e.g., pregnanalone; and a miscellaneous class including chemicals like the fungicide δ-hexachlorocyclohexane (Lindane). Lindane caused a widespread porphyria outbreak in Turkey when seed wheat was used to make bread (41).

The use of inhibitors of RNA and protein synthesis indicates that 5-βH steroids and DDC induce ALA synthetase by exerting their effects at the level of transcription while AIA and Lindane act at the level of translation (42). Quantitative immunochemistry has confirmed that the increases in ALA synthetase activity induced by DDC or AIA actually represent an increase in the amount of enzyme present (43).

Most of these chemicals also have other metabolic consequences. There is evidence that DDC and especially AIA promote destruction of cytochrome P_{450} (44,45), while Lindane inhibits the conversion of uro'gen III to copro'gen III (46). Phenobarbital increases ALA synthetase activity only after stimulating apocytochrome P_{450} synthesis (44).

b. Heme repression. There are strong indications that the primary control of ALA synthetase levels resides in the heme-dependent repression of mRNA translation of the enzyme. Cultured liver cells from chick embryos were induced by AIA and then treated with actinomycin D. Subsequent addition of hemin decreased the half-life of ALA

synthetase relative to controls, implying inhibition of translation of preformed mRNA (42). The results were the same when heme was generated endogenously by ALA loading (47). Again, immunochemical studies showed that heme specifically blocks the synthesis of ALA synthetase in cell cultures (43). Granick proposed that competition for heme by hemoproteins and the enzymes of heme catabolism could be balanced by heme synthesis through action of the heme repressor (10) (Figure 4). This was dramatically demonstrated by cyclic, complimentary oscillations in rat hepatic heme oxygenase and ALA synthetase following heme injections (48). Heme synthesis, blocked in cell cultures by addition of iron chelators, led to a synergistic increase in ALA synthetase upon AIA induction. This supports the hypothesis that many inducers act indirectly on ALA synthetase by decreasing the pool of heme available for repression (17,38). This pool is probably not free due to the high affinity of various proteins and membranes for heme (49). The interrelationships under discussion are diagrammed in Figure 4. For example, AIA is thought to induce ALA synthetase at the translational level. However, it also accelerates cytochrome P_{450} degradation, which could lead to a reduction in heme levels. Thus, the translational effect could in fact be release of heme repression. In this regard, it is interesting to note that cultured embryonic heart cells which do not have an active cytochrome P_{450} drug metabolizing system also do not synthesize ALA synthetase in response to AIA or DDC

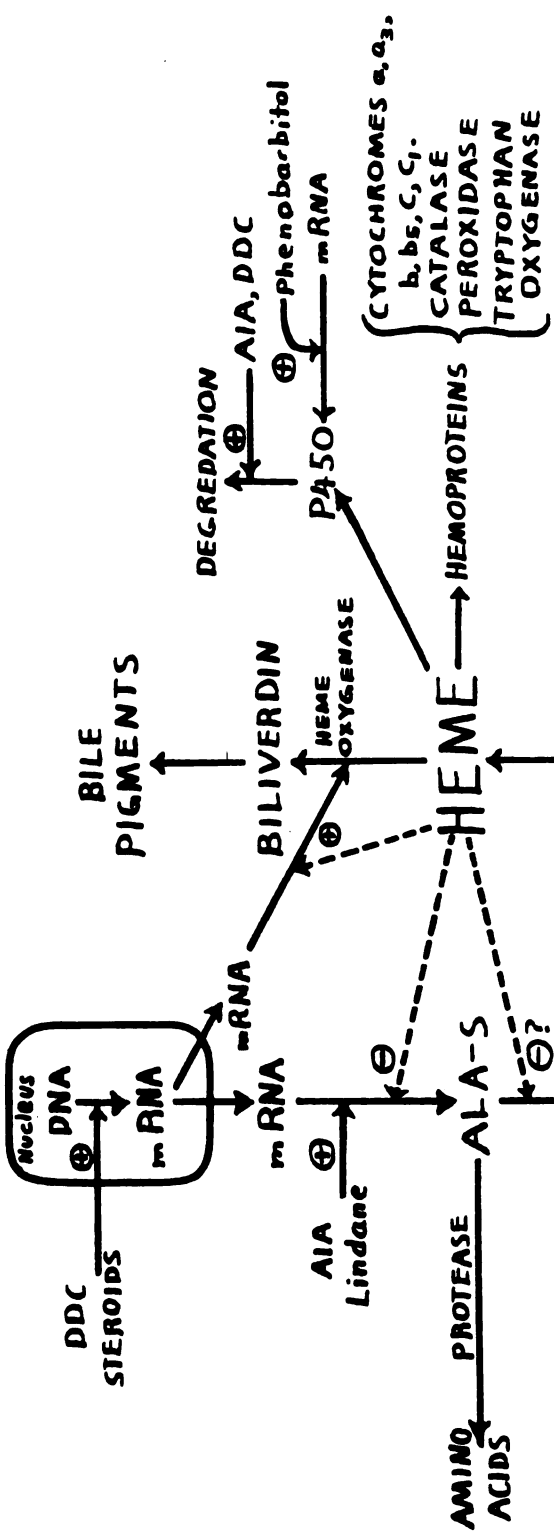

Figure 4. Regulation of heme synthesis and utilization.

(50). The buffering ability of the system is shown by heme's coordinated repression of ALA synthetase and induction of heme oxygenase synthesis which would tend to return excessive heme levels to normal. An apparent exception to this pattern occurs upon cobalt treatment of the liver. Cobalt inhibition of ferrochelatase reduces heme levels but there is no concomitant stimulation of ALA synthetase (38).

Release of heme repression also may be involved in the porphyrias, which are characterized by excretion of excess porphyrin intermediates. In some cases the primary event may be excessive ALA synthetase activity, but a block anywhere in the pathway would also cause intermediates to accumulate. The resulting reduction in heme could in turn reverse ALA synthetase repression (30). For example, congenital erythropoietic porphyria is distinguished by red urine and bones due to uroporphyrin I deposits resulting from limiting uro'gen III cosynthetase levels (51). In this disease, increased ALA synthetase activity enhances the accumulation of porphyrin intermediates due to the reduced capacity to convert them to uro'gen III. A comprehensive review of the porphyrias is available (30).

c. Glucose repression. Glucose also interferes with the induction of ALA synthetase by chemicals. This is reminiscent of microbial "catabolite repression" of enzyme induction by glucose. The mechanism is unknown

but the effect may be mediated by elevated ATP levels (52). Another hypothesis is that drug metabolism is more effective due to increased glucuronidation (10).

d. Compartmentalization. ALA synthetase is synthesized on cytoplasmic ribosomes and is only functional in the mitochondria, where succinyl CoA is available (53). The amount of functional enzyme could therefore depend on a transport process. Based on experiments in rats, it has been proposed that heme "represses" ALA synthetase activity by preventing transport of the enzyme from the cytoplasm to the mitochondria (54). These results have been questioned since the enzyme does not accumulate in the cytosol of hemin treated embryonic chick liver cells (17,43).

e. Degradation. The extremely short half-life of ALA synthetase in mammalian liver (70 minutes) suggests control by inhibition of degradation (55). A new inactivating enzyme for apopyridoxal phosphate enzymes has recently been found in rat muscle and intestine (56). Also, there is a pattern of inducible, rapid turnover enzymes which are degraded in the cytosol upon coenzyme dissociation (57). In spite of this, neither AIA nor hemin "induce" ALA synthetase in embryonic chick liver by inhibiting the enzyme's degradation (42). This situation needs to be reinvestigated in erythroid tissues due to the discovery of a low activity apo-ALA synthetase protease in bone marrow (58).

2. Control of enzyme activity

a. Heme inhibition. In early experiments with guinea pig liver mitochondria, Granick found no inhibition of ALA synthetase by hemin (59). However, hemin inhibited partially purified cytoplasmic enzyme with a K_i of 20 μM (60). The inhibition could be reversed by addition of albumin or cytosol protein, thus explaining the lack of inhibition in crude systems (53). A K_i of 10 μM was reported for hemin inhibition of partially purified mitochondrial ALA synthetase (13), while nearly homogeneous enzyme from embryonic chick liver exhibits a K_i of 35 μM (12). In light of the fact that hemin represses ALA synthetase synthesis with a K_i of 0.1 μM in the same tissue, Granick and co-workers feel that feedback inhibition is relatively unimportant in cellular regulation of ALA synthetase activity (12,17). Nevertheless, local heme concentrations could be higher due to proximity of ALA synthetase and ferrochelatase in the mitochondrion (15). Further complications in assessing physiological function arise from the tendency of hemin to aggregate into stacked dimers or polymers (45). Additionally, the porphyrin oxidation state in mitochondria is probably ferriheme (61), while in most inhibition experiments, hemin, the bis-chloro ferriheme, is used.

b. Substrate or cofactor availability. The high Km (ca 10 mM) for glycine has prompted the hypothesis that ALA synthetase activity could depend on the availability

of this substrate (62). Glycine loading increases porphyrin excretion in both healthy controls and porphyric patients and doubles porphyrin production in drug induced chick embryo liver (63). Glycine loading in normal embryos resulted in a minimal increase in porphyrin concentration. Thus, increased glycine levels would not lead to porphyria in animals but could be a controlling factor in already porphyric subjects.

Pyridoxine responsive anemia in humans and animals implies regulation of heme synthesis by ALA synthetase cofactor availability (64,65). Although B_6 deficient ducklings have reduced heme synthesis, animals normally have sufficient vitamin (66). It is therefore unlikely that pyridoxal phosphate concentration has a regulatory function except in some disease states.

c. Effectors. A correlation exists between iron overload in patients with sideroblastic anemia and a decrease in heme synthesis (67). Ferrous iron, while slightly activating ALA synthetase at 1 μM, inhibits it 50% at 1 mM in mitochondrial suspensions (68). The situation is complicated by the ability of pyridoxine to facilitate iron mobilization (68).

Divalent and monovalent cations activate ALA synthetase optimally at 50 and 400 mM, respectively (12). This is a higher concentration than is physiologically normal and may be related to a general ability of high salt concentration to stabilize and solubilize lipophilic enzymes.

II. HEME SYNTHESIS IN THE DEVELOPING GUINEA PIG

A. Introduction

Porphyrias are pathological conditions characterized by the accumulation of porphyrins resulting in excretion of the excess heme precursors in the urine and feces (30). The porphyrias affecting animals are separated into two major groups, hepatic or erythroid, depending on the site of porphyrin overproduction (30,69). In contrast to these usually deleterious disease states, the naturally porphyric fox squirrel (*Sciurus niger*) is quite healthy. This animal has huge porphyrin concentrations such that its bones and often its urine are red (70).

Dr. Hyram Kitchen has recently found that the bones of fetal animals have a high porphyrin content, identifiable by their red fluorescence under ultraviolet light (71,72). As in the fox squirrel, there appears to be no detrimental effect from this transient, perinatal porphyria. A literature search revealed that others had studied these fetal deposits over 50 years ago. Fetal serum was found to have two to ten times the porphyrin of newborn serum (73). Coproporphyrin was found in human amniotic fluid (125 da of gestation) (74). Porphyrins were also found in the bones of certain porphyric patients (75). Fraenkel experimentally reproduced the porphyrin build-up in bone by

injecting young guinea pigs with porphyrins. He also showed that if pregnant animals were given porphyrin injections, no fetal deposits could be found (76). These results indicate that the source of the excess porphyrin metabolites is the fetus and not the mother.

More direct evidence for a distinctly fetal regulation of heme synthesis has been presented recently. Woods reported that ALA synthetase in guinea pigs decreases in activity to the low adult level in the last two weeks of gestation. Rabbits and rats display the same decline (77). Doyle finds a similar pattern for ALA dehydratase in developing mice (78). While Doyle feels that the decline in ALA dehydratase levels is correlated with a decline in liver erythropoiesis, Woods believes that in the case of ALA synthetase it represents the development of adult-type control in maturing hepatocytes.

Thus, this transient porphyria presents a model for the study of developing control mechanisms of porphyrin metabolism. It should complement the current knowledge of control mechanisms acquired from the disease state and chemical induction models.

In the present study, ALA synthetase levels are measured at much earlier times of gestation giving a more complete developmental profile. The results show that the striking fetal porphyria is associated with erythropoietic activity in the liver rather than with maturing parenchymal cells.

B. Materials and methods

1. Chemicals - see Section IV.B.1

2. Animals

English short-hair guinea pigs of the Hartley strain were obtained from a closed, random-bred colony established in 1934 by Cannaught's Laboratories Limited, Willowdale, Ontario. Housing was provided by stainless steel or galvanized cages with wire mesh floors or by polyethylene tubs with wood chip bedding. They were fed, *ad libitum*, Wayne guinea pig diet from Allied Mills, Inc., and Triumph Rabbit Pellets from John A. Van Den Bosch Co. Distilled water was provided containing 2 g/liter ascorbic acid. Vitamin C was administered to compensate for insufficient levels in the diet, an important point considering its involvement in heme synthesis (79).

Guinea pigs were housed three to five females per male throughout the experiments. A technician examined the females daily for estrus, which begins with the rupture of the vaginal membrane. Day 1 of gestation was either the day following the appearance of a vaginal plug or, in its absence, the second day of vaginal opening. This is in accordance with Minot (80), who reports conception within 24 hours of coitus. Absence of estrus during the following 16 day cycle and the use of palpation to determine the presence of embryos allowed confirmation of pregnancy by the 18th day of gestation.

Pregnant pigs were ether-anesthetized and bled by heart puncture. The comatose animals were killed by cervical dislocation and pneumothorax. The uterus and its contents were removed from an abdominal incision and the amniotic fluid was aspirated by syringe. Blood was collected from the fetuses either by heart puncture or decapitation. The liver was then removed, blotted, weighed and placed in ice-cold isotonic buffer until subsequent homogenization and assay. This homogenization buffer contained 0.25 M sucrose, 0.02 M potassium phosphate, pH 7.5, and 0.05 mM pyridoxal-5'-phosphate.

3. ALA synthetase assay

To initiate the reaction, 0.1 ml of the isolated mitochondrial suspension[5] was added to 0.4 ml of assay mixture to yield the following concentrations: 100 mM

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[5]Livers were blotted and 0.5 gm from each fetus or newborn was homogenized with 5 ml of homogenization buffer in a 10 ml Potter-Elvejhem homogenizer (A. H. Thomas) operated at 300 rpm. The undiced liver was disrupted by two passes of the pestle and was cooled again in an ice bath. After 8 more passes, the homogenate was sedimented at 1000 rpm (about 100 x g) for 5 min in a Beckman J-21 centrifuge with a 50 ml head (Beckman JA-20) and 12 ml Delrin adapters (Sorval #402). The supernatant was weighed and then sedimented at 10,000 rpm (10,000 x g) for 10 min. The mitochondrial pellet was resuspended in 1.0 ml of homogenization buffer and the suspension weighed. The weighings allowed conversion of mitochondrial ALA synthetase activity into units per gram liver according to the following expression:

$$\text{U/gm liver} = \frac{\text{Units}}{\text{mitochondria}} \cdot \frac{\text{wt. mitochondria}}{\text{wt. 100 x g supernatant}} \cdot \frac{5.5 \text{ ml homogenate}}{0.5 \text{ gm liver}}$$

This equation assumes identity between the homogenate and the 100 x g supernatant with respect to distribution of activity.

glycine, 50 mM HEPES, 10 mM $MgCl_2$, 2 mM ATP, 1.1 mM CoA, 1.1 mM succinic acid, 1 mM DTT, 0.05 mM PLP, 1 µci ^{14}C-succinic acid (13 µci/µmole) and 0.3 units of succinyl CoA synthetase (20 µg) prepared from *E. coli* (see Sec. IV). This mixture was incubated in a water bath at 30°C in 10 x 75 mm disposable culture tubes (Corning) for 30 min. The reaction was stopped and deproteinized by addition of 0.05 ml 30% trichloroacetic acid (TCA). After at least 30 min the coagulated protein was sedimented for 7 min in an IEC clinical centrifuge and the supernatant carefully drawn off with a Pasteur pipette.

Two hundred microliters of the deproteinized supernatant was analyzed for ^{14}C-ALA in a modified Beckman 121 automatic amino acid analyzer. This linear and reproducible method is described in Sec. IV.

4. Determination of erythropoiesis

The extent of erythropoietic activity based on relative numbers of nucleated red blood cells in stained liver slices was determined by Mr. Roger Cook from Dr. Kitchen's laboratory.

5. Statistics

Each ALA synthetase determination is derived from one fetus or newborn for which 1 to 4 replicate assays were carried out. The average was 1.94 $\pm$ 0.04 replicates. The deviations are given as $\pm$ standard error of the mean (SEM) where SEM = Standard Deviation/$\sqrt{\text{no. of determinations}}$. Exceptions occur between 30-38 days of gestation where some

determinations are pools of 3-9 fetal livers. This was done in order to get 0.5 gm of liver for the standard assay. The overall average of the % deviation from the means of the replicates was 5.4 $\pm$ 0.8%. Two to eleven determinations were carried out for each date with an average of 4.6 $\pm$ 0.4 determinations per date. Fetuses of the same age, whether littermates or not, were lumped together. Graphical data represent SEM's as vertical bars.

C. Results

1. ALA synthetase activity during development

The specific activity of mitochondrial ALA synthetase expressed in U/gm liver rises to a maximum at 43 days of gestation. It declines to adult levels at birth (68th day), followed by a slight rise in the neonate (Figure 5). The activity of a normal adult male is around 2.3 U/gm liver. At 30 days of gestation the liver weight is about 50 mg. This represents the earliest time at which the methodology developed for this developmental survey could reliably assay ALA synthetase. At this time, the livers varied from nearly translucent in appearance through slightly reddish mottled, to nearly completely red.

If the liver weight is taken into account, the maximum ALA synthetic capacity of the fetus occurs at 53 to 54 days of gestation (Figure 6).

During the period of maximal ALA synthetic capacity, there was some increase in maternal liver ALA synthetase

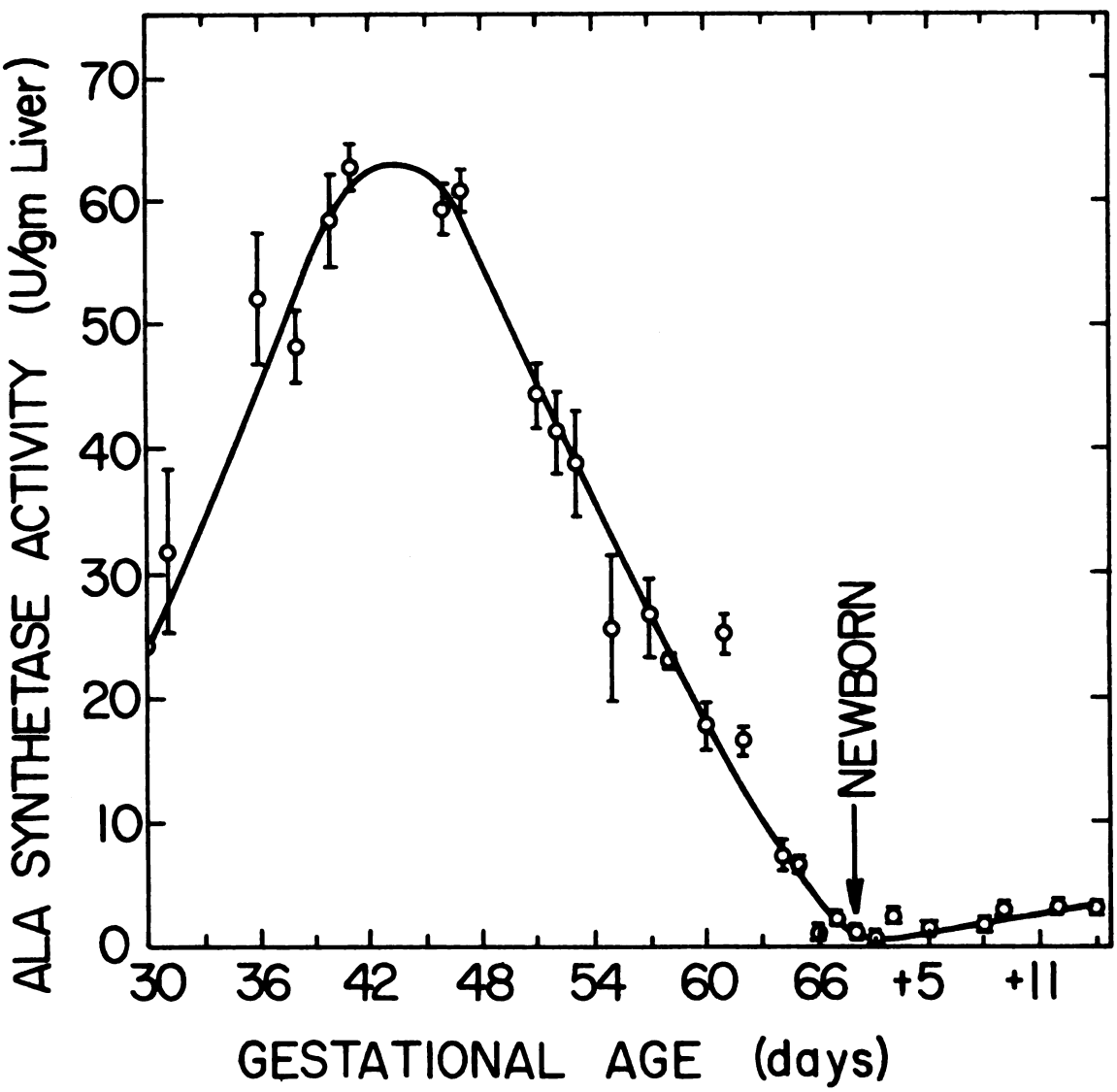

Figure 5. ALA synthetase specific activity during development. The assay, tissue isolation, statistical and dating procedures are described in Methods. The bars denote S.E.M.

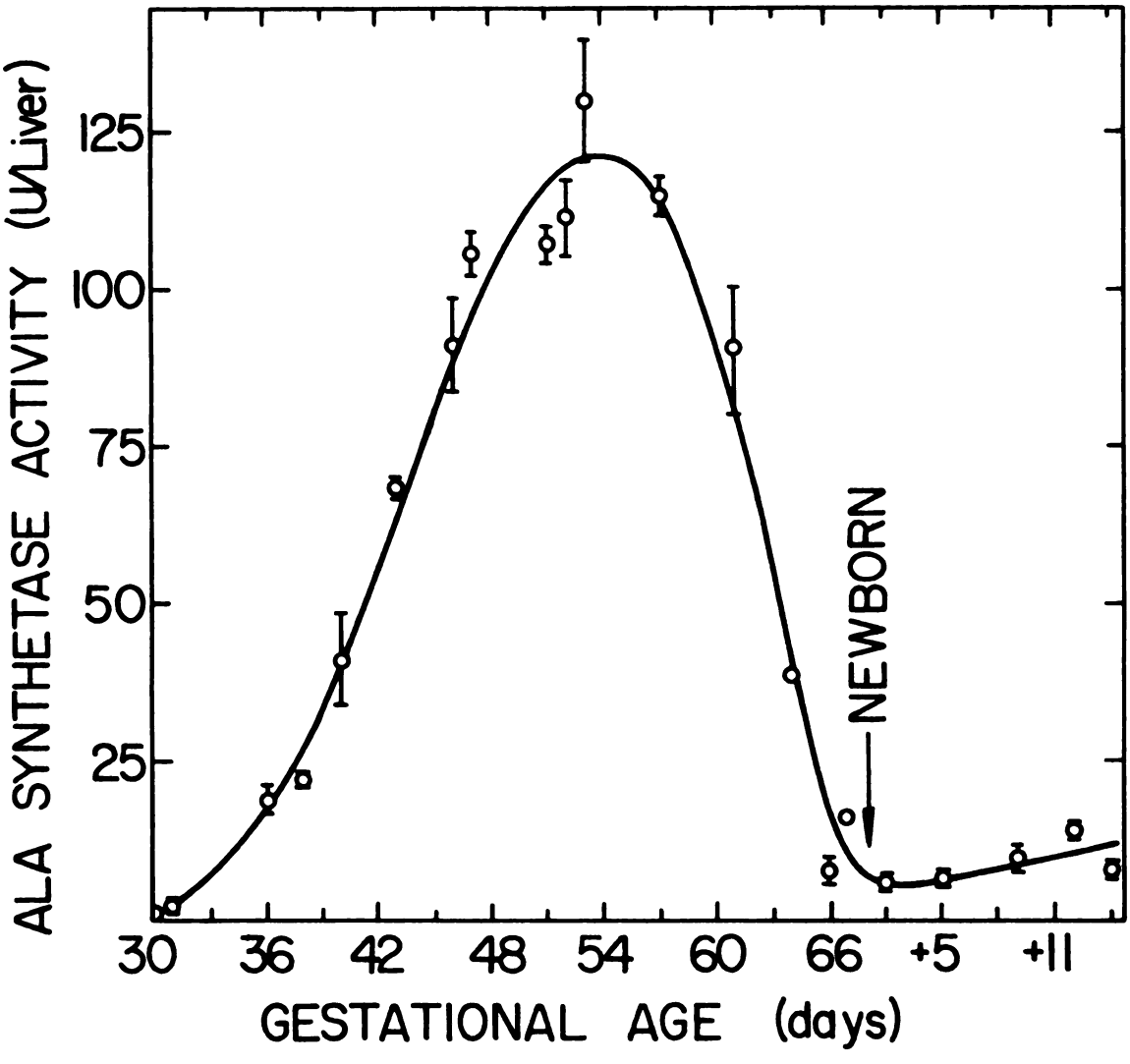

Figure 6. ALA synthetase total activity during development. The assay, tissue isolation, statistical and dating procedures are described in Methods. The bars denote S.E.M.

activity (Table 1). However, the highest measured value, 9 U/gm, amounts to only 15% of the highest fetal activity.

TABLE 1

Maternal ALA Synthetase Activity

Age (da)[1]	Activity (U/gm)[2]
38, 45, 46	0.0
47	9.0
53	2.0
58	4.7
62	1.3
64, +8, +11	0.0

[1]Age of litter. Days after birth denoted by (+).

[2]ALA synthetase assay of mitochondria from maternal liver, isolated and assayed as described for fetal tissue in Methods.

The assay for fetal ALA synthetase activity was linear for about 15 min with a consistent 15-20% reduction in product by 30 min (Figure 7A). The amount of ALA produced with varying protein concentration was linear for either high or low levels of enzyme activity (Figure 7B). Throughout the experiment, 30 min assays were run with 100 µl of mitochondrial suspension.

Under the conditions in which the mitochondrial ALA synthetase was isolated, the mitochondrial activity made up 62% of the total (Table 2). The remaining activity was

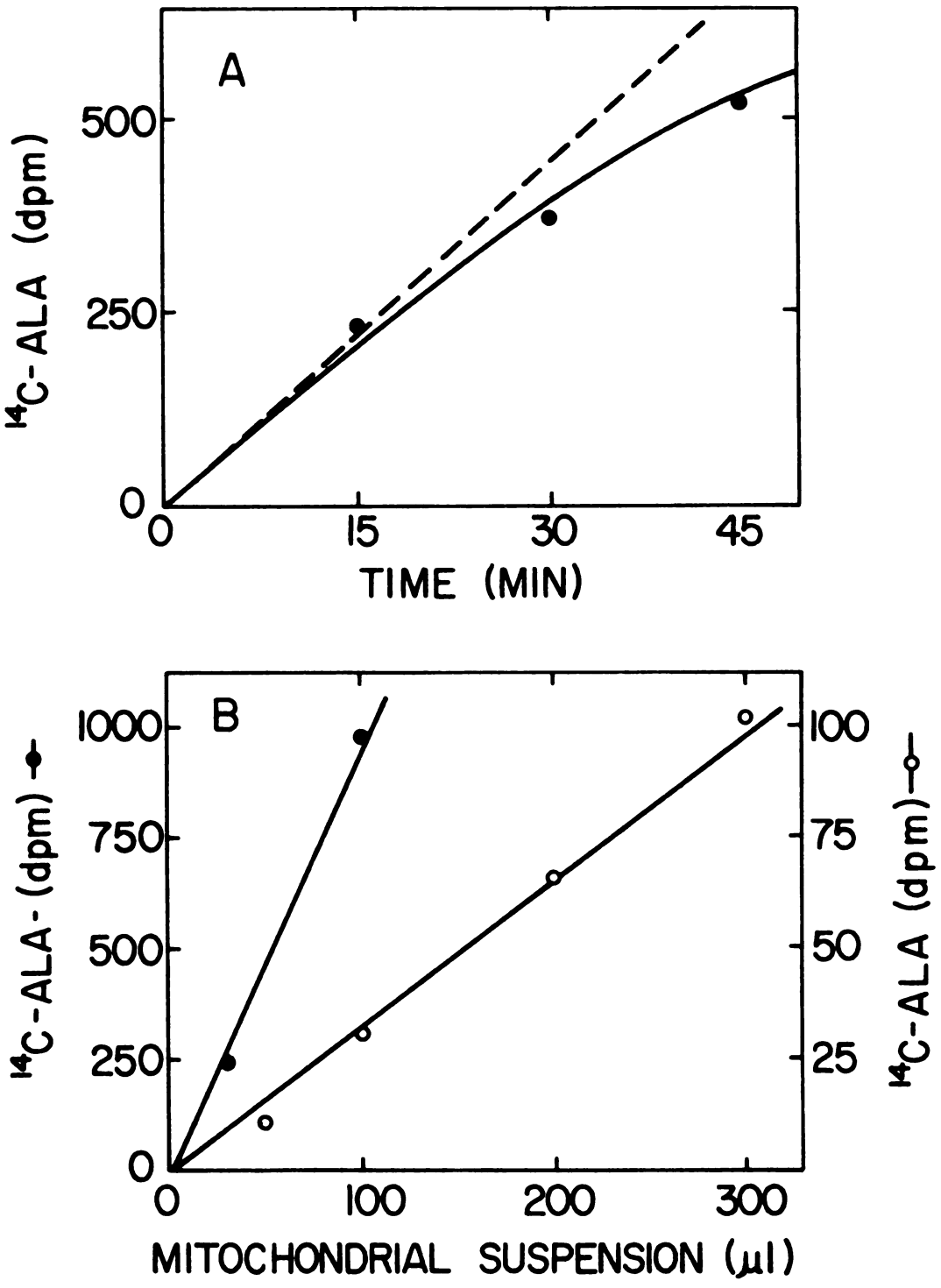

Figure 7. ALA synthetase assay linearity. (A) Linearity with time. Mitochondria (100 µl) from fetal liver (45th day). (B) Linearity with protein. Fetal liver (●), adult liver (○). Assay described in Methods.

primarily in the low speed pellet which contains unbroken cells, cell debris and nuclei.

TABLE 2

Subcellular ALA Synthetase Distribution

Source		% Distribution[2]		
Litter[1]	Fetus	Nuclear	Mitochondrial	Cytoplasmic
1	A	35.6	58.8	5.6
	B	35.0	64.2	0.8
2	A	38.9	60.2	0.9
	B	37.3	61.8	0.9
	C	36.2	63.8	0.0
3	A	40.0	59.1	0.9
	B	35.6	63.7	0.7
		36.9 ± 0.7	61.7 ± 0.9	1.4 ± 0.7

[1]Tissue treated as described in Methods, 47th day of gestation.

[2]Isolated and assayed as described in Methods. The nuclear assay is of the 100 x g pellet resuspended in homogenization buffer. The 10,000 x g supernatant is the cytoplasmic fraction. The statistic is S.E.M.

2. Erythropoiesis during development

Data from 37 litters show maximal erythropoiesis from the 38th day of gestation until about the 45th day, at which point erythropoiesis wanes, becoming virtually nonexistent at birth (Figure 8).

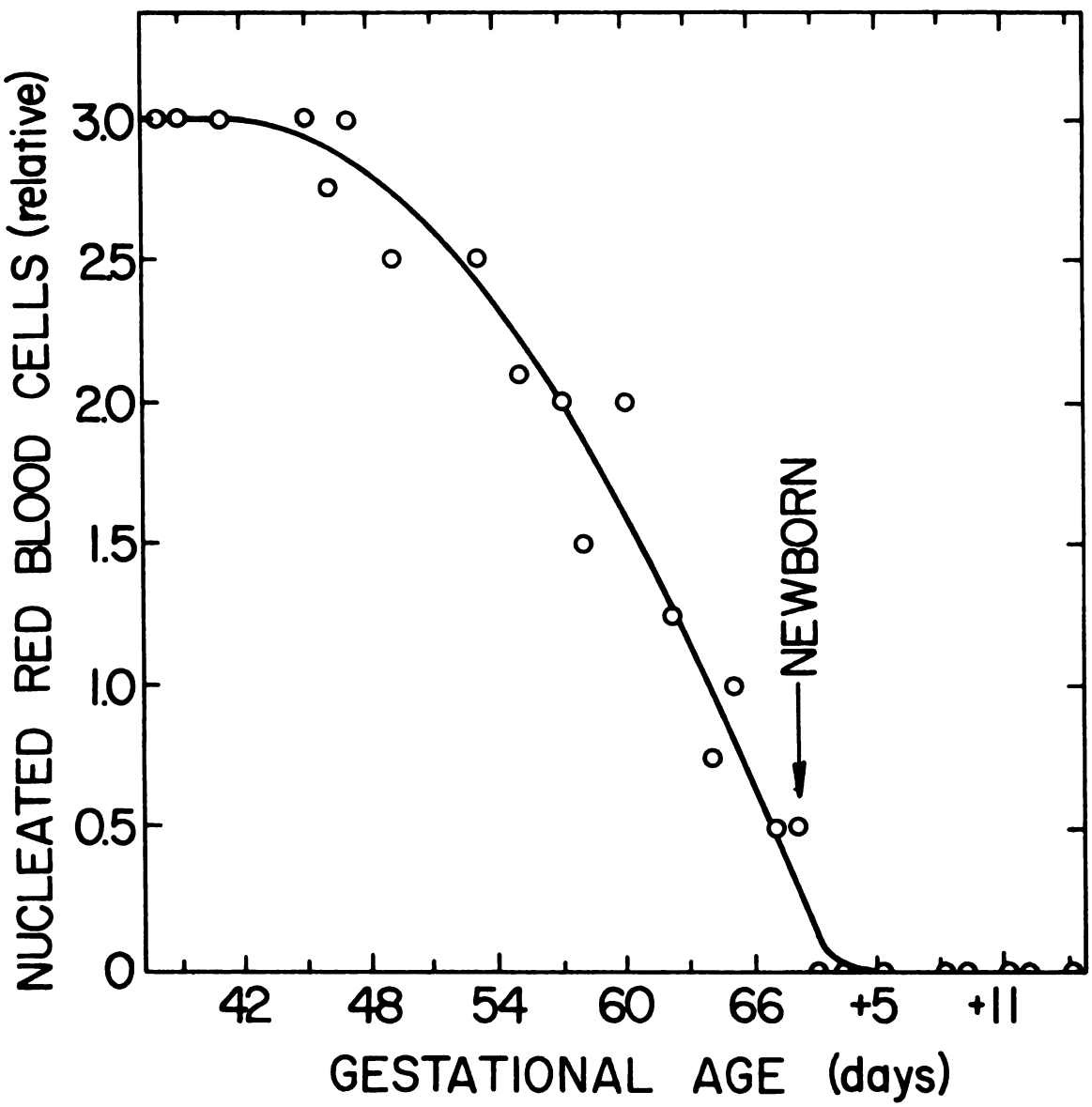

Figure 8. Erythropoietic activity during development. Hepatic erythropoiesis was estimated from the 38th day of gestation to 14 days post partum based on the number of nucleated red blood cells relative to parenchymal cells in each liver slice. Each point represents one to three slices (average = 1.4).

3. Guinea pig development

For 16 guinea pigs that went to term, the average gestational period as measured from conception (day 1) to birth (last day) was 68.1 ± 0.5 days. The average number of fetuses per litter for 80 females was 2.90 ± 0.08 fetuses.

Fetal weight showed little variation from a smooth parabolic growth curve (Figure 9). Liver weight likewise increased uniformly with gestational age except for some increased variability in the final quarter of gestation (Figure 9).

D. Discussion

The results of this study strongly support the hypothesis that the elevation of fetal ALA synthetase levels is due to the induction of erythropoiesis in the developing liver. Thus, the enzyme is the product of an erythroid cell type and not that of a parenchymal cell.

The mottled red appearance of the livers around the 30th day of gestation suggests that erythropoiesis was just getting under way. That only half of the liver looked erythropoietic at this time corresponds with the discovery that ALA synthetase activity was also only one-half of its maximal value. At the time when the number of nucleated red blood cells is the highest, the fetal liver is very fragile and under microscopic examination has no parenchymal structure. The abatement of erythropoiesis, as measured by the relative amount of nucleated red blood cells, closely parallels the decline in specific

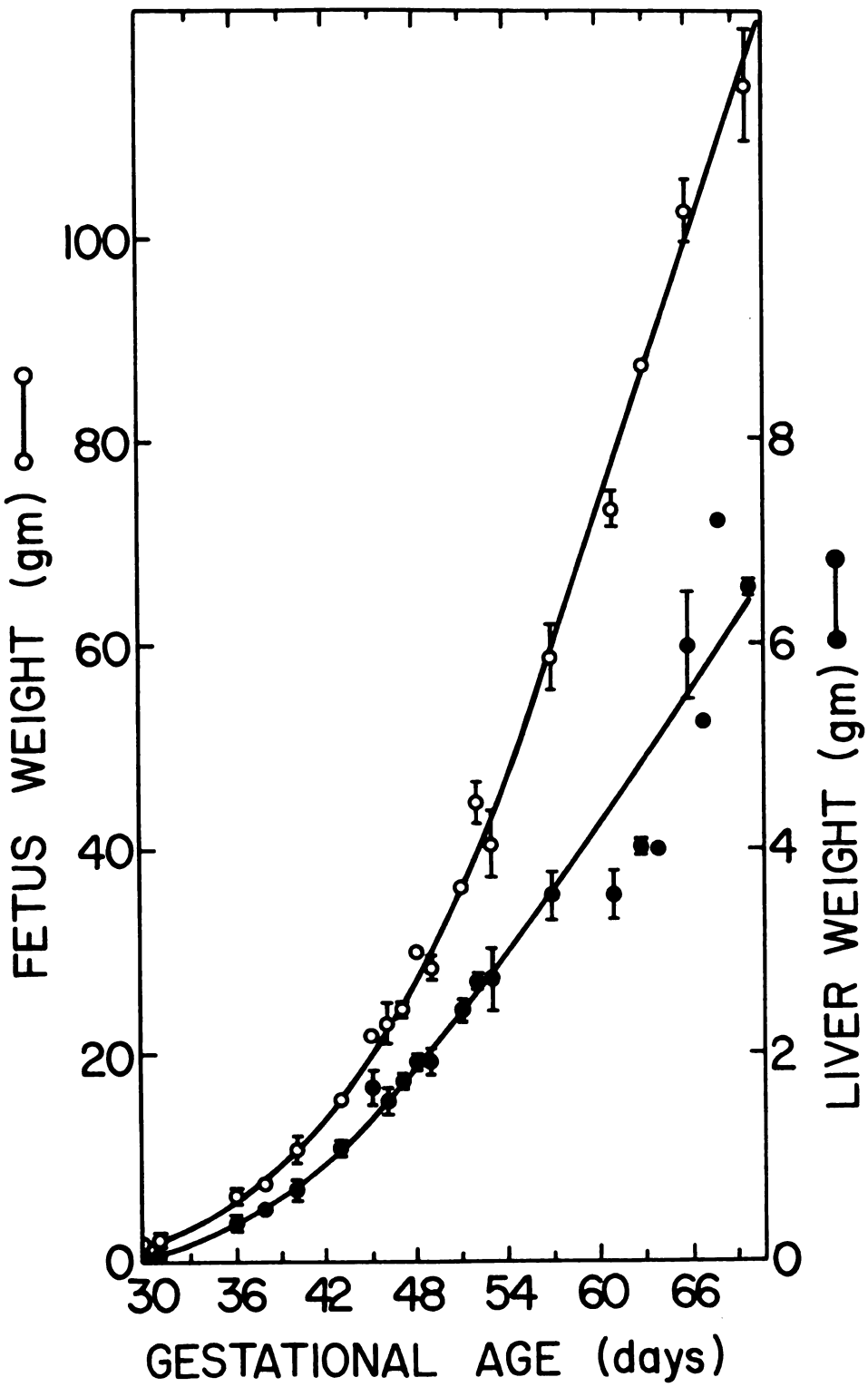

Figure 9. Growth curves for guinea pig fetus and fetal liver. Fetus weight is minus placenta. Livers were blotted before weighing. Bars indicate S.E.M. for determinations using more than one fetus or liver.

activity of ALA synthetase in this tissue. Furthermore, heme synthesis in these animals, as reflected in the accumulation of porphyrins in the amniotic fluid, also correlates with the observed ALA synthetase and erythropoiesis levels (81). These associated events (Figure 10) are best explained as arising from the transient establishment of the liver as an erythropoietic organ during development.

On the other hand, the hypothesis has been put forward that the elevated ALA synthetase activity in the late gestational period of rats and other animals is the product of an immature parenchymal cell type. Induction of ALA synthetase by DDC in perinatal rats was reduced relative to induction in adults, as was repression by hemin (82, 83). Fetal ALA synthetase could, therefore, be subject to different control mechanisms in an immature hepatocyte than in the mature cell. This hypothesis has also been proposed by Paul et al. (84) to explain similar results in neonatal rats. Based on the absence of hemin inhibition, the fetal enzyme was also proposed by Woods to undergo changes in its molecular structure accompanying mitochondrial maturation and the consequent development of hemin repression (83). The possible contribution of erythropoiesis to the elevated fetal ALA synthetase level was discounted due to an apparent lack of correlation between erythropoiesis levels and ALA synthetase levels and due to an insufficient number of erythroid mitochondria in the

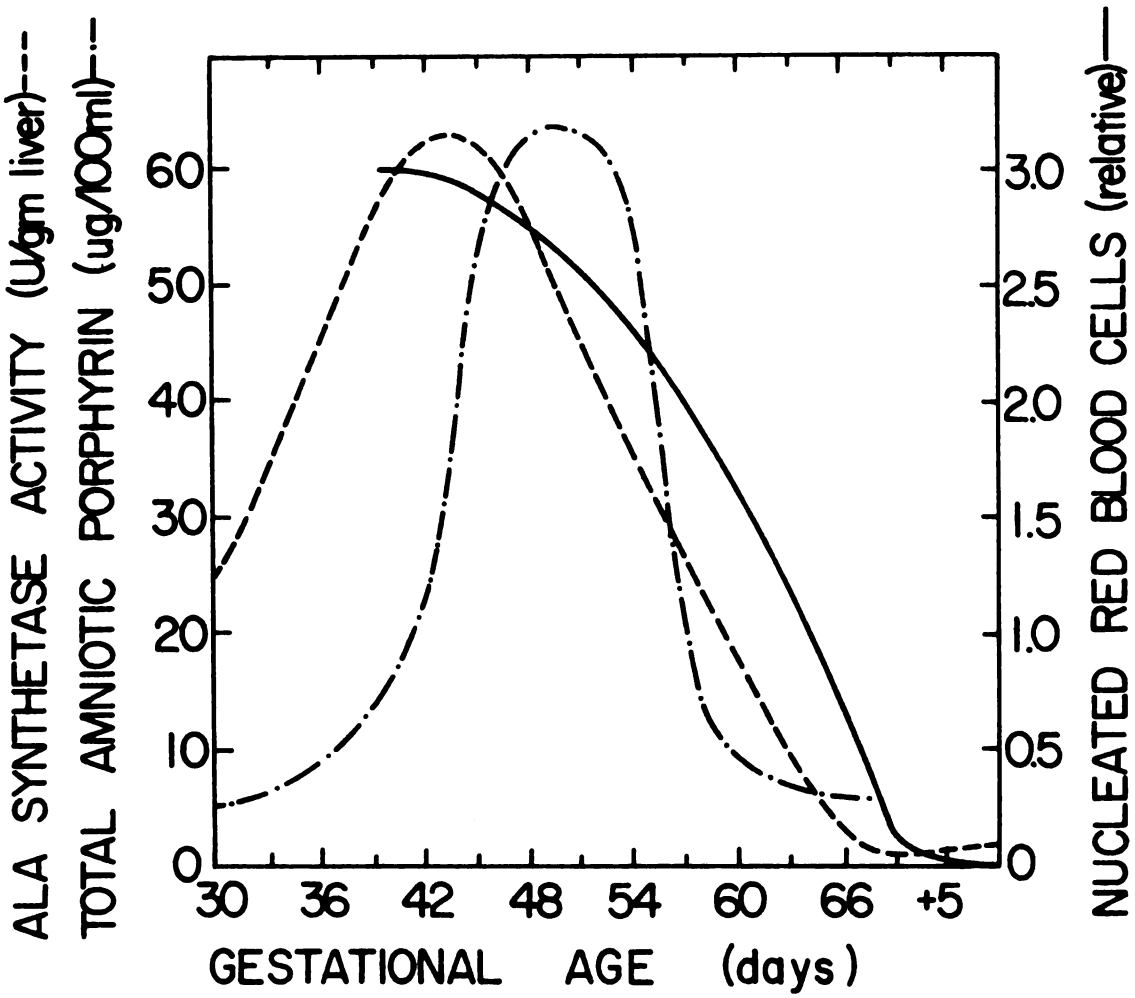

Figure 10. Correlation of porphyrin synthesis and erythropoiesis in guinea pig fetal liver. ALA synthetase and red blood cell data are from Figures 5 and 8. Porphyrins were determined by personnel from Dr. Hyram Kitchen's laboratory. The variation in total amniotic fluid volume does not significantly alter the porphyrin profile if expressed as µg/fetus.

erythroid cells to account for the greatly elevated enzyme levels (82,85).

Nevertheless, other evidence, in addition to that presented in this dissertation, mitigates against that interpretation. First, there are many examples of a strong correlation between levels of heme synthesis and erythropoiesis in developing animals. The data of Lucarelli et al. (86) on erythropoiesis levels in developing rat liver correlates quite well with the activity levels of ALA synthetase in perinatal rats as reported by Woods and Dixon (77). Others have recently correlated levels of ALA synthetase activity with maturation of erythroid differentiation in fetal mice (87,88) and with human erythropoiesis in fetal liver (89). Doyle and Schimke find a very close relationship between ALA dehydratase and erythropoiesis in mice (78). They also show a similar correlation with uroporphyrinogen I synthetase levels. The perinatal decline in ALA dehydratase activity has also been demonstrated in guinea pigs (90).

Second, though there does seem to be a distinctly fetal regulation of ALA synthetase activity, this difference appears more closely associated with an erythroid/non-erythroid distinction than with a fetal/adult hepatic distinction. Wada et al. compared the behavior of ALA synthetase in erythroid mouse spleen to that in non-erythroid liver (91). They found that the spleen enzyme was induced in response to hypoxia while the liver enzyme was not. Hemin and DDC induced liver, but not spleen,

ALA synthetase activity. Finally, the absence of fetal inhibition by hemin has its counterpart in the lack of significant hemin inhibition of the enzyme in the erythroid mitochondria of rabbit bone marrow (92,93). Thus, the weight of evidence suggests the existence of erythroid and non-erythroid isozymes of ALA synthetase.

The guinea pigs used in this study were apparently free of disease and exhibited no abnormal behavior or physiology. The growth curve of the fetuses studied (Figure 9) here was nearly identical to that established by Draper (94). The present curve rises slightly faster in the last quarter of gestation, perhaps due to better nutrition. Fetal weight is therefore an excellent indicator of gestational age and can be relied upon to aid in differentiation between uncertain conception dates if the estrous cycles were observed. The average gestational age of 68 days agrees with earlier studies where 68 days was also the mean value (95,96). The peak in fetal ALA synthetase activity cannot be due to a corresponding decrease in fetal liver weight since the latter increases smoothly throughout gestation.

In the conclusions of this study it is assumed that the mitochondrial activity is a faithful reflection of the total liver activity. Mitochondrial activity was measured due to the difficulty of reproducibly pipetting 0.1 ml of homogenate with uniform distribution of the various sized chunks. It also eliminated contamination by the soluble dehydratase which would prevent accumulation

of ALA. The proportion of non-mitochondrial activity found was consistently 35-40%. Others find this to hold true in DDC induced mice throughout the induction period (97,98). For these reasons and since the decline in ALA synthetase activity in the last quarter of gestation agrees with results on whole homogenates (77), and since porphyrins were found to accumulate when ALA synthetase levels were high, it is considered unlikely that the profile of enzyme activity is an artifact of enzyme compartmentalization.

The discovery of elevated ALA synthetase in maternal liver near the time of elevated fetal ALA synthetase activity suggests that there may be some factor common to these events. It is possible, however, that the cause is related to the complex endocrine mechanisms of pregnancy. Other laboratories have established that ALA synthetase is not only induced by certain sex steroids but its induction by various drugs is facilitated by glucocorticoids (99, 100). Jones has found a peak in plasma glucocorticoid concentration in pregnant guinea pigs around the 60th day of gestation (101). Paul et al. finds that the inducibility of ALA synthetase by allylisopropylacetamide in maternal liver decreases sharply before parturition in rats (84). These factors must be carefully considered in any explanation of the maternal ALA synthetase activity.

In conclusion, while there is still a possibility of maturing hepatocyte function and metabolism in the perinate, the earlier fetal ALA synthetase activity peak is

most likely due to erythropoietic activity. The existence of a different cell type than is found in the adult liver raises the question of possible differences not only in regulation of ALA synthetase levels but also in the molecular parameters affecting its activity.

III. PROPERTIES OF ERYTHROID AND NON-ERYTHROID ALA SYNTHETASE

A. Introduction

The liver of the fetal guinea pig is an erythropoietic organ from 30 to 60 days of gestation. Results reported in the previous section show that ALA synthetase activity is elevated during this period of transient erythropoiesis. The much lower activity associated with adult liver is non-erythroid since erythropoiesis in the adult is confined to bone marrow and spleen. Thus, it is proposed that there is a transition, during development, from erythroid to non-erythroid ALA synthetase. This is most likely accompanied by a changeover in liver cell type from erythrocyte precursors to parenchymal cells.

An alternate explanation has been proposed for elevated fetal ALA synthetase levels in the livers of rats, guinea pigs and rabbits. This hypothesis states that there is a fetal/adult transition in parenchymal ALA synthetase (82-84). In light of the demonstrated erythroid nature of fetal ALA synthetase, this hypothesis is replaced by the one in the previous paragraph. If there is a fetal/adult transition, it should be looked for in differences between fetal liver erythroid enzyme and adult bone marrow erythroid enzyme. This would be a transition analogous to

the known fetal/adult transition of another erythroid protein, hemoglobin.

To further substantiate the claim that fetal ALA synthetase is erythroid, comparisons need to be made with enzyme known to be erythroid such as that from bone marrow. While reports exist on the properties of ALA synthetase from erythroid and non-erythroid sources, comparisons are difficult to make and interpret since different species are used. In this section the isolation and characterization of ALA synthetase from fetal liver, adult bone marrow and adult liver of the guinea pig are investigated in order to clarify the relationships between these enzymes. The results will again demonstrate the similarity of fetal and adult erythroid forms in contrast to the adult hepatic form.

B. Materials and methods

1. Chemicals - see Sec. IV.B.1

2. Isolation of ALA synthetase

Guinea pigs were sacrificed by cerebral concussion followed by decapitation. Exsanguination was permitted for a short time with the neck under cold running water. The livers were quickly excised and placed in ice-cold isotonic homogenizing medium which contained: 0.25 M sucrose, 20 mM potassium phosphate, pH 7.5, and 50 µM pyridoxal-5'-phosphate. After blotting and weighing, the liver was diced with razor blades in a petri dish resting on ice. The diced liver, at

a concentration of 1.0 gm liver per 10 ml homogenizing medium, was homogenized in a Potter-Elvejhem homogenizer with a Teflon pestle driven at 300-500 rpm.

Bone marrow was scraped from the rear leg bones of guinea pigs with a Hayman microspatula (A. H. Thomas). It was collected in a small petri dish over ice and the resulting paste was weighed directly and homogenized as described above.

Mitochondria were isolated from these homogenates as described in Sec. II.B.3. The mitochondrial pellet was taken up in extract buffer equal in milliliters to twice the original tissue weight in grams. The extract buffer contained: 50 mM potassium phosphate, pH 7.5, 0.3% Lubrol WX (Sigma), 1.0 mM dithiothreitol and 100 μM pyridoxal-5'-phosphate. The mitochondrial suspension was stirred gently for one hour in a small beaker with a magnetic stir bar at 0°C. This solution was then centrifuged at 100,000-150,000 x g for one hour. The supernatant, containing solubilized ALA synthetase, was carefully drawn off leaving behind the upper lipid layer. The enzyme solution was stored at -15°C, usually in 1.5 ml disposable polypropylene centrifuge tubes (Brinkman). This extraction procedure is a modification of that of Murthy and Woods (14).

3. Protein determination

The following procedure is a modification of that reported by Böhlen et al. (102). The fluorimeter, filters and test tubes are identical to those reported. Boric

acid (0.2 M), titrated to pH 9.0 with sodium hydroxide, is the buffer. The fluorescamine reagent contains 30 mg Fluram (Hoffmann-La Roche) in 100 ml of acetonitrile (Mallinkrodt Nanograde) dried over Drierite (W. H. Hammond) and gravity filtered through a cone of Drierite in Whatman #1 filter paper. This reagent was stable at room temperature for at least three months if kept dry. Bovine serum albumin (Pentex Fraction V from Calbiochem), 25 mg per 100 ml of 0.05 M potassium phosphate pH 7.5, was the standard. This reagent lost only 2% of its fluorescent yield over a one year period when stored at 4°C.

The samples consisted of 1-50 µl aliquots of standard or unknown plus borate buffer to 1.0 ml. A five point standard curve was generated each time using 2.5-12.5 µg standard protein per tube. The mixed samples were placed on a vortex mixer and 0.35 ml of fluorescamine reagent was rapidly added from a 1.0 ml disposable tuberculin syringe (Becton-Dickinson). After fluorescamine addition was completed, all tubes were mixed again until no schlieren lines were visible. After 5 min, the fluorescence was measured with an Aminco Fluoro-Colorimeter. The zero point was set with the zero-bovine serum albumin blank and the relative fluorescence was adjusted to 80% of full scale with the 12.5 µg sample.

A typical standard curve is shown in Figure 11. As reported by Böhlen et al. (102), this method also measures free amino acids and other low molecular weight primary

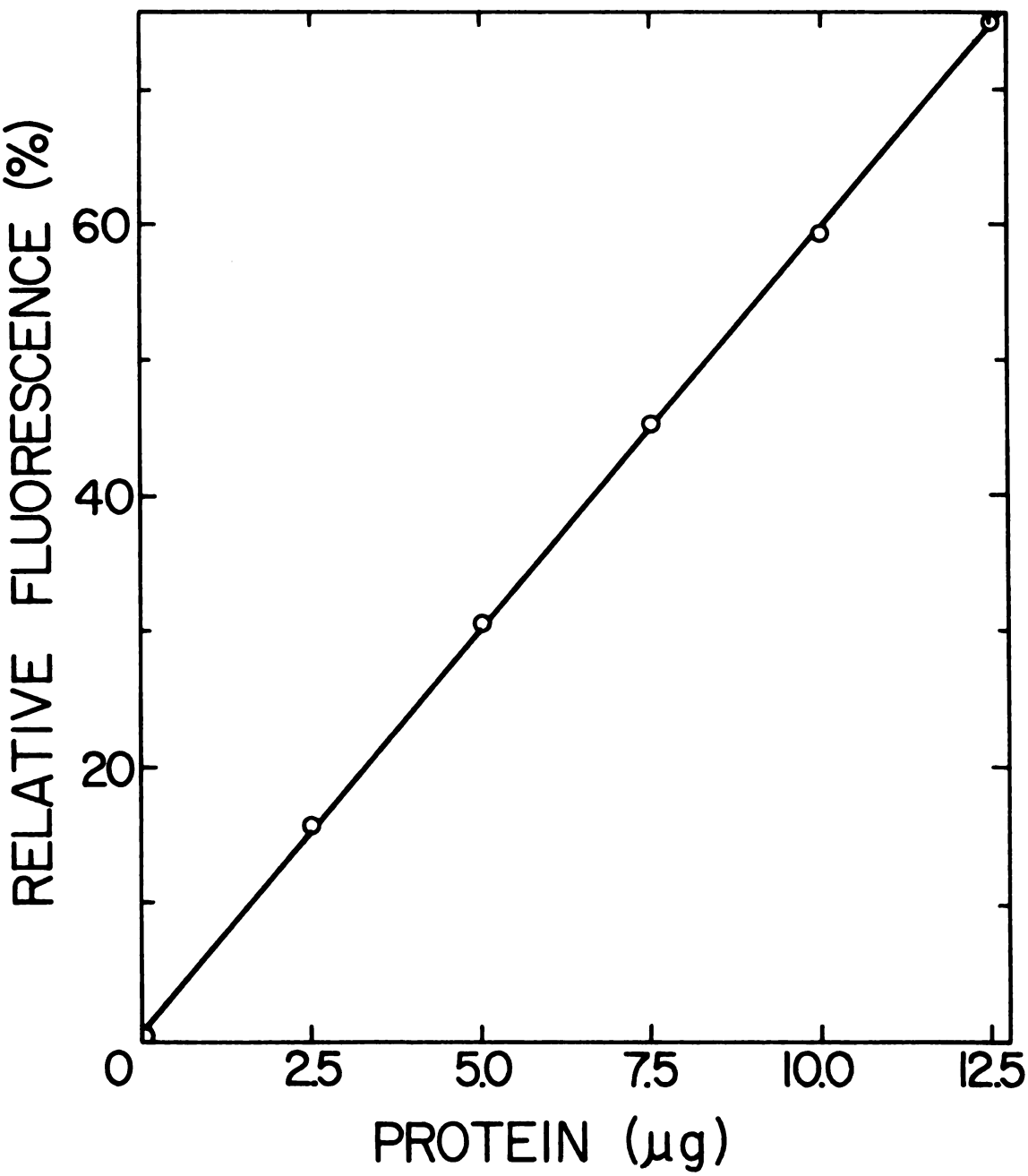

Figure 11. Fluorescamine-protein standard curve. See Methods for experimental details.

amines, which may amount to 50% of the fluorescence in crude tissue samples. In most assays of purified enzyme, these contaminants were removed by gel filtration with Sephadex G-25, medium mesh (Pharmacia) before protein determination.

4. Affinity chromatography

Sepharose beads with immobilized ligands were packed in 0.75 x 8.0 cm disposable plastic columns (Kontes) mounted in Pharmaseal K-75 three way Luer valves (Figure 12). The bed volume was 2.15 ml. The beads were packed, washed and used at 23°C. Between uses they were washed with distilled water. The AMP-Sepharose columns were quite stable under these conditions. The thioester linked CoA-Sepharose columns were somewhat more susceptible to bacterial or mold action and to leaching of the ligand. An attempt to remove protein buildup with 5 M guanidine HCl, pH 7.0, completely removed the CoA ligand.

Enzyme samples were applied in a volume of 0.6 ml and the displaced buffer was collected as fraction 1. Each succeeding fraction consisted of 0.6 ml of wash or elution buffer applied to the column and allowed to drain into a small test tube until the flow stopped. The test tubes were immediately transferred to an ice bucket for storage until assay.

The standard buffer used in all phases of the chromatography contains 50 mM potassium phosphate, pH 7.5, 0.3% Lubrol WX, 1 mM dithiothreitol, and 50 μM pyridoxal-5'-

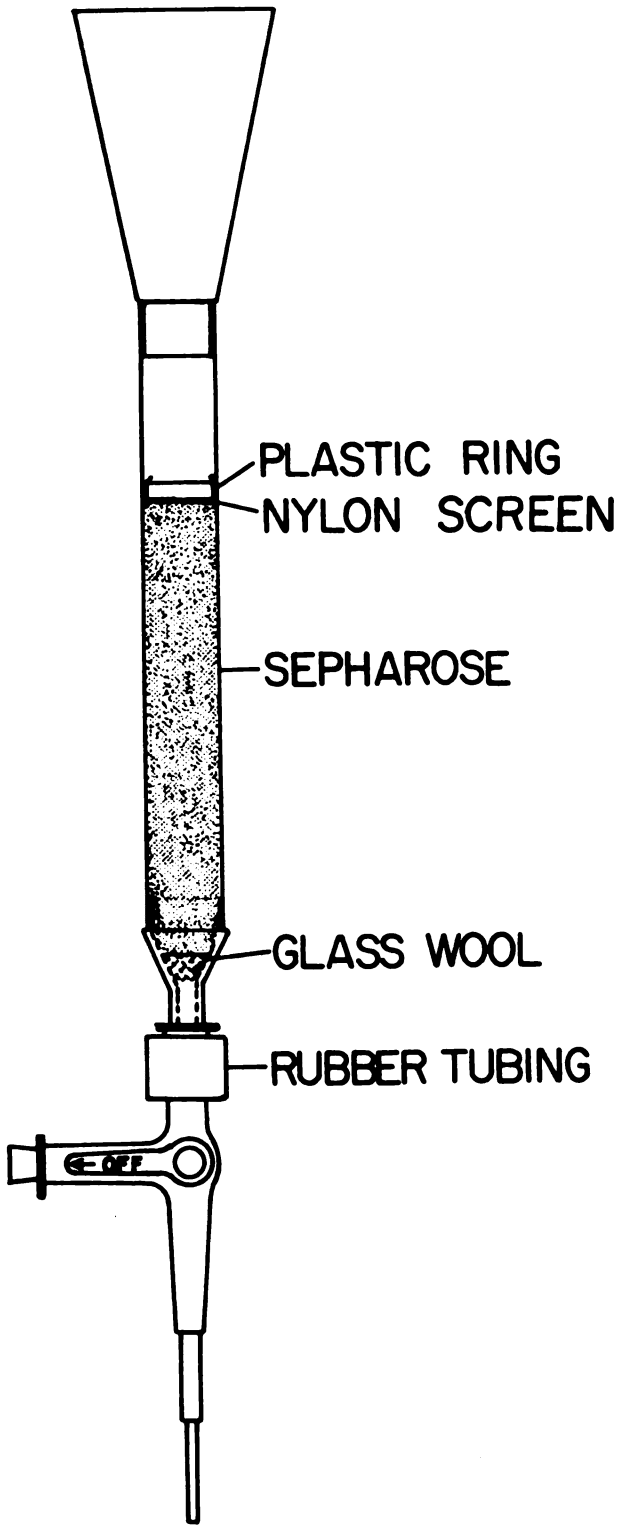

Figure 12. Column used for affinity chromatography. The plastic ring which holds a nylon screen is cut from the bottom of the filling cone. A short piece of heavy-walled rubber tubing is placed on the valve to facilitate holding by a thermometer clamp.

phosphate. Glycine at 100 mM was added to the wash buffer in some experiments. The elution buffer contained, in addition, 50 mM 5'-AMP and was 100 mM in potassium phosphate. This latter increase in ionic strength had no observable effect on elution.

Protein concentration was determined by the fluorescamine method which used 5 µl or less of the fraction. 5'AMP did not interfere with the assay. When glycine was present, protein was monitored by absorption at 280 nm in a Gilford spectrophotometer using a 0.3 ml microcuvette with a 1.0 cm light path. Protein was not measured in the AMP eluate of these experiments due to the presence of both AMP and small amounts of glycine.

5. Lubrol WX purification

Lubrol WX (palmityl-$[O\text{-}CH_2\text{-}CH_2]_{\sim 16}$-OH) was purified by the following modification of the method of Nakao et al. (103). Lubrol WX (100 gm) was melted in a beaker over a low flame and glass distilled water was slowly added, with stirring, to a volume of 900 ml. This solution was filtered while hot through a Whatman No. 1 pre-filter and a 0.45 µ Millipore filter. The solution was repeatedly filtered as it cooled. Water was added to 1000 ml and the solution stored overnight at 4°C. After slowly warming up to room temperature, it was filtered one more time. This solution was passed over a 3 x 20 cm column of a 50/50 mixture of Dowex 2 and Dowex 50 washed with glass distilled water and packed by tamping. The eluate was stored at 4°C

and swirled to mix before use. Though slightly more dilute, the mixture is assumed to be 10% Lubrol WX.

6. Heat denaturation

Heat denaturation of the various enzyme preparations was carried out in 10 x 75 mm test tubes. Identical aliquots of enzyme were distributed to each tube in an ice bath. To begin the experiment, the samples were placed in a test tube rack and then in a Dubnoff shaking water bath (Precision Instruments) at the prescribed temperature. Tubes were removed at intervals and placed on ice until they were assayed.

7. Sucrose density centrifugation

Molecular weights of ALA synthetase isozymes were estimated using the sucrose density sedimentation method of Martin and Ames (104). The gradients were made up 1 to 2 hours before use. They were made from 5 and 20% sucrose solutions containing 50 mM potassium phosphate, pH 7.5, 0.3% Lubrol WX, 1 mM dithiothreitol and 50 µM pyridoxal-5'-phosphate. A Pharmacia P-3 peristaltic pump was used to maintain the flow rate at 0.5 ml/min from the gradient-forming block to the centrifuge tubes. A motor driven 250 µl Hamilton syringe was used to layer the sample on the gradient. The gradients were unloaded using the method of Bubel and Riley (105). The 5 ml gradient was divided into 30 fractions, 170 µl each. The chase solution was 50% wt/vol sucrose with Blue Dextran 2000 (Pharmacia) added to aid in identification of the last fraction.

The following enzymes were used for molecular weight standards: yeast hexokinase (Sigma type C-300) MW = 51,000 (106,107);[6] pig heart lipoyl dehydrogenase (Sigma type III) MW = 102,000 (108,109); rabbit muscle lactic dehydrogenase (Sigma type II) MW = 142,000 (110,111).

Standard curves of migration distance versus $(MW)^{2/3}$ were plotted to permit calculation of the molecular weight of each ALA synthetase isozyme (104).

8. Induction of porphyria in guinea pigs

Adult male guinea pigs (600-900 gm) were given only water for 48 hr prior to drug induction. The drug mixture was given via stomach tube with the animal under ether anesthesia. The drug mixture contained: 1.0 gm 3,5-dicarbethoxy-1,4-dihydrocollidine, 1.0 gm sucrose, and 0.02 gm gum tragacanth in 10 ml H_2O. The mixture was sonicated before use. Two doses were given, 9 hr apart, and the animals sacrificed 15 hr after the second dose.

C. Results

1. Isolation and assay of ALA synthetase isozymes

The specific activity of ALA synthetase in the maturing guinea pig fetus reaches a maximum around 44 days of gestation. The fetal ALA synthetase used in this study

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[6]The second bibliographic citation is for the assay method.

was isolated from livers of fetuses in the 45th to 47th day of gestation.

Bone marrow ALA synthetase was isolated from 600-700 gm male guinea pigs. They were rendered anemic, to induce erythropoiesis, by removing 6 ml of blood per animal by heart puncture. After 5 days the animals were sacrificed and the marrow collected from the femur and tibia.

Chemical porphyria was induced in adult guinea pigs as described under Methods.

Typical activities of crude mitochondrial extracts from these sources are listed in Table 3.

TABLE 3

Crude Extract Activities

ALA synthetase source[1]	Activity[2] U/ml extract	Protein[3] mg/ml	Specific Activity U/mg
Fetal liver	9.5	5.3	1.8
Bone marrow	59.0	4.2	14.0
Adult liver	67.0	15.0	4.5

[1]See text above. For extraction procedure see Methods.

[2]Procedure described in Methods, 30 min assays, 50 µl enzyme.

[3]Fluorescamine procedure, see Methods.

When stored in extract buffer at -15°C, all three enzymes are stable for at least 5 months.

Both fetal and bone marrow enzyme rates were constant for 15 min, while the adult liver enzyme lost 15% of its activity within 10 min (Figure 13). In those experiments requiring kinetically valid assays, the incubation time was 10 min.

2. Affinity chromatography

While surveying various immobilized ligands for suitability in enzyme purification, it was discovered that mitochondrial ALA synthetase from the livers of adult induced guinea pigs binds strongly to both 5'-AMP (C_6-amino) and CoA (sulfhydryl)-carboxyhexyl Sepharose (Figures 14A and 15A). In contrast to this, both fetal and bone marrow ALA synthetases were only slightly retarded on either column (Figures 14B,C and 15B,C). The adult enzyme was eluted immediately in a sharp peak by 50 mM 5'-AMP. ATP (5 mM) eventually eluted the enzyme, but in a broad peak, probably due to the lower concentration of nucleotide.

The difference in behavior of the fetal and adult forms is not due to any substance in the respective enzyme solutions since when applied as a mixture the enzymes still eluted separately (Figure 16A). The adult enzyme is not retarded by unsubstituted Sepharose 4B (Figure 16B). The AMP-Sepharose affinity for ALA synthetase from a crude extract (Figure 16C) confirms that the 10-fold purified adult preparation used in Figure 14A had not merely lost a factor which could have inhibited the enzymes' binding to AMP-Sepharose.

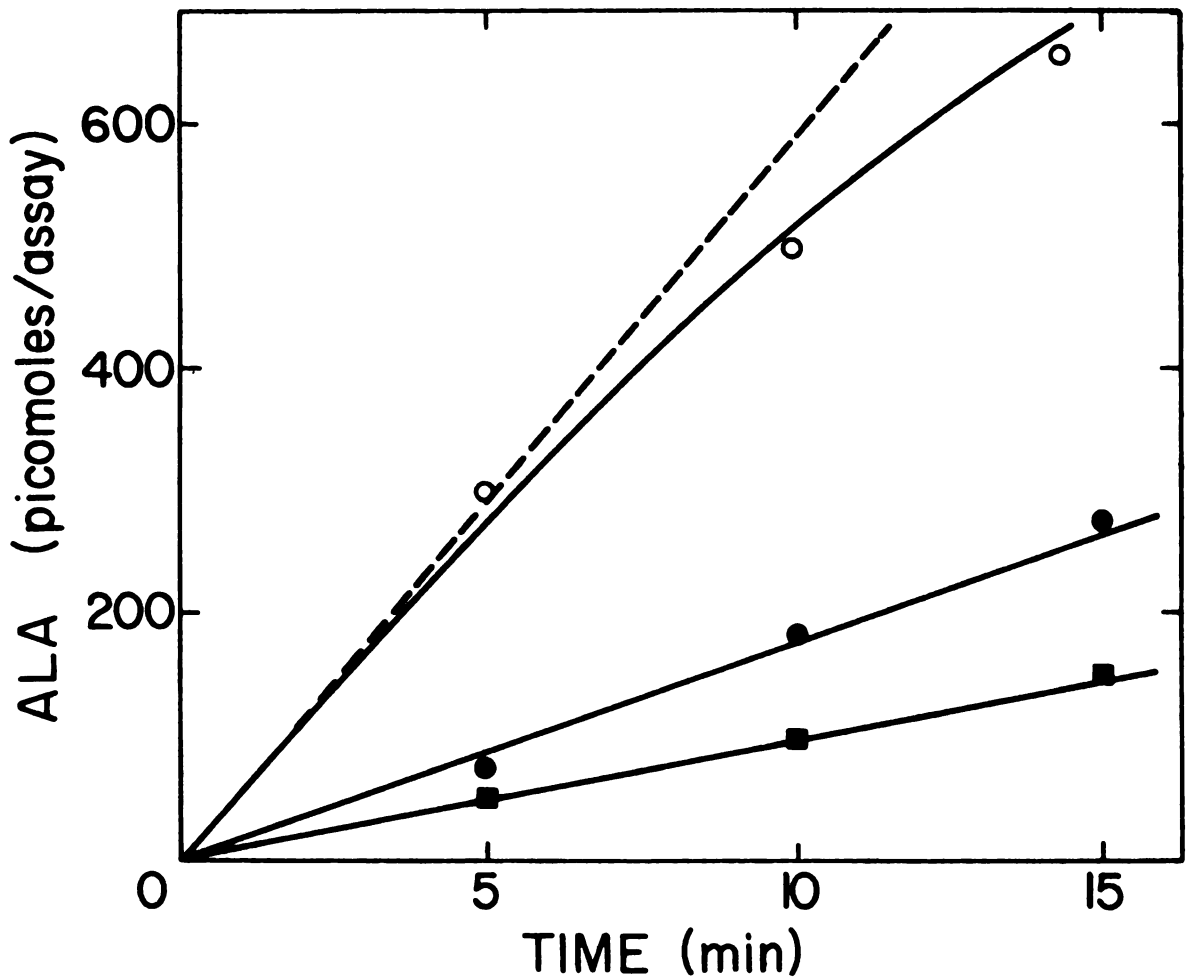

Figure 13. Linearity of the ALA synthetase assay with time. The assay procedure is described in Methods. The mitochondrial enzyme samples were from: adult induced liver (O), 52 U/ml, 4.9 U/mg; bone marrow (●), 44 U/ml, 29 U/mg; fetal liver (■), 12 U/ml, 4.9 U/mg.

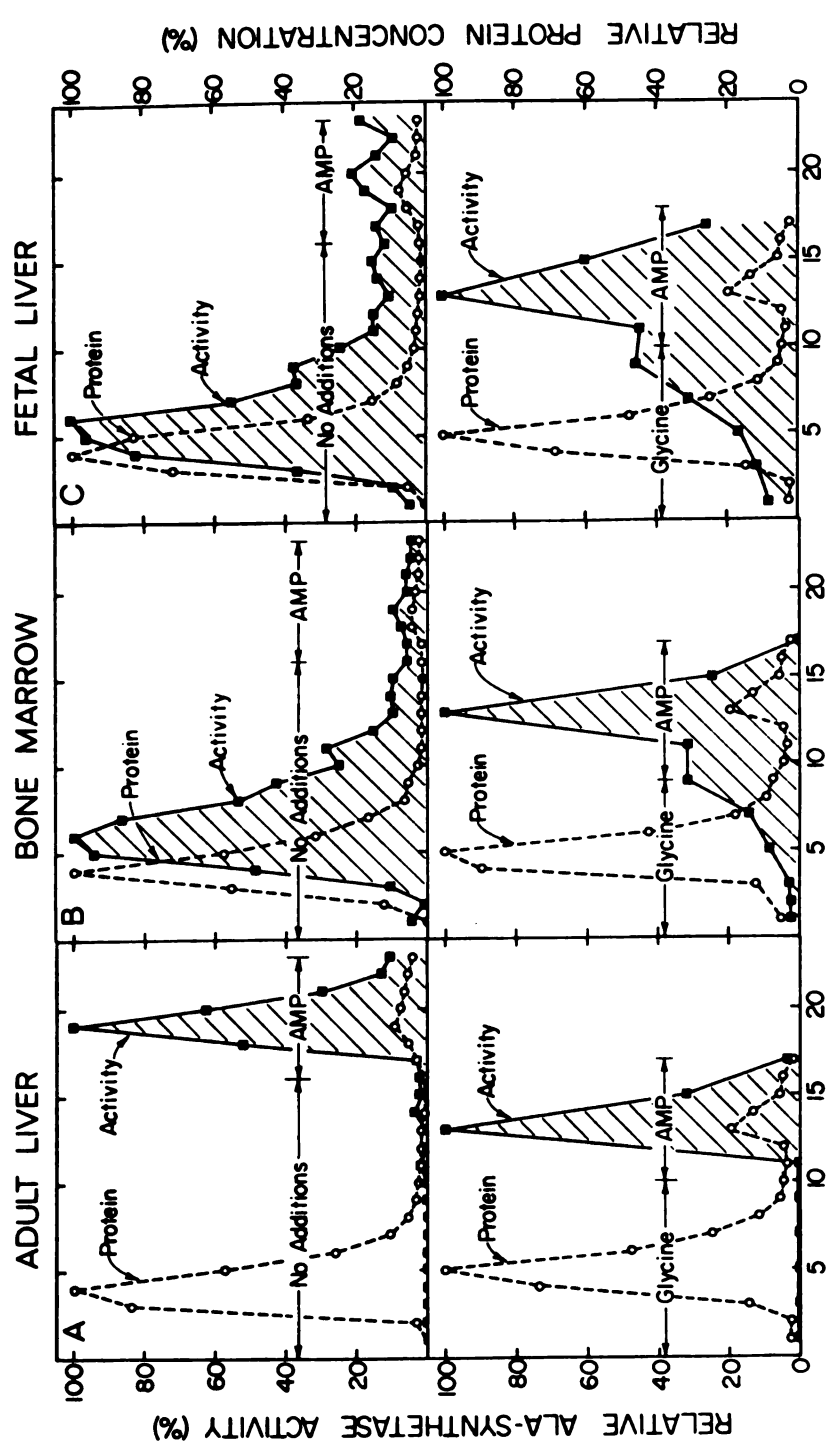

Figure 14. Behavior of ALA synthetase isozymes on AMP-Sepharose. The experimental procedure is described in Methods. Upper panels: (A) Adult enzyme from 500 to 800 gm male guinea pigs induced with DDC; 2.0 U, 8.5 U/mg. (B) Bone marrow enzyme from the hind leg bones of adult male guinea pigs made anemic by removal of 6 ml of blood via heart puncture 5 days prior to sacrifice; 2.6 U, 1.4 U/mg. (C) Fetal enzyme from the 45th to 47th day of gestation; 2.5 U, 1.7 U/mg. Lower panels: same enzyme sources as in the upper panels. (A) 16 U, 33 U/mg; (B) 0.75 U, 29 U/mg; (C) 1.2 U, 4.9 U/mg.

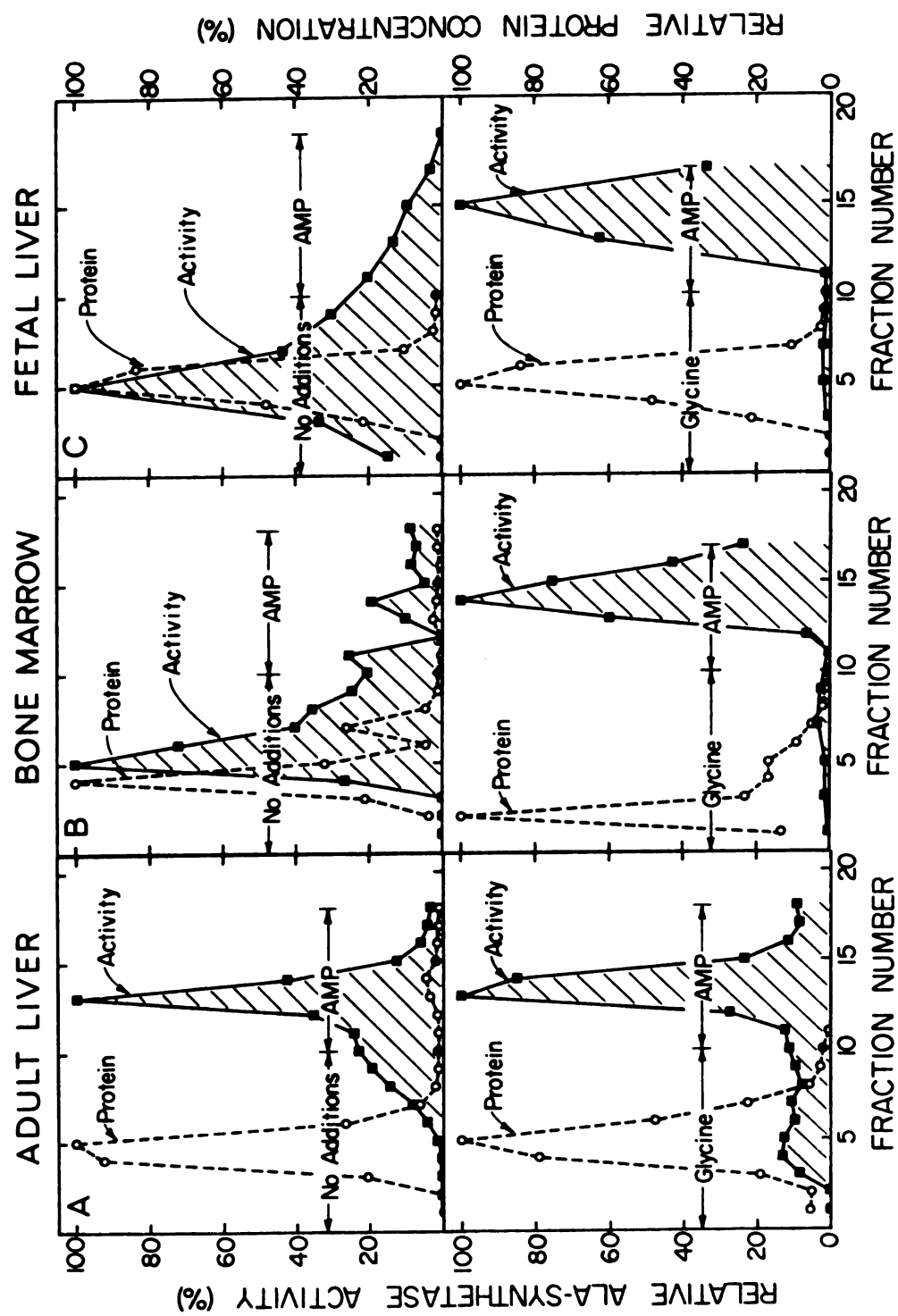

Figure 15. Behavior of ALA synthetase isozymes on CoA-Sepharose. For details see Figure 14. Upper panels: (A) 12 U, 6.7 U/mg; (B) 2.3 U, 14 U/mg; (C) 0.6 U, 3.3 U/mg. Lower panels: (A) 12 U, 6.7 U/mg; (B) 2.2 U, 29 U/mg; (C) 3.5 U, 1.2 U/mg.

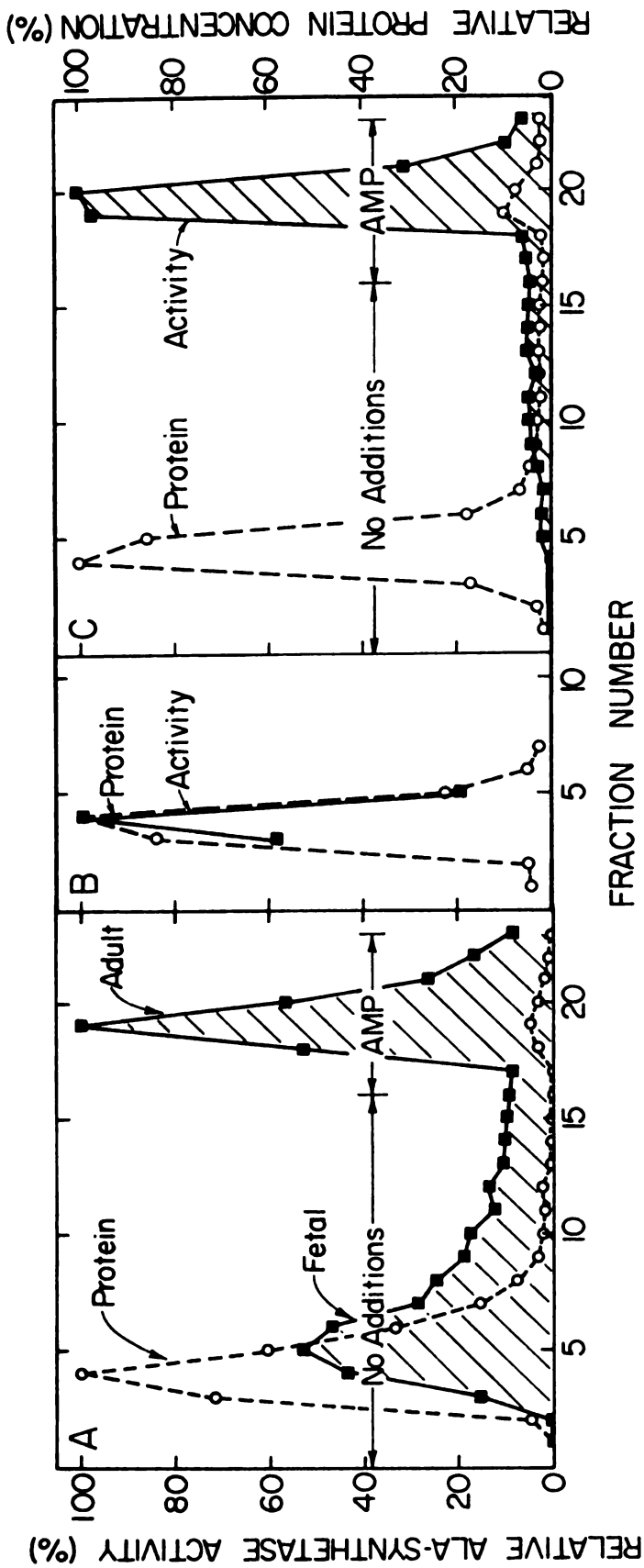

Figure 16. Affinity chromatography of ALA synthetase isozymes. See Figure 14 for experimental details. (A) Behavior of a mixture of fetal and adult liver isozymes on AMP-Sepharose. Fetal; 2.5 U, 1.7 U/mg. Adult; 2.0 U, 8.5 U/mg. (B) Adult liver enzyme on unsubstituted Sepharose 4B; 4.5 U, 9.5 U/mg. (C) Crude adult liver enzyme on AMP-Sepharose; 3.4 U, 0.79 U/mg.

Since ALA synthetase from livers of uninduced adult guinea pigs is also quantitatively bound by AMP-Sepharose (Figure 17), this property must be a distinctly hepatic one and not a drug induced aberration. This property clearly allows one to distinguish between enzymes from erythroid and non-erythroid sources.

A further distinguishing characteristic is the fact that in the presence of a substrate, glycine, both erythroid forms are significantly retarded on either CoA- or AMP-Sepharose (Figures 14B,C and 15B,C). The behavior of the adult enzyme is unchanged (Figures 14A and 15A). The glycine induced binding of the erythroid forms to either CoA- or AMP-Sepharose seems weaker than the binding of the adult non-erythroid enzyme. It is possible, however, that the capacity is reduced due to the presence of other AMP or CoA binding proteins in the erythroid samples. The fetal and bone marrow enzymes are, however, bound more strongly to the CoA beads than to the AMP beads. This is demonstrated by the absence of pre-elution leakage on the CoA columns (Figures 15B and 15C). In fact, twice the units were bound to the CoA column and it had only half the ligand concentration of the AMP column.

The CoA column with glycine is still not an effective means of purifying crude erythroid ALA synthetase. Application of 12 units of fetal enzyme resulted in 50% break-through (Figure 18A). Reapplication of fraction 6 of the break-through resulted in complete binding (Figure 18B).

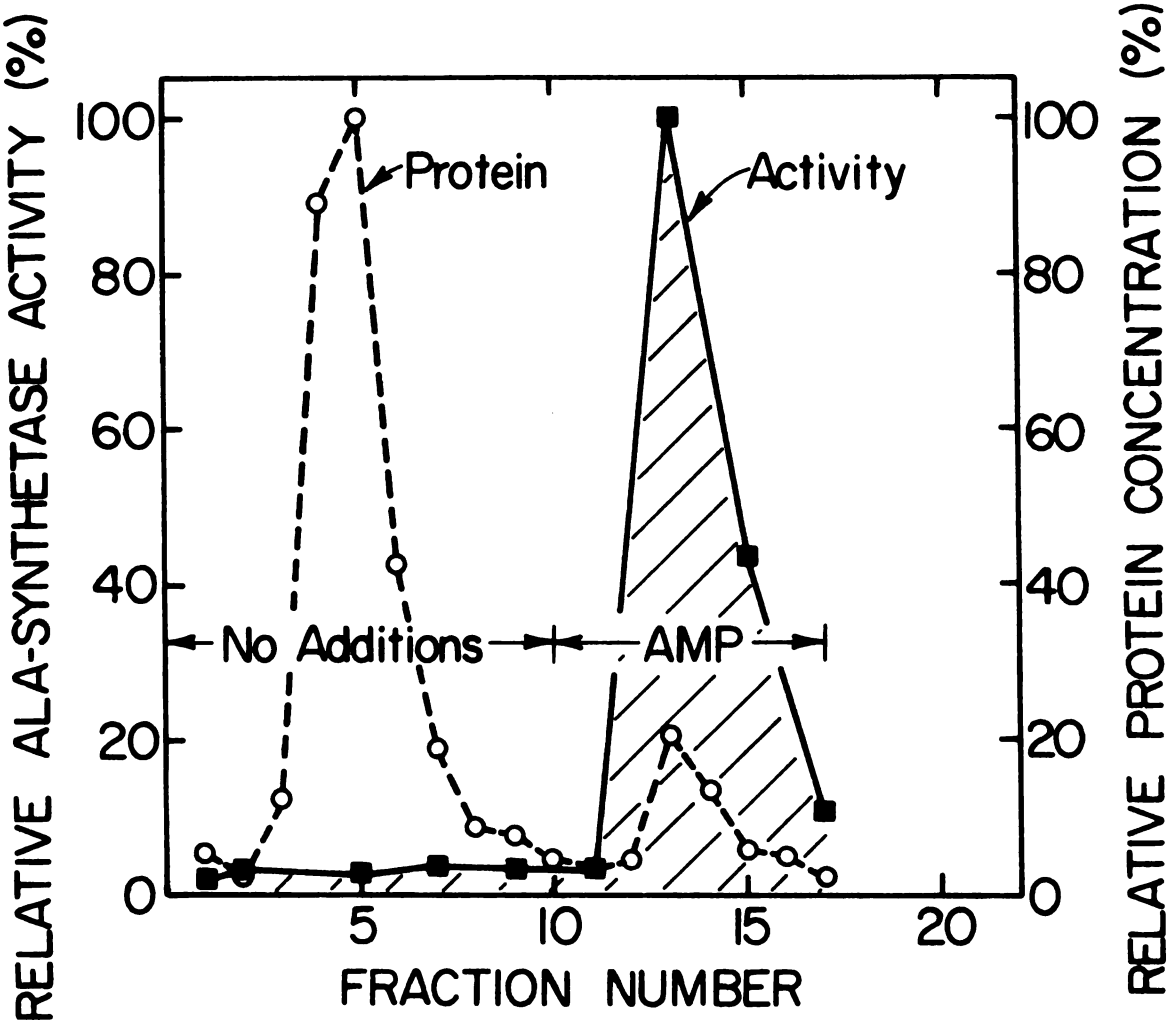

Figure 17. Native adult ALA synthetase on AMP-Sepharose. See Methods for experimental procedures. The unlabeled succinate was omitted from the assays to increase the sensitivity (see Sec. IV). The enzyme was isolated from the liver of an 815 gm adult male guinea pig; 0.42 U, 0.052 U/mg.

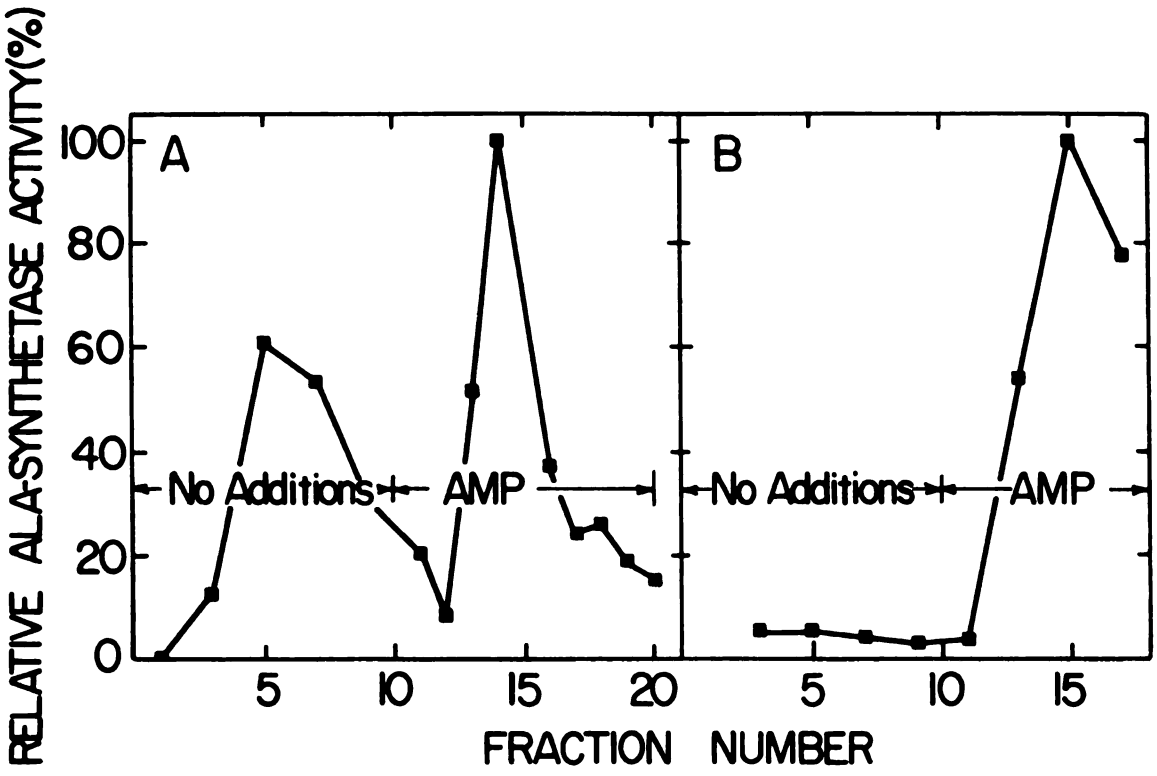

Figure 18. Glycine-induced binding of fetal ALA synthetase to CoA-Sepharose. See Methods for experimental procedures. The unlabeled succinate was omitted from the assays (see Figure 17). (A) The enzyme was isolated from the livers of 47 day old fetuses; about 12 U, 6 mg was applied to the column. Fractions 13 to 20 were multiplied by 4 due to 75% inhibition by AMP. (B) Fraction 6 from Figure 18A was reanalyzed on the CoA-Sepharose column.

In later stages of purification the sequential application of this technique first without and then with glycine should be very useful.

The AMP-Sepharose column was quite useful in purifying the adult enzyme, even from crude extracts. The results of one such purification are shown in Table 4.

TABLE 4

Purification of Adult Liver ALA Synthetase[1]

Step	Volume (ml)	Activity (U/ml)	(U)	Protein (mg/ml)	S.A. (U/mg)	Fold
Crude extract[2]	25.8	49.6	1280	14.7	3.37	--
Sephadex G-50[3]	67.0	24.3	1630	3.79	6.41	1.9
5'-AMP-Sepharose[4]	22.8	74.1	1690	1.42	52.2	15.5

[1]See Methods for activity and protein assays.

[2]Male guinea pig, 620 gm; 12 gm liver (50%); DDC induced.

[3]4.5 x 42 cm, medium mesh (Pharmacia).

[4]1.5 x 4 cm, 0.63 ml/min.

The increase in specific activity after the G-50 step could be due to the removal of fluorescamine-positive material or of something inhibitory to ALA synthetase. The elution profile from the AMP column is shown in Figure 19.

In another experiment, AMP Sepharose affinity chromatography after Sephadex G-200 and pyridoxamine-5'-phosphate-Sepharose steps gave a final specific activity of 420 U/mg.

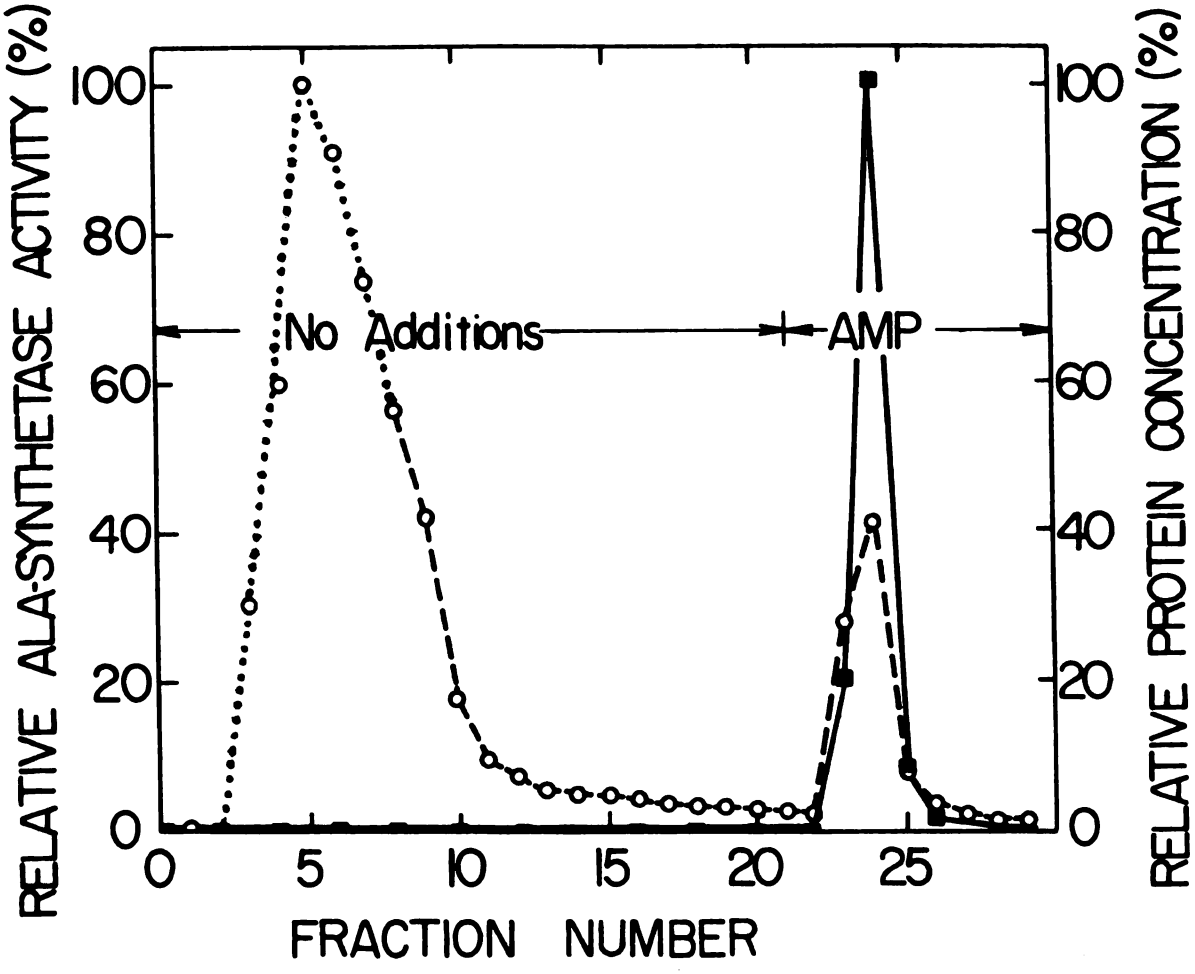

Figure 19. ALA synthetase purification by AMP-Sepharose. For experimental procedures see Methods and Table 4. (O) Protein concentration, 100% = 4.75 mg/ml. The fractions in the dotted portion of the profile were pooled and assayed for protein. While the shape of the curve is an estimate, the total protein is correct. (■) ALA synthetase activity, 100% = 163 U/ml. The fraction volume is 8 ml.

No further attempt was made to optimize the conditions for purification of the enzyme.

3. Salt effects

Others have shown that NaCl activates adult ALA synthetase and inhibits the fetal enzyme (12,13,83,112). The results reported here (Table 5) confirm the strong inhibition of the fetal enzyme but indicate partial inhibition of the adult enzyme. In addition, the bone marrow enzyme is inhibited in a manner similar to the fetal enzyme. Comparable results were found with crude extracts of all three enzymes.

TABLE 5

Effect of Salts on ALA Synthetase Activity[1]

Enzyme Source	No Additions U/mg	No Additions U/ml	+0.4N KCl % Decrease[5]	+0.4N NaCl % Decrease	+0.4N KF % Decrease
Fetal liver[2]	3.2	5.6	67	63	6
Bone marrow[3]	22	33	56	57	2
Adult liver[4]	24	38	16	13	7

[1]The assays were conducted as described in Methods with the addition of 50 µl of 4 N salt to the appropriate assays.

[2]Partially purified by CoA-Sepharose.

[3]Crude mitochondrial extract, desalted on Sephadex G-50.

[4]Partially purified from DDC induced liver by AMP-Sepharose.

[5]Per cent decrease in activity over starting activity (U/ml).

The reversal of inhibition of ALA synthetase by potassium fluoride is consistent with the report by Yoda et al. (113) showing similar effects in chicken liver homogenates and particulate fractions. The effect was thought to be due primarily to control of ATPase activity which would influence the production of succinyl-CoA by the ATP-requiring succinyl CoA synthetase. However, it is unlikely that Na^+/K^+ activated ATPase is in the mitochondrial preparations used in the present study since the enzyme is specifically associated with microsomes (103). This ATPase was present in the particulate fractions of Yoda et al. since they were 105,000 x g precipitates. There is still the possibility that KF inhibits the oligomycin-sensitive Mg^{++}-ATPase of mitochondria (114).

On the other hand, incubation of fetal ALA synthetase with KCl did not significantly lower the production of succinyl CoA (Table 6), suggesting that the effect is indeed due to the anions.

4. Hemin effects

Contrary to expectations, with partially purified preparations and short assay times, both erythroid and non-erythroid ALA synthetase enzymes were activated by high concentrations of hemin (Table 7). The hemin solution gave a spectrum similar to that of monomeric hemin in 80% dimethyl sulfoxide (117). This might be due to micellar intercalation of hemin by Lubrol like that obtained with Triton X-100 (118). In another experiment

TABLE 6

Effect of KCl on Succinyl-CoA Levels

Incubation Time[1] (min)	Fetal ALA Synthetase[2]	0.4M[3] KCl	Acyl CoA Remaining (μmoles)[4]
0	-	-	0.42
10	-	-	0.31
0	+	+	0.34
10	+	+	0.29

[1]ALA synthetase assay as described in Methods.

[2](+) = added the same sample as used in Table 5.

[3](+) = with 50 μl of 4 M KCl in the assay.

[4]Hydroxamic acid assay (115,116).

TABLE 7

Effect of Hemin on ALA Synthetase Activity[1]

Enzyme Source	S.A. (U/mg)	Protein (mg/ml)	Activity (U/ml)	Activation by Hemin (%)[2] 100 μM Hemin	200 μM Hemin
Fetal liver	3.5	1.8	4.3	37	41
Bone marrow	30.5	1.5	23.0	51	74
Adult liver	34.0	1.6	30.0	38	49

[1]The enzymes were partially purified, dialyzed against extract buffer and assayed as described in Methods. Incubation time was 10 min.

[2]Stock 1.0 mM hemin was prepared as follows: 6.5 mg of hemin was dissolved in 0.1 ml 1.0 N NaOH and diluted to 1.0 ml with water. Potassium phosphate, 0.05 M, pH 7.4, containing 0.3% Lubrol was added to a volume of 10 ml followed by 1.0 N HCl to pH 7.4. This was diluted in the assay to the stated amount.

with crude extracts of the three enzymes, hemin in the range of 1 to 100 μM had little effect on the activity.

While the activation of the erythroid fetal enzyme is similar to that reported by Woods and Murthy (83), the activation of the adult enzyme was contrary to previous demonstrations of inhibition by hemin (13,119). The interpretation of the effects of hemin on ALA synthetase is difficult at present due to the lack of control on the experimental variables. Hemin spectra show monomer-polymer equilibria and coordination ligand differences in various solutions depending on pH, dielectric constant, oxidation state and available ligands (120,121). A much more thorough study is needed once highly purified ALA synthetase isozymes are available.

5. Heat denaturation

Each of the three enzymes had different denaturation rates at 45°C (Figure 20A). These results are also summarized in Table 8. In each case there was a fast initial decay followed by a slower one. Subtraction of the second rate from the first gave faster initial decays but did not change their ranking (Figure 20B). A mixture of fetal and bone marrow enzymes decayed at an intermediate rate close to that of the theoretical summation (Figure 20C). Thus, there is little evidence for the presence of proteases or stabilizers in either enzyme preparation.

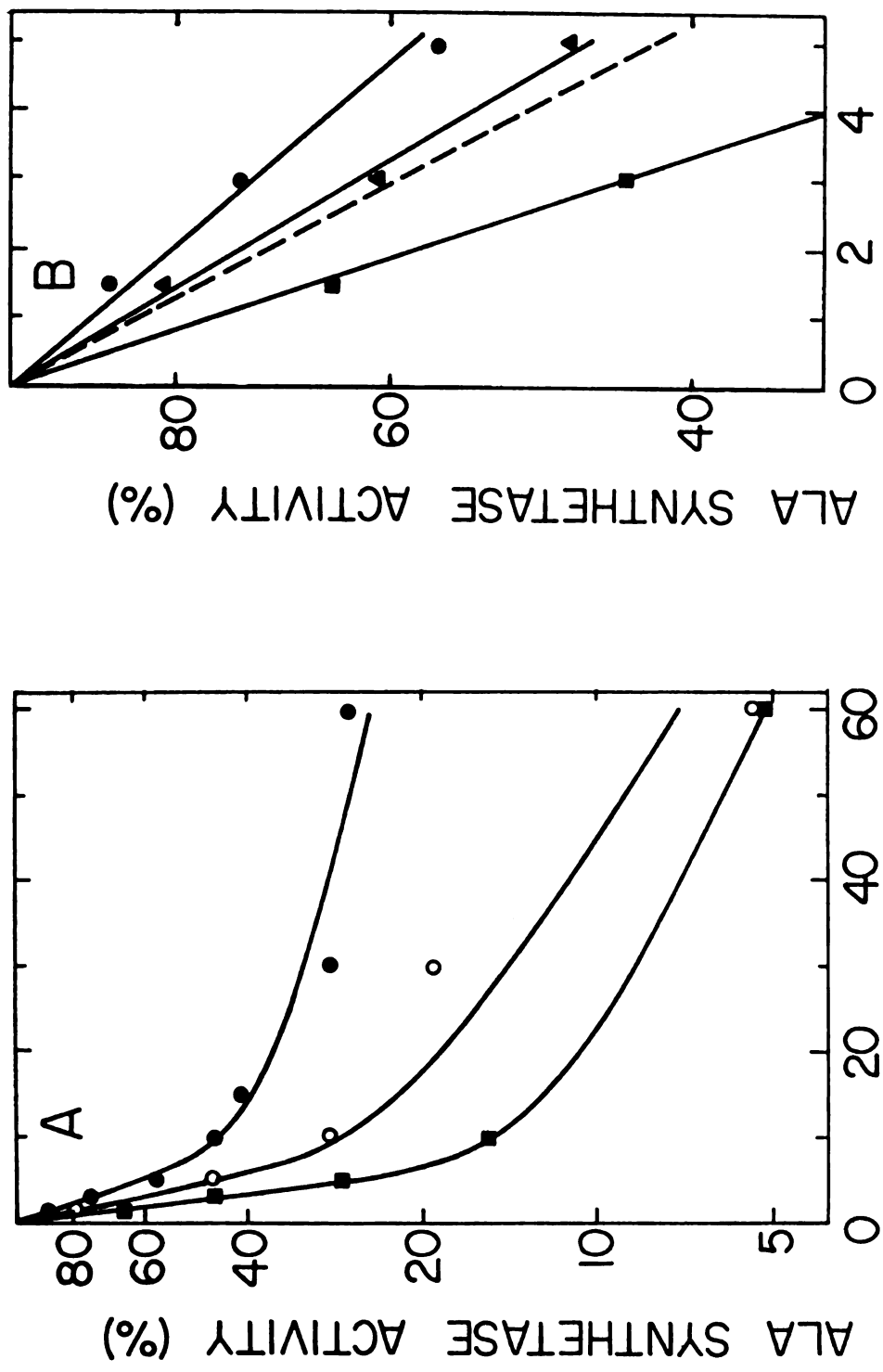

Figure 20. Heat denaturation rates of ALA synthetase isozymes. See Methods and Table 8 for additional information. (A) Decay of ALA synthetase from fetal liver (●), adult liver (○) and bone marrow (■). (B) Same as A plus a mixture of the fetal and bone marrow enzymes (▲). The dotted line is for the expected theoretical mixture.

TABLE 8

Heat Denaturation of ALA Synthetase Isozymes[1]

Enzyme	Initial Activity (U/ml)	Protein (mg/ml)	Measured Half-life (min)			Calculated Half-life (min)
			20A	20B	20C	
Fetal liver	5.9	1.8	6.8	3.8	6.8	...
Adult liver	32.0	1.5	4.5	2.9	...	...
Bone marrow	33.0	1.6	2.7	2.0	2.7	...
Fetal/Bone	10.0	1.8	...	...	4.7	4.3

[1]See Methods and Figure 20 for experimental details.

6. Molecular weight

The molecular weights of the ALA synthetase enzymes were determined by a modification of the method of Martin and Ames (see Methods). The adult enzyme moved slightly faster than the lipoyl dehydrogenase marker (Figure 21A), corresponding to a molecular weight of 113,000 daltons (Figure 21B). In the same experiment, the molecular weight of the fetal enzyme was 109,000 daltons. In another experiment comparing the fetal and bone marrow enzymes, the molecular weights were both 105,000. These results support the hypothesis that the erythroid fetal and bone marrow enzymes are similar and do not indicate any significant molecular weight differences between the erythroid and non-erythroid enzymes.

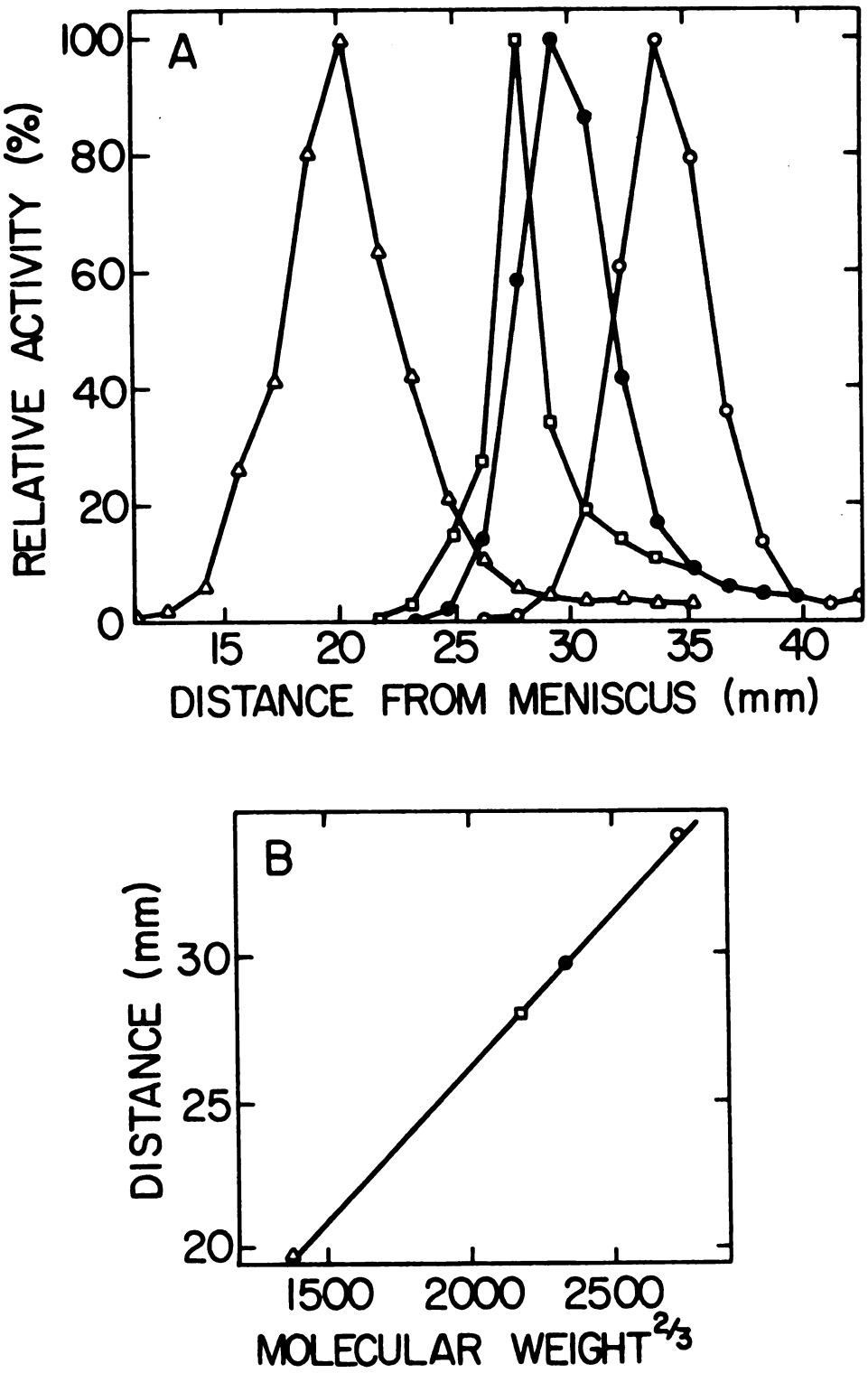

Figure 21. Sucrose density molecular weight determination. See Methods for details. (A) Migration profiles for adult liver ALA synthetase (●) and marker enzymes: (Δ) hexokinase, (□) lipoyl dehydrogenase, and (O) lactate dehydrogenase. (B) Replot of migration distance versus molecular weight.

The observed adult liver ALA synthetase molecular weight is close to the values of 113,000 and 110,000 for sucrose density determinations on the rat and chicken liver enzymes, respectively (122,123). Whiting reports a molecular weight of 77,000 for the adult guinea pig enzyme on Sephadex G-150 in high salt (13).

The molecular weight of the fetal guinea pig liver enzyme is double that reported for the fetal rat liver enzyme (83). Besides the species difference, the rat enzyme was analyzed in Tris buffer instead of phosphate as used here. Since Tris binds pyridoxal-5'-phosphate, which could be necessary for maintaining a dimeric structure, fetal and bone marrow enzymes were analyzed in the same Tris buffer solution as was reported for the G-200 MW determination (83). The calculated molecular weights of 102,000 and 100,000, respectively, suggest a small density change, but not dissociation to monomers. Either a species difference or a difference in isolation procedure is most likely responsible for the observed disparity between the results reported here and those of Woods and Murthy.

7. Kinetics

The three ALA synthetase isozymes were compared with regard to their affinity for glycine. The Km's of the erythroid enzymes were 2- to 3-fold higher than that of the adult liver enzyme (Table 9). Also, the non-erythroid adult enzyme exhibited negative cooperativity

TABLE 9

Kinetic Properties of ALA Synthetase Isozymes

Enzyme Source	Nucleotide (25 mM)	Hill[2] no.	Km (Gly) (mM)	Vmax[3] (rel.)	$V_{0.5}$[4] (rel.)	Corr. Coef.
Fetal liver	None	1.00	10.8	100	50	0.998
Fetal liver	AMP	0.78	38.1	161	44	0.991
Bone marrow	None	1.00	16.1	100	50	0.990
Bone marrow	AMP	0.89	33.7	126	43	1.000
Adult liver	None	0.82	3.33	100	50	0.997
Adult liver	AMP	0.73	6.07	145	57	0.999
Adult liver	GMP	0.64	3.72	69	33	1.000
Adult liver	ADP	0.89	7.41	90	30	0.997
Adult liver	ATP	0.75	3.88	101	48	0.993

[1]Approximately one unit each of fetal liver and bone marrow enzyme and 4 units of adult liver enzyme were used in each assay with maximal velocities of 3.93, 34.7 and 6.16 units per mg protein, respectively.

[2]Hill n and other kinetic parameters were estimated by a computer program which selects the best least squares fit to the equation $v = -v\,(\frac{km}{s})^n + Vmax$

[3]Per cent of Vmax for no nucleotides.

[4]Per cent of Vmax with substrate concentration equal to the $Km_{(Gly)}$ for no nucleotides.

while the erythroid enzymes did not. The Km's found here for the erythroid enzymes were similar to the Km for glycine of 17 mM reported for fetal rat liver (83). The reported Km of 19 mM for adult rat liver ALA synthetase is higher than that found here (13).

The effect of AMP and other nucleotides on the Km for glycine was assessed for the three isozymes. AMP (25 mM)

induced negative cooperativity with an increase in Km and Vmax for all 3 isozymes (Table 9). Other nucleotides have varying effects in the kinetics with GMP giving a pronounced suppression of Vmax (Table 9). The nucleotide GMP is the mammalian mitochondrial product of succinyl-CoA and adenylate kinase and could play a role in coordinating ALA synthetase activity with succinyl-CoA levels. While the experiments reported do not define a role for nucleotides, the affinity of the adult enzyme for AMP, and its kinetic modulation by AMP, suggest that nucleotides may have some regulatory function. A more complete picture awaits detailed studies with pure enzyme. It is possible that the nucleotide effects were overlooked thus far by others due to the lack of a valid assay.

8. AMP influence on enzyme stability

In many of the experiments involving AMP, there was an apparent activation of the adult enzyme. This was more often true of older enzyme solutions than fresh ones, suggesting that AMP reversed some denaturation process. It has been demonstrated that AMP can affect the oligomerization state of numerous enzymes (124). In the present experiments, heat inactivation of ALA synthetase in the presence of AMP is 40% less than in its absence. The initial decay of the adult enzyme had a half-life of 4.5 min (Table 8). In the presence of AMP, however, it increased to 7.3 min (Figure 22). Discounting the possibility of isozymes, the two-phase decay profile could

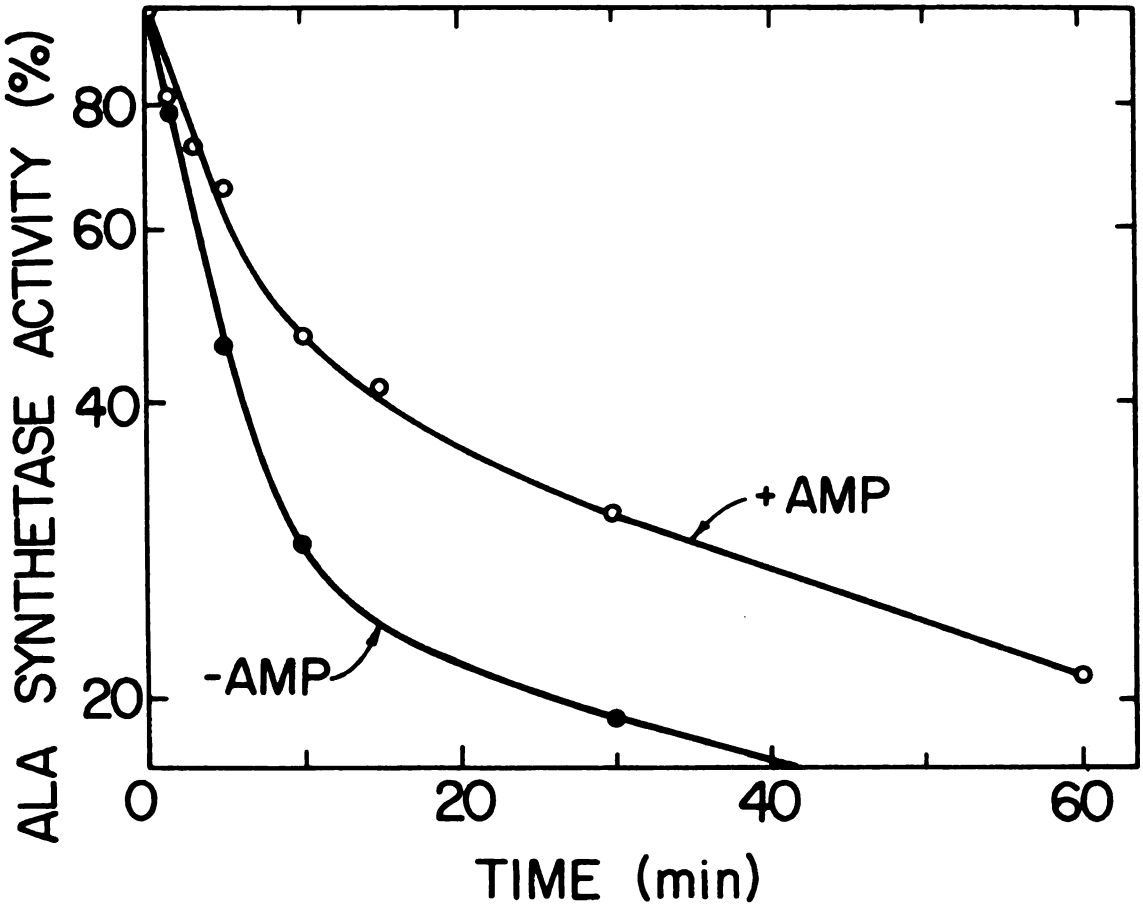

Figure 22. Heat denaturation rate for adult liver ALA synthetase with and without AMP. See Methods for experimental details. The AMP concentration was 25 mM.

arise from a fast initial process such as dissociation into subunits, followed by a slower secondary process like subunit denaturation.

9. Basal levels of ALA synthetase

As will be discussed in Section IV, the basal level of ALA synthetase activity in crude extracts of induced enzyme from adult guinea pig liver is 100-fold higher than the uninduced level. For comparison, the basal ALA synthetase activities of crude mitochondrial extracts of fetal liver, bone marrow and normal adult liver are 60, 120 and 3.0 units/gm tissue, respectively. This again demonstrates the erythroid/non-erythroid differences of ALA synthetase isozymes.

D. Discussion

The central thrust of this section has been to substantiate the hypothesis of the previous section that since the fetal and bone marrow enzymes are associated with erythroid tissue, they should be considered as a separate class from the adult non-erythroid enzyme. To this end, properties of the three ALA synthetase isozymes have been investigated and are briefly summarized in Table 10. The results indeed show a similarity in erythroid enzyme behavior in contrast to non-erythroid for most properties investigated. On the other hand, there is some support for a similarity in fetal and adult hepatic enzymes with respect to hemin activation, heat denaturation and

TABLE 10

Properties of ALA Synthetase Isozymes

Property or Treatment	ALA Synthetase Isozyme		
	Fetal Liver	Bone Marrow	Adult Liver[1]
Binding to AMP or CoA Sepharose	-	-	+
Glycine induced binding to AMP or CoA Sepharose	+	+	-
Inhibition by 0.4 N NaCl or KCl (%)	65	57	15
Activation by 200 μM hemin (%)	41	74	49
Half-life at 45°C (min)	6.8	2.7	4.5
Molecular weight	107,000	105,000	113,000
Km for glycine (mM)	11	16	3.3
Km for glycine at 25 mM AMP (mM)	38	34	6.1
Negative cooperativity in the absence of AMP	-	-	+
S.A. of crude extract (U/gm tissue)	60	120	3.0[2]

[1]DDC induced.

[2]Uninduced.

molecular weight. The results of the molecular weight determinations are consistent with either classification since all three values are essentially identical within experimental error. The more significant difference in heat stability between the fetal and bone marrow enzymes suggests that, although it may be possible on the basis

of other properties to designate them as erythroid types, they are probably not identical. In this regard there may be a fetal and adult erythroid ALA synthetase, as there are developmental hemoglobin types.

Since the AMP-binding phenomenon of the adult liver enzyme is so important to the concept of erythroid/non-erythroid isozymes of ALA synthetase, it was important to demonstrate that this drug induced enzyme was the same as that normally found in liver. This was satisfactorily demonstrated with the aid of the ultrasensitive ^{14}C-ALA assay procedure (described in Sec. IV).

The use of AMP Sepharose thus permits a straightforward differentiation between erythroid and non-erythroid isozymes of ALA synthetase. This technique could be profitably applied to analysis of perinatal liver ALA synthetase to establish the chronology of switch-over between erythroid and non-erythroid isozyme synthesis. In addition, the binding constant was high enough for the adult enzyme to permit the use of AMP Sepharose in enzyme purification.

In speculating on the possible role of the AMP-binding property, two possibilities need to be considered. First, AMP may simply be similar enough to CoA to bind to a succinyl-CoA site on the enzyme. If that were the case, however, one would expect to see inhibition of ALA synthetase activity due to competition by AMP for the substrate site. Instead the kinetics show an increase in Vmax. In addition, one would expect, in the simplest case, that the

erythroid isozymes would also bind to AMP Sepharose through their succinyl-CoA sites. This does not happen unless the substrate glycine is present.

The alternative hypothesis which now appears more attractive is that there is a separate AMP or nucleotide binding site on adult liver ALA synthetase which is not present on the erythroid isozymes. There are at least two additional lines of thought which give credence to this hypothesis. First, there is the well established inhibition of bacterial ALA synthetase by ATP (125), providing a precedent for nucleotide sites on ALA synthetase. If, as some investigators feel, mitochondria are indeed of bacterial origin, there may be some evolutionary pressure for maintaining a nucleotide site in the mitochondrial enzyme due to some metabolic requirement. This leads to the second consideration, namely the centrality of nucleotide regulation of enzyme activity in the mitochondria and the close association of a GTP-requiring enzyme to ALA synthesis. The various effects of nucleotides on ALA synthetase activity as reported here open the possibility of regulation by energy charge or by simple allosteric regulation by a specific nucleotide. As suggested by the heat denaturation studies, these effects could be mediated by ligand induced association-dissociation phenomena of enzyme subunits. Since the mitochondria of liver cells have a much greater number of metabolic tasks than those of the more highly specialized reticulocytes, it is also reasonable to expect the occurrence of more complicated

regulation of ALA synthetase in the liver where heme is required for a variety of metabolic functions.

The glycine-induced binding of erythroid ALA synthetase is informative kinetically in that it has provided a straightforward way to determine order of substrate binding without application of Cleland's rules (126) to an involved series of kinetic experiments. Clearly, glycine binding is obligatory for CoA binding, establishing the reaction as an ordered bi bi mechanism. If CO_2 release is enzyme catalyzed, then the mechanism would be ordered bi ter.

IV. SEMIAUTOMATIC ASSAY FOR PICOMOLE LEVELS OF ^{14}C-ALA

A. Introduction

δ-Aminolevulinic acid synthetase (ALA synthetase)[7] catalyzes the first committed reaction of heme biosynthesis in animals (3,127). The condensation of succinyl CoA and glycine to yield δ-aminolevulinate and CO_2 is essentially irreversible and is considered to be the rate determining step of the pathway (16,37). While there is still uncertainty regarding the existence of feedback inhibition by heme (12,59,83), its synthesis is clearly represed by hemin at 10^{-6} M (17,43,48). In addition, glycine availability may be a regulatory factor (63,128).

Precise knowledge of control mechanisms for this enzyme may help in devising treatments for certain human porphyrias in which liver ALA synthetase activity is elevated (30,129). Induction of the enzyme in liver by a variety of chemicals has proved to be a useful tool for study of the regulation of ALA synthetase (59,130,131). The discovery that ALA synthetase levels increase during fetal life (85) correlates with the known accumulation of heme precursors in perinates (8,71,72) and presents another model for the study of regulatory properties of ALA synthetase.

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[1] See abbreviation list, p. x.

One of the limiting factors in investigations of this sort has been the lack of a rapid, specific and sensitive assay. Until recently, δ-aminolevulinic acid (ALA)[7] formation was estimated by condensation with 2,4-pentanedione followed by colorimetric determination with Ehrlich's reagent (132). Subsequent modifications improved specificity and sensitivity (55,59,66,92,133,134). However, careful control of reaction times and column washing protocol is necessary to prevent large errors. The major drawbacks are interference by aminoacetone, precipitation of potassium perchlorate and a sensitivity limit of only 1-10 nanomoles of ALA per ml of reaction mixture. Low sensitivity restricts the use of the assay in homogenates of uninduced tissue as well as in biopsies and column monitoring.

The introduction of radiochemical assays utilizing ^{14}C-succinyl-CoA has lowered the detection limit to 10-100 picomoles of ALA per ml of reaction mixture (119,135-137). This gives adequate sensitivity, but the use of multiple columns is tedious and has up to 10% error due to variability of recovery (48). The single column methods are sensitive and more reproducible but led in this laboratory to large errors due to the presence of contaminants with mobilities on Dowex 50 which were very similar to that of ALA. The use of ^{14}C-glycine as a substrate is not practical since 0.1 M concentrations are required to saturate the enzyme.

To eliminate these problems, an assay was developed using a Beckman model 121 amino acid analyzer with automatic sampling capabilities as a means of isolating ^{14}C-ALA. The modification of the analyzer is simple and the assay is quite specific and sensitive. This report demonstrates the capability of the assay to detect ^{14}C-ALA specifically at the limit of 1-10 picomoles/ml of reaction mixture in up to 30 samples per day.

B. Methods

1. Reagents

Dowex 50-X8, 200-400 mesh was purchased from J. T. Baker Chemical Co.; 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) was obtained from Eastman Kodak Co.; succinic and octanoic acids were obtained from Mallinkrodt Chemical Works; dithiothreitol was from Nutritional Biochemicals Corp., Blue Dextran-2000 was purchased from Pharmacia Fine Chemicals, Triton X-100 was obtained from Rohm and Haas Co.; ATP, ALA, glycine, HEPES, lithium CoA and pyridoxal-5'-phosphate were purchased from Sigma Chemical Co.

^{3}H-succinic acid was obtained from Dhom; ^{14}C-ALA, ^{14}C-succinic acid, ^{14}C-phenylalanine and ^{14}C-serine were purchased from New England Nuclear Corp. All other chemicals were of analytical reagent grade.

2. High voltage paper electrophoresis

Samples were applied to discs cut from Whatman 3MM paper strips with a paper punch. After wetting the 5 x 57 cm strips with the electrophoresis solution, 8.8% formic acid, the discs with dried samples were returned to their holes and the strips were electrophoresed on a Savent flat-bed electrophoresis unit at 2000 VDC for one hour.

3. Scintillation counting

The scintillation solution contained: 500 ml toluene, 500 ml Triton X-100, 2 gm PPO and 50 mg dimethyl POPOP (138). Ten milliliters of this solution, thoroughly shaken with 5.3 ml citrate buffer (0.35 N, pH 4.25), forms a clear gel which is stable indefinitely at -8 to +6°C, but which separates into two phases at room temperature. Samples were counted in a Packard Tricarb Scintillation Counter Model 3324 using the channels ratio method for quench correction (139). The ^{14}C counting efficiency was 60-65%. Radioactivity was measured to give a maximum of 2.5% counting error (2000 cpm) and the results are expressed in dpm.

4. Purification of commercial ^{14}C-succinate

An 8 mm diameter Kontes plastic column was packed to a 3.0 cm depth with Dowex 50 x 8 H^+ 200-400 mesh resin. The resin was washed with 10 ml of water, 10 ml of NH_4OAc (1.0 M in acetate) pH 5.0, 10 ml of 0.1 M NaOAc pH 4.0, 5 ml of 2 parts methanol, 1 part 0.1 M NaOAc pH 4.0, and

30 ml of 0.01 N HCl. Up to 20 μmoles of ^{14}C-succinate in 10% EtOH 0.01 N HCl was applied to the column and eluted with 0.01 N HCl. The unretarded succinate peak was collected with about 95% recovery and stored at 4°C.

5. Purification of succinyl-CoA synthetase

Succinyl-CoA synthetase was isolated in a nearly homogeneous state by the procedures of Ramaley et al. (140,141) followed through the first DEAE-Sephadex stage. The specific activity was 21 U/mg. This preparation was stable at -15°C for two years in 0.1 M HEPES, pH 7.2 and 0.1 M KCl. No ALA synthetase activity was detected. One unit of activity equals 1 μmole succinyl-CoA formed per min at 30°C (141).

6. ALA synthetase assay

a. Incubation conditions. The incubation mixture contains 100 mM glycine, 50 mM HEPES, 10 mM $MgCl_2$, 2.0 mM ATP, 1.1 mM succinate, 1.1 mM lithium CoA, 1.0 mM dithiothreitol, 50 μM pyridoxal-5'-phosphate, KOH to pH 7.5, 0.5 μCi purified ^{14}C-succinate (22.5 μCi/μmole), 0.3 units succinyl-CoA synthetase, up to 0.4 ml ALA synthetase in a final volume of 0.5 ml. The reaction is usually started by addition of ALA synthetase. Incubation proceeds for 10-30 min at 30°C and is stopped with 50 μl 50% trichloroacetic acid (TCA).

The equilibrium of the succinyl-CoA synthetase reaction was determined to be 3.3 in the direction of hydrolysis

under the conditions of the assay. Thus, the initial succinyl-CoA concentration is 0.33 mM. Higher concentrations were inhibitory.

HEPES buffer is used because of its pKa of 7.55 and its lack of primary amino groups which in a buffer like Tris form Schiff's bases with pyrodixal-5'-phosphate. Phosphate is not used since it shifts the succinyl-CoA synthetase equilibrium more towards hydrolysis.

Ten-fold concentrated assay mixture minus CoA, dithiothreitol, ^{14}C-succinate and enzymes is stored at -20°C in small aliquots for up to one month.

b. Isolation of ^{14}C-ALA. ^{14}C-ALA is separated from other compounds in the reaction mixture on the short basic column of Spackman et al. (142). For this purpose a Beckman model 121 amino acid analyzer was modified as diagrammed in Figure 23.

The 0.35 N citrate buffer (20 liters) contains the following per liter: 34.33 gm sodium citrate dihydrate, 14.3 ml concentrated HCl, distilled water to one liter and 0.15 ml octanoic acid. After the mixture is stirred 2-4 hours the octanoic acid dissolves and the final pH should be 4.25 $\pm$ 0.1. The entire solution is filtered through a sterile millipore filter (0.45 μ) into a sterile bottle. The 0.2 N NaOH is also filtered before use. Water is fed into the two unused ports at valve A to prevent air bubbles from entering the line during switching. The pump is adjusted to give a flow rate of 1.33 ml/min for the

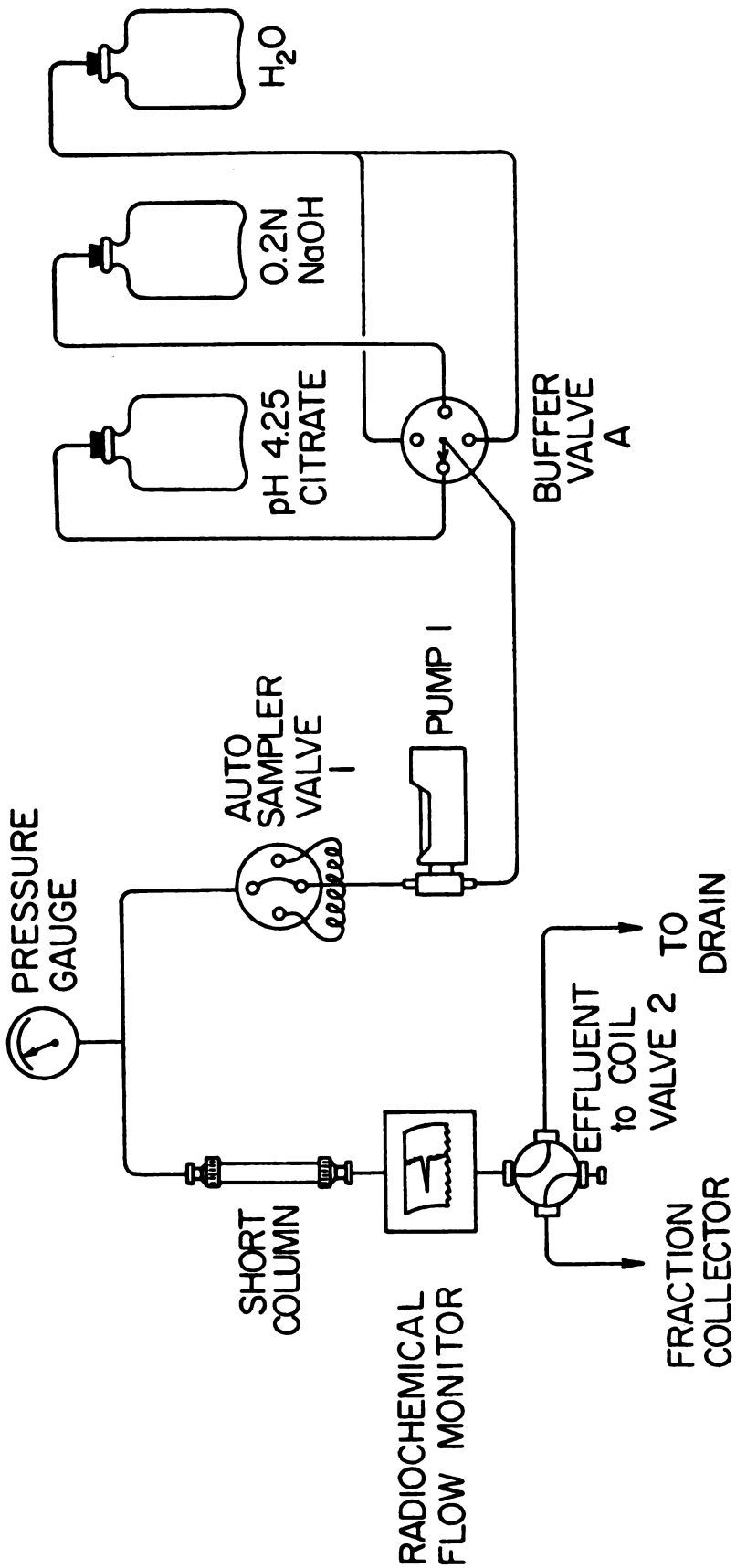

Figure 23. Diagram of the modified Beckman model 121 amino acid analyzer.

0.9 cm column at a pressure of about 100 psi. Samples are diluted to 0.6 ml with water in 1.5 ml polypropylene centrifuge tubes. This amount is completely transferred to a 1.0 ml Beckman sample coil for the automatic sampler. A 0.2-1.0 cm air bubble is introduced just before the last drop is transferred to prevent siphoning of the sample before analysis. A 500 µl metering coil is used at valve 1. Beckman PA-35 resin is packed in the 0.9 x 23 cm column to a height of 9.1 cm. Column temperature is maintained at 50°C by the circulating water bath. Radioactivity of the effluent is determined by a Packard model 3022 Radiochemical Flow Monitor. However, monitoring the effluent for radioactivity is a convenience rather than a necessity. The line from valve 2 to the fraction collector should be as short as possible. The ALA peak is collected over a 4.0 min period in a volume of about 5.3 ml. The entire fraction is poured into 10 ml of scintillation solution and counted for 10-50 min as described above. Each time fresh buffer is made, or once a month, the flow rate is adjusted so that the radioactive ALA peak is evenly divided between two fractions collected for 2 min each. Table 11 gives the nominal operating parameters.

A new program tape for the analyzer must be prepared; the program steps are listed in Table 12. The program tape is advanced to step 1 and one or two old samples are run through the complete cycle to stabilize the system. The inject cycle (see Table 12) is started before the transfer cycle is complete in order to minimize high

TABLE 11

Nominal Settings of the Controls on the Beckman Analyzer

Control	Setting
Valve safety	Auto
Buffer valve A	Auto
Pump 1 switch	Interrupt
Buffer pump 1	Auto
Pump 1 flow	975
All inj./sample controls	Auto
Tank N_2 pressure	25 lbs
Inlet N_2 pressure	5 lbs
Sample transfer pump	910
Sample inject sequence	ODD
Inject cycle delays	
Buffer	0 sec
Inject	6 sec
Wash	35 sec
Purge	40 sec
Valve	Max
Bath temperature	52°C
Effluent to coil valve 2	Auto
Recorder safety	Off
Flow safety	Off

TABLE 12

Steps for the Beckman Programer[1]

Step	Program Statement	Step Time (min)	Elapsed Time (min)
1	Initiate sample transfer; stop slew	0.1	0139.0
2	Sample transfer time	1.5	0.2
3	Initiate inject cycle; pump 1 off	0.1	1.8
4	Pump 1 off	0.2	2.0
5	Pump 1 on	10.0	2.3
6	Valve A to NaOH (column regeneration)	5.0	12.4
7	Valve A to citrate buffer	7.9	17.5
8	Effluent to fraction collector (valve 2)	4.0	25.5
9	Effluent to drain (valve 2)	9.1	29.6
10	Start slew	0.1	38.8

[1]These statements and times are coded onto the program tape according to the instructions in the analyzer manual of the Beckman Instrument Co.

transient pressures during valve switching. The NaOH regeneration step is timed so that the NaOH arrives at the column about 3 min after the ALA peak is collected. Since the entire cycle takes 39 min, the fraction collector is also set for this interval and is manually indexed at step 6 at the beginning of the run.

7. Modified assay of Ebert et al.

The method of Ebert et al. (136) was used with the following modifications. The bed height was 1.8 cm. The volume of each washing reagent was one-half of the volume specified in the paper. Elution was accomplished with NH_4OAc (1.1 M in acetate) pH 5.0. One milliliter aliquots were counted in 10 ml Bray's scintillation solution (143) at 70% efficiency.

C. Results

1. Interference by compounds similar to ALA

In the course of using the modified assay of Ebert et al. (136), it was found that ^{14}C-α-ketoglutarate gave a much higher apparent ALA synthetase activity than did the substrate, ^{14}C-succinate. This ultimately led to the discovery that ^{14}C-glutamate contaminated the ^{14}C-ALA containing eluates of the small Dowex 50 columns used in that method. Amino acid analysis using a scintillation flow monitor showed that, in addition to ^{14}C-glutamate, the effluent contained ^{14}C-aspartate and two other radioactive contaminants with mobilities slightly greater than alanine and valine, respectively (Figure 24A,B). An incubation mixture containing ^{14}C-ALA did not give rise to any of these contaminants.

Patton and Beattie also reported difficulties in reproducibility with Ebert's method (145). Repetition of their procedures with ^{14}C-succinate as substrate also yielded large quantities of the valine-like material in

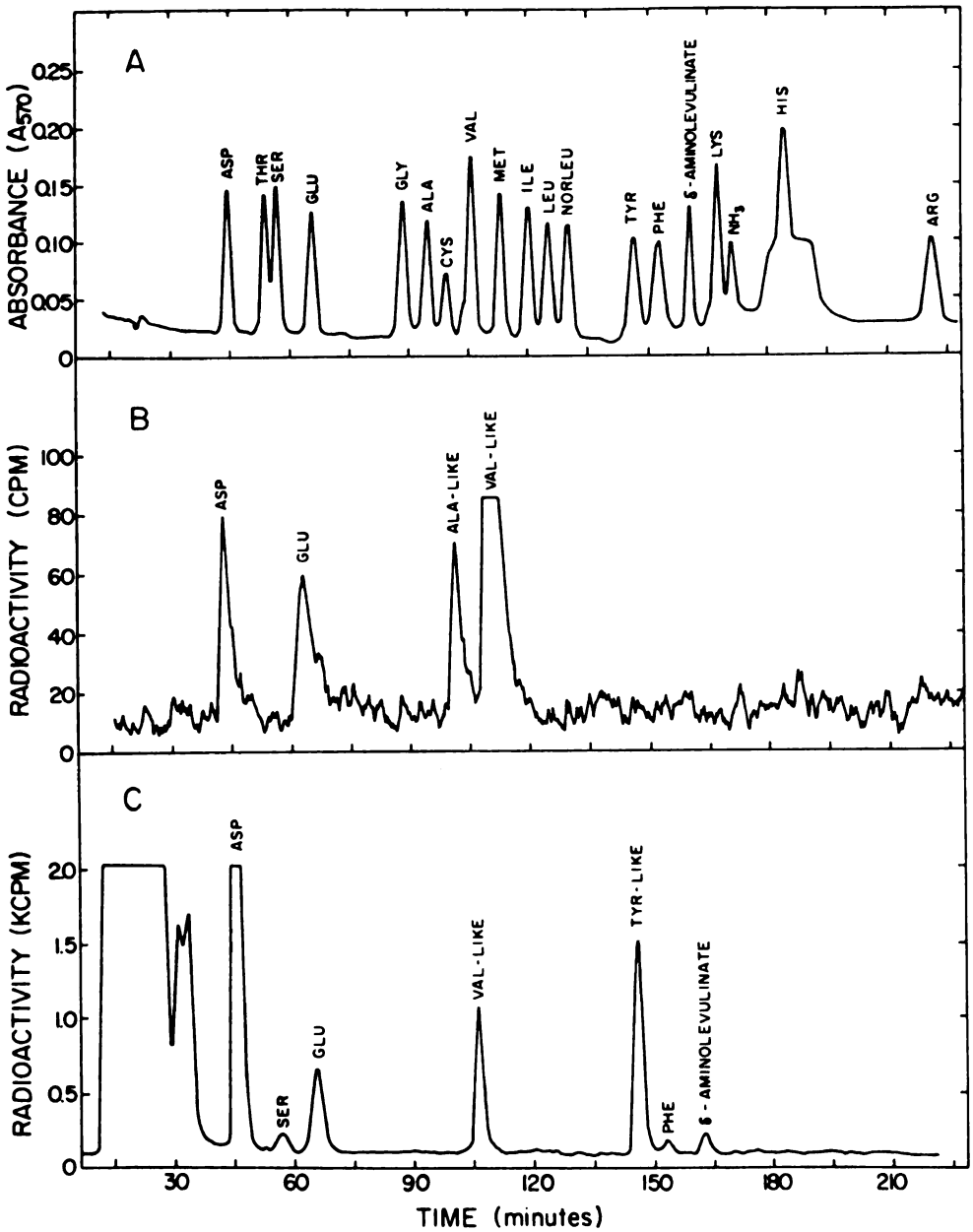

Figure 24. Amino acid analyzer profiles. The 2 hr accelerated system of Robertson et al. (144) was used. (A) Ninhydrin detection of an amino acid calibration mixture. (B) Radiochemical flow monitor detection of ^{14}C-amino acids. The sample was an acidified and concentrated eluate from the short Dowex 50 columns of the modified ALA synthetase assay of Ebert et al. (see Methods). Mitochondria were isolated from the liver of a normal adult guinea pig. Full scale deflection was 100 cpm. (C) Radiochemical flow monitor detection of ^{14}C-metabolites of ^{14}C-succinate of the TCA supernatant from an assay of rat liver mitochondria as described by Patton and Beattie (145). ^{14}C-Serine and ^{14}C-phenylalanine standards were added to the sample. Full scale deflection was 2000 cpm.

the trichloroacetic acid supernatants. In addition, a new contaminant with a mobility similar to that of tyrosine was present in 10-fold greater concentration than the ^{14}C-ALA produced in the assay (Figure 24C). Sonication of the rat liver mitochondria reduced the valine-like peak but increased the tyrosine-like peak nearly four-fold with respect to the ^{14}C-ALA peak. These results suggest an alternative explanation for the substrate and sonication dependent differences in ALA synthetase activity reported by Patton and Beattie. No further study of these compounds was undertaken; however, the presence of the tyrosine-like compound was correlated with the presence of Tris in the assay.

2. Elimination of contaminants

In order to eliminate the problem of metabolites of ^{14}C-succinate in the ALA-containing eluates from the short Dowex 50 columns, a trial assay was performed using the column for basic amino acids of the Spackman et al. system (142). The resolving power of this column was shown to separate ALA from other radioactive materials, including the contaminants with mobilities similar to valine and tyrosine (Figure 25). After this success, the Beckman analyzer was modified as described earlier. An example of the high resolution achieved is given in Figure 26.

Aminoacetone is not considered a possible contaminant since it is not synthesized directly from ^{14}C-succinate. In addition, it was found to have a mobility quite

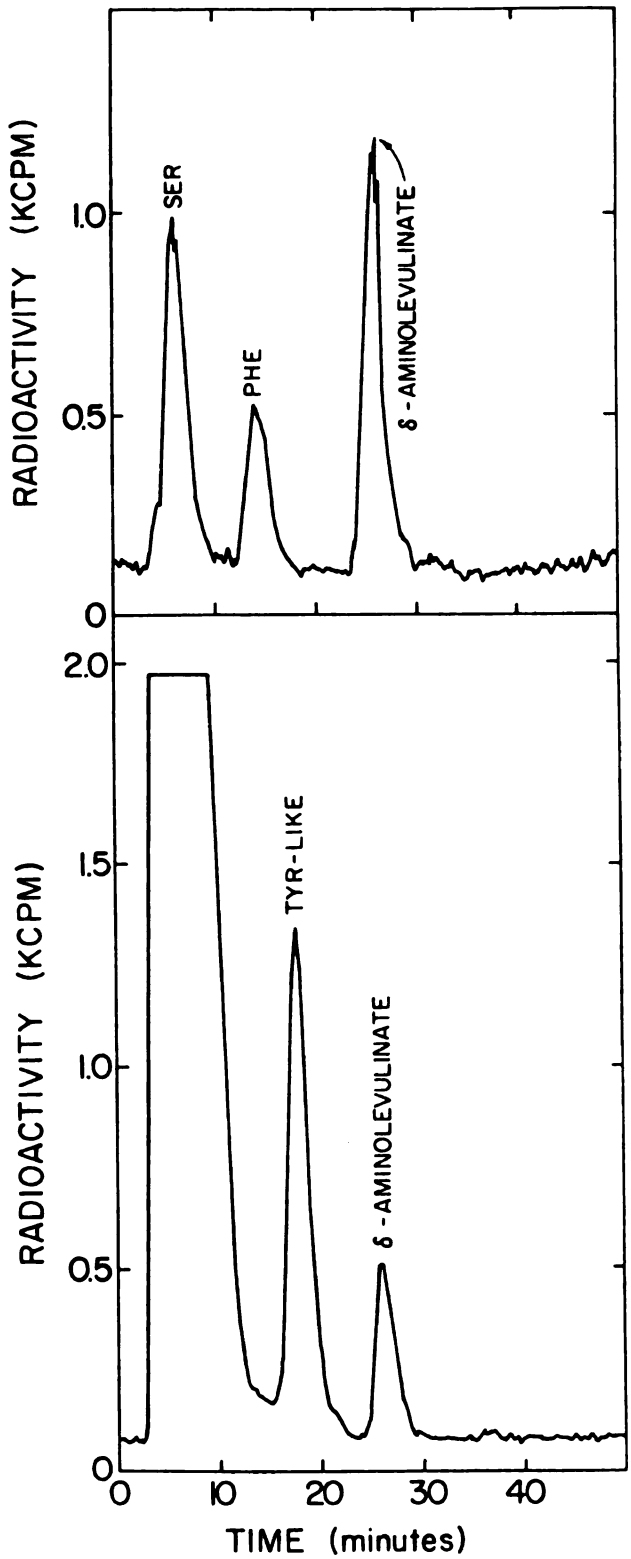

Figure 25. Radiochromatograms of standards and samples. The basic amino acid column of Spackman et al. (142) was used. (A) The sample contained added ^{14}C-serine, ^{14}C-phenylalanine and ^{14}C-ALA. (B) The sample is similar to that in Figure 24C except that the serine and phenylalanine standards are omitted.

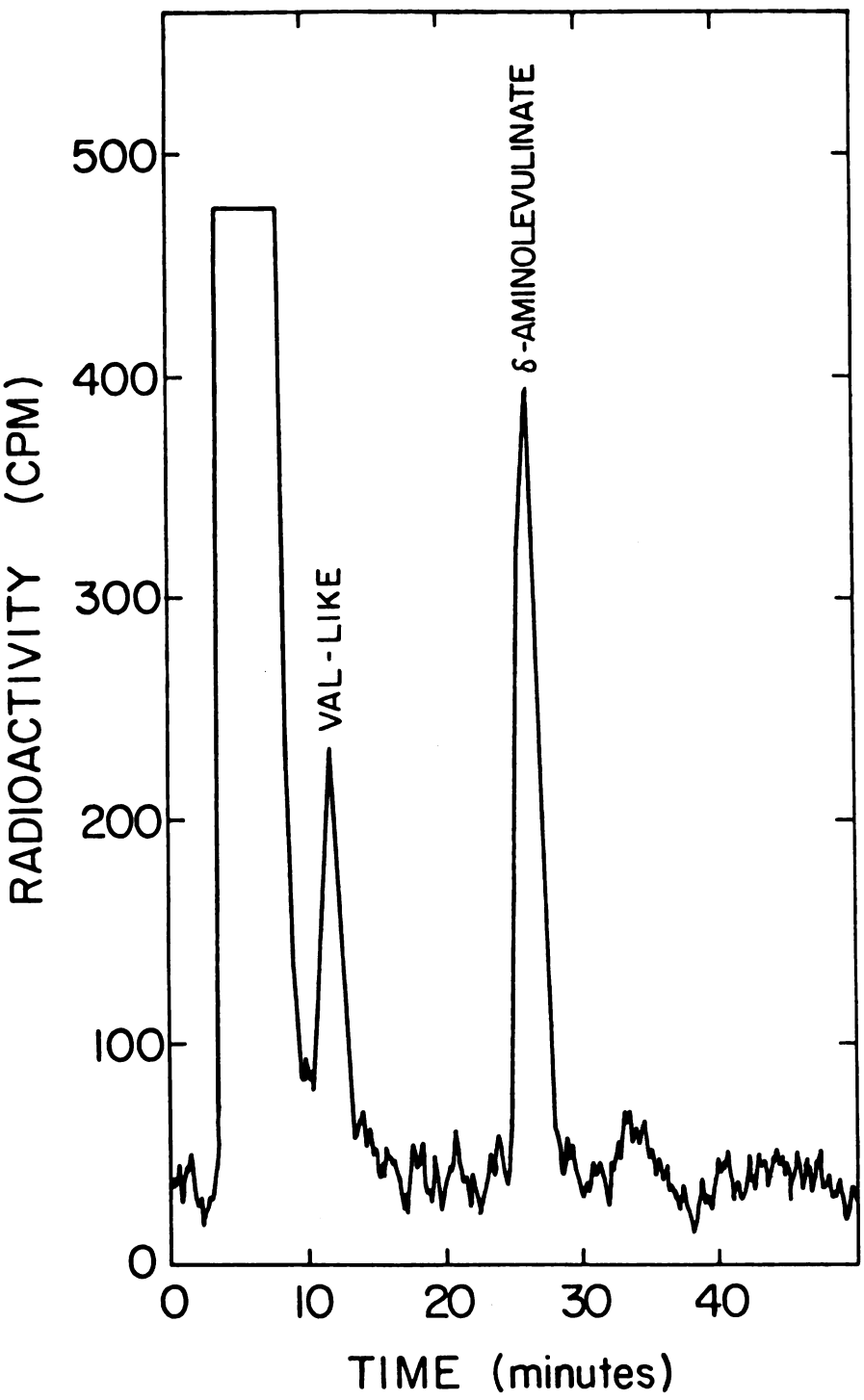

Figure 26. Radiochromatogram from a routine run on the modified Beckman 121 amino acid analyzer. The sample was a TCA supernatant from an incubation of mitochondria from the liver of a DDC induced adult guinea pig. The assay was carried out as described in Methods using 100 µl of mitochondrial suspension incubated for 30 min. The ALA synthetase activity of the suspension was 140 U/ml.

different from that of ALA when analyzed on a 4 hr single column system (146).

3. Purity of the ^{14}C-ALA standard

While one commercial preparation of ^{14}C-ALA was only 68% pure, that from the source used in these experiments showed two contaminants on high voltage paper electrophoresis amounting to only 1.5% of the total activity. Further purification was accomplished on the Spackman et al. (142) basic column. The peak material in 0.35 N citrate was acidified to pH 1 with HCl. This served as the standard in experiments on recovery and linearity.

4. Recovery of ALA on the modified amino acid analyzer

Since the automatic sampler does not transfer all of the solution in the sample coil to the column, the fraction reaching the column was determined by using samples containing Blue Dextran-2000. The column top flow adapter was removed and during program control the Blue Dextran was collected at this point. From the collected sample's absorbance at 610 nm in 0.35 N citrate, pH 4.25 (A_{610} = 0.540), the fraction of the applied sample reaching the column was calculated. From the sample coil to the top of the column the % recovery was 85.8% $\pm$ 2.5% S.D., n = 7.

The overall recovery was determined by mimicking the actual assay conditions. Numerous 0.6 ml aliquots, with

and without additions were analyzed. The 85.9% recovery shown in Table 13 indicates that all losses occur prior to ion exchange chromatography, that is, in the automatic

TABLE 13

Recovery of ALA from the Beckman Analyzer[1]

Additions	No. of Assays	^{14}C-ALA (dpm ± S.D.)	Net (dpm)	Recovery (%)
None[2]	3	123 ± 2	-	-
^{14}C-ALA[3]	5	1954 ± 18	1831	87.2
^{14}C-ALA and carrier[4]	5	1926 ± 8	1803	85.9

[1]Conditions as described in Methods.

[2]Pooled TCA supernatants of assays with low ALA-synthetase activity.

[3]2099 ± 9 dpm S.D., n = 3.

[4]10 nmoles ALA.

sampler and transmission to the column. Omission of carrier ALA gave an increasing trend in consecutive sample counts for replicates. Since in either case the error was less than 1%, carrier is omitted from the procedure.

5. Linearity, precision and sensitivity

The ALA synthetase assay is made up of two parts. In the first part ^{14}C-ALA is generated during incubation of enzyme and substrates. In the second, a modified amino acid analyzer is used to isolate the ^{14}C-ALA which is then

quantitated. Linearity of the ^{14}C-ALA analysis part of the assay was assessed by adding ^{14}C-ALA standard to pooled, low activity supernatants from the incubation part of the assay. Recovery of added ^{14}C-ALA was linear over a range of 15 to 950 pmoles ALA analysis (Figure 27). This represents a 63-fold change in ALA concentration.

The sensitivity and linearity of the entire assay were determined using dilutions of adult guinea pig ALA synthetase which gave 3-150 pmoles ALA per analysis per 15 min incubation (Figure 28). One hour counting times are necessary to achieve a 2.5% counting error for these low activities. The average deviation from the least squares line was 3.2%. The activity of the dilution giving 3 pmoles ALA per analysis is 0.125 U/ml for a 15 min incubation. This represents a 5-fold improvement in sensitivity over the method of Strand et al. (137), with half the error. If the enzyme used is stable in the assay for longer than 15 min, proportionately greater sensitivity is possible.

6. Background interference

The sensitivity of the assay is limited by the percentage of starting radioactivity which is recovered in the blank. As seen in Table 14, this background noise is less than one part in 10,000. The noise appears to be more dependent on the total amount of succinate applied than on its specific activity, suggesting that a minute

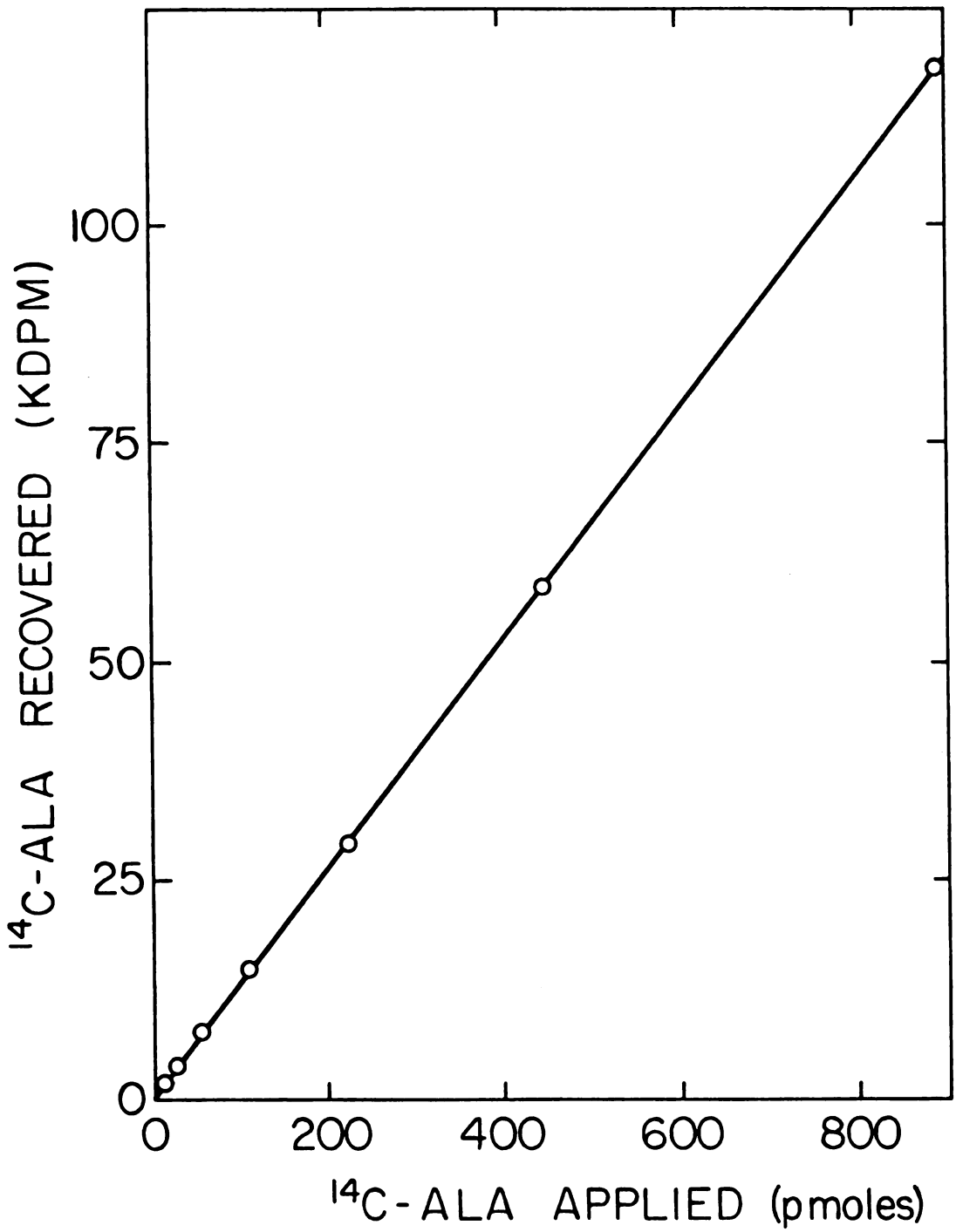

Figure 27. Linearity of analysis with added ^{14}C-ALA. Samples were taken from a pool of trichloroacetic acid supernatants of assays with no detectable ALA synthetase activity. ^{14}C-ALA (59 μCi/μmole) was added to the samples as indicated. The ^{14}C-ALA recovered was corrected for the 15% loss in the automatic sampling system. The preparation of pure ^{14}C-ALA and the isolation procedure on the analyzer are described in Methods.

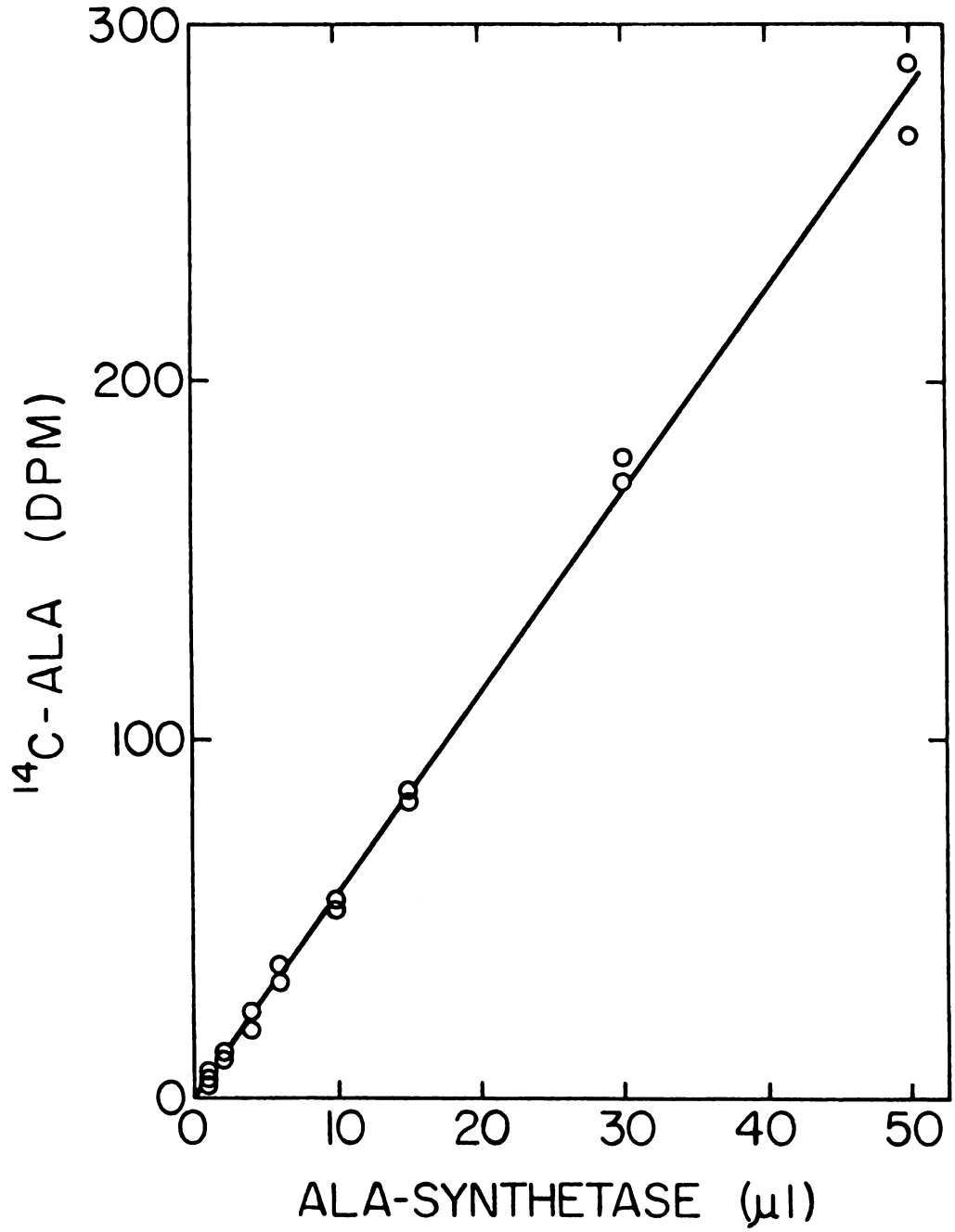

Figure 28. Linearity and sensitivity of the assay. One to 50 µl ALA synthetase (49 U/ml) from adult guinea pig liver was assayed (see Methods). The incubation period was 10 min. For each assay, 250 µl of the TCA supernatant was analyzed.

amount of succinate carries over into the ALA elution position.

TABLE 14

Contamination of the Blank by Substrate

Label[1]	Amount (nmoles)	Radio-activity (kdpm)[2]	Sp.Act. (μCi/ μmole)	Net Recovery (dpm)[3]	Contamina-tion (%)
^{14}C	235	423	0.81	38	0.0066
^{14}C	198	821	1.87	43	0.0052
^{3}H	273	40,400	66.6	3720	0.0092

[1]Both ^{14}C and ^{14}H succinate were purified on Dowex 50 as described in Methods.

[2]kdpm = 1000 dpm.

[3]Net counts recovered in the ^{14}C-ALA peak for assays stopped at zero time with TCA. The counts for citrate buffer alone were 40 dpm.

With a constant percent background contamination, an increase in the specific activity will permit shorter counting times at the same error level but will not increase the sensitivity.

Sensitivity may be increased by reducing the concentration of succinate in the assay while keeping the level of ^{14}C-succinate constant. With suboptimal substrate concentrations, however, enzyme quantification and kinetics are unreliable.

7. Application of the assay to guinea pig liver

The average level of mitochondrial ALA synthetase activity in the livers of normal adult male guinea pigs was 2.9 U/gm liver as determined by this new method (Table 15).

TABLE 15

Activity of Uninduced ALA Synthetase

Experiment[1]	Volume Mitochondrial Suspension[2] (µl)	Activity[3] (U/ml)	Average Activity (U/ml)	Average Activity (U/gm liver)
	100	1.23		
1	200	1.32		
	300	1.36		
			1.46	2.9
2	100	1.56		
	100	1.66		

[1]Adult male English short-hair guinea pigs of the Hartley strain (700 gm).

[2]Homogenized 0.5 gm liver in 5 ml ice cold buffer containing: 0.25 M sucrose, 20 mM potassium phosphate, pH 7.5, and 50 µM pyridoxal-5'-phosphate. The mitochondrial pellet was resuspended in 1.0 ml homogenizing buffer.

[3]Assayed as in Methods. Incubation time for experiment 1 = 30 min, for experiment 2 = 15 min.

Induction of the enzyme with 3,5-dicarbethoxy-1,4-dihydrocollidine (13) in guinea pigs of similar age resulted in activities of 150-350 U/gm liver. This level of activity

is essentially the same as that (200-350 U/gm) obtained by Whiting and Elliott (13). Thus, it is estimated that this drug induces a 100-fold increase in liver ALA synthetase activity in guinea pigs.

8. Increased assay sensitivity as applied to analysis of sucrose gradients

As discussed earlier, it is possible to increase the sensitivity of the assay at the expense of saturating substrate conditions. This is useful when only a qualitative indication of enzyme presence is needed. A sample of ALA synthetase assayed as described in Methods yielded a net activity of 280 dpm ^{14}C-ALA/25 µl enzyme/20 min. The same sample assayed as described minus cold succinic acid gave 3450 dpm ^{14}C-ALA/25 µl enzyme/20 min with no increase in background dpm. This allows ALA synthetase to be qualitatively determined with activities generating as low as 2 pmoles ALA/hr/ml enzyme. This technique was used to determine the subcellular distribution of ALA synthetase activity in a sucrose density gradient (Figure 29). Approximately 96% of the activity is found in the mitochondrial fraction, while the remaining 4% is associated with the soluble fraction.

9. Other applications

The behavior of fetal liver, adult liver, and adult bone marrow ALA synthetases was monitored using this assay in both its normal and increased sensitivity modes (148). The assay has also been used to measure the levels

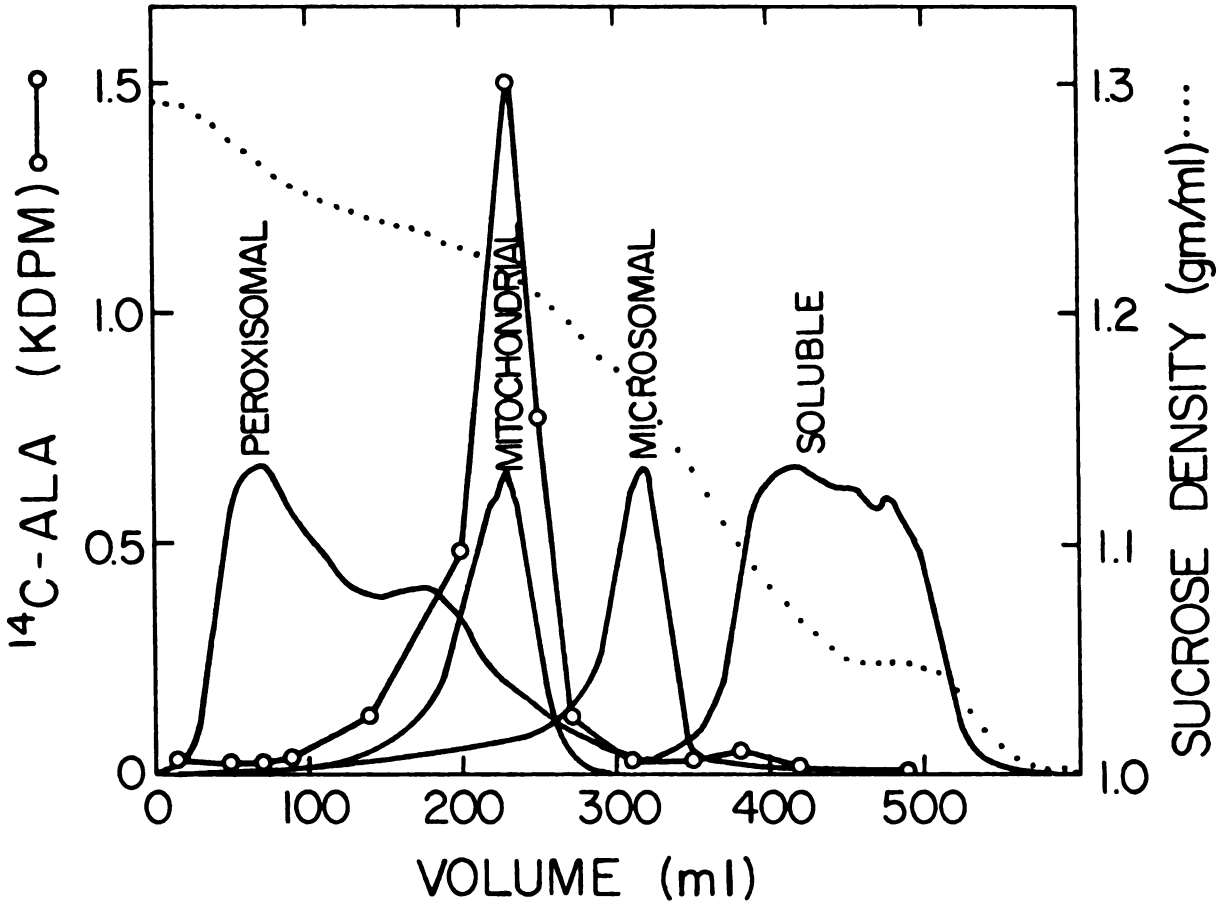

Figure 29. Subcellular location of ALA synthetase. ALA synthetase activity was determined using the increased sensitivity procedure described in Methods. The zonal gradient was generated and assayed by Dr. N. E. Tolbert's laboratory personnel (147) using liver from phenobarbital treated rats. The peak activities for the various marker enzymes were: peroxisomal catalase, 4.7 mmoles/min/ml; mitochondrial glutamate dehydrogenase, 0.79 μmoles/min/ml; microsomal cytochrome reductase, 1.6 μmoles/min/ml; and soluble catalase, 9.5 mmoles/min/ml.

of ALA synthetase activity in fetal liver during the development of the guinea pig (81).

D. Discussion

In our studies of ALA synthetase, satisfactory adoption of reported procedures which use short columns of Dowex-50 was frustrated by contamination with metabolites of ^{14}C-ALA. Other investigators have reported similar difficulties (145,149,150). These problems were eliminated by making use of the superior resolution and specificity of an amino acid analyzer to isolate the ^{14}C-ALA produced during the assay incubation. This is apparently the first use of an amino acid analyzer for routine assays of ALA at such low levels. Previously, an analyzer was used to determine the concentration of ALA in the 10 nanomole region in potato sprout extracts (151). ALA was also qualitatively identified in relation to 186 other ninhydrin positive substances by Hamilton's single column procedure (152).

The modification of the Beckman analyzer described in this report is simple and is easily interchanged with standard procedures. It requires only a free short column system, separate effluent control valves, fraction collector, and program tape.

Samples containing ALA synthetase activities in the range of 0.1-100 U/ml were routinely assayed. ^{14}C-ALA was specifically determined in the presence of many other metabolites of ^{14}C-succinate. Only ampholytes used in

isoelectric focusing were found to generate compounds migrating similarly to ^{14}C-ALA in this system. A further 10-fold increase in sensitivity at the expense of saturating substrate conditions permits location of low concentrations of ALA synthetase in chromatography fractions and other separation methods. The automatic sampling capability allows up to 30 samples to be assayed per day with minimal attendance. Automation also brings the benefit of excellent reproducibility.

This method has permitted the first reported measurement of mitochondrial ALA synthetase levels in the livers of normal guinea pigs. This activity has previously proven difficult to measure. Using colorimetric methods which do not discriminate between ALA and aminoacetone, levels of 8-25 U/gm liver were determined (85,135,153). However, it has been shown that these activities are most likely due to aminoacetone synthetase which is present in normal guinea pig liver (135). No ALA synthetase activity was reported with either the ALA-specific colorimetric assay of Granick and Urata (37), or the radiochemical assay of Irving and Elliott (135).

Much evidence has accumulated favoring the hypothesis that ALA synthetase is synthesized on cytoplasmic ribosomes and then transported into the mitochondria where it functions in heme synthesis (53,54,154). The mitochondrial location of ALA synthetase has been demonstrated previously by differential sedimentation methods (15,155). We confirm the mitochondrial location of ALA synthetase using

zonal centrifugation, a technique demonstrated to separate lysosomes and peroxisomes from mitochondria.

Many laboratories will not have access to an amino acid analyzer for routine analysis of ALA synthetase. However, in the light of the many problems of specificity, those laboratories using radiochemical assays could profit by determining the purity of their final effluents by such a reference method. For this purpose a completely manual chromatographic separation by the method of Spackman et al. (142) would suffice.

V. SUMMARY AND CONCLUSIONS

The widespread occurrence in animals of a transient fetal porphyria raises the issue of differential regulation of fetal and adult ALA-synthetase. The present study was directed toward an understanding of the structure and function of the two enzymes with reference to features consistent with the observed physiological differences. The level of ALA synthetase was surveyed in the livers of guinea pig embryos throughout most of the significant growth and development of that organ. This study clearly identified the 40th to 47th days of gestation as the period of maximal specific activity for fetal liver ALA synthetase. The activity subsequently diminished until by birth it had reached the low level found in adults. Erythropoietic activity paralleled ALA synthetase activity, strongly suggesting that the enzyme was associated with the transient erythroid cell line of the fetal liver. Fetal ALA synthetase was partially purified from livers of the period of peak activity for comparison with the adult enzyme.

In anticipation of a possible erythroid/non-erythroid classification of fetal and adult ALA synthetase isozymes, the enzyme was also isolated from adult bone marrow, the mature counterpart of early fetal liver. The properties

of all three enzymes were compared. The results support the hypothesis that fetal ALA synthetase is an erythroid enzyme with properties similar, but not identical, to those of the adult erythroid enzyme of bone marrow. They may exist as developmental erythroid isozymes. The adult hepatic enzyme is in a non-erythroid class though there are of course similarities in the structure and function of the enzymes of the two classes.

No dramatic differences in regulation of activity were observed which could account for the high levels of fetal porphyrin production as compared with that of the adult. The apparent activation of fetal ALA synthetase by hemin, however, is compatible with the high fetal activity of the enzyme. In an organ such as bone marrow or fetal liver where high activity levels are necessary to insure a sufficient supply of heme for hemoglobin synthesis, it would be counterproductive for this enzyme to be strongly inhibited by hemin. Hemin should probably be deemphasized as an allosteric regulator of mammalian ALA synthetase since it also has little effect on the adult hepatic enzyme. Furthermore, all the observed effects were apparent only at hemin levels much higher than physiologically normal. It would be more to the point if hemin could be shown not to inhibit the synthesis of the fetal enzyme in contrast to its well documented repression of adult hepatic ALA synthetase.

While the nucleotide effects discovered in this study do not account for the gross differences in enzyme activity

during development, they may have important consequences for control of adult hepatic ALA synthetase. The possible control of the non-erythroid isozyme by nucleotides suggests that the control over enzyme synthesis and degradation is not responsive enough to handle rapid metabolic changes in the liver. It is not clear, however, that heme synthesis exerts a big enough load on the mitochondrial machinery to require its regulation by energy charge. Thus, the nucleotide effect may be a clue that ALA has other metabolic fates than porphyrin synthesis. This is also suggested by the location of the heme pathway partly in the mitochondrion and partly in the cytoplasm with the first enzyme in the cytoplasm part of the pathway also having the characteristics of a regulatory enzyme (156). The recent discovery that in plants ALA is synthesized from α-ketoglutarate instead of succinyl-CoA and glycine (157) supports the possibility of alternative pathways of ALA metabolism as does the discovery of an NADH-dependent ALA dehydrogenase in *Rhodospirillum rubrum* (158,159).

Very little progress could be made on this research before the development of a satisfactory assay for ALA synthetase was completed. Much of one year's work had to be discarded after radioactive metabolites other than ^{14}C-ALA were found in the column eluates of the method then in use, modified as described in Methods, Sec. III. The sensitive and specific assay described in Sec. IV was quite reliable and eliminated most of these problems.

VI. RECOMMENDATIONS

Outlined below are several lines of research and groups of experiments suggested by the results of this dissertation.

A. Isolation of erythroid cells

The erythroid/non-erythroid classification of ALA synthetase isozymes would be put on more solid footing if the various cell types of fetal liver were isolated and the ALA synthetase of fetal erythroid cells shown to be identical with the one isolated from fetal liver in this report.

B. Salt effects

Inhibition of fetal and bone marrow ALA synthetase by KCl and NaCl but not by KF suggested that either there was specific inhibition by Cl^- or some indirect effect mediated by something else in the crude enzyme preparations. Assay in the presence of trimethyl ammonium chloride may help confirm a chloride effect while a mixing experiment of fetal and adult enzyme plus KCl or NaCl should show if the effects are additive or not.

C. Classification of ALA synthetase isozymes

The AMP-Sepharose column presents a tool for investigating many interesting questions concerning structure and function of ALA synthetase. First, there has been a long-standing hypothesis that mitochondrial ALA synthetase has a precursor of larger molecular weight in the cytoplasm. Their respective AMP-Sepharose affinities might shed some light on their relationship. Second, AMP binding should be surveyed with ALA synthetase isozyme from muscle, heart, brain and other tissues in order to help pin down the metabolic relationships of this property. Third, this tool could be applied to the isozyme from yolk blood islands to see if this earliest erythroid enzyme behaves similarly to the other erythroid enzymes. Fourth, Whiting has shown that ALA synthetase from chick embryo livers is inducible by drugs and has a Km for glycine of around 2 mM (290), both properties being similar to those of ALA synthetase from adult rats and guinea pigs. One might predict, therefore, that this enzyme would also bind to AMP-Sepharose. Last, the ATP inhibition of bacterial ALA synthetase suggests this enzyme would also bind to the affinity resin. Of course, this method may also provide a key step in the purification (in some cases for the first time) of the enzyme from these various sources. In this regard it should be pointed out that all affinity experiments were done at 25°C. Binding is often much stronger at 0°C and thus should be tried for all isozymes.

D. Delineation of the nucleotide effect

As mentioned earlier in the text, the important work of clarifying the role of nucleotides in regulation of ALA synthetase is yet to be done. In addition to the enzyme kinetics approach, something may be learned of the nature of the nucleotide binding site by using nucleotide-Sepharoses with different ligand attachments.

E. Effector induced changes in subunit association

The sucrose density experiments should be repeated in the presence of AMP and KCl to see if there are any changes in the state of association of the enzyme subunits.

F. Developmental pattern of ALA synthetase isozymes

The developmental survey should be repeated to determine if it is possible to show an erythroid switch-over in isozyme type on the basis of AMP-Sepharose binding.

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