The University of Manitoba

Cycloheximide Enhancement of Hepatic Porphyrin in Chick Embryos With Induced Levels of δ-Aminolevulinic Acid Synthetase, Mediated by a Raised Glycine Pool

by

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A Thesis submitted to the Faculty of Graduate Studies In Partial Fulfillment of the Requirements for the Degree of Master of Science

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Winnipeg, Manitoba

October, 1972.

ACKNOWLEDGEMENTS

The author wishes to express his indebtedness and thanks to Dr. L.G. Israels for his advice, intellectual stimulation and humour during this project. They have helped make the past two years a rewarding experience. Special thanks are extended to Dr. B. Yoda, who aided in the solving of several problems, and Miss Jacqueline Anderson for her technical assistance.

ABSTRACT

Small doses of cycloheximide, given to fifteen day old chick embryos, one hour before an injection of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), a potent δ-aminolevulinic acid synthetase inducer, caused a two fold increase in the hepatic porphyrin within five hours, as compared with embryos given only DDC. A corresponding increase in the level of δ-aminolevulinic acid synthetase was not present. The conversion of exogenous δ-aminolevulinic acid into porphyrin was not enhanced by cycloheximide. Glycine loading of chick embryos given DDC raised hepatic porphyrin levels much above those given DDC alone, and no further increase accompanied the addition of cycloheximide at the time of glycine loading. Evidence indicates that low levels of cycloheximide produce an increase in the glycine pool available to δ-aminolevulinic acid synthetase, thereby causing an increased production of δ-aminolevulinic acid, which is quickly converted to porphyrin.

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INTRODUCTION

HEME BIOSYNTHESIS

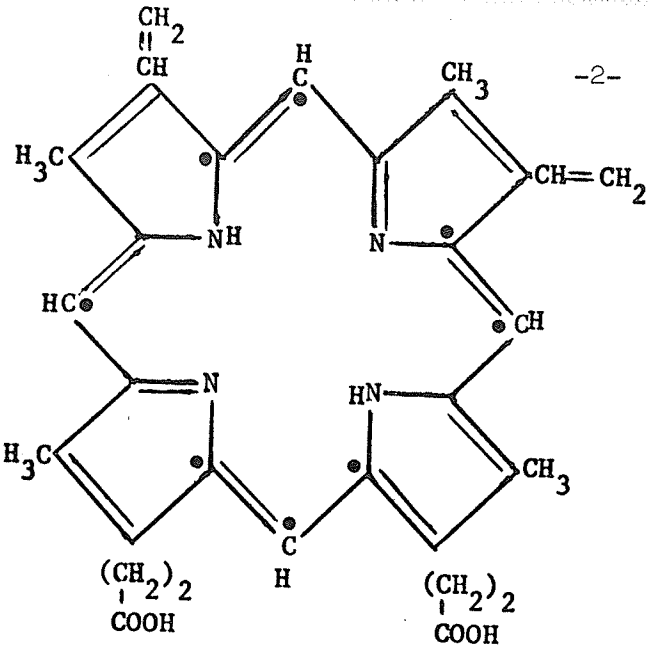
Little was known of porphyrin and hemoglobin biosynthesis prior to 1945. In that year Shemin and Rittenberg (1,2) showed that ^{15}N labelled glycine was rapidly incorporated into the heme of hemoglobin, in man and other animals.

Later studies, from various laboratories, showed that blood from ducks and chickens (3,4) which contain nucleated erythrocytes, or blood from anemic rabbits (5) containing an increased number of reticulocytes were able to synthesize labelled heme from glycine ^{-14}C *in vitro*. These systems revealed that all four nitrogen atoms (6) and eight carbon atoms of protoporphyrin (7) were derived from glycine. The carboxyl carbon was not utilized in heme biosynthesis (8), while the remaining 26 carbon atoms arose from citric acid cycle intermediates (9).

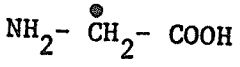
From these results it was deduced that the synthesis of porphyrin required 8 moles of glycine and 8 moles of a four carbon intermediate of the citric acid cycle, presumably succinate (10). Since the carbon of glycine is always utilized equally for both the pyrrole ring and the methene bridge carbon atoms (Fig. 1), the possible ways succinate and glycine could combine was limited.

These findings led Shemin and others to suggest δ-amino levulinic acid (ALA) as the product of condensing one molecule of glycine and one of succinate (10,11) and that it is an intermediate in the biosynthesis of heme. This has now been firmly established by experiments using radio-active precursors (12,13,18).

The principal pathway for the utilization of ALA is the formation of porphobilinogen (PBG), a heme precursor (14). However, it has been demonstrated ($\Delta^{-14}C$)-ALA can label formate, uric acid, and the ureido group of guanine, as well as



PROTOPORPHYRIN 9



GLYCINE

Figure 1. C^{-14} Labelling pattern in protoporphyrin 9, following the ingestion of glycine 2-C^{-14}.

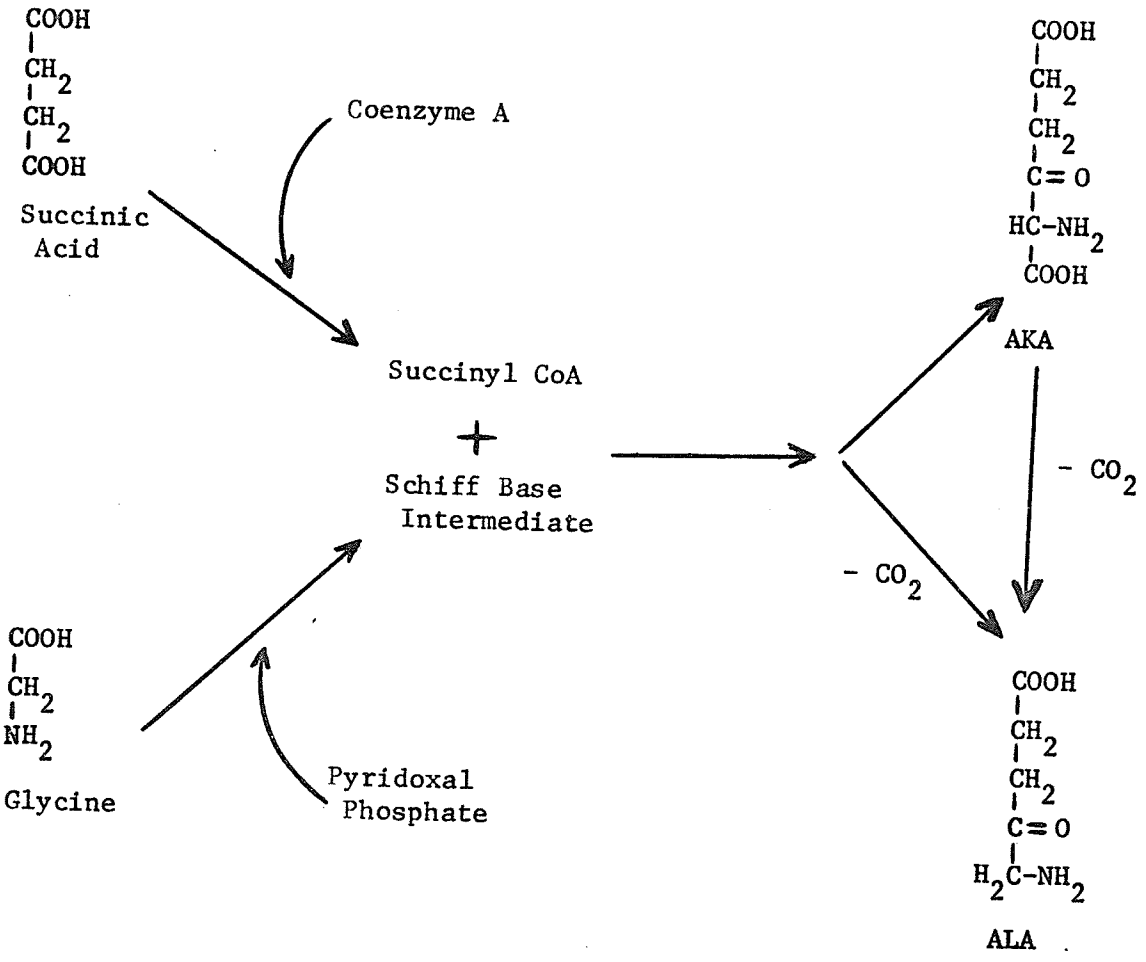


Figure 2. The condensation of succinate with glycine in the presence of coenzyme A, pyridoxal phosphate, and ALA synthetase to form ALA.

PBG and porphyrin (15,16). This so-called succinate-glycine cycle has been shown to be quantitatively unimportant in mammals.

ALA SYNTHESIS:

Condensation of glycine with succinyl coenzyme A to form ALA has been demonstrated in nucleated avian red blood cells, mammalian reticulocytes, liver and kidney (17,18), but not in mature mammalian erythrocytes (5). In the mammalian cell, ALA is formed only within the mitochondria (20), since this organelle is uniquely endowed with the capacity to generate succinyl-CoA and contains the major fraction of intracellular ALA synthetase (21). The activity of ALA synthetase is regulated. This regulation plays a critical role in the determination of intracellular heme levels. Aspects of this control will be discussed later.

Pyridoxal phosphate enhances the production of ALA and porphyrin *in vitro*, while partially purified ALA synthetase shows an absolute dependence on the vitamin for activity. The condensation of glycine with succinyl-CoA requires pyridoxal phosphate, which is likely bound to the enzyme proper *in vivo* (22).

Studies by Gibson et al (23), Kikuchi et al (24), Granick (25), and others have conclusively shown that succinyl-CoA is indeed the intermediate that condenses with glycine, as originally suggested by Shemin (9). Succinyl-CoA., a member of the citric acid cycle, can be produced from either α-ketoglutarate or succinate (23,24).

The primary product of condensing glycine with succinyl-CoA was expected to be α-amino-β-ketoadipic acid (AKA) (15). It has been suggested that decarboxylation of the pyridoxal phosphate derivative of glycine and condensation with succinyl-CoA may be simultaneous reactions, since the labile intermediate, AKA, has never been identified (Fig. 2)(24).

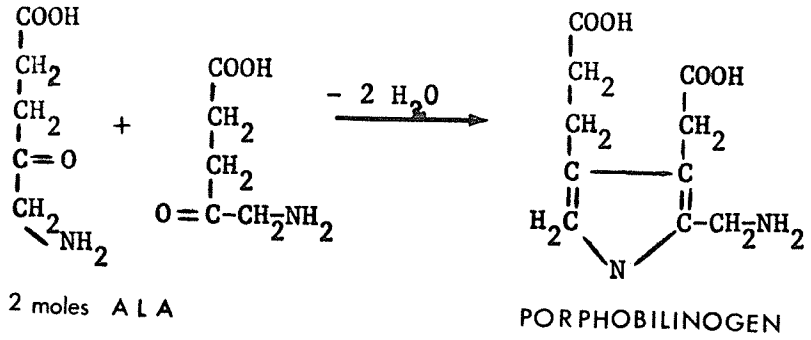


Figure 3. The synthesis of porphobilinogen (PBG), catalyzed by ALA dehydratase.

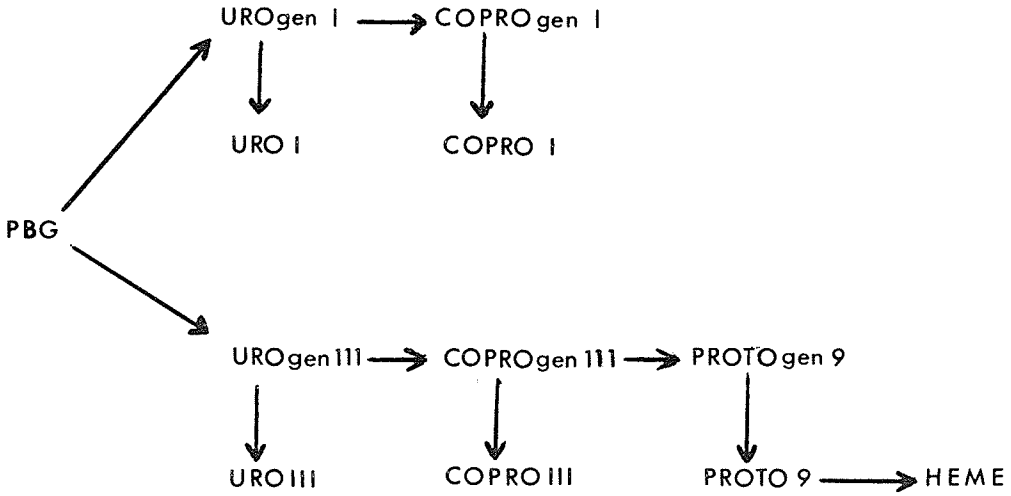


Figure 4. Scheme of heme biosynthesis from porphobilinogen (PBG). Uroporphyrin III, coproporphyrin III, the porphyrins and porphyrininogens of the type I isomer are side products, not utilized for heme biosynthesis.

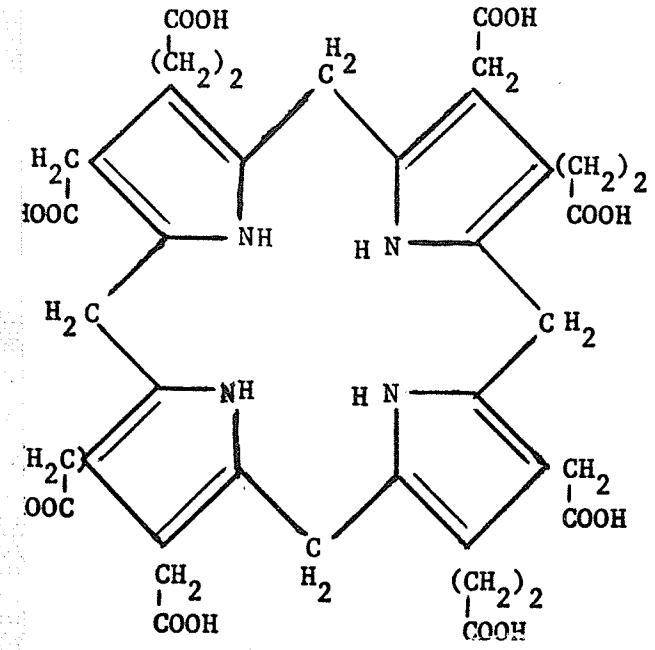
In summary: The biosynthesis of ALA involves succinyl-CoA, generated by the tricarboxylic acid cycle, and glycine. ALA synthetase, primarily an intramitochondrial enzyme, requiring pyridoxal phosphate, catalyses the production of ALA. The lack of mitochondria in mature mammalian erythrocytes precludes formation of succinyl-CoA and therefore ALA.

PBG SYNTHESIS: With ALA established as an intermediate in heme biosynthesis, studies on subsequent enzymatic steps were greatly facilitated. The condensation of two molecules of ALA, forming the pyrrole porphobilinogen (PBG), has been shown to be catalyzed by ALA dehydratase, a cytoplasmic enzyme (26) (Fig. 3).

The enzyme has been purified from bacteria (27), mammals (28), and plants (29). It requires glutathione for activation (27,29). Marked inhibition by ethylenediaminetetraacetic acid (EDTA) has been noted (30), but fully active preparations have been prepared in the absence of copper or iron (31, 32). The enzyme is allosterically activated by potassium (27), and inhibited by heme, suggesting ALA dehydratase, like ALA synthetase, may have some regulatory role in heme biosynthesis.

Heme has been synthesized from labelled PBG (33), confirming the earlier reports that PBG was a specific precursor (34,35).

PORPHYRIN SYNTHESIS: Early studies assumed that condensation of four PBG units would lead to the formation of the next intermediate, uroporphyrin. Stepwise decarboxylation was thought to then convert uroporphyrin into protoporphyrin. However, a number of systems, capable of converting PBG into protoporphyrin, were unable to utilize uroporphyrin (36). The true intermediate was then suggested to be uroporphyrinogen, the reduced, colorless form of uroporphyrin (37). Subsequent work (29,38) clearly showed the oxidized, fluorescent porphyrins to be by-products, resulting from irreversible oxidation of their respective



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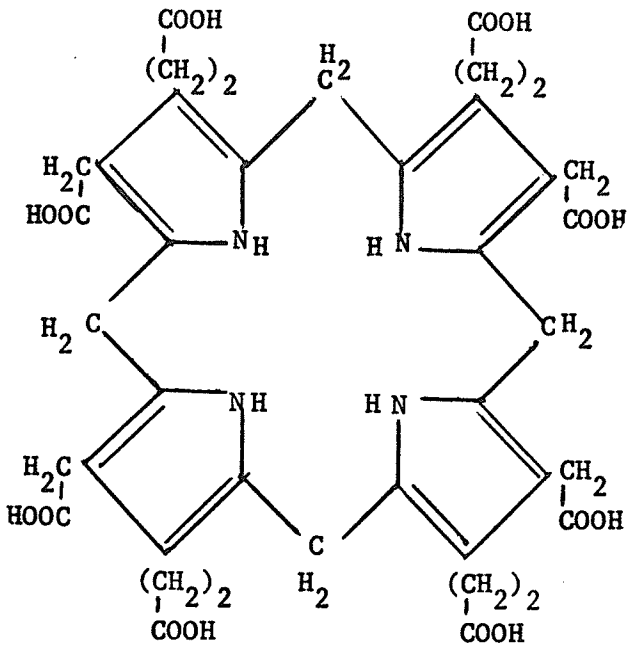

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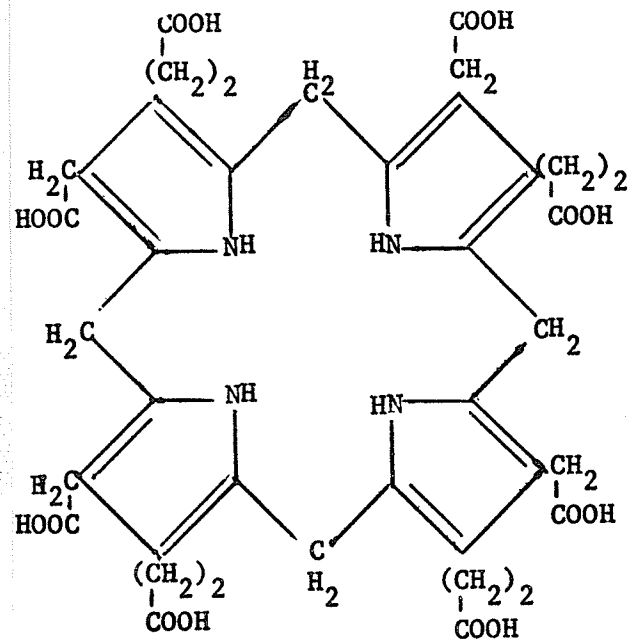

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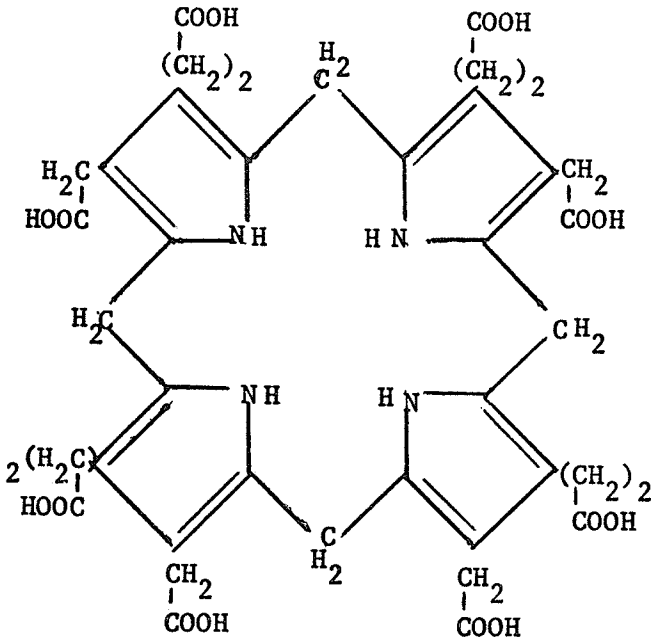


IV

Figure 5. The four isomeric forms of uroporphyrinogen. Only types I and III have been identified in nature, and only the type III isomer is processed into heme.

porphyrinogens (Fig. 4).

Four isomers of uroporphyrinogen are structurally possible (Fig. 5). In nature, however, only the type I and type III are found. Type I is not on the path to heme synthesis and is excreted in the urine, usually in trace amounts, while protoporphyrin 9 and heme are derived from uroporphyrinogen III.

Incubation of PBG with enzyme systems derived from bacteria (39), red cell hemolyzates (30) or mouse spleen (41) result in uroporphyrin III production. If the system is heated to 60^0C, prior to incubation, only the type I isomer is synthesized. Two separate enzyme fractions, uroporphyrinogen I synthetase, which is relatively heat stable, and uroporphyrinogen III cosynthetase, which is heat labile (42,43), have been separated. Uroporphyrinogen I synthetase alone will remove the amino group of PBG and condense the individual monopyrroles into the symmetrical uroporphyrinogen I (43). The addition of uroporphyrinogen III cosynthetase to the system will produce uroporphyrinogen III (44). By itself, uroporphyrinogen III cosynthetase reacts with neither PBG nor uroporphyrinogen I (44,45). It seems clear the cosynthetase permits isomerization of an intermediate in the conversion of PBG to uroporphyrinogen I, producing the type III isomer (46).

Uroporphyrinogen III has two possible fates: it can be irreversibly oxidized to uroporphyrin III, or enzymatically decarboxylated to porphyrinogens with less than eight carboxyl groups (37). Uroporphyrin III is a side product and not a substrate for uroporphyrinogen decarboxylase. The amount of uroporphyrin excreted in the urine is normally very small, indicating oxidation is not a significant reaction, under normal circumstances.

Enzyme preparations of uroporphyrinogen decarboxylase, under anaerobic conditions, catalyze the formation of coproporphyrinogen III. ALA, PBG, and uroporphyrin cannot replace uroporphyrinogen as substrate (45). Antioxidants such as glutathione and cysteine are of importance in keeping the tetrapyrroles

in a reduced state (44,45). The removal of the four acetic acid groups is random, giving low concentrations of intermediates with 7,6, and 5 carboxyl groups and finally coproporphyrinogen III with 4 such groups (Fig. 4), (47).

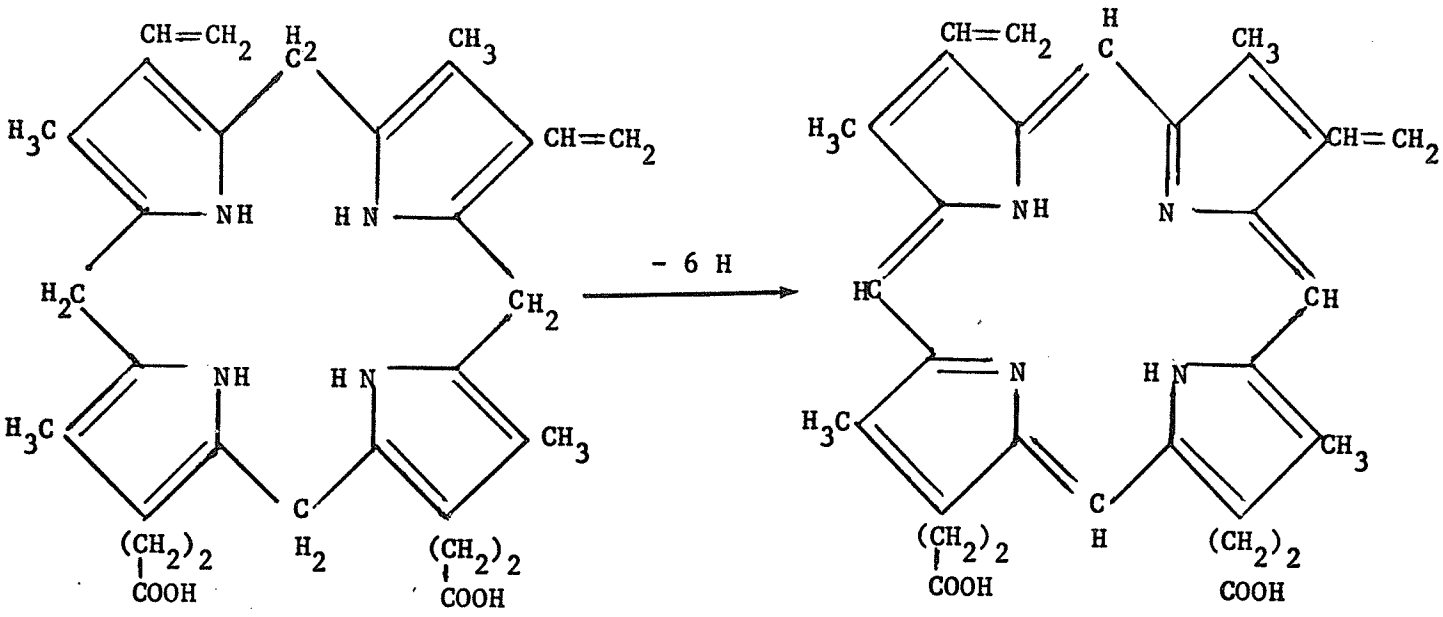
Protoporphyrin or heme corresponding to the type I isomer has never been demonstrated in nature (48), while coproporphyrinogen III is quickly converted into protoporphyrin 9. This enzymatic process involves decarboxylation and oxidation of the two propionate groups at positions 2 and 4, yielding vinyl groups. The resulting protoporphyrinogen 9 is then oxidized to protoporphyrin 9 by the removal of 6 hydrogens (Fig. 6), (49). The enzyme is found in the mitochondria, with highest activity in tissues exhibiting a rapid heme turnover (49). The oxidation most likely occurs simultaneously with the decarboxylation (49,50), but whether the oxidation is spontaneous in the presence of oxygen, or under enzymatic control is not known.

The incorporation of iron into the protoporphyrin ring has been accomplished nonenzymatically by chemical means (51). However, convincing evidence (52,53), indicates the <u>in vivo</u> incorporation of iron is done in the presence of the intramitochondrial enzyme, ferrochelatase, or heme synthetase. Activity depends on the presence of reducing substances such as glutathione, cysteine, or ascorbic acid (53). The enzyme is specific for porphyrin with free carboxyl groups on the propionic acid side chains in positions 6 and 7. Copro- and uroporphyrin are not utilized by the enzyme, possibly because of steric effects.

Most of this evidence for heme synthesis has been obtained from systems concerned with hemoglobin formation. Other heme proteins and related pigments are likely formed in a similar fashion (54,55).

<u>SUMMARY</u>

1. ALA and PBG are obligatory intermediates in the biosynthesis of porphyrins and heme.



PROTOPORPHYRINOGEN 9 PROTOPORPHYRIN 9

Figure 6. The conversion of reduced, colorless protoporphyrinogen 9 into the oxidized, fluorescent protoporphyrin 9. This reaction occurs in the mitochondria.

2. Pyridoxal phosphate and coenzyme A are essential for the condensation of succinate with glycine.

3. Three enzymatic steps require aerobic conditions: a) formation of succinyl-CoA, b) oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen 9, c) oxidation of protoporphyrinogen 9 to protoporphyrin 9.

4. The oxidative steps are catalysed by enzymes bound to the mitochondria and therefore absent in the mature, non-nucleated erythrocyte.

5. The steps from ALA to coproporphyrinogen are catalysed by cytoplasmic enzymes.

6. Porphyrinogens and porphyrins of the type I isomer have no physiological role, and are normally produced in insignificant amounts.

7. Porphyrins of the type III isomer, with the exception of protoporphyrin 9, are not intermediates in the synthesis of heme.

MECHANISM OF HEME REGULATION

Regulation of a sequence of metabolic events leading from precursor to an end product several stages removed can be a function of several factors:

1. The activity and quantity of the individual enzymes in the sequence are important, particularly the rate controlling enzyme (ALA synthetase in the case of heme biosynthesis (64,66)).

2. The ability of the system to control the concentration of the initial substrates and subsequent intermediates.

3. The availability of alternate pathways for the utilization of these intermediates.

Enzymatic activity can be influenced in several ways:

1. Modification of pre-existing protein without changing the quantity of enzyme present (allosteric changes). "Inhibition" or "activation" implies an effect on the preformed enzyme.
2. Changes in the rate of enzyme degradation, causing either an increase or decrease in the net amount of enzyme present.

   Both 1 and 2 provide mechanisms for altered enzyme function, without changing the rate of protein synthesis.
3. Changes in the rate of enzyme production. "Induction" or "repression" implies a modification in the rate of protein synthesis. Regulation of protein synthesis may involve control at any level from transcription in the nucleus to translation on the ribosomes.

Heme exhibits negative feedback inhibition on three of its enzymes: ALA synthetase, ALA dehydratase and ferrochelatase (56,57). Reversibility of

the inhibition has been noted, as would be expected with an allosteric effect.

Neither pyridoxal phosphate nor the various cations that have been recently shown to activate ALA synthetase in vitro (71), appear to play a regulatory role in the production of ALA in vivo.

The activity of ALA dehydratase and ferrochelatase in erythrocytes, are both inhibited by lead (61). Lead is presumed to combine with sulfhydryl groups essential for enzymatic activity. Manifestations of these blocks can be seen in cases of chronic lead poisoning.

Control of enzyme synthesis provides a more "economical" and efficient control. Jacob and Monod (60), on the basis of bacterial studies, proposed the last molecule in a biosynthetic sequence could react with an apo-repressor forming a repressor. This repressor would be capable of impeding the production of the messenger RNA responsible for certain enzymes in the metabolic pathway. It is not yet certain that this system is operable in animal cells.

Heme repression has been demonstrated in some laboratories; i.e. heme has been able to interfere with the chemical induction of ALA synthetase (61,62). ALA synthetase has a short half life (approximately 70 minutes in the rat liver system (72)) providing a sensitive control mechanism. Indirect data has been interpreted to indicate heme regulation of ALA synthetase at the level of translation (73).

Tschudy and other workers (70,21) have reported that the ingestion of carbohydrate or protein reduces the excretion of porphyrin precursors in chemically induced porphyria and acute intermittent porphyria. The level of ALA synthetase under this "glucose effect" is measurably lower, but the mechanism remains unexplained.

Following the administration of certain drugs, animals will exhibit

increased hepatic porphyrin levels. Granick and Urata demonstrated that induction of ALA synthetase was the cause of this increase (63). A number of laboratories have extended this work (64,65). A method of quantitating the induction of ALA synthetase by determining the porphyrin fluorescence in primary avian hepatocyte cultures was later introduced by Granick (66). Representative inducers are illustrated in figure 7. These drugs do not cause increased levels of ALA synthetase by a common mechanism.

Phenobarbital induces cytochrome P_{450} as part of an adaptive response, enhancing the livers capacity to detoxify phenobarbital and many other chemicals (67). The demand P_{450} makes for heme is postulated to induce high levels of ALA synthetase.

The mechanism by which allylisopropylacetamide (AIA) and similar compounds works has not been fully explained. An increased rate of cytochrome P_{450} turnover is a common denominator. The drain on heme may tend to inhibit its role as a repressor, thereby increasing the induction of ALA synthetase (68). Sassa provides some evidence that AIA acts primarily at the level of translation (73).

A mechanism for the inductive effect of 3,5-diethoxycarbonyl-1,4-dihyro-2,4,6-trimethylpyridine (DDC) on ALA synthetase may involve the inhibition of the enzyme, ferrochelatase, thus preventing the synthesis of heme (69). The lowered heme levels would then derepress ALA synthetase. The magnitude of this mechanism is thought to be insufficient to account for the potent inducing capacity of this agent. Other work indicates DDC acts at the level of transcription (73).

Another important group of recently described inducers (66), are some of the reduced steroids. Evidence has been presented showing the primary effect to be induction at the level of transcription (73). Unlike other active chemicals, steroids that induce ALA synthetase in avian liver also stimulate heme biosynthesis in mammalian marrow. (Fig. 8)

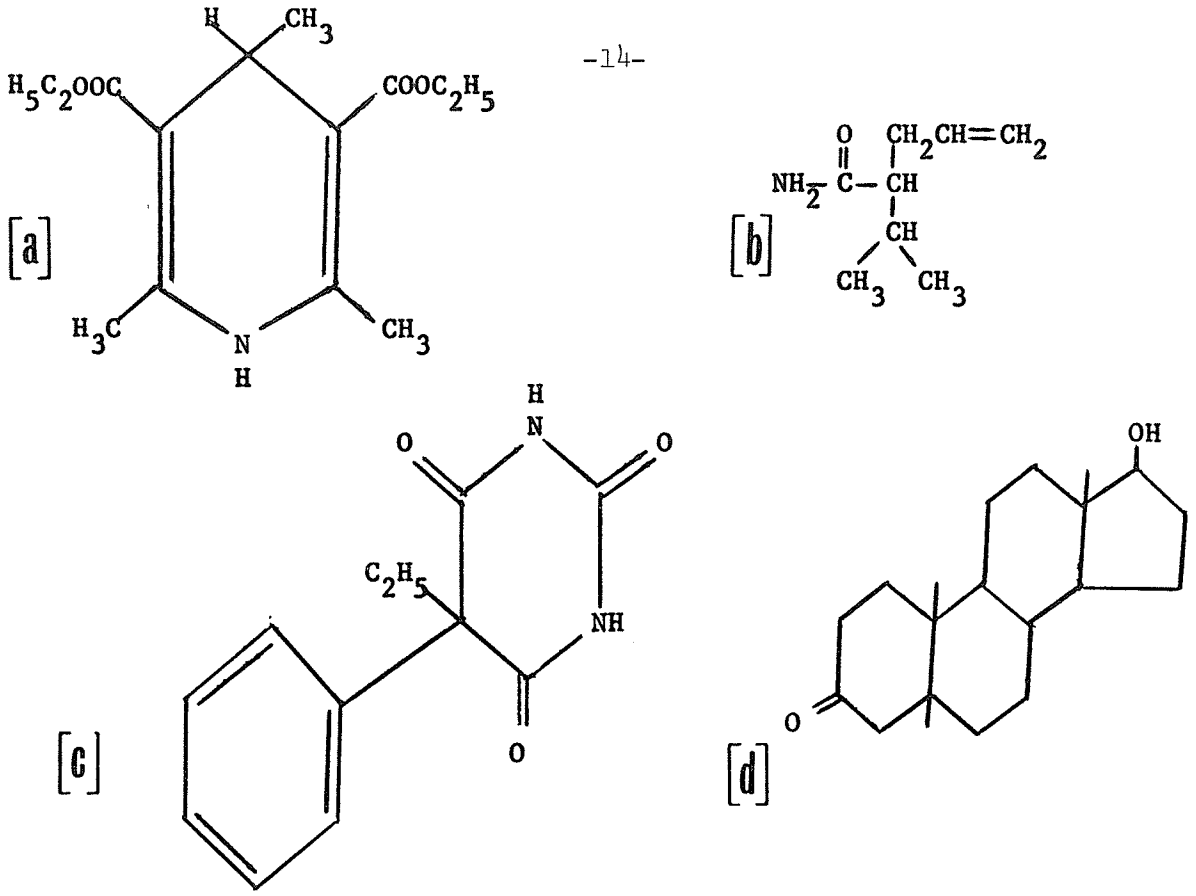


Figure 7. Structures of several representative compounds known to induce hepatic ALA synthetase. a) 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) b) Allyl-isopropylacetamide (AIA) c) Phenobarbital d) Etiocholanalone-17β

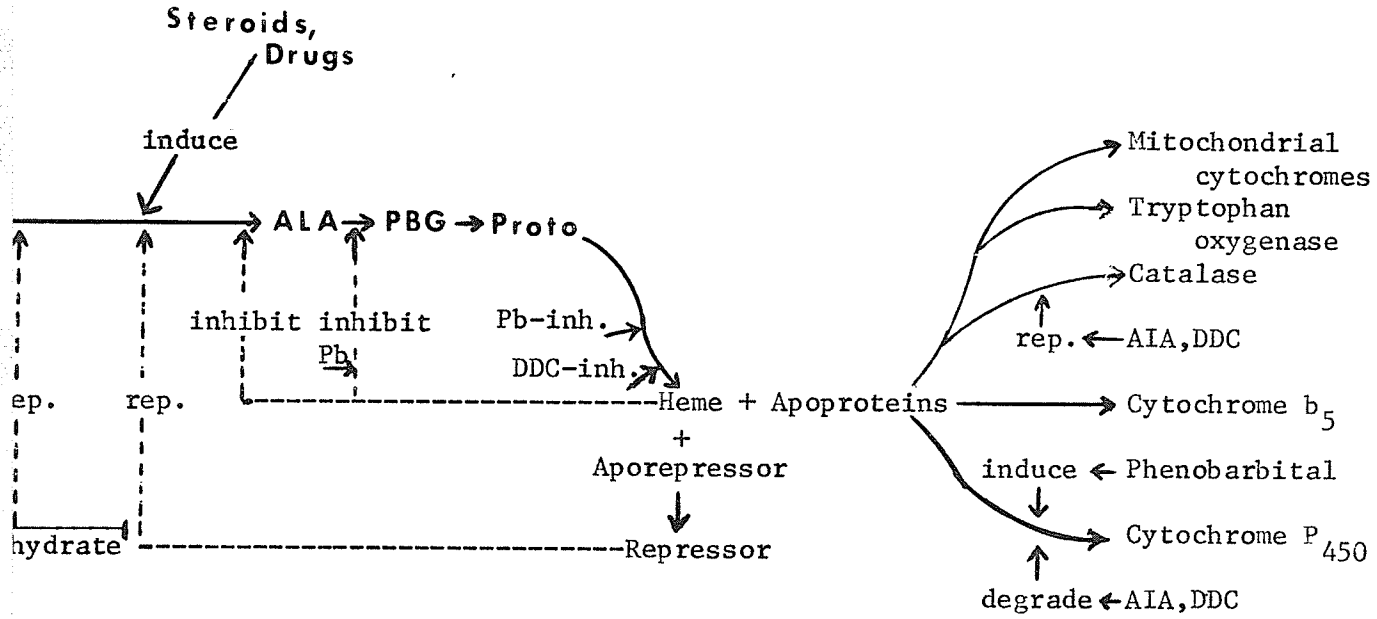


Figure 8. Summary of the specific sites and mechanisms known to influence hepatic heme and hemoprotein biosynthesis.

ALA synthetase appears to be rate limiting in heme biosynthesis (64,66), however, recent work has shown the enzyme to have a relatively large glycine Michaelis constant (14). The implication is that porphyrin biosynthesis might be modified by the intracellular availability of glycine. Under ordinary circumstances, an increase in the amount of glycine would not be expected to augment the rate of ALA production sufficiently to account for the symptoms of experimental or genetic porphyria. In general, the overall velocity of a multi-enzyme system is limited by the least active step (74). If the substrate concentrations are not saturating, then the velocity will be regulated by both substrate and enzyme concentrations.

Small doses of cycloheximide were observed to cause a two-fold increase in the hepatic porphyrin of 15 day chick embryos treated with DDC, a potent ALA synthetase inducer, as compared to controls given only the inducing drug.

Evidence presented in this thesis indicates the primary effect of the low level of cycloheximide was to raise the intracellular glycine pool, thereby causing an increased production of ALA, which was quickly converted to porphyrin.

METHODS AND MATERIALS

1. Chick Embryos

Fifteen day chick embryos of the Commercial White Leghorn hybrid stock, second generation, were used unless specified otherwise. White Leghorn, Shaver strain, were used in some experiments when the original hybrid strain was not available. Both strains were obtained from the Poultry Department of the University of Manitoba.

2. Chemicals

Coproporphyrin standard was purchased from Harleco. Cycloheximide and δ-aminolevulinic acid were obtained from Nutritional Biochemicals Corporation, while 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) was a gift from Professor G. S. Marks (Queens University, Kingston).

3. Injection Technique

The method used was a modification of that of Racz and Marks (75), and Granick (66). Sterile technique was used during the administration of all chemicals. The drugs, with the exception of DDC, were prepared in normal saline and Millipore filtered (porosity 0.45μ) prior to injection. DDC was dissolved in 0.1 ml of dimethylsulfoxide (DMSO), because of its solubilizing properties and the apparent nontoxicity of DMSO in low concentrations (75).

A sterile tuberculin syringe with 1¼ inch, 21-gauge disposable needle was used for injection. A small hole was made in the egg shell above the air sac and the drugs introduced through the chorioallantois into the embryonic fluids. δ-Aminolevulinic acid (ALA), when used, was placed on the surface of the air sac. The opening in the shell was covered with cellophane tape and the embryo incubated at 38°C in an upright position.

4. Assay for δ-Aminolevulinic Acid Synthetase Activity

The method of Hayashi et al (76) was used, modified to use E. coli, Crooke's strain, as a source of succinyl-CoA synthetase (E.C. 6.2.1.5) instead of R. spheroides (77). ALA is converted into a pyrrole by condensing with acetylacetone in a boiling water bath. It is then separated from other pyrroles by ion exchange chromatography. The ALA monopyrrole then is allowed to react with p-dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent) to produce a red compound that can be quantitated at 553 mμ. The method is specific for ALA (76,19).

Livers were removed from 15 day chick embryos and homogenized in three volumes of 0.25 M sucrose containing 0.1 mM EDTA[1], 0.1 mM PALP[2], and 20 mM Tris-HCl (pH 7.6). The homogenate, containing 5 - 8 mg protein as determined by the Biuret method (78), was used for the δ-aminolevulinic acid synthetase assay.

The incubation mixture per determination was as follows:

Tris-HCl (pH 8.0)	100 μ moles
ATP	20.0 μ moles
Coenzyme A	0.2 μ moles
PALP	0.2 μ moles
EDTA	8.0 μ moles
$MgCl_2$	10.0 μ moles
β-Mercaptoethanol	10.0 μ moles
Succinyl-CoA synthetase	Catalysing 3 μ moles succinyl-CoA/hr (79)
Succinate	20.0 μ moles
Glycine*	200 μ moles

* The incomplete incubation mix, when used, did not contain glycine.

1) ethylenediaminetetraacetic acid
2) pyridoxal-5'-phosphate

note: substrate concentrations are saturating for ALA synthetase. The final volume of each determination was adjusted to 2 ml with water.

The homogenate and incubation mixture were normally kept separate at 4°C until the initiation of incubation, at which time they were combined. When the glycine Michaelis constant was determined for ALA synthetase, the method was modified. Glycine concentrates were made up in double distilled water, while the homogenate was added to the incomplete incubation mix and held at 4°C. Incubation was initiated when the two were combined.

The homogenate and incubation mixture were incubated in 14 ml test tubes in a Dubnoff Metabolic Shaking Incubator at 37°C for 30 minutes. The reaction was terminated by the addition of 0.5 ml of 25% TCA. As a control to measure any interference, 0.5 ml of TCA was added to one sample prior to incubation. Following centrifugation at 3,000 rpm for 10 minutes, the supernatant was decanted.

Two ml of the supernatant was added to a solution of 0.05 ml of acetylacetone in 2.0 ml of 1 M acetate buffer (pH 4.6). Following 10 minutes in a 100°C water bath, the mixture was cooled and applied to a Dowex 1x2-400, 200-400 dry mesh, acetate form, ion exchange column (0.8 cm x 2.5 cm). The tubes were then washed with 4 ml of water and the contents added to their respective columns. Washing with 4 ml of 50% methanol was followed by 2 ml of 1 M acetic acid. ALA was eluted with 2.5 ml of glacial acetic acid.

Ehrlich-Hg reagent (2.5 ml) was added and the solution agitated. After a 10 minute development period at room temperature, the optical density was read at 553 mμ, using equal parts of glacial acetic acid and Ehrlich-Hg reagent as a blank.

The initial velocity of ALA synthetase was calculated according to the method of Hayashi et al (76). Extinction coefficient (ϵ mM) = 53

Velocity = mμ moles ALA produced/ hour/ mg protein

$$= \frac{\text{Optical Density}}{53} \times 5 \text{ ml sample} \times 10^{-3} \text{ mμ moles} \times \frac{60 \text{ min}}{30 \text{ min}} \times \frac{1}{\text{mg protein}}$$

Results express: mean ± standard error, as calculated with an Olivetti Underwood 101 computer, using a program outlined by Cochrane and Cox (82).

The K_m for glycine was calculated as follows:

The Lineweaver-Burk reciprocal equation was used (74).

$$\frac{1}{v} = \frac{K_m}{V_{max} s} + \frac{1}{V_{max}}$$ which has the form of a straight line with:

$\frac{1}{V_{max}}$ = y intercept, $\frac{K_m}{V_{max}}$ = slope of line

v = velocity of reaction (mμ moles ALA/hr/mg protein)

V_{max} = maximum velocity

s = concentration of glycine (mM)

K_m = Michaelis constant for glycine

The y intercept and slope were calculated with a Linear Regression program as outlined in the Olivetti Underwood Programma 101 (statistical analysis), page 65, using an Olivetti Underwood 101 computer.

Results express: mean ± standard error, as given by the program.

5. Procedure for Extraction and Separation of Porphyrin

The method used was a modification of Schwartz et al (80). A Turner model 110 fluorometer fitted with a 405 mμ band pass primary filter and a Wratten No. 25 (595 mμ) sharp cut secondary filter was employed for all fluorometric determinations.

A standard curve (Fig. 9) was constructed by using appropriate dilutions of a coproporphyrin standard. The standard was made up with concentrated HCl to 3 N. Since protoporphyrin and uroporphyrin exhibit different fluor-

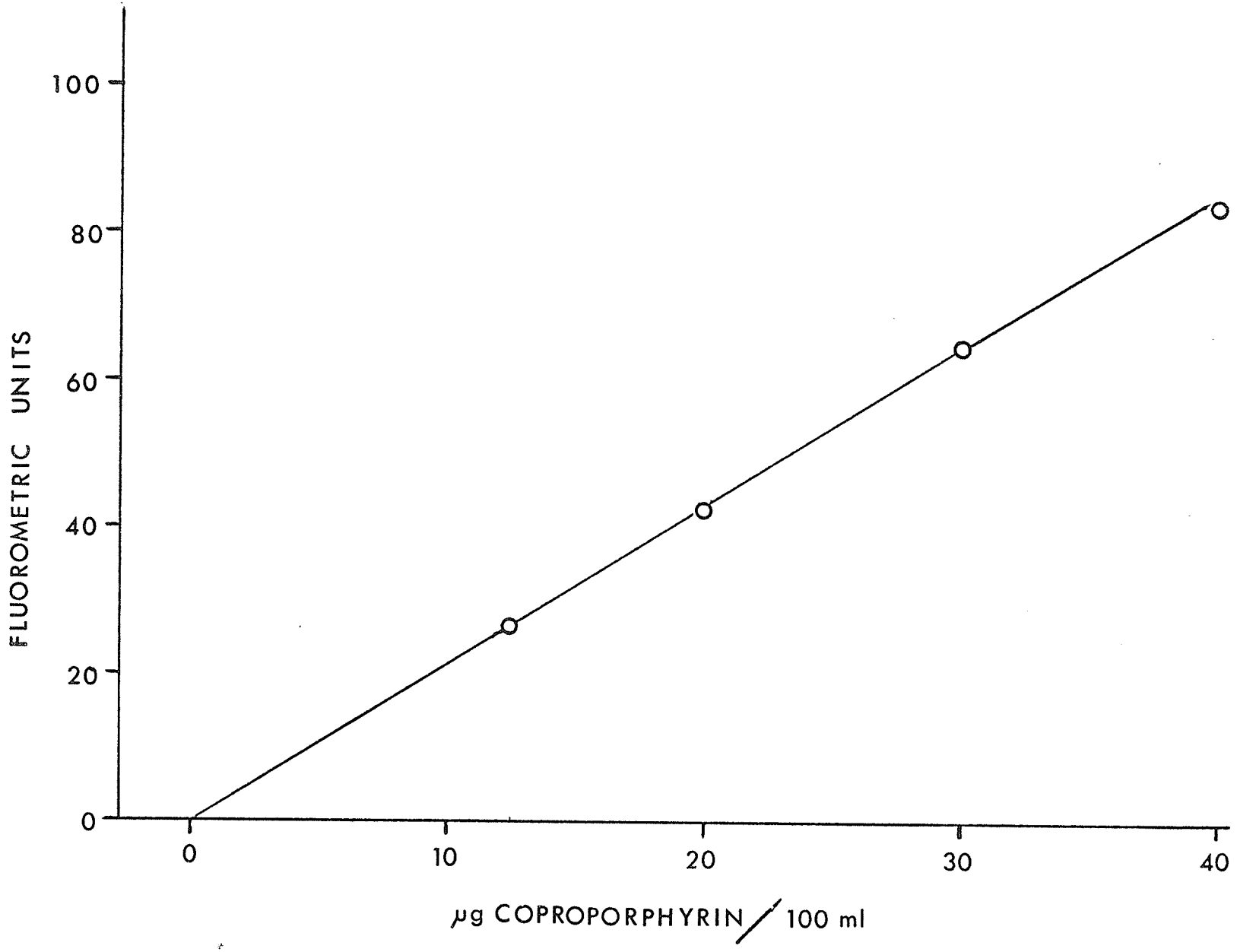


Figure 9. Standard curve for the determination of coproporphyrin by fluorophotometry, constructed by using appropriate dilutions of a coproporphyrin standard, purchased from Harleco.

escent intensities, correction factors as outlined by Schwartz et al (80) were used when these compounds were measured.

Total porphyrin extraction into ethyl acetate: acetic acid (4:1) was done as follows:

The livers (approximately 0.3 g) were weighed and then homogenized in a Potter-Elvehjem homogenizer with 5 ml of ethyl acetate: acetic acid (4:1). The homogenate was spun at 2,500 rpm for three minutes. The supernatant was decanted into a separatory funnel and the precipitate resuspended in 5 ml of the ethyl acetate: acetic acid solution. Following centrifugation as before, the supernatant was added to the first and the extraction repeated a third time.

The ethyl acetate: acetic acid mixture was then shaken with 10 ml of 3% sodium acetate containing one drop of 0.1% iodine (in ethanol). The lower aqueous phase was removed and saved. The organic phase was washed once with 3% sodium acetate (10 ml) and the washings added to the first aqueous portion.

Uroporphyrin: The sodium acetate washes were filtered through a column of aluminum oxide (prepared with 3% acetic acid) to adsorb the porphyrin. The column was next washed with 3% acetic acid until the eluate showed no trace of fluorescence. The uroporphyrin was then extracted with 1.5 N HCl, and estimated fluorometrically by comparison with the coproporphyrin standard (correction factor 0.75).

Coproporphyrin: The washed ethyl acetate: acetic acid layer was extracted with 5 ml portions of 3 N HCl until the eluate showed no visible fluorescence under a long wave ultra-violet light. The HCl extract was neutalized to congo red with saturated sodium acetate, and extracted into ethyl acetate (3 extractions of 30 ml each). The combined ethyl acetate extracts were washed with water (discard) and extracted exhaustively with 5 ml portions of 0.1 N HCl to remove the coproporphyrin.

The 0.1 N HCl solution was washed twice with 5 ml portions of chloroform to remove traces of chloroform soluble porphyrins. After allowing the last chloroform extract to settle completly, 1/15 volume of 3 N HCl was added to the aqueous HCl solution to increase its HCl concentration to approximately 0.3 N and to clear the solution of opalescence. The coproporphyrin concentration was determined fluorometrically.

Protoporphyrin: The ethyl acetate fraction left after the 0.1 N HCl extractions were now extracted exhaustively with 5 ml aliquots of 1.5 N HCl. The protoporphyrin concentration was determined by fluorometer, using the coproporphyrin standard (correction factor 1.25).

In the DDC induced chick embryo system, 99% of the extracted porphyrin was found to be protoporphyrin (Table 4). Routine extractions, as described in the next section, were therefore used that remove only the coproporphyrin and protoporphyrin.

6. Assay of Chick Embryo Liver for Porphyrin

The method used was that of Racz and Marks (75). The livers were removed, weighed, and a total porphyrin extraction performed, using three volumes of 5 ml ethyl acetate: acetic acid (4:1), as explained in section 5.

Three percent sodium acetate solution (10 ml) and one drop of 0.1% iodine in ethanol were added to the ethyl acetate: acetic acid extracts in the separatory funnel. After thorough agitation, the lower aqueous phase was removed and discarded. The organic layer was then washed once with 3% sodium acetate (10 ml).

Coproporphyrin and protoporphyrin were removed from the organic phase with three extractions of 3 N HCl, in 5 ml portions. The completness of the extraction was confirmed by lack of fluorescence under a long wave ultra-violet source. If fluorescence was present, the extraction with 5 ml of 3 N HCl was repeated. The samples were diluted with 3 N HCl until they could be read in the

fluorometer.

Hepatic porphyrin concentration was calculated as follows:

$$\mu g \text{ porphyrin/g liver} = \mu g \text{ porphyrin (measured)} \times \frac{\text{total volume}}{100 \text{ ml}} \times \frac{1}{\text{g liver}}$$

Results express: mean ± standard error

7. Micro-Determination of Glycine

The method used was that of Alexander et al (81) and is based on the detection of formaldehyde, released specifically when glycine reacts with ninhydrin. The formaldehyde is then distilled over into a collection tube and identified by its reaction with chromotropic acid. One gram of glycine was dissolved in one liter of water, and then diluted to form a stock solution of 10 µg glycine/ml for use in the construction of a calibration curve(Fig. 10).

Sufficient stock solution was added to an all glass distillation apparatus to cover the range of concentrations from 5 µg to 40 µg glycine. Water was added to a final volume of 5 ml. Two ml of a pH 5.5 phosphate buffer (3.5 g of K_3PO_4 was added to 100 ml of a 20% solution of KH_2PO_4) and one ml of 1% ninhydrin was then added. Using a glass bead to prevent bumping, the solution was quickly distilled into a test tube calibrated to 10 ml. After about 7 ml of liquid was distilled, the flask was cooled to room temperature in a water bath, 2 ml of water added and distillation continued to dryness. Total time for distillation never exceeded 15 minutes. The distillate was made up to 10 ml with water. After mixing, 5 ml was pipetted into a test tube and 4 ml of concentrated H_2SO_4 was added slowly with cooling in an ice bath. When the solution attained room temperature, 0.1 ml of chromotropic acid solution (5%) was added, the mixture shaken, stoppered, and placed in a boiling water bath for 30 minutes. The absorbance of the cooled solution was read at 575 mµ. The blank determination substituted 1 ml of water for the ninhydrin. All determinations were done in triplicate.

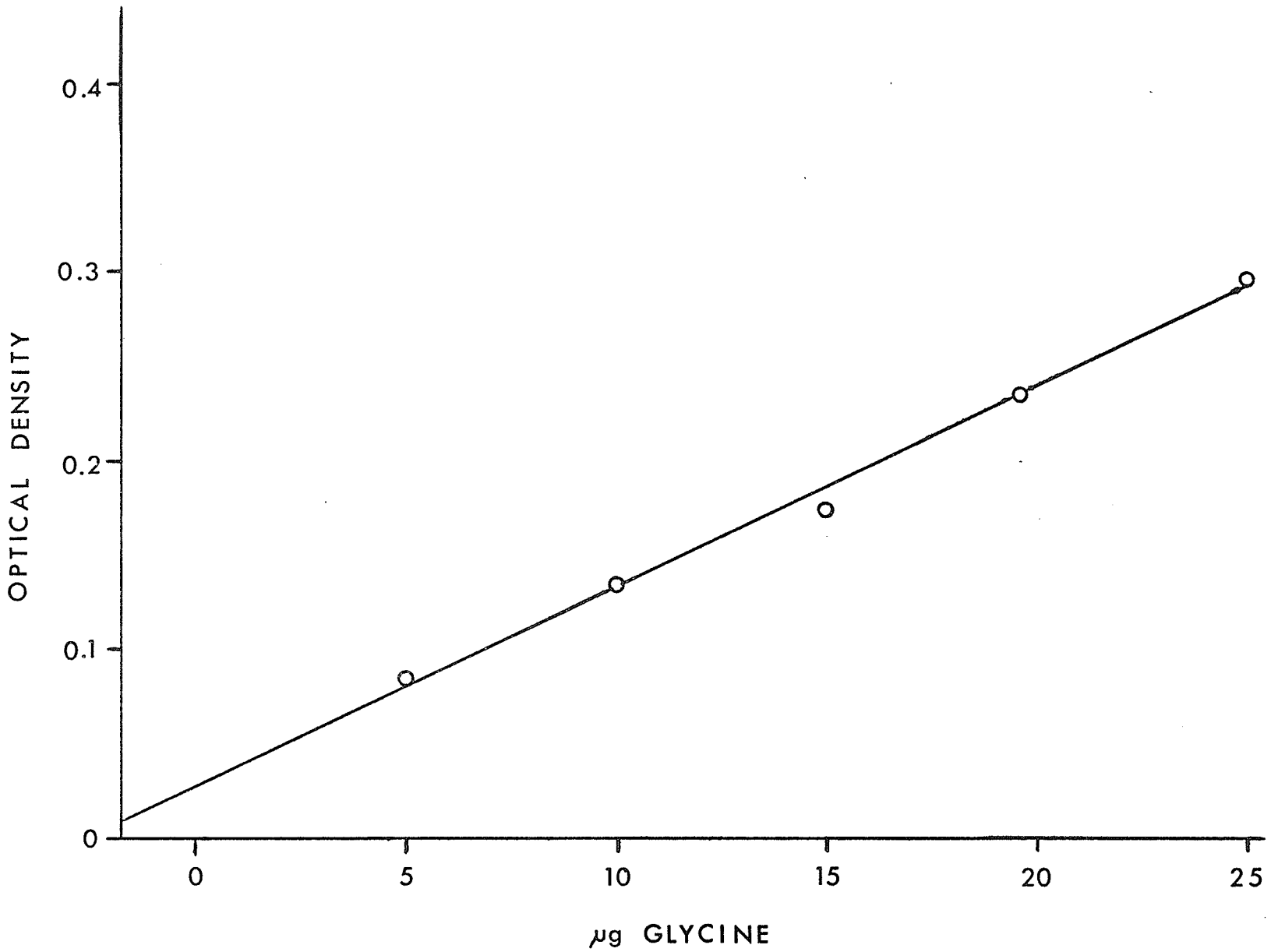


Figure 10. Standard curve for the micro-determination of glycine. The method is that of Alexander et al (81), and is based on the detection of formaldehyde, released when glycine reacts with ninhydrin.

The glycine concentration of embryonic liver was determined as follows:

Chick embryo livers were removed, weighed, and then homogenized with 5 ml of water. One ml of 25% TCA (1.52 M) was added to the homogenate to cause protein precipitation. Following centrifugation at 2,500 rpm for 10 minutes, the supernatant was removed and the pellet resuspended in 5 ml of water, centrifuged as before and the wash added to the first supernatant. The solution was neutralized with 1 ml of 1.52 M KOH, and the volume adjusted to 15 ml.

Five ml of the deproteinized solution was then added to an all glass distillation apparatus and treated in the same manner as the glycine solutions used in establishing the calibration curve.

Hepatic glycine concentration was calculated as follows:

$$\text{mM glycine} = \frac{\text{mg glycine}}{75} \times \frac{1}{\text{liver wt (Kg)} \times 0.7 \text{ (water fraction)}}$$

Results express: mean ± standard error

EXPERIMENTAL

1. Cycloheximide Enhancement of DDC Induced Hepatic Porphyrin Levels

Eggs containing 15 day chick embryos were injected with cycloheximide, 1 µg to 10 µg, one hour prior to an injection of 3 mg of DDC. The livers were removed five hours later and the porphyrin extracted and assayed as described. Other embryos were given only 3 mg of DDC and incubated for five hours.

Control embryos had a basal level of 1.81 ± 0.08 µg porphyrin/ g liver (Fig. 11, Table 1). Induction with 3 mg of DDC raised this to 29.6 ± 2.4 µg porphyrin/ g liver in five hours. Ten µg of cycloheximide effectively blocked the inducing action of DDC (3.2 ± 0.3 µg porphyrin/ g liver), while pretreatment with 3 µg of cycloheximide produced an enhanced level of 65.6 ± 9.6 µg porphyrin/ g liver. Three µg cycloheximide alone had no effect on the porphyrin content (1.75 ± 0.17 µg porphyrin/ g liver).

2. A Time Study of the Cycloheximide Effect on Porphyrin Induction by DDC

As 3 µg of cycloheximide was found to enhance the DDC induction of porphyrin at 5 hours, a study was undertaken to examine the effect of this dose of cycloheximide on DDC induction over a 12 hour period. Eggs containing 15 day chick embryos were injected with 3 µg of cycloheximide, followed in one hour with 3 mg of DDC. The eggs were then incubated at 38°C for times ranging from 3 - 12 hours. Following incubation, the livers were removed and the porphyrin extracted and assayed. These were compared with embryos injected with 3 mg of DDC alone.

Embryos given only DDC showed an exponential increase in porphyrin for the first 12 hours, reaching a mean of 189 ± 34 µg porphyrin/ g liver. The embryos treated with cycloheximide previous to induction with DDC reached a maximum value of 127 ± 25 µg porphyrin/ g liver at 8 hours, and then fell to

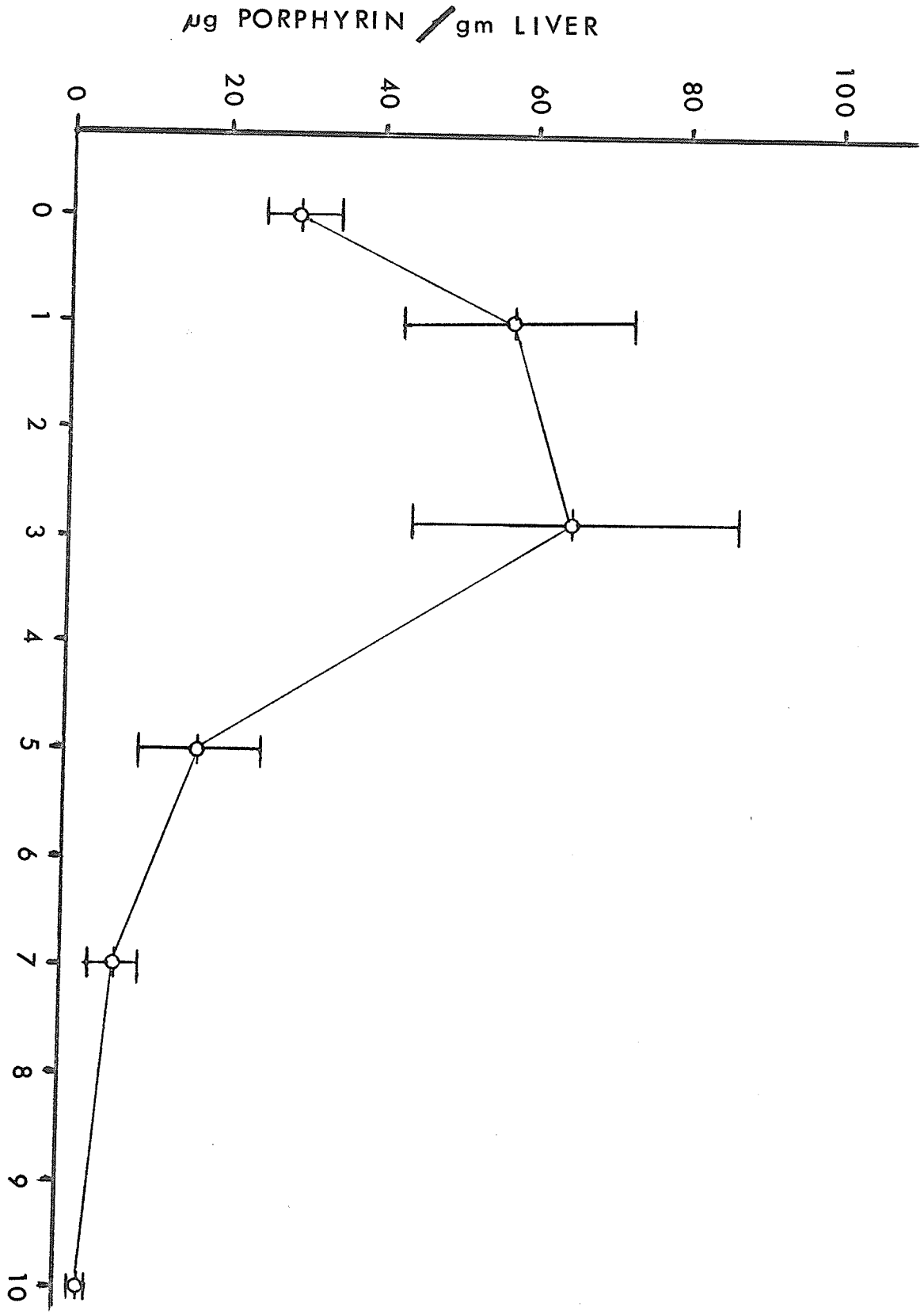


Figure 11. Hepatic porphyrin levels of 15 day chick embryos given variable doses of cycloheximide, followed in one hour with 3 mg of DDC, then sacrificed after 5 hours incubation at 38°C.

TABLE 1

Hepatic porphyrin levels of 15 day chick embryos, given variable doses of cycloheximide, followed in one hour with 3 mg of DDC, and sacrificed five hours later.

	Porphyrin Concentration (µg/g liver)		
	Mean	Standard Error*	No. of embryos
Drug free control	1.81	±0.08	16
3 mg DDC	29.6	±2.4	23
1 µg cycloheximide plus 3 mg DDC	57.8	±6.4	8
3 µg cycloheximide plus 3 mg DDC	65.6	±9.6	12
5 µg cycloheximide plus 3 mg DDC	17.5	±3.4	9
7 µg cycloheximide plus 3 mg DDC	6.9	±1.4	8
10 µg cycloheximide plus 3 mg DDC	3.2	±0.3	4
3 µg cycloheximide only	1.75	±0.17	10

67.8 ± 12.4 μg porphyrin/ g liver at 12 hours (Fig. 12, Table 2). Thus, cycloheximide caused only a transient enhancement of porphyrin production that was maximal at a concentration of 3 μg and a time of 5 - 6 hours.

3. Cycloheximide Effect on the DDC Induced Level of δ-Aminolevulinic Acid Synthetase

Granick and other workers have often used hepatic porphyrin levels as an indirect measure of ALA synthetase activity (66,67), since the enzyme is considered to be rate limiting. As 3 μg of cycloheximide had produced a two-fold increase in the hepatic porphyrin content of "DDC-induced"* chick embryos at 5 hours, it was next decided to examine the effect of cycloheximide on ALA synthetase activity.

Eggs containing 15 day chick embryos were injected with cycloheximide (0 - 10 μg), one hour prior to receiving 3 mg of DDC. The eggs were incubated at 38°C for five hours, following which the livers were removed. ALA synthetase activity was assayed according to the method of Hayashi et al (76).

The livers of the embryos given only DDC showed maximal activity of 5.78 ± 0.89 mμ moles ALA/ hr/ mg protein. Chick embryos pretreated with 3 μg of cycloheximide had a lower mean of 4.54 ± 1.11 mμ moles ALA/ hr/ mg protein, while larger doses of cycloheximide caused a more dramatic drop in ALA synthetase activity (Fig. 13, Table 3).

Pretreatment with 3 μg of cycloheximide was seen to cause an enhanced level of hepatic porphyrin in embryos induced with DDC. However, this enhancement was not accompanied by an increased amount of ALA synthetase.

4. Differential Analysis of Embryonic Hepatic Porphyrins

Three chick embryos (15 days) were treated with 3 μg of cycloheximide,

* refers to the inducing effect of DDC on hepatic ALA synthetase

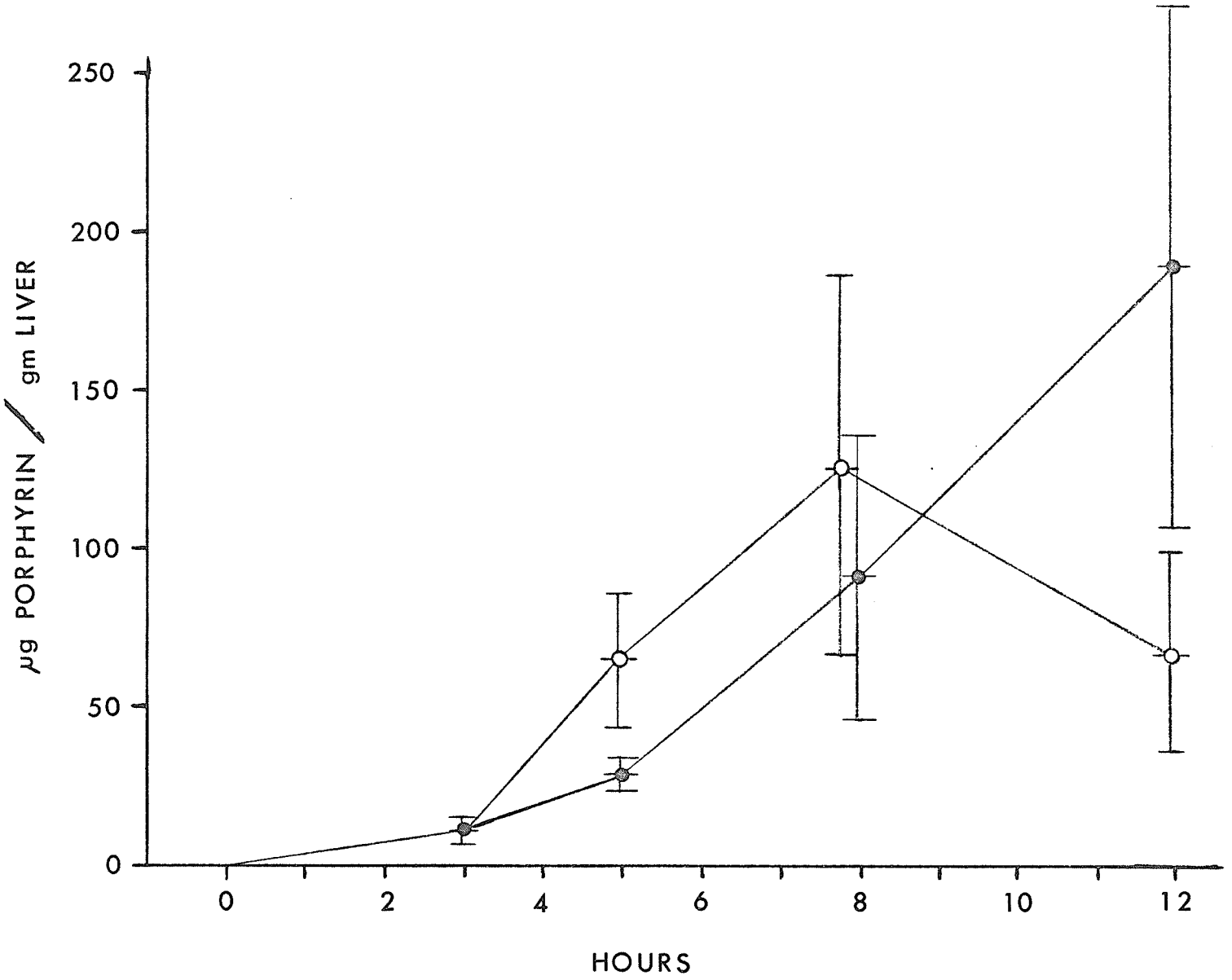


Figure 12. Hepatic porphyrin levels of 15 day chick embryos given 3 µg of cycloheximide, followed in one hour with 3 mg of DDC. Another group were given only 3 mg of DDC. After variable times of incubation, the chicks were sacrificed. ⊕ 3 mg DDC only; ○ 3 µg cycloheximide plus 3 mg DDC.

TABLE 2

Time study of the cycloheximide enhancement of DDC induced hepatic porphyria in 15 day chick embryos.

		Porphyrin Concentration (µg/g liver)		
	Time (hr)	Mean	Standard Error	No of embryos
Drug free control	-	1.81	±0.08	16
3 mg DDC	3	11.0	±2.0	8
"	5	29.6	±2.4	23
"	8	91	±19	8
"	12	189	±34	7
3 µg cycloheximide plus 3 mg DDC	3	10.4	±1.2	8
"	5	65.6	±9.6	12
"	8	127	±25	7
"	12	67.8	±12.4	6

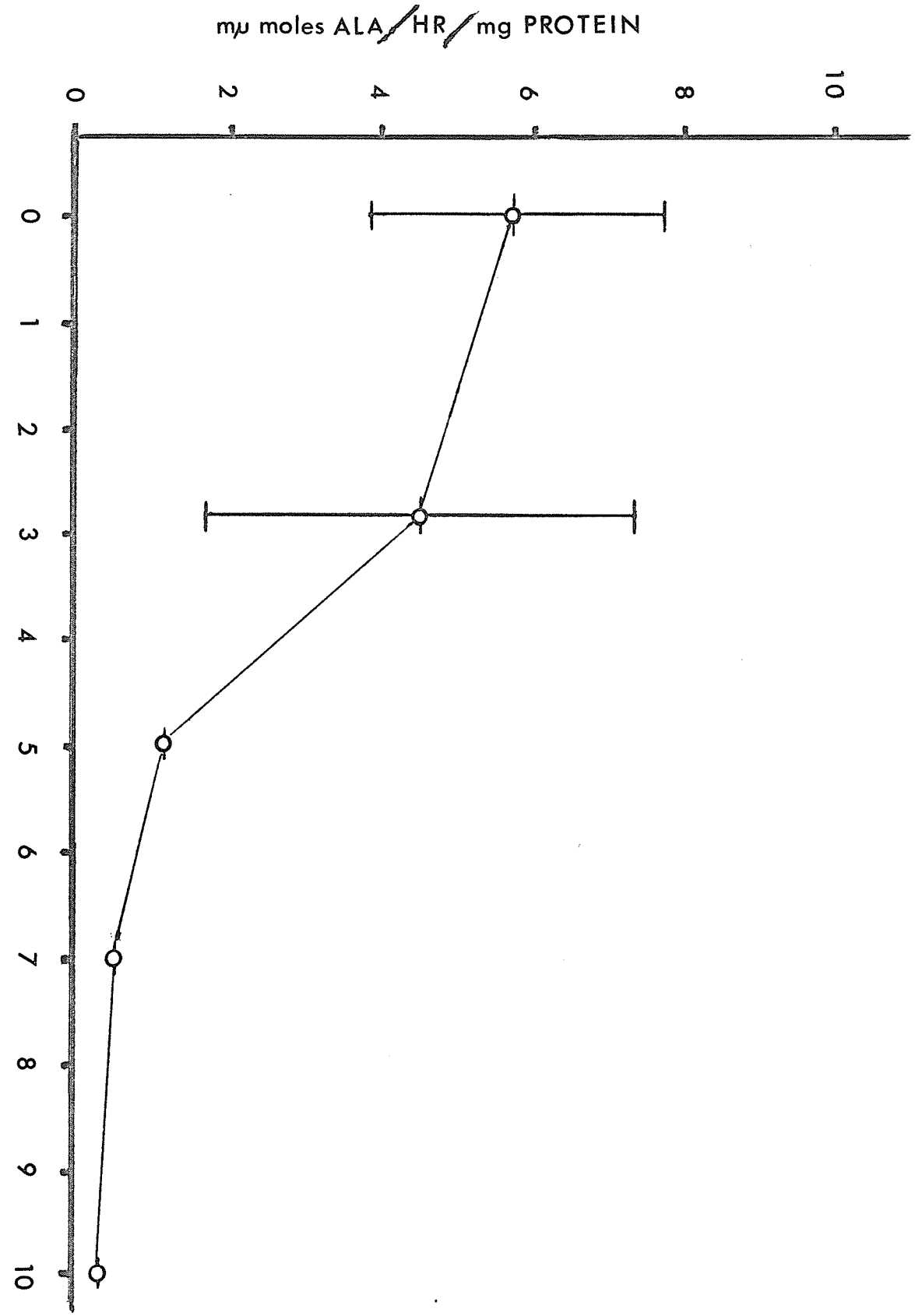


Figure 13. Hepatic ALA synthetase activities of 15 day chick embryos given variable doses of cycloheximide, followed in one hour with 3 mg of DDC, then sacrificed after 5 hours incubation at 38°C.

TABLE 3

Hepatic ALA synthetase levels of 15 day chick embryos given variable doses of cycloheximide, followed in 1 hour with 3 mg of DDC, and sacrificed five hours later.

	ALA SYNTHETASE ACTIVITY*		
	Mean	Standard Error	No. of embryos
3 mg DDC	5.78	±0.89	12
3 µg cycloheximide plus 3 mg DDC	4.54	±1.11	6
5 µg cycloheximide plus 3 mg DDC	1.18	--#	4
7 µg cycloheximide plus 3 mg DDC	0.52	--	3
10 µg cycloheximide plus 3 mg DDC	0.32	--	4

* expressed as mµ moles ALA/hr/mg protein

pooled livers were used for these determinations

followed in one hour with 3 mg of DDC. Other embryos were given 3 mg of DDC. After five hours of incubation at 38°C, the livers were removed and the porphyrin extracted. The differential method of Schwartz et al (80) was used to separate and quantitate the uroporphyrin, coproporphyrin, and protoporphyrin.

The total porphyrin pool was composed 99% of protoporphyrin (Table 4). Coproporphyrin was responsible for about 1% of the porphyrin, while only traces of uroporphyrin were found. This is in contrast to the in vitro chick embryo liver cell system of Granick (66), where the principle pigment was coproporphyrin.

No significant difference could be found between the percentage porphyrin distributions in the embryos given only DDC and the cycloheximide treated, DDC induced embryos. Cycloheximide was therefore not blocking the synthesis of protoporphyrin, or channeling porphyrin precursors into inactive, type I isomers (Fig. 4).

5. Cycloheximide Effect on the Conversion of Exogenous ALA into Porphyrin

It was of importance to establish whether the cycloheximide effect was mediated prior to, or after the synthesis of ALA. To do this it was necessary to establish that the amount of porphyrin synthesized was proportional to the ALA in the system.

ALA in amounts ranging from 0.1 mg to 2.0 mg were placed on the air sacs of eggs containing 15 day chick embryos and incubated at 38°C. Five hours later the embryos were sacrificed, the livers removed and the porphyrin extracted and assayed.

The porphyrin content was found to be directly proportional to the amount of ALA administered, except at the highest concentration. ALA in the amount of 0.1 mg gave a mean value of 40.3 ± 4.3 µg porphyrin/ g liver, while 2 mg of ALA produced 385 ± 17 µg porphyrin/ g liver (Fig. 14, Table 5). This supports previous

TABLE 4

Analysis of hepatic porphyrins present in 15 day chick embryos following either 3 mg of DDC and 5 hours incubation, or 3 mg DDC one hour after pretreatment with 3 µg cycloheximide, then followed by a further five hours incubation.

	3 mg DDC				3 ug Cycloheximide plus 3 mg DDC			
	Uro	Copro	Proto	Total	Uro	Copro	Proto	Total
Porphyrin Concentration*	0.06	0.46	32.0	32.5	0.07	0.45	50.0	50.5
% of total	trace	1%	99%	--	trace	1%	99%	--

*concentration expressed as µg porphyrin/g liver

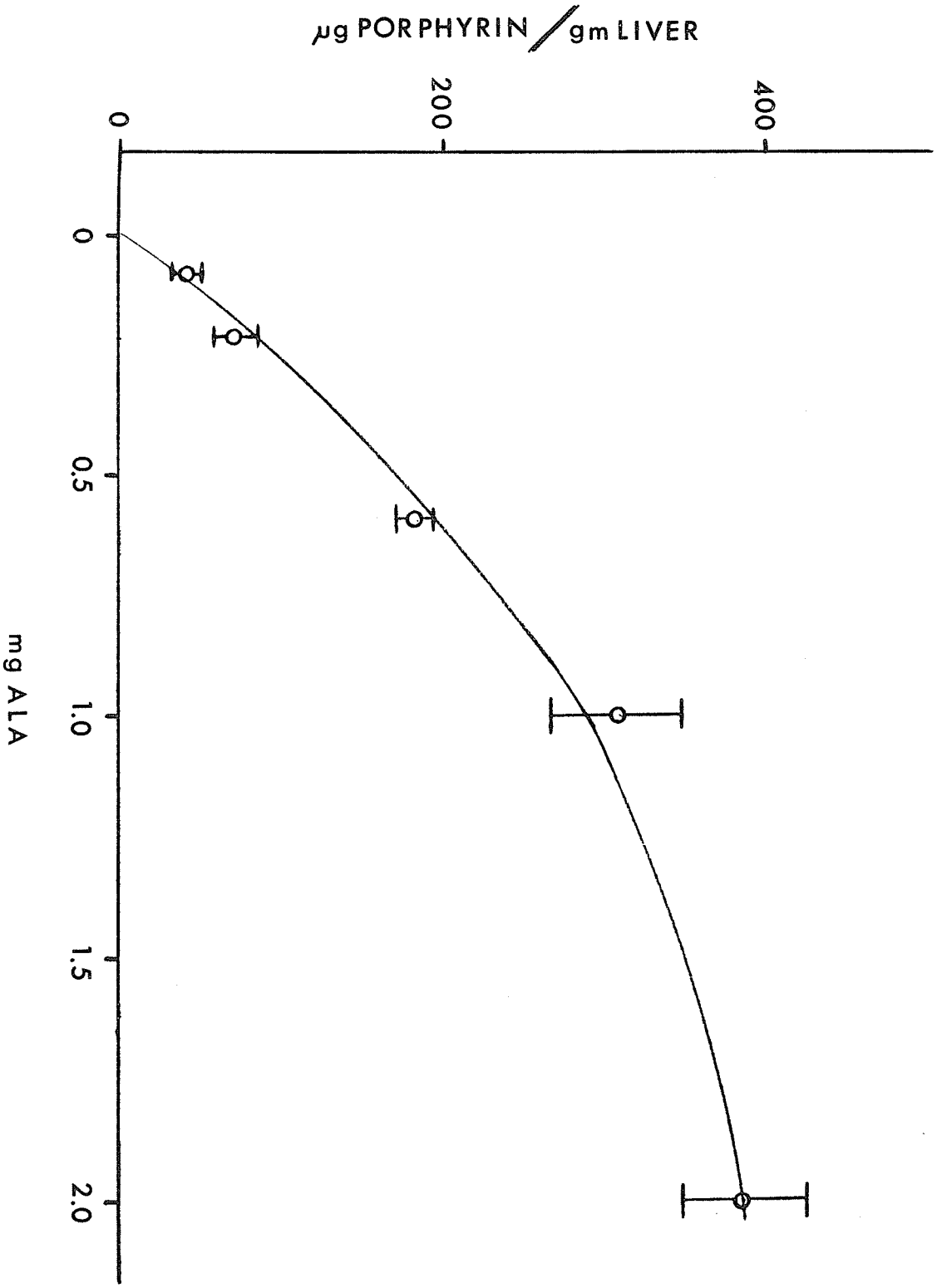


Figure 14. Hepatic porphyrin levels of 15 day chick embryos five hours after receiving variable doses of exogenous ALA.

TABLE 5

Hepatic conversion of exogenous ALA into porphyrin during a five hour incubation

	Porphyrin Concentration (µg/g liver)		
	Mean	Standard Error	No of embryos
Drug free control	1.81	±0.08	16
0.1 mg ALA	40.3	±4.3	12
0.2 mg ALA	72.0	±5.7	8
0.6 mg ALA	183	±5	10
1.0 mg ALA	309	±17	7
2.0 mg ALA	386	±17	9

findings that the rate limiting step in the synthesis of porphyrin precedes the presence of ALA in the cytosol (64,66,21).

The effect of cycloheximide on the conversion of ALA into porphyrin was then considered. ALA in amounts of 0.1 or 0.2 mg were placed on the air sacs of 15 day chick embryos one hour after an injection of 3 µg of cycloheximide. Following a five hour incubation at 38°C, the livers were removed, extracted for porphyrin and assayed. The results were compared with those of chick embryos receiving only ALA.

Embryos given only 0.1 mg ALA had a mean level of 40.3 ± 4.3 µg porphyrin/ g liver as compared with a mean of 26.4 ± 4.0 µg porphyrin/ g liver for chick embryos pretreated with cycloheximide (Fig. 15, Table 6). Embryos receiving only 0.2 mg ALA had a level of 72.0 ± 5.7 µg porphyrin/ g liver, while those pretreated with cycloheximide had a mean of 69.2 ± 4.9 µg porphyrin/ g liver.

Thus, cycloheximide did not enhance the conversion of exogenous ALA into porphyrin. The drug must then be exerting its primary effect before ALA enters the cytoplasm.

6. Effect of Glycine Loading on Hepatic Porphyrin Levels

Recent literature has suggested glycine may not be present in sufficient concentration *in vivo* to saturate ALA synthetase (71). Increasing the available glycine might then be expected to increase the production of ALA and porphyrin.

Eggs containing 15 day chick embryos were injected with either 10 mg or 100 mg of glycine. After a five hour incubation at 38°C, the livers were removed, extracted for porphyrin and assayed.

Ten mg of glycine raised the mean control level of 1.81 ± 0.08 µg porph-

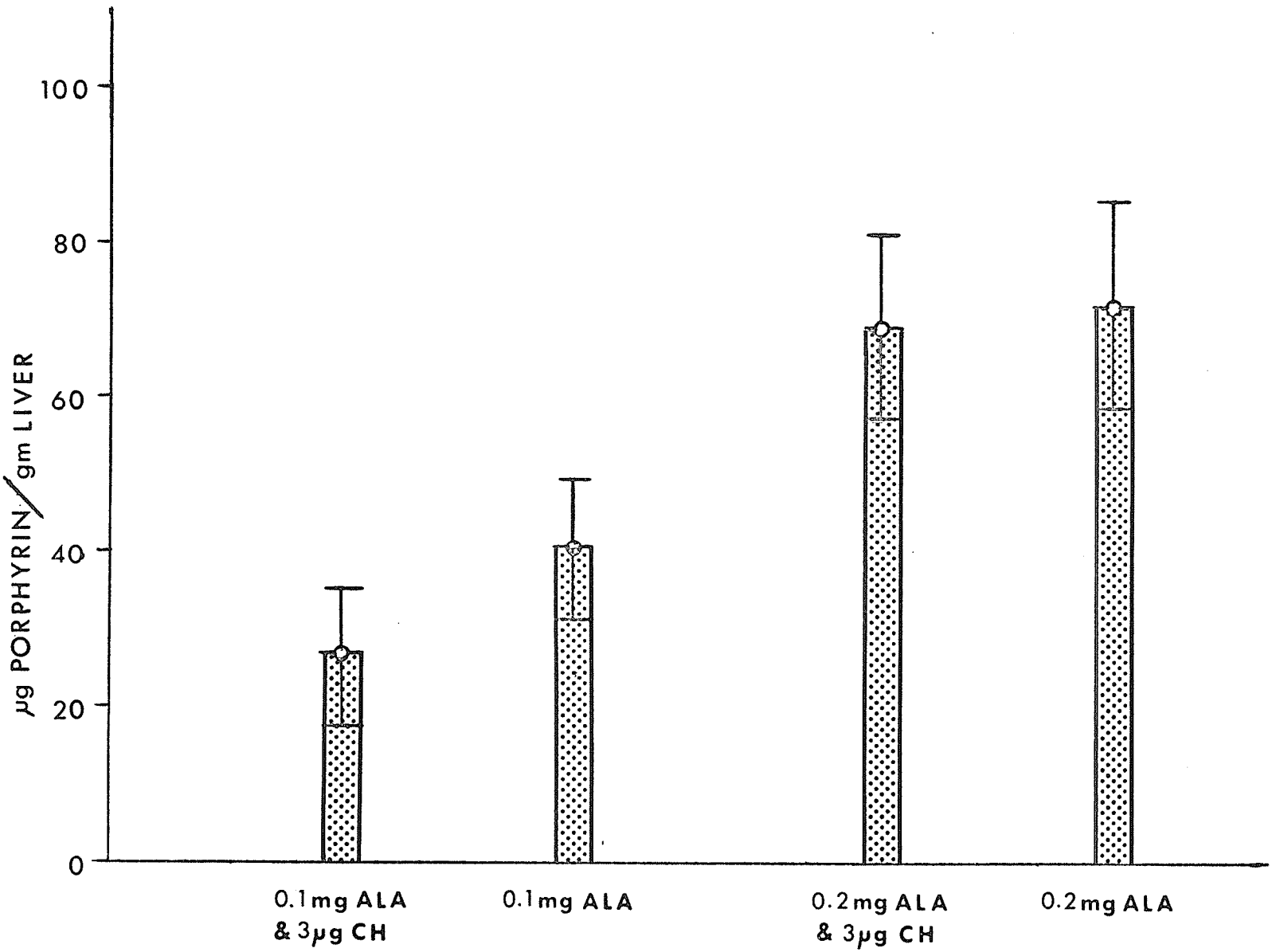


Figure 15. Hepatic porphyrin levels of 15 day chick embryos given 3 µg of cycloheximide followed in one hour with either 0.1 mg or 0.2 mg ALA. Other chick embryos were given only ALA. After five hours incubation at 38°C, the embryos were sacrificed.

TABLE 6

Hepatic porphyrin levels of 15 day chick embryos given 3 µg cycloheximide, followed one hour later with either 0.1 or 0.2 mg ALA, then sacrificed five hours later.

	Porphyrin Concentration (µg/g/liver)		
	Mean	Standard Error	No. of embryos
0.1 mg ALA only	40.3	±4.3	12
3 µg cycloheximide plus 0.1 mg ALA	26.4	±4.0	13
0.2 mg ALA only	72.0	±5.7	8
3 µg cycloheximide plus 0.2 mg ALA	69.2	±4.9	8

yrin/g liver to 2.14 ± 0.20 µg porphyrin/g liver and to a mean value of 2.81 ± 0.32 µg porphyrin/g liver with 100 mg of glycine (Fig. 16, Table 7).

The ability of glycine to raise the basal porphyrin level lent credence to the suggestion that ALA synthetase is not saturated with glycine in vivo.

7. Porphyrin Content of DDC Induced Chick Embryonic Liver, Pretreated With Either Glycine, Succinate, or Cycloheximide and Glycine

Since glycine had been able to raise the basal porphyrin level, its effect on the "DDC-induced" liver was of interest.

Glycine (10 mg) or succinate (10 mg) was injected into the amniotic fluid surrounding 15 day chick embryos, followed 1 hour later with 3 mg of DDC. Other experiments involved the simultaneous injection of 10 mg of glycine and 3 µg of cycloheximide 1 hour prior to the administration of 3 mg of DDC. Following a five hour incubation at 38°C, the livers were removed, extracted for porphyrin and assayed.

Pretreatment with glycine raised the DDC induced mean of 29.6 ± 2.4 µg porphyrin/g liver two-fold to a value of 56.3 ± 5.2 µg porphyrin/g liver. Succinate had a negligible effect (mean 31.7 ± 3.2 µg porphyrin/g liver). The combination of glycine and cycloheximide with DDC produced a mean of 51.9 ± 6.2 µg porphyrin/g liver, which was not greater than that of either cycloheximide or glycine alone (Fig. 17, Table 8). The possibility of a common mechanism was then considered.

8. The Affinity of Glycine for Chick Embryo δ-Aminolevulinic Acid Synthetase

ALA synthetase, in the rat liver system has a relatively large glycine K_m (4 - 5mM (14,76)), but the Michaelis constant in the chick system was unknown.

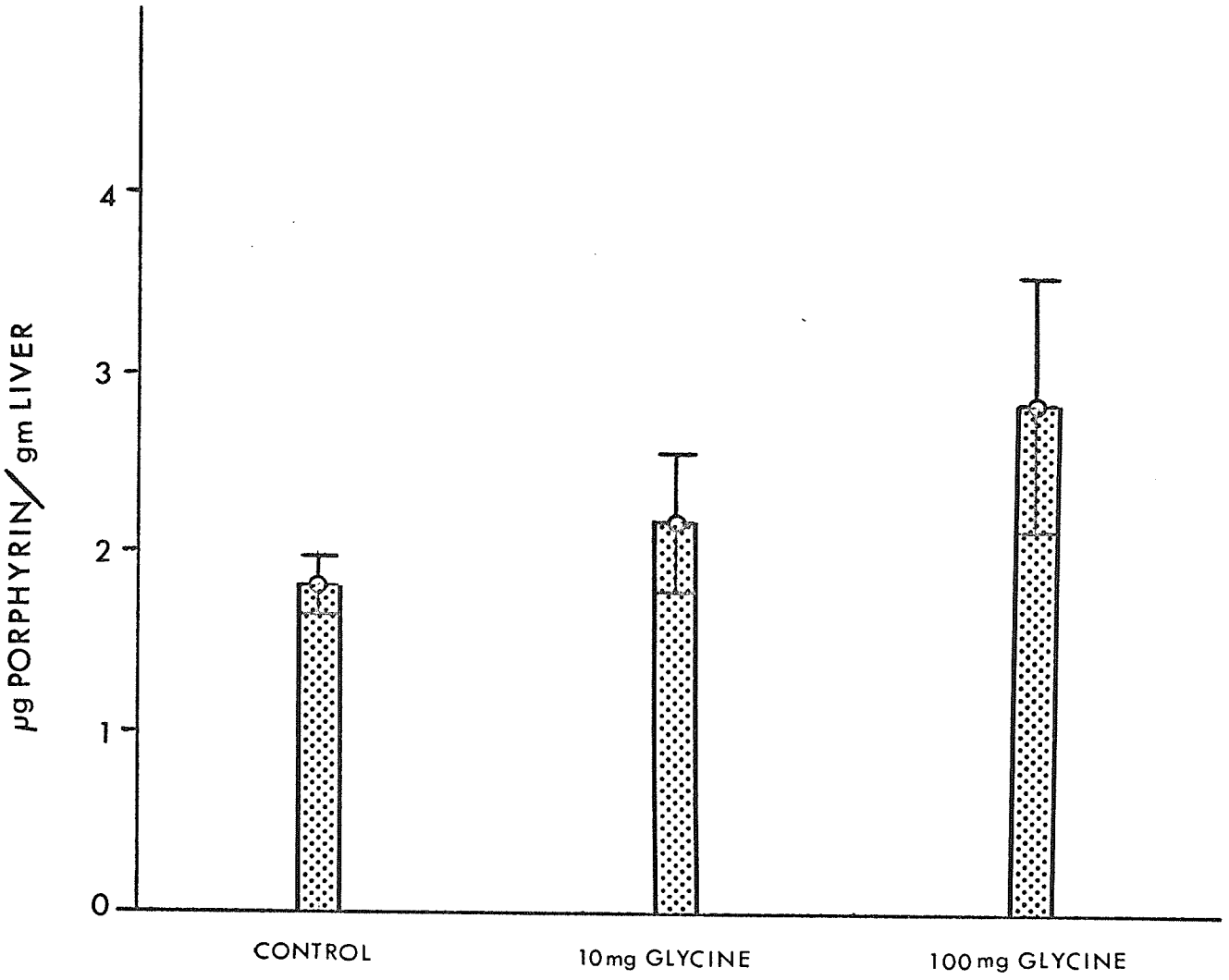


Figure 16. Hepatic porphyrin levels of 15 day chick embryos after five hours incubation at 38°C with either 10 mg or 100 mg of glycine.

TABLE 7

Hepatic porphyrin levels of 15 day chick embryos five hours after the administration of either 10 or 100 mg of glycine

	Porphyrin Concentration (µg/g liver)		
	Mean	Standard Error	No. of embryos
Drug free control	1.81	±0.08	16
10 mg glycine	2.14	±0.20	13
100 mg glycine	2.81	±0.32	9

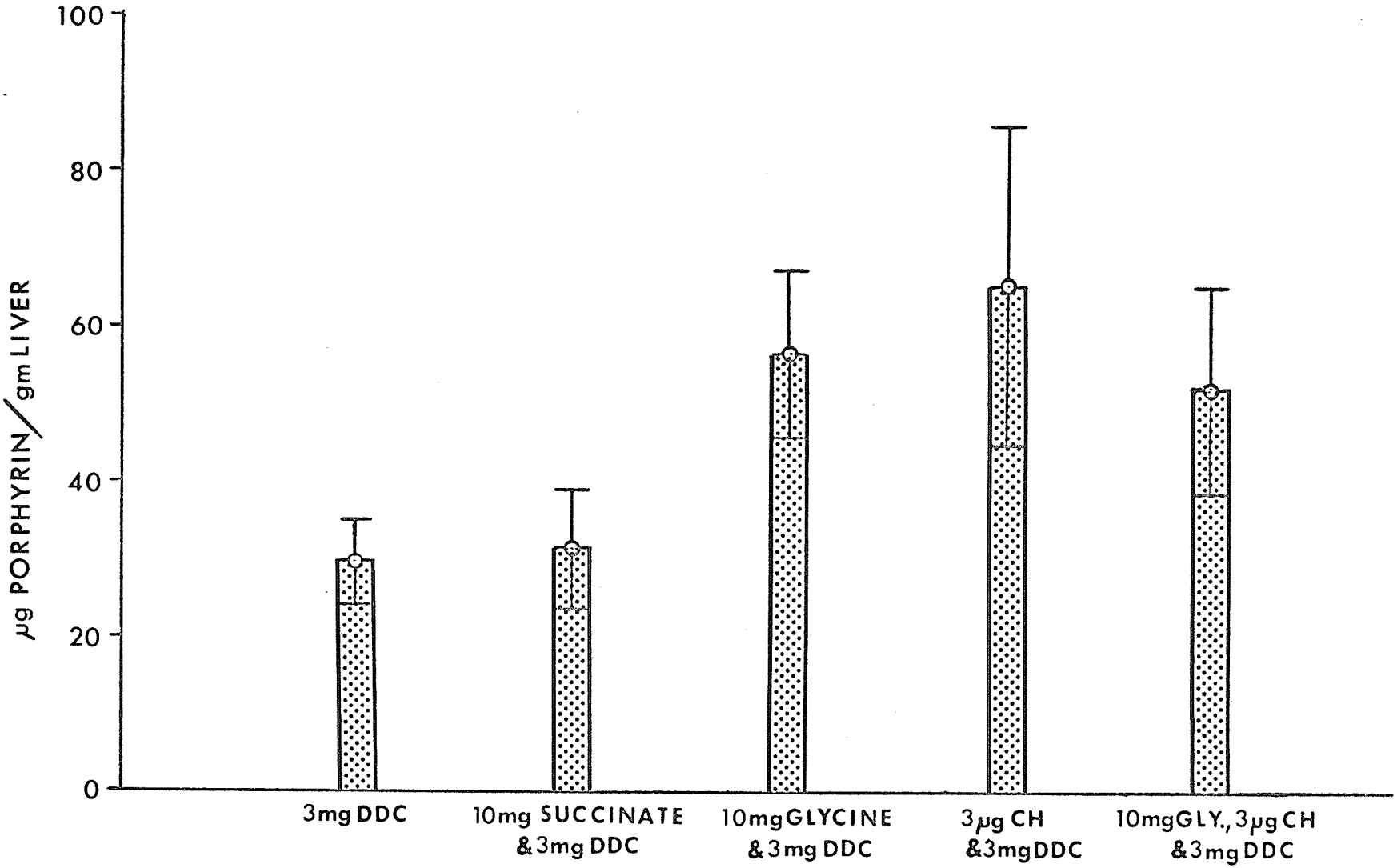


Figure 17. Hepatic porphyrin levels of 15 day chick embryos treated with either: 10 mg succinate, 10 mg glycine, 3 μg cycloheximide, or 10 mg glycine and 3 μg cycloheximide, then followed in one hour with 3 mg DDC. Livers were removed after a five hour incubation at 38°C.

TABLE 8

Hepatic porphyrin levels of 15 day chick embryos treated with either 10 mg succinate, 10 mg glycine, 3 µg cycloheximide, or 10 mg glycine and 3 µg cycloheximide, followed in one hour with 3 mg DDC, then sacrificed five hours later.

	Porphyrin Concentration (µg/g liver)		
	Mean	Standard Error	No. of embryos
3 mg DDC only	29.6	±2.4	23
10 mg succinate plus 3 mg DDC	31.7	±3.2	8
10 mg glycine plus 3 mg DDC	56.3	±5.2	18
3 µg cycloheximide plus 3 mg DDC	65.6	±9.6	12
3 µg cycloheximide and 10 mg glycine plus 3 mg DDC	51.9	±6.2	13

Sixteen eggs containing 15 day chick embryos were each injected with 3 mg of DDC. Following a 5 hour incubation at 38°C, the livers were removed, pooled and homogenized in a sucrose solution as explained in Methods. ALA synthetase activity was assayed as a function of the glycine concentration (0-300mM). A Lineweaver-Burk reciprocal plot was constructed using the initial velocities and their respective glycine concentrations (Fig. 18, 19, and Methods).

The calculated glycine K_m of 4.2 ± 0.1 mM (deduced from one set of points) agrees closely with the known literature values for the rat system, illustrating the low affinity of ALA synthetase for this substrate.

9. The Effect of DDC and Cycloheximide on the Hepatic Glycine Content of Chick Embryos

The low affinity of ALA synthetase for glycine had suggested the enzyme might not be saturated *in vivo*. In order to pursue this idea, a knowledge of the hepatic concentration of glycine was needed.

Eggs containing 15 day chick embryos were injected with 3 µg of cycloheximide and incubated for 6 hours or given 3 mg of DDC and incubated for 5 hours. Other eggs were given 3 µg of cycloheximide followed in 1 hour with 3 mg DDC and incubated for 5 hours. The livers were then removed and the glycine content assayed by the method of Alexander et al (81).

Untreated chick embryos had a mean glycine level of 0.85 ± 0.18 mM, not significantly different from those given DDC alone (0.83 ± 0.19 mM). The mean value of embryos given cycloheximide alone was 2.83 ± 0.17 mM glycine while those treated with both cycloheximide and DDC had a glycine level of 3.11 ± 0.32 mM (Fig. 20, Table 9).

Thus the intrahepatic concentration of glycine was seen to be well below that of saturation, indicating that changes in the glycine pool might be expected to affect the amounts of ALA produced, and a 6 hour incubation with cyclo-

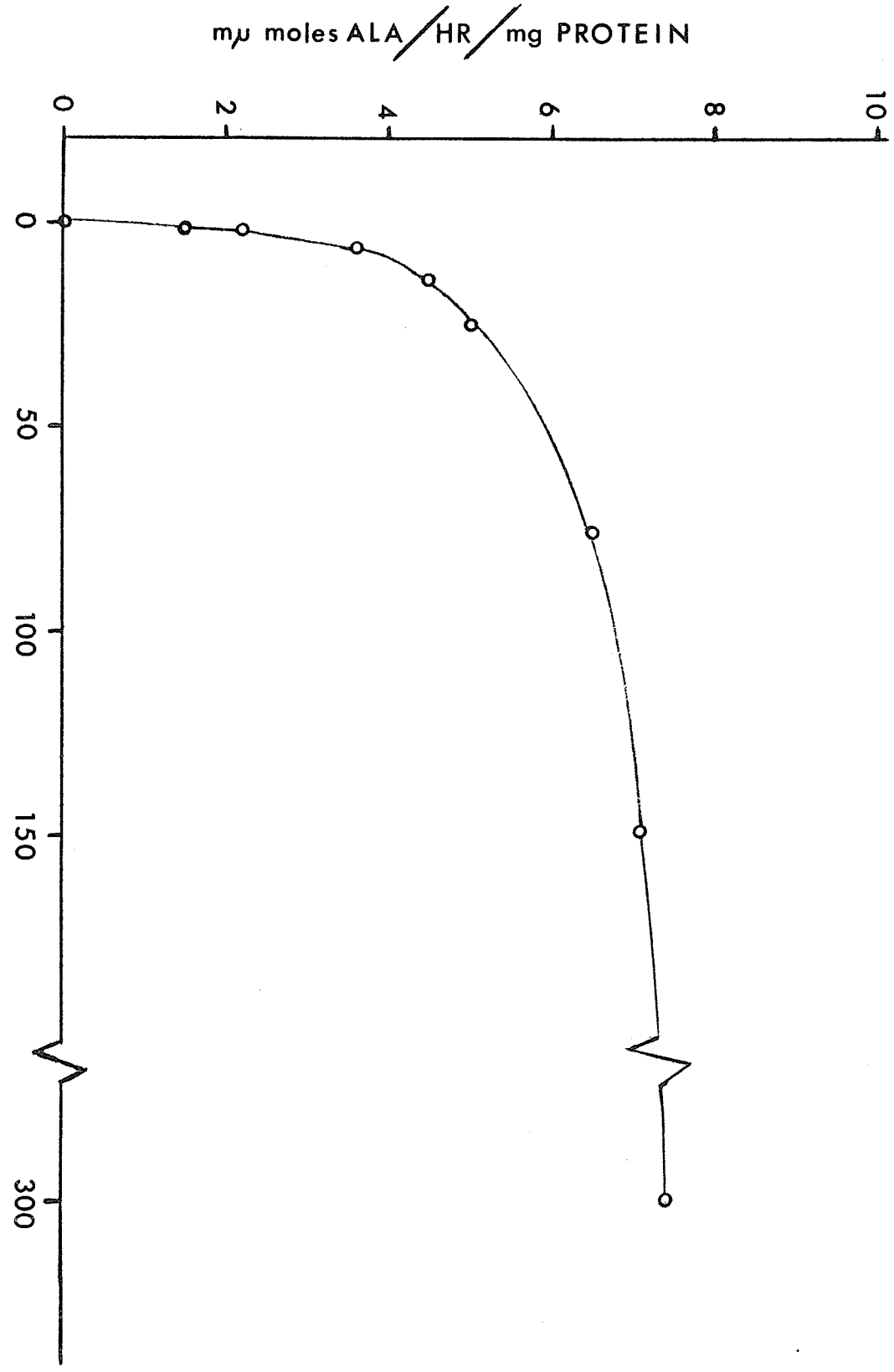


Figure 18. ALA synthetase activity of DDC induced chick embryo liver homogenate as a function of the final glycine concentration in the incubation mixture.

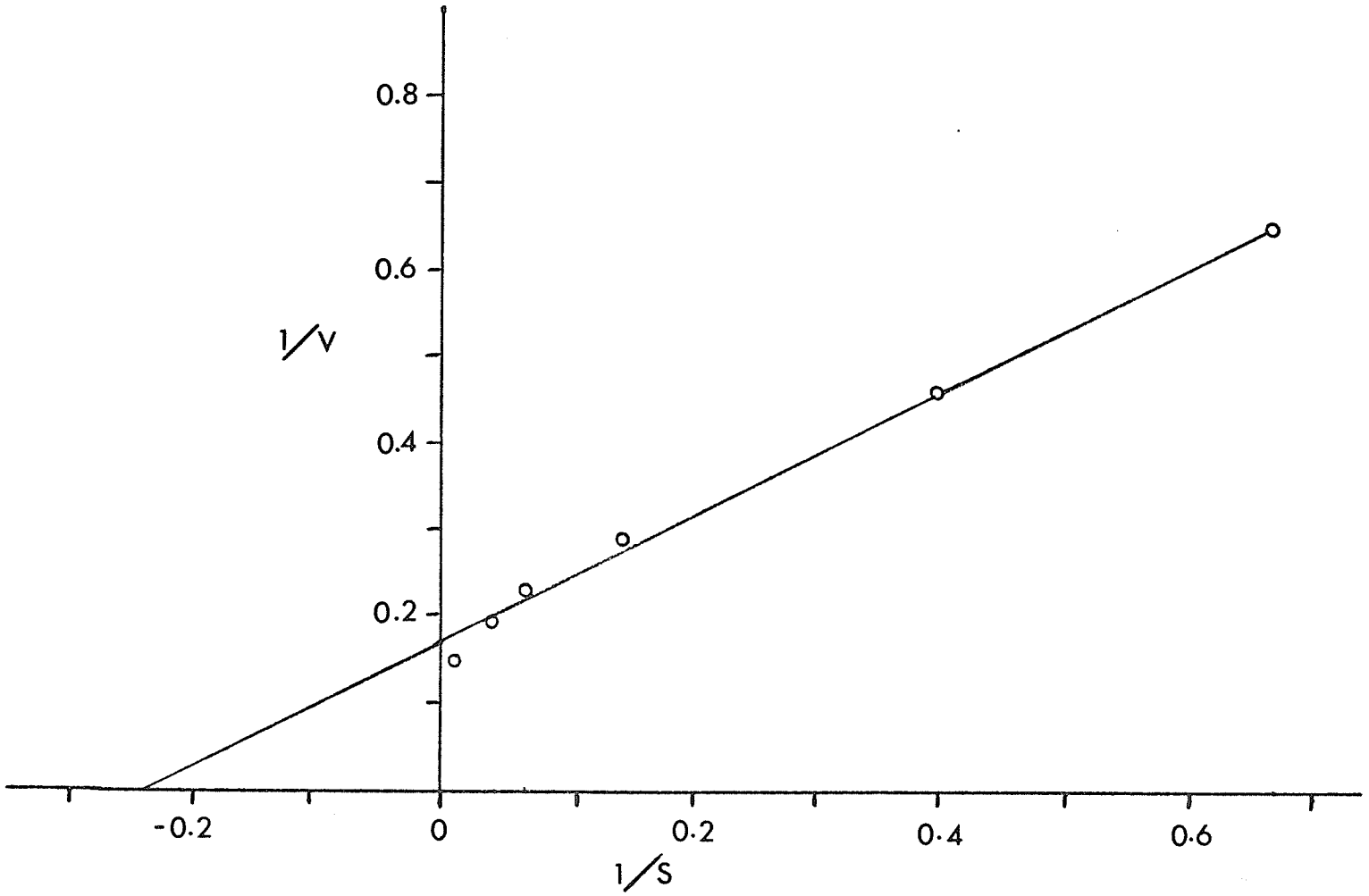


Figure 19. Lineweaver-Burk reciprocal plot used in the calculation of the glycine Michaelis constant for hepatic chick embryo ALA synthetase. V= mμ moles ALA/ hr/ mg protein, S= mM glycine

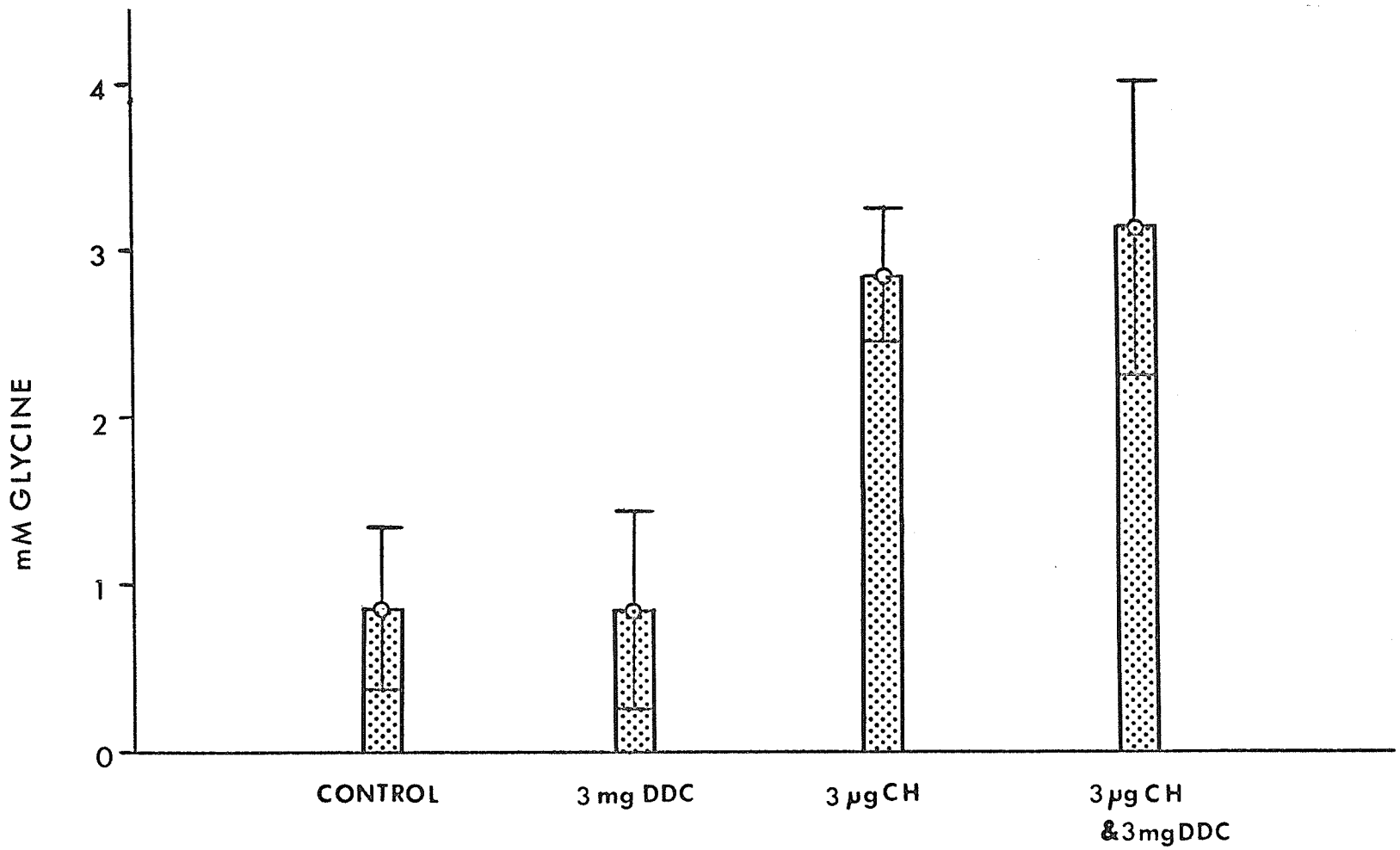


Figure 20. Hepatic glycine concentrations of 15 day chick embryos given either: 3 mg of DDC and incubated for five hours, 3 µg cycloheximide and incubated for six hours, or 3 mg DDC one hour after receiving 3 µg cycloheximide and incubated a further five hours.

TABLE 9

Hepatic glycine concentrations of 15 day chick embryos given either 3 mg DDC and incubated for five hours, 3 µg cycloheximide and incubated for six hours, or 3 mg DDC one hour after receiving 3 µg cycloheximide and incubated a further five hours.

	Glycine Concentration (mM)		
	Mean	Standard Error	No. of Embryos
Drug free control	0.85	±0.18	5
3 mg DDC only	0.83	±0.19	4
3 µg cycloheximide	2.83	±0.17	6
3 µg cycloheximide plus 3 mg DDC	3.11	±0.32	5

heximide caused a three-fold increase in the chick embryo hepatic glycine pool.

This raised glycine level is postulated to augment the production of ALA and porphyrin to the observed levels.

DISCUSSION

The role of ALA synthetase in heme biosynthesis has been studied by many workers. As reviewed in the Introduction, the enzyme is rate limiting for the biosynthesis of heme. Heme has been reported to allosterically inhibit ALA synthetase (56,57) and to repress DNA transcription, when the enzyme is induced by chemical mediators (61,62). The short half life of ALA synthetase offers another sensitive control (72). The interest in ALA synthetase as the rate limiting enzyme has neglected other possible rate controlling factors in the synthesis of ALA, including the availability of substrate. Recent work in a rat liver system has shown ALA synthetase to have a Michaelis constant for glycine of about 5 mM (14,76). Since the intracellular glycine concentration of rat liver is in that range (84,96), the implication that porphyrin biosynthesis might be modified by changes in the intramitochondrial availability of glycine was made (71).

The inducers, allylisopropylacetamide (ALA) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), produce large amounts of porphyrin in the livers of whole chick embryos by induction of ALA synthetase (66,75). The porphyrin increase is evident in 3 - 5 hours and has been used as a model system to study porphyria in man.

While using the chick embryo system, it was noted that 3 mg of DDC caused an induced porphyrin level of 29.6 ± 2.4 µg porphyrin/g liver in 5 hours. If the chick embryos were pretreated with 3 µg of cycloheximide 1 hour before the inducing dose of DDC, a porphyrin level of 65.6 ± 9.6 µg porphyrin/g liver was obtained. Granick (66) and others (75) have often used porphyrin levels as an indirect measure of ALA synthetase activity. However, 3 µg of cycloheximide caused a slight inhibition of the induced enzyme activity, relative to embryos receiving only DDC (Fig. 13, Table 3). Somehow, less enzyme (ALA synthetase) was

producing more product (porphyrin).

The Introduction outlined the many ways enzyme function could be altered. A metabolic block may have prevented the further metabolism of porphyrin to heme, resulting in porphyrin accumulation. Another possibility was interference with uroporphyrinogen III cosynthetase, channeling porphyrin precursors into type I isomers. The latter possibility was ruled out when 99% of the porphyrin was found to be protoporphyrin (Table 4) which is not a product of type I synthesis (Fig. 4). The conversion of ALA into porphyrin was a simple function of the substrate concentration (Fig. 14), indicating the enzymes following ALA synthetase were not saturated or rate limiting. Cycloheximide treated embryos given exogenous ALA did not produce enhanced levels of porphyrin, as compared to embryos given ALA only. From this it was concluded the cycloheximide effect was before the appearance of ALA in the cytoplasm.

It has been frequently suggested that the distribution of enzymes concerned in the biosynthesis of heme between the mitochondria and cytosol may be important in biosynthetic regulation (49). Jones and Jones have recently shown rat liver mitochondria to be freely permeable to glycine, but not to exogenous ALA, although a measurable efflux from the mitochondria of synthesized ALA was observed (83). They noted that chemically induced porphyria did not affect the ALA permeability, but added that the barrier could be important in the regulation of heme synthesis. An increase in the mitochondrial ALA permeability would lower the intramitochondrial concentration, and could increase the rate of ALA production.

Cycloheximide, as a protein inhibitor, might have been expected to cause some changes in the mitochondrial membrane. However, since the ALA synthetase activity assay (76) used a liver homogenate with intact, viable mitochondria, any <u>in vivo</u> increase in the ALA permeability producing an enhanced

ALA production, would have been measured with the in vitro assay. This was not observed.

ALA synthetase activity is routinely measured using saturating amounts of succinate (10 mM) and glycine (100 mM) (76). The glycine K_m for ALA synthetase from one set of points in the chick embryo liver system was 4.2 ± 0.1 mM, agreeing closely with values of 4 - 5 mM in the rat liver system (14,76). However, micro-assay of the free glycine pool in normal chick liver revealed a mean concentration of only 0.85 ± 0.18 mM (Fig. 20, Table 9), although the hepatic intracellular glycine concentration may be somewhat higher. Nukada states rat brain mitochondria have a limited ability to concentrate glycine, but this was not shared by rat liver or kidney mitochondria (84). This is supported by Jones and Jones (83). Literature could not be found regarding glycine permeability that related to hepatic chick mitochondria specifically, but 100 mg of glycine given to otherwise untreated chick embryos increased the basal porphyrin level by 55%, from 1.81 ± 0.08 µg porphyrin/gm liver to 2.81 ± 0.32 µg porphyrin/g liver in a period of 5 hours (Table 16). The evidence suggests hepatic chick embryo ALA synthetase is not saturated with glycine in vivo.

Succinate (10 mg) given 1 hour before an inducing dose of DDC had no effect on the production of porphyrin, while pretreatment with 10 mg of glycine raised the mean porphyrin level of embryos given only DDC from 29.6 ± 2.4 µg porphyrin/g liver to 56.3 ± 5.2 µg porphyrin/g liver during a 5 hour incubation (Fig. 17, Table 8). Three µg of cycloheximide given at the same time as the glycine in the above experiment, did not augment the porphyrin level (51.9 ± 6.2 µg porphyrin/g liver).

The ability of cycloheximide to increase the pool of free glycine has been well documented in work with various fungi (85,86). Grenson et al (87) postulated an increased amino acid pool in Saccharomyces cerevisiae, caused by cycloheximide's inhibition of protein synthesis, while Seglen has suggested

cycloheximide augments the amount of free amino acid in isolated, perfused rat liver (88).

Three μg of cycloheximide in the chick embryo system raised the hepatic glycine concentration of untreated controls from 0.85 ± 0.18 mM to 2.83 ± 0.17 mM in 6 hours (Fig. 20, Table 9). Similar levels of glycine were obtained when DDC (3 mg) was injected 1 hour after the cycloheximide in the above experiment (3.11 ± 0.32 mM). Chick embryos given only 3 mg of DDC showed no appreciable change from control values in 5 hours (0.83 ± 0.19 mM glycine).

It is proposed that the raised glycine pool is responsible for the observed high levels of porphyrin in chick embryos pretreated with low doses of cycloheximide, before receiving DDC. Cycloheximide has two conflicting effects in this system. One is the inhibition of ALA synthetase production, while the other causes a concomitant rise in the glycine pool. The first produced a decrease in the amount of ALA synthetase, as measured in the ALA synthetase assay (Fig. 13). The second effect allowed the remaining enzyme to work at a greater velocity, producing large amounts of ALA that were quickly converted to porphyrin (Fig. 11). The optimum balance between these two effects was produced with 3 μg of cycloheximide in a period of 5 - 6 hours.

Acute intermittent porphyria is a rare genetic disease characterized by abdominal and neurologic manifestations which are frequently intermittent in nature. The defect involves the overproduction of the porphyrin precursor ALA in the liver, resulting in increased excretion of ALA and PBG in the urine (89).

Increased urinary levels of ALA and PBG have been observed following the oral administration of 25 g of glycine, in both healthy controls and patients suffering from acute intermittent porphyria. The porphyric patients usually showed dramatic increases and the diagnostic significance of this loading technique has been discussed (90,91).

The control of hepatic heme biosynthesis involves several factors, in-

cluding diet (21,92,93), hormones (94,95,66) and chemical agents (66,61,64). A diet high in carbohydrate or protein has been shown to inhibit ALA synthetase induction by ALA (21). However, calories given in the form of fat did not inhibit the chemical induction. Similar results have been noted with patients suffering with acute intermittent porphyria, where diets rich in protein or carbohydrate diminished the urinary excretion of ALA and PBG, while a fat diet caused increased excretion (92,93).

Wiss has done studies on the effect of diet on rat liver (96)(Table 10). Rats starved for 20 hours had a level of 25 mg glycine/100 gm liver, which was increased by a fat diet to 29 mg glycine/100 gm liver, and decreased to 20 mg glycine/100 gm liver with protein feeding. Serine, which is readily converted to glycine by intramitochondrial enzymes (97) was also raised by the fat diet and decreased by the protein relative to fasted controls.

It is interesting to speculate whether the inhibitory effect of a protein diet and the enhancing effect of fat on the urinary excretion of porphyrin precursors by patients with acute intermittent porphyria is related in any way to the respective hepatic glycine pools.

Carbohydrate also caused an increase in the glycine pool relative to the fasted control, although the serine level was lower. However, this "glucose effect" has been known for some time to inhibit the synthesis of several enzymes (98) and is likely unrelated to the glycine level. It has been suggested that carbohydrate mediates its effect by glucose catabolite repression, or by hormonal changes (95). Heme oxygenase, the enzyme responsible for the degradation of heme, is induced by hypoglycemia (99). The effect would seem to be mediated through glucagon. Carbohydrate would therefore inhibit the degradation of heme, and via mechanisms discussed earlier, inhibit the induction of ALA synthetase.

In conclusion, ALA synthetase, the rate limiting enzyme in the biosynthesis of heme, is not considered to be saturated with glycine <u>in vivo</u> and

TABLE 10

The effects of various diets on the free glycine and serine pool of rat liver. #

Diet	20 hr starvation	Fat	Protein	Carbohydrate
glycine level	25*	29	20	29
Serine level	20	26	12	18

*Concentration expressed as mg amino acid/100 mg liver

Wiss, O. (96)

the production of porphyrin is therefore a function of both the intramitochondrial glycine concentration and the amount of ALA synthetase present. The porphyrin enhancement produced in chick embryos liver by pretreatment with 3 µg of cycloheximide, before induction with 3 mg of DDC, is postulated to be caused by an increase in the intracellular free glycine pool.

Under ordinary conditions, a mere increase in the amount of glycine available would not augment the rate of ALA production sufficiently to account for the development of experimental or human genetic porphyria. However, variation in the hepatic glycine pool of porphyric patients may be of significance in the expression of their disease.

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