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Regulation of δ-Aminolevulinic Acid Synthase and Heme Oxygenase in Cultured Chick Embryo Liver Cells: Synergistic Induction of Both Enzymes by Glutathimide and Iron and Repression of δ-Aminolevulinic Acid Synthase by Metalloporphyrins and Heme: A Dissertation

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University of Massachusetts Medical School

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A Dissertation Presented

By

Edward Earl Cable
B.S. Grove City College, Grove City Pennsylvania

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES
April 1993
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REGULATION OF δ-AMINOLEVULINIC ACID SYNTHASE AND HEME OXYGENASE IN CULTURED CHICK EMBRYO LIVER CELLS: SYNERGISTIC INDUCTION OF BOTH ENZYMES BY GLUTETHIMIDE AND IRON AND REPRESSION OF δ-AMINOLEVULINIC ACID SYNTHASE BY METALLOPORPHYRINS AND HEME

A Dissertation Presented

By

Edward Earl Cable

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April 1993
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Primary chick embryo liver cells were used to explore the regulation of δ-aminolevulinic acid synthase and heme oxygenase, the enzymes that catalyze the rate-limiting reactions of heme anabolism and catabolism, respectively. The general focus of the work was the exploration of the novel observation in which glutethimide and iron synergistically induced both δ-aminolevulinic acid synthase and heme oxygenase, a phenomenon that would not be predicted \textit{a priori}. The course of events appeared to be: first, that heme synthesis was increased after addition of the glutethimide and that iron potentiated heme synthesis; second, the heme induced heme oxygenase five to ten fold; and third, that heme oxygenase degraded the heme permitting an uncontrolled induction of δ-aminolevulinic acid synthase. This induction of δ-aminolevulinic acid synthase could be prevented by the addition of a metalloporphyrin inhibitor of heme oxygenase. Induced δ-aminolevulinic acid synthase activity could be dramatically reduced by the addition of nanomolar concentrations of a metalloporphyrin, inhibitory for heme oxygenase, and heme.

Specific observations related to the synergistic induction of heme oxygenase by glutethimide and iron was that the induction of heme oxygenase activity by glutethimide and iron occurred rapidly, with maximal increases occurring four to six hours after original treatment. Induction of heme oxygenase by glutethimide and iron was shown to be dependent on \textit{de novo} heme synthesis since 4,6-dioxoheptanoic acid, a potent and specific inhibitor of heme biosynthesis, prevented the activity of
heme oxygenase from increasing in the presence of glutethimide and iron. Induction of activity was associated with increases in heme oxygenase mRNA and protein; and, when induction was prevented by 4,6-dioxoheptanoic acid, no increase in either mRNA or immunoreactive protein was observed.

δ-Aminolevulinic acid synthase activity was also synergistically increased by glutethimide and iron; this increase occurred 4-6 hours after maximal heme oxygenase activity had been attained. The temporal relationship between the induction of δ-aminolevulinic acid synthase and heme oxygenase suggested that the oxygenase depleted a regulatory heme pool that would normally prevent uncontrolled induction of the synthase. When cultures were exposed to tin-mesoporphyrin, a potent inhibitor of heme oxygenase, induction of δ-aminolevulinic acid synthase, normally produced by glutethimide and iron, was prevented. Addition of tin-mesoporphyrin after δ-aminolevulinic acid synthase induction had already been established promptly halted any further induction. When heme or a combination of heme and tin-mesoporphyrin was added after induction of δ-aminolevulinic acid synthase was established, activity of the synthase was rapidly reduced.

Finally, experiments in primary chick embryo liver cells with tin-, zinc- and copper-chelated porphyrins were done to assess their effects on activities of δ-aminolevulinic acid synthase, induced by prior treatment of cells with glutethimide and iron. Nanomolar concentrations of zinc- or tin-porphyrins reduced δ-aminolevulinic acid synthase activities, while copper-chelated porphyrins did not. When nanomolar concentrations of heme
were added with zinc- or tin-porphyrins, δ-aminolevulinic acid synthase activity was further reduced. Effects of the non-heme metalloporphyrins on δ-aminolevulinic acid synthase were closely correlated with their abilities to inhibit heme oxygenase (r=0.78). The largest decrease of δ-aminolevulinic acid synthase (67%) was obtained with zinc-mesoporphyrin and heme. There was a rapid appearance of the cytosolic, precursor form of δ-aminolevulinic acid synthase in the presence of both 10 µM heme or 50 nM zinc-mesoporphyrin and 200 nM heme. Reduction of the half-life of the mRNA from 5.2 hours to 2.2-2.5 hours was observed in the presence of both 10 µM heme or 50 nM zinc-mesoporphyrin and 200 nM heme.

In summary, the chick embryo liver cell culture model treated with glutethimide and iron may serve as one experimental model for patients suffering from acute porphyrias, in whom uncontrolled induction of hepatic δ-aminolevulinic acid synthase plays a key role in pathogenesis of disease. The synergistic induction of δ-aminolevulinic acid synthase in the presence of glutethimide and iron may serve as an experimental paradigm for this disease. The reduction of δ-aminolevulinic acid synthase by low doses of zinc-mesoporphyrin and heme may help form the experimental foundation for eventual studies in patients suffering from acute porphyrias.
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ABBREVIATIONS

$A^*$ Acetate
AIA Allylisopropylacetimide
AIP Acute intermittent porphyria
ALA 8-Aminolevulcinic Acid
ALA-pyrrole 2-methyl-3-acetyl-4-propionic acid pyrrole
ALAD ALA dehydratase (PBG synthase)
ALAS ALA synthase
ATP Adenosine triphosphate
BCIP 5-bromo-4-chloro-3-indoyl phosphate
BVR Biliverdin reductase
cDNA complementary DNA
CEP Congenital erythropoietic porphyria
CoA Coenzyme-A
Copro Coproporphyrin
Copro'gen Coproporphyrinogen
DDC 3,5-diethoxycarbonyl-1,4-dihydrocollidine
DHA 4,6-dioxoheptanoic acid
DMAB p-Dimethylaminobenzaldehyde
DMF Dimethylformamide
DOVA 4,5-dioxovaleric acid
$E^*$ Ethyl
EDTA Ethylenediaminetetraacetic acid
EPP Erythropoietic protoporphyrina
HAT 2-hydroxy-3-aminotetrahydoxypyran-1-one
HCP Hereditary coproporphyria
HEP Hepatoerythropoietic porphyria
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC High-performance liquid chromatography
IRE Iron responsive element
IRE-bp Iron responsive element binding protein
IUB International Union of Biochemistry
<table>
<thead>
<tr>
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<tr>
<td>IUPAC</td>
<td>International Union for Pure and Applied Chemistry</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole Limpet Hemocyanin</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis Menten Constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;r&lt;/sub&gt;</td>
<td>Concentration where biosynthesis of heme is reduced by one-half</td>
</tr>
<tr>
<td>MBS</td>
<td>m-maleimidobenzoic acid-N-hydroxysuccinimide ester</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro blue tetrazolium</td>
</tr>
<tr>
<td>NC</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NRF-1 RE</td>
<td>Nuclear respiratory factor-1 responsive element</td>
</tr>
<tr>
<td>M*</td>
<td>Methyl</td>
</tr>
<tr>
<td>P*</td>
<td>Propionate</td>
</tr>
<tr>
<td>PBG</td>
<td>Porphobilinogen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline (10 mM sodium phosphate, pH 7.4; 150 mM NaCl)</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>PCT</td>
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<td>pH</td>
<td>-log hydrogen ion concentration</td>
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<td>Proto</td>
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<td>Succinyl-CoA</td>
<td>Succinyl Coenzyme A</td>
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<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3,5,3'-triiodo-thyronine</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Uro</td>
<td>Uroporphyrin</td>
</tr>
<tr>
<td>Uro'gen</td>
<td>Uroporphyrinogen</td>
</tr>
<tr>
<td>V*</td>
<td>Vinyl</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Velocity of an enzymatic reaction at infinite substrate concentration</td>
</tr>
<tr>
<td>VP</td>
<td>Variegate porphyria</td>
</tr>
</tbody>
</table>

* Italized abbreviations are found in chemical structures only.
CHAPTER I
INTRODUCTION

Historical background and porphyrin biosynthesis

Porphyrins, the foundation of life. Porphyrins are a class of biological compounds essential to the sustenance of every kind of life. Metalloporphyrins are the catalytic molecules supplying all the energy needs for all living organisms, because they function as the chemical catalysts converting sunlight into biochemically stored energy. Eukaryotic aerobes satisfy their high energy needs through oxidative respiration, which is catalytically supported by the various mitochondrial cytochromes, all of which contain iron-protoporphyrin IX* (Figure 1.1 [1]§) as the catalytic species. Iron-protoporphyrin IX also serves as the functional prosthetic group in oxygen transporting proteins, and as the catalytic group in cytochrome P-450 dependent mono-oxygenase system. These central roles of pyrrole-derived compounds underscore their importance, and suggest the importance of understanding the regulatory features of pyrrole synthesis and degradation (for reviews see 1-8)

* Iron-protoporphyrin IX and heme will be used interchangeably and are not meant to imply any particular oxidation state of the iron. Although, traditionally, heme implies ferrous-protoporphyrin IX and hemin implies ferric-protoporphyrin IX with a chloride counter ion, this convention will not be followed, since most of the discussion of heme will be independent of oxidation state. When the oxidation state of the protoporphyrin IX-chelated iron is important to the discussion, this will be clearly described.

§ The bracketed numbers, i.e. [1], refer to compound structures while references are referred to with superscripted numbers.
Figure 1.1: Structure of ferric protoporphyrin IX (heme) [1]. Heme is a cyclic tetrapyrrole chelating an iron in either a low-spin octahedral coordination, in which the iron lies essentially within the place of the porphyrin ring, or a high-spin square pyramidal coordination, in which the iron lies as much as 8 Å out of the porphyrin ring. The valence of the iron modulates between divalent and trivalent states and permits some marvelous redox chemistry to occur. The numbering system shown is the official numbering system of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry.9.
[1]
Chemical Isolations and Characterizations of Porphyrins.

Porphyrin research originated in the 1840's with the isolation of "Eisenfreies hämatin" (iron-free hematin) from dried and powdered blood (for a review of the history of porphyrin research see 10). Later efforts showed that the main constituent of "Eisenfreies hämatin" was "hämatoporphyrin". Independently, uroporphyrin was isolated from the urine of a patient who, in retrospect, suffered from congenital porphyria. Studies of different compounds led to the discovery of the strong spectrophotometric absorbance of this class of compounds by Soret (see 11).

Due to the complexity of the heme biosynthetic pathway, many different compounds were isolated from porphyric patients, and many erroneous theories were presented. Although the delineation of the biosynthetic pathway would wait until the advent of isotopic labelling, the correct chemical structure of heme was proposed in 1912. The chemical separations of the three non-metallated cyclic tetrapyrroles were accomplished during this time: Stokvis\(^{12}\) and Fischer were two of several who chemically isolated uroporphyrin [2], Saillet and Fischer isolated coproporphyrin [3], and protoporphyrin [4] was, unknowingly, prepared by Laidlaw (Figure 1.2). Perhaps one of the reasons that Fischer was able to isolate two of these compounds was the successful recruitment of Matthias Petry as a laboratory aide. Mr. Petry suffered from congenital erythropoietic protoporphyrria, and he not only worked in the laboratory, but provided a constant source of porphyrins, until his death in 1925. He then became the subject of an autopsy that described the porphyrin deposits in
**Figure 1.2:** Structures of three naturally occurring porphyrins [2-4] and porphobilinogen [5]. The isomers shown are the ones that were first chemically isolated, as these are the normally occurring porphyrin isomers, and the porphyrins are listed in the order of metabolic synthesis.
<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>[n]</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
<th>R₇</th>
<th>R₈</th>
</tr>
</thead>
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<td>P</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>P</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>Coproporphyrin III</td>
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<td>M</td>
<td>P</td>
<td>M</td>
<td>P</td>
<td>M</td>
<td>P</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>4</td>
<td>M</td>
<td>V</td>
<td>M</td>
<td>V</td>
<td>M</td>
<td>P</td>
<td>P</td>
<td>M</td>
</tr>
</tbody>
</table>

A = Acetate  
M = Methyl  
P = Propionate  
V = Vinyl
developing erythropoietic cells, bones, and organs. Although the porphyrins were isolated from the excrement of porphyria patients, the porphyrins are not substrates for the heme biosynthetic enzymes, except for ferrochelatase. Rather, the porphyrinogens are the substrates\(^ {13}\) but are easily oxidized to porphyrins upon exposure to air.

**The heme biosynthetic pathway.** The discovery of the monopyrrole, porphobilinogen [5], occurred in the 1930's\(^ {10}\) and represented the last of the pyrroles in the pathway to be described. This characterization was also the last major breakthrough in biological porphyrin synthesis independent of the biochemical delineation of the heme biosynthetic pathway. Further description of the pathway came via the use of radiolabelled precursors, either \(^ {14}\)C-acetate or \(^ {15}\)N-glycine and accurately described glycine and succinyl-CoA as the precursors of \(\delta\)-aminolevulinate, which subsequently formed the monopyrrole porphobilinogen, and ultimately the tetrapyroles.\(^ {14-17}\) David Shemin even used himself as an experimental subject to show the utilization of \(^ {15}\)N-glycine in the formation of heme.\(^ {18}\) The tetrapyrrrole uroporphyrinogen III is the intermediate that is common among all tetrapyrrrole molecules.\(^ {19,20}\) This molecule can be either oxidized to form coproporphyrinogen III or methylated to eventually form cobyrinic acid [6], a precursor of vitamin B\(_{12}\) [7] (Figure 1.3).\(^ {21}\) Coproporphyrinogen III is oxidatively decarboxylated to protoporphyrinogen IX, which is then oxidized to protoporphyrin IX, the first and only non-metallated porphyrin that is biologically active in the metabolic pathway.\(^ {13}\) Protoporphyrin IX is the metabolic branch-point of chlorophyll and heme. Chlorophyll contains...
Figure 1.3: Structures of cobyric acid [6] and vitamin B$_{12}$ [7]. These compounds are derived from the heme precursor, uroporphyrinogen. These compounds serve to demonstrate the multifarious functions of tetrapyrroles.
A = Acetate
Pr = Propionate
AA = Acetamide
PA = Propionamide
magnesium, heme contains iron\textsuperscript{6,7} and the post-chelation modifications are quite different.

\textbf{δ-Aminolevulinic acid, the first committed intermediate.} δ-Aminolevulinic acid [8] is the first committed intermediate in the biosynthesis of tetrapyrroles. The formation of \textit{ALA}\textsuperscript{*} can occur either via the glycine pathway or the C5 pathway. The glycine pathway, also referred to as the Shemin pathway, produces \textit{ALA} from glycine and succinyl-CoA using pyridoxal phosphate as a cofactor. \textit{ALA} may also be formed from glutamate via the C5 or 4,5 dioxovaleric acid (DOVA) pathway, which forms \textit{ALA} from glutamic acid via a glutamyl-tRNA intermediate, and requires the action of an NADPH-dependent dehydrogenase, and glutamate-1-semialdehyde 2,1-aminotransferase.\textsuperscript{22,23} These reaction schemes show the complexity of the formation of \textit{ALA}, and suggest many possibilities for regulation of \textit{ALA} formation.

\textbf{Mechanism of the glycine reaction.} The mechanism of the condensation of glycine and succinyl-CoA includes the formation of the glycine-pyridoxal phosphate Schiff base, which is preceded by the deprotonation of the amine (Figure 1.4, reaction 1).\textsuperscript{24-26} The \textit{pro-R} hydrogen is exclusively removed and the \textit{pro-S} hydrogen of the glycine becomes the \textit{pro-S} hydrogen of the aminolevulinic acid.\textsuperscript{27} This reaction mechanism requires that the enzyme perform both the condensation and the decarboxylation steps and that the 2-amino-3-ketoadipic acid intermediate remain as the pyridoxal phosphate complex.\textsuperscript{28} The energy

\* See pages xvii-xviii for abbreviations
Figure 1.4: Naturally occurring reactions synthesizing δ-aminolevulinic acid. Reaction 1 shows the formation of ALA [8] from glycine and succinyl-CoA, the pathway occurring in all animals, and some plants. This reaction shows the role of pyridoxal phosphate (PLP). Reaction 2 shows the C5 pathway that is used by almost all prokaryotes and in many plants. The mechanism whereby tRNA$_{\text{glu}}$ is utilized is demonstrated.
Reaction 1

Glycine

Decarboxylation

Hydrolysis

Reaction 2

Glycine

Aminolevulinic acid

6-Aminolevulinic acid

4,5-Dioxo-5-carboxylate acid

4,5-Dioxo-5-carboxylate acid

2-Hydroxy-2-carboxylate acid

2-Hydroxy-2-carboxylate acid

8-Hydroxy-8-carboxylate acid

Hydroxypyran-1-one

NADPH

Dehydrogenase

NADPH

Dehydrogenase

ATP, Mg2+

(9)

(10)

Dioxovaleric acid

Aminolevulinic acid

Aminolevulinic acid
required for the complete synthesis of heme is provided by the succinyl-CoA substrate.

**Mechanism of the glutamate reaction.** The formation of ALA via the C5 pathway utilizes a mechanism that was discovered in plants in 1974 after years of research that showed plants poorly incorporated glycine and succinyl-CoA into tetrapyroles. The first step in the C5 pathway is the glutamyl-tRNA synthetase which charges the tRNA. This reaction requires ATP and Mg²⁺ and the tRNA substrate appears to be the same tRNA used for both ALA and protein formation (Figure 1.4, reaction 2). The tRNA-bound glutamate is reduced in a pyridine nucleotide dependent reaction to form a glutamate-1-semialdehyde type of product. The exact nature of the intermediate has yet to be delineated but it could be glutamate-1-semialdehyde, or its hydrated hemiacetal. Study of the synthetic glutamate-1-semialdehyde synthesized by Kannangara and co-workers appears to show that the compound is the cyclic ester between the γ-carboxyl and the hydrated aldehyde group, 2-hydroxy-3-aminotetrahydroxopyran-1-one (HAT) [9]. HAT is much more stable to heat and in aqueous solution since this compound contains no free aldehyde or carboxyl groups and HAT does form ALA. The next compound could be 4,5-dioxovaleric acid [10], but if this compound is an intermediate, it appears to remain enzyme bound. Transamination is the final step to ALA and appears to be an intermolecular transfer, suggesting that 4,5-dioxovaleric acid could indeed be an intermediate.

**Glycine or glutamate pathway?** The formation of ALA via two different pathways indicates that either one, the other, or both may be
involved in formation of heme in animals. The glycine pathway was
discovered in animals first, and the C5 pathway discovered in plants,
suggesting that the two pathways are segregated between the plant and
animal kingdoms. However, ALA synthase has been shown to be
functional in \textit{Rhodobacter spheroides} \textsuperscript{45} and \textit{Rhodopseudomonas palustris} \textsuperscript{46,47} with no evidence of the C5 pathway. The green
photosynthetic phytoflagellate \textit{Euglena gracilis} appears to contain both
synthetic pathways, with the C5 pathway supplying ALA exclusively for the
formation of plastid pyrroles, while the Shemin pathway supplies ALA for
all of the other pyrroles.\textsuperscript{48-50} Despite the existence of ALA synthase in \textit{R. spheroides}, and \textit{R. palustris}, it appears that ALA synthase does not
significantly contribute to ALA formation in the higher photosynthetic
plants, although certain plant cells do contain ALA synthase.\textsuperscript{51-60} A
survey of photosynthetic bacteria showed that the C5 pathway is distributed
among all the bacterial groups and may be the more primitive pathway,
while the glycine pathway is only found in the purple nonsulfur bacteria, \textit{R. spheroides}, \textit{R. palustris}, and \textit{Rhodospirillum rubrum}.\textsuperscript{61} The widespread
existence of dual pathways in higher plants has yet to be unequivocally
demonstrated.

The formation of ALA in animals is obviously supported by the
glycine pathway, since animals were used to discover the steps in
porphyrin biosynthesis. However, since the formation of ALA can be
accomplished using two different mechanisms utilizing common
substrates, the possibility exists that the glutamate pathway could also
support porphyrin biosynthesis in animals. The enzyme L-alanine:4,5-
dioxovaleric acid (DOVA) aminotransferase (E.C. 2.6.1.43) does exist in the mitochondrial matrix of mammalian cells, except for humans, where it is localized to the peroxisome. DOVA aminotransferase can convert 4,5-dioxovaleric acid to ALA in vitro. DOVA aminotransferase, in vitro, forms ALA at a much higher rate than produced by basal ALA synthase in vitro, 24 nmole ALA formed/hr/mg protein for DOVA aminotransferase and 0.64 nmole ALA formed/hr/mg protein for ALA synthase. However it is not clear that the required substrate, 4,5-dioxovaleric acid, is present in the mitochondrial matrix, or peroxisome, and there is no increase in DOVA aminotransferase in the presence of porphyrinogenic compounds as seen with ALA synthase. There is only one unconfirmed report describing the utilization of glutamate for the formation of ALA in animals. Despite the high activity for DOVA aminotransferase in vitro there is no evidence to support the hypothesis that DOVA aminotransferase functions to provide ALA for animals. The lack of induction of DOVA aminotransferase in the presence of porphyrinogenic compounds, and the induction of ALA synthase, argues against the function of DOVA aminotransferase as a supplier of endogenous ALA. The primary activity of this enzyme appears to be an L-alanine:glyoxylate aminotransferase (E.C. 2.6.1.44). In humans, type 1 hyperoxaluria is caused by the incorrect localization of this protein into the mitochondria instead of the peroxisome. The glycine pathway appears to be the only physiologic pathway for ALA formation in mammals.

**Formation of porphobilinogen.** Porphobilinogen [5] (PBG) is formed from the condensation of two molecules of ALA by ALA dehydratase, a zinc
containing cytosolic enzyme.\textsuperscript{70-72} The ALA dehydratase is a homo-octamer that condenses two molecules of ALA in a Knorr type reaction with the ellision of two molecules of water. The mechanism of reaction has been investigated for mammalian enzymes as well as bacterial enzymes, and is identical for all dehydratases investigated.\textsuperscript{73-75} Zinc appears to play a role as a structural element, not a catalytic center\textsuperscript{76} and is required for the enzyme binding a second molecule of ALA.\textsuperscript{74}

\textbf{Formation of hydroxymethylbilane.} Porphobilinogen deaminase (hydroxymethylbilane synthase) (E.C. 4.3.1.8) catalyzes the head to tail polymerization of four molecules of porphobilinogen to form 1-hydroxymethylbilane, a linear tetrpyrrole, and the last intermediate of the heme biosynthetic pathway to be discovered. The mechanism of the reaction is complex and created many postulated, but erroneous reaction products such as the headless dipyrrrole,\textsuperscript{78} the N-alkylporphyrinogen,\textsuperscript{79} and the aminomethylbilane.\textsuperscript{80-82} The latter is the closest to reality and was only eliminated after transient NMR experiments on enzymatically formed products.\textsuperscript{83,84} The proposed product, 1-hydroxymethylbilane (HMB), was synthesized and found to be the substrate for uroporphyrinogen III synthase.\textsuperscript{85} This intermediate is quite unstable with a half-life of 4.5 minutes at 37°C\textsuperscript{84} and rapidly undergoes uncatalyzed cyclization to uroporphyrinogen I. The I isomer of uroporphyrinogen is not the natural substrate for uroporphyrinogen decarboxylase, although it will be decarboxylated to form coproporphyrinogen I; however the I isomers will not lead to the formation of heme. The naturally occurring III isomers will be preferentially formed in acid solutions of porphobilinogen, with the
Figure 1.5: Formation of porphobilinogen from δ-aminolevulinic acid. Even though ALA dehydratase contains zinc, this metal does not appear to be directly involved in the catalysis, and the mechanism shown reflects the absence of the zinc at a catalytic center. The enzyme is involved in the formation of a Schiff base with the first substrate. The lysine that functions as the catalytic site has been identified.77
molar ratio of isomers; III:IV:II:I::4:2:1:1,\textsuperscript{85,86} which indicates that the thermodynamically favored uroporphyrinogen is the III isomer. The syntheses of heme, chlorophyll and cobyric acid depend solely on the enzymatic steps leading to the formation of uroporphyrinogen III. One interesting aspect of this reaction is the utilization of a dipyrromethane cofactor \cite{11} to provide an anchor on which to build the tetrapyrrole hydroxymethylbilane. The dipyrromethane is assembled from two molecules of PBG, remains enzyme bound once formed, and catalyzes the formation of numerous molecules of hydroxymethylbilane (Figure 1.6).\textsuperscript{87,88}

**Formation of uroporphyrinogen III.** Uroporphyrinogen III synthase (E.C. 4.2.1.75) must not only cyclize the hydroxymethylbilane substrate but rearrange the orientation of the D-ring of the uroporphyrinogen I \cite{13} to form the III isomer \cite{15} (Figure 1.7). A spirane rearrangement has been postulated as the mechanism\textsuperscript{89} and synthetic studies have supported this hypothesis.\textsuperscript{90} Studies of the enzyme suggest that the cyclization of the 1-hydroxymethylbilane \cite{12} and the rearrangement occurs at the same site on the enzyme (Figure 1.8).\textsuperscript{91}

**Formation of vitamin B\textsubscript{12}.** Uroporphyrinogen III is a universal intermediate for the formation of tetrapyrroles, and in animals, but not in humans, uroporphyrinogen is a precursor of cobyric acid \cite{6}, the macrocycle of vitamin B\textsubscript{12} \cite{7} The natural synthesis of this compound, although not completely delineated, is quite complex and is reviewed elsewhere.\textsuperscript{6,7}

**Formation of coproporphyrinogen III.** The formation of coproporphyrinogen III \cite{22} is accomplished by the decarboxylation of the
Figure 1.6: Formation of the dipyrromethane cofactor and 1-hydroxymethylbilane from porphobilinogen. This reaction scheme shows the utilization of porphobilinogen to form the dipyrromethane cofactor (C) [11] and then the head to tail condensation to form an enzyme-bound hexapyrrole. Each step requires the deamination of one molecule of porphobilinogen, and once the hexapyrrole is formed the product is hydrolyzed to leave the dipyrromethane cofactor enzyme-bound, yielding 1-hydroxymethylbilane [12].
Figure 1.7: Structures of various porphyrinogens. The tetrapyrrrole derived macrocycle depicts a general porphyrinogen. The different isomers and compounds are generated by changing either the positioning or substitutions at any of the eight bridging monopyrrrole derived carbons. The table will be used here and throughout and the numbers referring to the compounds in the text are listed in order through the table, and not necessarily in order through the text, in order that identification of individual porphyrinogens is facilitated.
<table>
<thead>
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<th>[n]</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
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</tr>
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<td>M</td>
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<td>V</td>
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<td>P</td>
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</tr>
</tbody>
</table>

A = Acetate  
E = Ethyl  
He = -CHOH-CH₃  
M = Methyl  
P = Propionate  
V = Vinyl
Figure 1.8: Reaction mechanism for uroporphyrinogen III synthase. The reaction mechanism demonstrates the proposed spirane rearrangement of the D-ring leading to the III isomers for uroporphyrinogen and coproporphyrinogen. The chemical cyclization of 1-hydroxymethylbilane would lead to the I isomers for uroporphyrinogen and coproporphyrinogen, neither of which is a substrate for the heme biosynthetic pathway.
acetate side chains of uroporphyrinogen III by uroporphyrinogen III decarboxylase (UROD) (E.C. 4.1.1.37). UROD catalyzes the decarboxylation of each of the acetic acid side chains to methyl groups. The natural substrate is the III isomer of uroporphyrinogen, but the I [13] isomer functions as a substrate, albeit at a lower rate. The synthetic II [14] and IV [16] isomers can also function as substrates.\textsuperscript{92} The substrate binding site of UROD must accommodate not only the 8-carboxyl porphyrinogen [15], but the 7- [17], 6- [18], and 5- [19] carboxyl porphyrinogen substrates, each of which is increasingly more hydrophobic. In order to catalyze each successive decarboxylation the product of the previous reaction must be completely released, and rebound in a different configuration. The impairment of the rebinding step may lead to accumulation of specific porphyrins under certain conditions, and will be discussed later. The reaction catalyzed by UROD was thought to be specific with the first decarboxylation occurring on the acetate of the D-ring, next the A-ring, then the B-ring and finally the C-ring acetate is decarboxylated.\textsuperscript{93,94} However recent data indicating that decarboxylation of the porphyrinogen occurs randomly have challenged this theory.\textsuperscript{95-97} The issue remains unresolved.\textsuperscript{98} In the proposed mechanism, the protonated pyrrole ring functions as an electron withdrawing group, analogous to the pyridine ring of pyridoxal phosphate, and facilitates removal of the acetic carboxyl group as carbon dioxide (Figure 1.9). This mechanism would explain why the porphyrinogens, not the porphyrins, are substrates of this enzyme.\textsuperscript{99,100}

Formation of protoporphyrinogen IX. The oxidative decarboxylation of coproporphyrinogen III is the antepenultimate step in heme
biosynthesis. Coproporphyrinogen oxidase (E.C. 1.3.3.3) catalyzes the stepwise decarboxylation of the A- and B- ring propionate side chains to give harderoporphyrinogen [24], and subsequently protoporphyrinogen IX [26].\textsuperscript{101-104} In eukaryotes, this enzyme is located in the inter-membrane space of the mitochondria.\textsuperscript{105,106} The catalytic characterization demonstrated that only harderoporphyrinogen was formed, and not isoharderoporphyrinogen [25], with this intermediate remaining enzyme bound. Two postulated mechanisms can account for the enzymatic reaction, one involving a β-hydroxypropionate intermediate,\textsuperscript{107,108} and one involving an intermediate imine and a hydride ion (Figure 1.10).\textsuperscript{109} A distribution of the two different mechanisms between anaerobic and aerobic conditions has been proposed,\textsuperscript{6,108,110} since the β-hydroxypropionate intermediate can only be formed under aerobic conditions. Coproporphyrinogen oxidase substrate specificity precludes coproporphyrinogens I [20] and II [21] as substrates,\textsuperscript{101,104} but coproporphyrinogen IV [23] can be converted to protoporphyrinogen XIII [27].\textsuperscript{104,111,112} The lack of a requirement for a metal ion or cofactor suggests that a hydroxylation mechanism does not exist for coproporphyrinogen oxidase, and it is possible that the hydride ion pathway functions as the sole pathway for the oxidative decarboxylation of coproporphyrinogen.\textsuperscript{109}

**Formation of protoporphyrin IX.** Protoporphyrinogen IX is oxidized in an oxygen-dependent six electron oxidation catalyzed by the mitochondrial enzyme, protoporphyrinogen oxidase (E.C. 1.3.3.4).\textsuperscript{113,114} Although protoporphyrinogen IX is rapidly oxidized \textit{in vitro}, chemical
Figure 1.9: The reaction mechanism for uroporphyrinogen decarboxylase. The mechanism is displayed for only one ring, although each decarboxylation occurs via the same mechanistic pathway. The use of the pyrrole nitrogen as an electron donor precludes uroporphyrin as a substrate since the pyrrole carbon electrons can not function as an electron acceptor due to the extensive $\pi-\pi$ bonding in the conjugated porphyrin macrocycle.
oxidation in vivo is not a step in the heme biosynthetic pathway, although accumulation of protoporphyrin occurs under certain conditions. The sensitive nature of the substrate has made characterization of this enzyme quite difficult. Other substrates for protoporphyrinogen oxidase are mesoporphyrinogen IX [28], protoporphyrinogen XIII [27], and 2(4)vinyl-4(2)hydroxy-ethyl deuteroporphyrinogen [29,(30)]. Cobalt protoporphyrin, bilirubin, and the ditaurine conjugate of bilirubin have all been reported to be inhibitors of protoporphyrinogen oxidase activity.

**Formation of heme.** Ferrochelatase (E.C. 4.99.1.1), properly protoheme ferrolyase, catalyzes the insertion of ferrous iron into the protoporphyrin IX macrocycle. Although the reaction can occur non-enzymatically, there is no doubt of the physiological importance of ferrochelatase. The $K_m$ and $V_{max}$ for the porphyrin increase in the order protoporphyrin IX=hematoporphyrin IX<mesoporphyrin IX<deuteroporphyrin IX and the metals in the order of $Co^{2+}=Ni^{2+}<Fe^{2+}<Zn^{2+}$. Other metals, metalloporphyrins (including heme), and N-methylated porphyrins are inhibitory. Lead is particularly interesting since exposure of animals to lead leads to increased production of zinc-protoporphyrin. Ferrochelatase has an absolute requirement for ferrous iron, and it seems that the ferrochelatase is associated with the mitochondrial electron transport complex I, which has an NADH-dependent ferric iron reduction system, although the reduction of iron may occur elsewhere as well.

**Formation of chlorophylls.** The first step in chlorophyll synthesis is the insertion of magnesium into protoporphyrin IX by magnesium
Figure 1.10: Mechanism of the reaction catalyzed by coproporphyrinogen oxidase to form protoporphyrinogen. The mechanism depicted shows the hydride ion mechanism and not the β-hydroxypropionate intermediate that has also been proposed.
chelatase to form magnesium protoporphyrin.\textsuperscript{147} Since magnesium is naturally divalent, no reduction step is necessary; however, ATP is required for this activity.\textsuperscript{148} Zinc protoporphyrin is a product of this enzymatic activity as well.\textsuperscript{148} The formation of chlorophyll and other tetrapyrrole-derived photosynthetic pigments are completely dependent on the biosynthesis of protoporphyrin IX by the enzymes discussed previously. Reviews of chlorophyll metabolism may be found elsewhere.\textsuperscript{6,7,36}

**Formation of heme derivatives.** Heme derivatives are used in several cytochromes, such as cytochrome oxidase and cytochrome c. The biosynthetic steps required to form heme \(a\), contained in cytochrome oxidase, are unknown but it appears to require an intermediate derived from mevalonate, probably farnesyl phosphate, to form heme \(a\).\textsuperscript{149} Cytochrome c contains covalently bound heme attached via a thioether bond between protein cysteines and the vinyl groups of the A and B rings of the porphyrin.\textsuperscript{150}

**Summary of heme biosynthesis.** The complexity and diversity of the formation of tetrapyrroles are a marvel of nature, and present many interesting problems, from discovering the stereochemistry of substrates and products, to understanding the features involved in regulating the synthesis of tetrapyrroles. Understanding of the features of the heme biosynthetic pathway is important in understanding possible sites of regulation, and ultimately, in formulating methods to control the diseases associated with disorders of normal heme biosynthesis. The features of the regulatory mechanisms will be discussed, along with features of the
porphyrias, the regulation of heme degradation, and the selected properties of some of the compounds studied in this work.
Introduction. The regulation of heme biosynthesis in higher animals will be the focus of this discussion, although regulation of heme in bacteria and lower eukaryotes is also of great importance, especially relating to the field of herbicides. The regulation of heme in animals is divided into two different classifications, erythroid and non-erythroid regulation. The requirements for heme in these cell types is drastically different since erythroid cells need large amounts of heme to supply heme for hemoglobin, which accounts for about 80% of total body heme. The liver is a unique situation of a non-erythroid cell type, due to the large demand for heme by cytochrome P-450s.

Regulation of hepatic heme biosynthesis. Regulation of hepatic heme biosynthesis is accomplished through modulation of ALA synthase activity, since this activity is rate-limiting under normal conditions. The activity of PBG deaminase in human liver, under normal conditions, is about 2-fold greater than that of ALA synthase. The short half-life of ALA synthase protein, between 30 and 180 minutes, uniquely suits the rapidly changing needs for heme in the hepatocyte, since the average half-life of mitochondrial proteins is 5 days.

The major metabolite known to modulate ALA synthase activity is heme, the end product of the biosynthetic pathway. Many different mechanisms for this regulation have been proposed, including: direct inhibition by heme, repression of translation, increased degradation of the
mRNA, repression of transcription, and inhibition of mitochondrial translocation.

Inhibition of ALA synthase activity by heme. Heme at high, non-physiologic concentrations, was reported to inhibit ALA synthase in chick embryo liver cell cultures,\textsuperscript{168-170} and in a partially purified rat liver enzyme preparation.\textsuperscript{171,172} It had been proposed that the mitochondrial localization of both ALA synthase and ferrochelatase would allow locally high concentrations of heme,\textsuperscript{173-175} and that inhibition of the synthase might therefore be a regulatory mechanism. The three terminal enzymes in heme biosynthesis appear to form a metabolic channeling complex although there are no data to support the involvement of ALA synthase or heme-mediated inhibition of the synthase in such a complex.\textsuperscript{123,176} Other data have shown that increases in heme generation do not decrease ALA synthase activity\textsuperscript{177} and studies on the purified enzyme show that heme is not an inhibitor.\textsuperscript{178} The consensus opinion by investigators in the field is that direct inhibition of ALA synthase by heme is not physiologically important.\textsuperscript{178-180}

Repression of ALA synthase mRNA translation. Some investigators had reported that heme affected the translation of ALA synthase mRNA,\textsuperscript{169,181-183} and that in heterogeneous cell-free translation systems 20 \(\mu M\) heme inhibited elongation of ALA synthase protein.\textsuperscript{184} However, in post-mitochondrial supernatants from chick embryo liver 10 \(\mu M\) heme did not inhibit polysome elongation\textsuperscript{185} and in other heterogeneous elongation systems 100 \(\mu M\) heme did not inhibit polysome elongation.\textsuperscript{159} There are no data to show that heme, at any concentration prevents, or inhibits,
initiation of translation of ALA synthase mRNA, and the sparse, conflicting data published, strongly argue against a translational control mechanism for ALA synthase.

Increase of ALA synthase mRNA degradation. Reports suggesting transcriptional control of the ALA synthase gene were based on the observation that ALA synthase mRNA levels were decreased in the presence of heme. Recent data in chick embryo liver cell cultures show that the half-life of ALA synthase mRNA is decreased from 180-220 minutes to 70-90 minutes in the presence of heme (1-10μM), while no change in ALA synthase gene transcription was observed. The existence of a labile, heme-modulated RNase to mediate this effect was postulated since addition of cycloheximide prevented the reduction of the half-life of the ALA synthase mRNA by heme. Whether this phenomenon is due to a specific RNase or a more generalized effect of translation of mRNA stability remains to be clearly elucidated. Results obtained in rats contradict these results, in that others reported that the half-life of ALA synthase mRNA in rat liver was not changed in the presence of heme, but rather that ALA synthase gene transcription was decreased. These differences could be due to differences in the animal models, or an increase in the endogenous heme of rat liver due to allylisopropylacetimide (AIA) treatment. The reported half-life of the rat ALA synthase mRNA was 20 minutes suggesting that endogenous heme levels were already high and the half-life of the mRNA was already decreased.

Transcriptional repression of the ALA synthase gene. The half-life of the mRNA of hepatic ALA synthase is reported to be short, from 1-5
hours,\textsuperscript{169,176,189,190,193,194} and \textit{a priori} suggests that transcriptional regulation could be an effective site for the control of ALA synthase activity. Earlier reports presented indirect data suggesting transcription was a site of heme-mediated regulation,\textsuperscript{186-188} and some later reports presented nuclear transcription experiments supporting this hypothesis.\textsuperscript{192,195,196} In the chick embryo liver cell culture there is no effect of heme on the transcription of ALA synthase,\textsuperscript{189} while in the rat systems heme may affect transcription.\textsuperscript{192,195,196} The existence of a heme-activated transcription factor in yeast\textsuperscript{197} demonstrate that heme-binding proteins can modulate transcription in certain systems. The existence of a similar protein in animals has yet to be demonstrated. The differences between transcriptional regulation and decreases in mRNA stability required further investigation.

\textbf{Decrease of mitochondrial import of pre-ALA synthase.} ALA synthase is synthesized on cytosolic ribosomes\textsuperscript{165,173,185,198} and must be imported into the mitochondria in order to be physiologically active. Both the precursor (cytosolic) and mature (mitochondrial) forms are catalytically active \textit{in vitro},\textsuperscript{173,199-204} but \textit{in vivo} succinyl CoA is only found in the mitochondria, not in the cytosol. Heme has been found to inhibit the translocation and processing of the cytosolic, precursor form into the mitochondria,\textsuperscript{205-210} and this has been proposed as a site for regulation.

Recent data from Lathrop and Timko\textsuperscript{211} demonstrated the existence of a heme regulatory motif (HRM) in the amino terminal of the pre-ALA synthase protein. Heme inhibited the transport of both the purified mouse erythroid ALA synthase and a chimeric protein containing the HRM and
mitochondrial ornithine transcarbamoylase (OTC). The non-chimeric OTC translocation was not inhibited by heme. Site directed mutagenesis of the conserved cysteine (Cys\(^{10}\)→Ser \(^{10}\)) in ALA synthase produced a protein whose mitochondrial transport was unaffected by heme. The identified HRM (amino acid sequence: LCPVL) was inserted into the OTC protein and this again conferred heme regulation of the protein transport. Site directed mutagenesis of conserved cysteine (Cys\(^{10}\)→Ser \(^{10}\)) again produced a chimeric protein whose mitochondrial transport was unaffected by heme. Although the experiments were carried out with the mouse erythroid ALA synthase, the same sequences exist in hepatic forms of ALA synthase, rat hemopexin\(^{212}\) and the yeast heme-activated transcription factor.\(^{197,213,214}\) The existence of this motif throughout several heme-activated proteins suggests that this motif may be a general mechanism for metabolic control.

**Summary of regulatory mechanisms.** In summary, there is evidence for heme-dependent regulation of ALA synthase transcription,\(^{192,195,196}\) enhancement of mRNA degradation,\(^{189,190}\) and reduction of mitochondrial uptake of the precursor protein.\(^{205-208,210,215,216}\) One researcher studying the effects of heme on the half-life of ALA synthase states, "Addition of heme to the cultured hepatocytes decreased the steady-state level of ALA synthase mRNA by approximately 50%, whereas ALA synthase enzyme activity was almost completely suppressed."\(^{189}\) The most probable reason for these observations is the existence of two or more physiologically relevant sites of regulation. Although there is not enough data to form a general model describing the regulation of hepatic ALA synthase, recent evidence suggests that in chick
embryo liver cells the two major sites of regulation are the HRM-dependent mitochondrial import and the decrease in the half-life of the ALA synthase mRNA.189,206,207,209,215
The regulatory heme pool

Suppression of ALA synthase activity by excess heme. The observed effects of heme on ALA synthase led to the formulation of a concept referred to as the free, regulatory, or unassigned heme pool. Since heme is rapidly bound by proteins, the heme pool will not be referred to as "free" heme, although some small fraction of cellular heme must always be unbound (Figure 1.11). Since the major function of cellular heme that will be considered is its role in regulation of ALA synthase activity, this heme will be referred to as regulatory heme. Exogenous heme was found to rapidly inhibit heme synthesis in cultured chick embryo cells in serum free medium with $K_r=10^{-7}$ M, in media containing bovine serum albumin $K_r=2\times 10^{-6}$ M and with human serum albumin $K_r=2\times 10^{-8}$ M. Microsomal and mitochondrial cytochromes (P-450, b5, etc...) bind heme with a $K_d \leq 10^{-7}$ M whereas heme oxygenase activity is induced by heme concentrations $>10^{-6}$ M. These data suggest that under normal circumstances, heme will first supply the demand for cellular hemoproteins, next heme will modulate the activity of ALA synthase to prevent excess heme formation, and finally, if excess heme is present, heme oxygenase will be induced.

Induction of ALA synthase activity by heme depletion. A corollary to the regulatory heme pool model for ALA synthase repression is the hypothesis that a reduction of intracellular heme will result in the induction of ALA synthase. Chemicals that destroy heme (e.g., AIA, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), or
**Figure 1.11:** Schematic representation of the regulatory heme pool. Regulation of heme metabolism is thought to be regulated via a regulatory heme pool. This model proposes that increases in cellular heme concentration serve to decrease the activity of ALA synthase (light blue), the enzyme that catalyzes the rate limiting step in heme biosynthesis. This regulation can occur at the site of mitochondrial translocation of pre-ALA synthase, at the level of mRNA by altering message half-life, and at the level of transcription. If the amount of heme in the cell increases then heme oxygenase (green) will increase to degrade any excess heme. The largest factor affecting heme levels in the cell is the amount of apo-cytochrome P-450 (pink) that requires heme in order to be catalytically active. This enzyme can be induced by a variety of drugs and heme may act as a positive regulator of cytochrome P-450 gene expression. The theory of how metalloporphyrins function to decrease ALA synthase activity suggests that inhibition of heme oxygenase occurs first, which causes an increase in the regulatory heme pool. This heme would then exert a repressive effects on ALA synthase. The schematic also shows the sub-cellular distribution of the heme metabolic pathway (red).
Hepatocyte

Nucleus

Heme Oxygenase

HO mRNA

ALA Synthase

Cytochrome(s) P-450

Apo

Cytochrome P-450

Regulatory Heme Pool

Heme Oxygenase Protein (Microsomal)

Biliverdin

Carbon Monoxide

Ferrous Iron

Heme

Bilirubin

ALA Synthase mRNA

Turns Transcription ON

Increase mRNA Degradation

ALA Synthase mRNA

Turns Transcription OFF

Decrease Protein Translocation

ALA Synthase Pre-Protein

ALA

Proto

Proto'gen

COPRO'gen ← URO'gen ← PBG ← ALA

Heme

ALA

Glycine + Succinyl CoA

Fe^{2+}
griseofulvin\textsuperscript{236-238}, or chemicals that inhibit heme synthesis (e.g., 4,6-dioxoheptanoic acid (DHA),\textsuperscript{239-241} deferoxamine,\textsuperscript{242,243} or N-methylated porphyrins\textsuperscript{231,244}) can produce modest increases in ALA synthase activity. Inhibitors of heme biosynthesis are often used in conjunction with other porphyria-producing chemicals as an experimental model for porphyria.\textsuperscript{236,240,245,246} Experimental results with phenobarbital, and other porphyrinogenic chemicals demonstrated an increase in apocytochrome P-450 and ALA synthase activity,\textsuperscript{224,225,235,247-250} and induction of apohemoproteins was envisioned to cause an increase in demand of heme, thereby leading to an induction of ALA synthase. One study in chick liver cell suspensions reported an increase in ALA synthase activity after a transitory exposure to heme alone.\textsuperscript{251} These experiments have not been confirmed, and others have been unable to reproduce the results (E. Cable, unpublished results; P. Sinclair, personal communication). Recently, other experiments investigating the relationship between induction of cytochrome P-450 and ALA synthase have shown that mRNAs for P-450 and ALA synthase were simultaneously increased after treatment with phenobarbital-like drugs.\textsuperscript{252,253} Increases in ALA synthase mRNA were observed after AIA treatment, even in the presence of cycloheximide, indicating that synthesis of new molecules of apocytochrome P-450 is not required for induction of ALA synthase by AIA.

Recent transient transfection experiments with the rat hepatic ALA synthase cDNA have identified both positive and negative cis-acting sequences\textsuperscript{254} along with a consensus nuclear respiratory factor responsive element sequence (NRF-1 RE). This consensus sequence identified in other
respiratory proteins allows the cell to control the supply of proteins involved in cellular respiration.\textsuperscript{255-257} The existence of NRF-1 RE sequence in the ALA synthase 5' flanking region suggests the importance of this factor in controlling the availability of heme for mitochondrial cytochromes. This would indicate that ALA synthase is directly controlled via the NRF-1 RE instead of being elevated only after the mitochondrial apo-hemoproteins depleted the regulatory heme pool. The biological importance of this sequence is underscored by the fact that the erythroid ALA synthase gene lacks NRF-1 RE (see next section).\textsuperscript{254}

Although no "drug-responsive" elements have been identified to date in the hepatic ALA synthase gene, available data show that heme depletion alone may generate a small (≤ 2-fold) induction of ALA synthase, but that heme depletion \textit{per se} will not give rise to the high activities (≥ 5-fold) of ALA synthase observed in many different experimental systems.\textsuperscript{231,232,248,258-260} Heme depletion can lead to synergistic inductions of ALA synthase in the presence of porphyrinogenic compounds.\textsuperscript{236,240,245,246} Thus, the regulatory heme pool may be viewed as primarily functioning to decrease ALA synthase activity when replete while permitting, but not causing, increases of ALA synthase activity when depleted.

**Regulation of erythroid heme biosynthesis.** Erythroid heme biosynthesis represents a special case scenario for regulation of heme synthesis. In developing red blood cells, ALA synthase activities must remain elevated, compared to non-differentiated cells, even in the presence of high heme concentrations.\textsuperscript{261-264} Differential regulation of heme
biosynthesis in erythroid versus non-erythroid cells is accomplished primarily through two separate genes encoding ALA synthase.\textsuperscript{265-268} Induction of ALA synthase seems to play a key role in erythroid development.\textsuperscript{269} Erythropoietin\textsuperscript{270,271} and iron availability\textsuperscript{272-274} play key roles in induction of ALA synthase activity during differentiation of erythroid cells. Erythroid ALA synthase seems to be primarily under control of the iron responsive element binding protein (IRE-bp).\textsuperscript{272,274} This protein was first described for its ability to bind transferrin receptor mRNA\textsuperscript{275-277} and ferritin mRNA.\textsuperscript{278-281} In the absence of iron the protein remains bound to a sequence specific stem-loop mRNA structure and prevents translation of the ferritin message. However, in the presence of iron, the protein binds iron and its affinity for mRNA decreases rapidly. The identification of an IRE on erythroid specific ALA synthase mRNA\textsuperscript{272,274} provided a breakthrough in understanding the induction of erythroid ALA synthase even in the presence of large concentrations of heme. Several investigators have described an aconitase activity (E.C. 4.1.2.3) associated with the IRE-bp\textsuperscript{282-285} and the nucleotide sequences from \textit{E. coli} and bovine aconitase display a remarkable similarity to the human IRE-bp.\textsuperscript{286,287} Similarities between aconitase and the IRE-bp exists for the cytosolic aconitase only, and not the mitochondrial form.\textsuperscript{286} Switching between the aconitase activity and the IRE-bp function may be regulated by the change in an iron-sulfur cluster from 4Fe-4S to 3Fe-4S.\textsuperscript{283,288-290}

Both ALA dehydratase\textsuperscript{267,291} and PBG deaminase\textsuperscript{292-295} are also differentially regulated in erythroid cells versus non-erythroid cells, but via
differential mRNA splicing. The differences between hepatic and erythroid heme biosynthesis are necessary to supply the needs for each specific cell type. Understanding the differences in function between the different cell types gives further insight into the problems faced by people with hepatic or erythropoietic porphyrias, and the possible treatments for each type.
Porphyria

Introduction. The porphyrias are a group of inherited or acquired diseases in which the fundamental defect is in heme biosynthesis. These diseases present different symptoms, and are classified due to the enzymatic defect in the heme biosynthetic pathway. The first case of human porphyria was described just over 100 years ago by Stokvis\(^1\) in a woman who had taken sulfonal. Sulfonal precipitated porphyria in 5-10\% of female patients, and barbiturates were known to precipitate porphyria as well.\(^10,15\) Other patients presenting with port-wine colored urine, abdominal pain, and psychiatric disturbances appeared independent of precipitating chemical exposure.\(^296,297\)

Many chemicals were found to precipitate or exacerbate patients suffering from porphyria and are classified as porphyrinogenic, while chemicals that did not have a deleterious effects on patients suffering from porphyria are considered safe. There are other chemicals that have either given mixed results (e.g. deleterious only in some patients), or for which experimental data are inconsistent. Animal systems have been used as models for porphyria and to test chemicals for porphyrinogenic properties. The most widely-used and reliable model is the chick embryo liver cell culture system.\(^246-248,298-310\) Patients suffering from acute hepatic porphyrias should avoid exposure to porphyrinogenic compounds, because of the extreme and debilitating symptoms and signs that may occur after such exposure.
Table 1.1: List of drugs that have been determined as porphyrinogenic, possibly porphyrinogenic and non-porphyrinogenic in either humans or cell culture models (*). Persons suffering from acute porphyria, even in the latent phase, should avoid exposure to the porphyrinogenic chemicals. In cell culture systems however, some of these drugs can be used to produce perturbations in heme biosynthesis that serve as a paradigm for human porphyrias.
Porphyrinogenic Drugs

Alcohol248,311-319
Aminoglutethimide*320
Aminopyrine321
Amitriptyline*322
Amphetamines322
Barbiturates321,323-325
Benzodiazepines326
Busulfan*322
Carbamazepine*327
Carbromal328
Chlorambucil*322
Chloramphenicol299
Chlorambucil326
Clonidine*322
Cocaine333
Colchicine322
Cyclodextrins322
Cyclophosphamide*334-336
Cycloserine322
Danazol337
Dapsone329
Dichloroacetate322
Diphenylhydantoin322
Dipyrone322
Ergot preparations321,322
Ethambutol322
Ethchlorvynol339
Ethindone*322
Ethosuximide322
Ethotoin*322
Etomidate*322
Eucalyptol322
Flufenamic acid322
Flunitrazepam325
Furosemide322
Glucocorticoids340
Glutethimide258,322
Gold preparations*322
Griseofulvin341-346
Hexachlorobenzene347-355
Hydantoins322
Hyoscine322
Indomethacin356
Isoniazid322
Isopropyl
  meprobamate322,357
Ketoprofen322
Lidocaine*322
Mephenytoin322
Meprobamate357,358
Mercury compounds358
Methoxyflurane322
Methsuximide359
Methylprednisolone360
Methyprylon322
Metoclopramide329
Metronidazole322
Metyrapone361-363
Nalidixic acid364
Nikethamide322
Nitrazepam322
Novobiocin322
Oral contraceptives365-367
Pancuronium322
Paramethadione322
Pargyline*368
Pentazocine322
Pentylethylhexazol339
Phenelzine322
Phenoxybenzamine*368
Phensuximide322
Phenyldihydroxy322
Phenytoin327
Primidone322
Probenecid*322
Progesterone369-374
Pyrazinamide322
Pyrazolones322
Ranitidine322
Spironolactone*322
Succinimides322
Sulfonamides322
Sulfonylureas322
Theophylline375,376
Tolazamide328
Tolbutamide322
Tramadol322
Trimethadione322
Valproic acid330
Xylocaine322
Possibly Porphyrinogenic Drugs

- Chlormethiazole
- Chloroquine
- Clonazepam
- Diazepam
- Enflurane
- Erythromycin
- Estrogens
- Halothane
- Hydralazine
- Imipramine
- Ketamine
- Mefenamic acid
- Meperidine
- Nitrofurantoin
- Oxazepam
- Paraldehyde
- Pethidine
- Phenylbutazone
- Pyrimethamine
- Pyridoxine
- Rifampin
- Steroids
- Tetracyclines
Non-Porphyrinogenic Drugs

<table>
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<tr>
<th>Drug Name</th>
<th>Code</th>
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<tbody>
<tr>
<td>Acetaminophen</td>
<td>322</td>
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<tr>
<td>Acetazolamide</td>
<td>*322</td>
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<tr>
<td>ACTH</td>
<td>322</td>
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<tr>
<td>Adrenalin</td>
<td>322</td>
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<tr>
<td>Allopurinol</td>
<td>*322</td>
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<td>Aminoglycosides</td>
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<td>Bumetanide</td>
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<tr>
<td>Clofibrate</td>
<td>*195,396,397</td>
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Classification of porphyria. The porphyrias are generally grouped into hepatic and erythropoietic porphyrias depending upon the major site of overproduction of heme precursors, either hepatic or erythropoietic cells. Another categorizing scheme is based on the occurrence in some patients of acute symptomatic attacks interspersed by non-symptomatic latent periods. Thus, the porphyrias are categorized as either acute or non-acute (Table 1.2).
**Table 1.2:** Enzyme defects, clinical features and inheritance of human porphyria. Classifications of porphyrias, the enzyme defects and the inheritance are listed in Table 1.2. The table is from “The porphyrias”.¹⁶¹ Note that photosensitivity is a feature of all of the porphyrias that are associated with porphyrin accumulation, whereas neurological features occur in those with accumulation of ALA. Unless otherwise stated, the isomers are the physiologic porphyrin isomers.
<table>
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- Type II POT is inherited in an autosomal dominant fashion, while type I POT is acquired after exposure to precipitating factors.

<table>
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<tr>
<th>Enzyme Defect</th>
<th>Clinical Features</th>
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<td>Protoporphyrin</td>
<td>Dominant Neur...</td>
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- Enzyme Defects
  - Protoporphyrin: ALA Dehydratase
  - Coproporphyrin I: PBG Deaminase
  - Isocoproorphyrin: Uroporphyrinogen III Decarboxylase
  - Coproporphyrin: Uroporphyrinogen III Decarboxylase
  - Protoporphyrin: Erythropoeitic Synthase
  - Coproporphyrin I: Ferrochelatase

- Clinical Features
  - Recessive: Photosensitivity
  - Dominant: Photosensitivity
  - Variable: Photosensitivity
  - Recessive: Neurological Deficiency of ALA Oxidase
  - Dominant: Protoporphyrinogen Erythropoeitic Synthase
  - Recessive: Protoporphyrinogen Erythropoeitic Synthase
  - Dominant: Prot...
Hepatic porphyrias

Acute intermittent porphyria. Acute intermittent porphyria (AIP), the most common form of the acute hepatic porphyrias,\(^{161}\) is due to an autosomal dominantly inherited defect in PBG deaminase. Almost all AIP are heterozygous for this defect, and are asymptomatic prior to puberty and approximately 90% remain asymptomatic throughout their lifetimes. Homozygotes,\(^{410}\) however, present in infancy or childhood with severe symptoms. Clinical manifestations are more common in women, it is believed due to the porphyrinogenic effects of female sex hormones. Such hormones are probably the most common class of compounds which induce attacks in acute porphyrics.\(^{411}\) During the acute phase of AIP, urinary excretions of ALA and PBG increase 20-200 fold, and excretions of uroporphyrin and coproporphyrin are also increased.\(^{412}\) Precipitation of attacks of AIP are strongly dependent on exposure to precipitating factors and one important treatment of the disease is to remove the patient from exposure to porphyrinogenic compounds.\(^{413,414}\)

The most common clinical features of acute intermittent porphyria in relapse are abdominal pain, nausea and vomiting, and constipation.\(^{161,415,416}\) The psychiatric features of this disease, depression, delirium, disorientation, and paralysis, have led to an incorrect diagnosis of patients presenting with these symptoms. This has led to hospitalization of AIP patients in mental institutions.\(^{417}\) Treatment of the psychiatric features of the disease without proper diagnosis have led to administration of drugs that further exacerbated already severe conditions.\(^{418}\)
Photosensitivity never occurs in AIP and can be used to distinguish AIP from the other porphyrias.

**Hereditary coproporphyria.** Hereditary coproporphyria (HCP) is an autosomal dominantly inherited defect of coproporphyrinogen oxidase. HCP is an acute porphyria exhibiting acute and latent phases. When symptomatic, HCP is generally more mild than AIP with similar urinary levels of ALA and PBG, but coproporphyrin excretion can be dramatically increased during the acute phase. The symptoms and signs of HCP are clinically indistinguishable from AIP except that victims of HCP can exhibit cutaneous photosensitivity. Homozygous HCP is very rare (fewer than 10 cases reported), but clinical manifestations are severe and occur early in life. Another rare variant of HCP is harderoporphyria, named because of the high levels of excreted harderoporphyrin in the urine and feces.

**Variegate porphyria.** Variegate porphyria (VP) is another acute porphyria, inherited in an autosomal dominant fashion. VP is caused by a 50% decrease in the penultimate enzyme of heme biosynthesis, protoporphyrinogen oxidase. VP is most common among South Africa whites, with evidence that all affected individuals are the offspring of two Dutch settlers from 1680. Cases of VP have been reported worldwide, and probably reflect independently occurring mutations. Chester porphyria, from a kindred in Chester, UK, is the coexistence of both variegate and acute intermittent porphyrias and all the subjects with this disorder can be traced to one couple married in 1896. Homozygous cases of VP have presented with more severe clinical
symptoms than presented by heterozygotes. The clinical features of VP are similar to those of HCP, and include photosensitivity and excretion of a high level of fecal and bile protoporphyrin.

**Severe deficiency of ALA dehydratase.** Severe deficiency of ALA dehydratase (ALAD deficiency) is the rarest porphyria, with only 4 patients described to date. One diagnosed individual was an asymptomatic 68 year old male, and another confirmed homozygotic infant. Inheritance of ALAD deficiency is autosomal recessive. There is no cutaneous photosensitivity, but there are neurologic symptoms similar to those seen with AIP.

**Porphyria cutanea tarda.** Porphyria cutanea tarda (PCT) is the most common form of porphyria, and is characterized by a decrease in uroporphyrinogen decarboxylase activity. Homozygous decreases in UROD are known as hepatoerythropoietic porphyria (HEP). PCT appears in two forms: a sporadic form (type I), and a familial form (type II). PCT is a hepatic form of porphyria and appears later in life sometimes in conjunction with exposure to alcohol, other chemicals, like polychlorinated cyclic hydrocarbons, and frequently in conjunction with increases in hepatic iron. One possible explanation for the decrease in UROD activity is the impairment of rebinding of the porphyrinogen substrate. The name for the disease is derived from the clinical hallmarks of cutaneous photosensitivity due to the accumulation of uro- and 7-carboxy- porphyrins in the skin. Appearance of PCT in children virtually assures familial inheritance. PCT patients do not suffer the acute attacks seen in the other hepatic porphyrias, and
treatment is as simple as avoidance of precipitating factors\textsuperscript{475} and phlebotomy.\textsuperscript{476-480}
Erythropoietic porphyrias

**Congenital erythropoietic porphyria.** Congenital erythropoietic porphyria (CEP) is an homozygotic, autosomal recessive, disorder of uroporphyrinogen III cosynthase. CEP is very rare, less than 300 reported cases, and is characterized by the onset of the disease in infancy, and pronounced cutaneous photosensitivity.  CEP patients (like Matthias Petry, p. 4) suffer from deposits of large amounts of cutaneous uroporphyrin, which is an overflow from the erythroid system where a predominance of porphyrin deposits can be observed. The erythropoietic nature of this disease does not produce the latent and acute phases as seen with the acute hepatic porphyrias.

**Erythropoietic protoporphyria.** Erythropoietic protoporphyria (EPP) is a heterozygotic, autosomal dominant, defect of ferrochelatase. Ferrochelatase activities half of normal probably allow the clinical symptoms of EPP to be milder than CEP although onset of EPP is still in childhood. Patients with EPP excrete protoporphyrin in the feces, although high protoporphyrin concentrations can be found in erythrocytes, plasma and bile. In some patients increases in hepatic ALA synthase may contribute to the high levels of protoporphyrin. There are no precipitating factor associated with EPP and no neurological symptoms exist.

**Additional porphyrias.** There exist examples where two different porphyrias were present in the same patient, such as Chester
porphyria and other types of dual porphyrias. Perhaps the most shocking types of porphyrias that appear are the chemically-induced porphyrias, such as the outbreak in Turkey due to ingestion of hexachlorobenzene-treated seed grain, and those that sporadically occur in workers exposed to various chemicals. Treatment of these porphyrias consist of removing the offending compound from the environment of the patient, however correct diagnosis of porphyria and correct identification of the offending compound tends to be more problematic.
Treatment of porphyria

Treatment for the erythroid types of porphyria generally consists of nothing more than preventative treatments for the symptoms, however heme therapy, discussed later, has shown some promise.\textsuperscript{498,499} Since ALA synthase in erythroid cells is not normally under tight metabolic control, the best treatment seems to be prevention of the damage that can easily occur due to the deposition of porphyrins in the skin, such as avoidance of sunlight exposure or treatment with β-carotene.\textsuperscript{500-510} These diseases could be a good model for eventually testing the effectiveness of stem cell gene therapy due to the heterozygous nature of the inheritance of the diseases.

The nature of the acute porphyrias, AIP, HCP, and VP, and the similarity of the clinical manifestations, first led investigators to postulate a defect in ALA synthase.\textsuperscript{162,511,512} Elevated ALA synthase plays a key role in the recrudescence of the acute phase of porphyria and an overproduction of heme precursors. Reversion of ALA synthase activities to normal is associated with an asymptomatic state.

Prior to the introduction of heme as a treatment for the acute porphyrias,\textsuperscript{513} the only treatment that existed was placing the patient on a high carbohydrate diet, oral glycerol\textsuperscript{514} or corn oil\textsuperscript{515} or i.v. infusion of glucose\textsuperscript{516} or fructose.\textsuperscript{517} The effects of carbohydrate on reducing ALA synthase activity were not reproducible in all situations,\textsuperscript{516} even in models of experimental porphyria.\textsuperscript{247,518-520} The use of i.v. hemin as a therapeutic
agent has become widespread and is the standard treatment for patients in the acute phase. Panhematin® (Abbott Laboratories) is the only preparation of heme approved by the FDA for use in the United States. Unfortunately, aqueous solutions of heme are notoriously unstable. Treatment of patients with the standard dose of 4 mg/kg every 12-24 hours leads to thrombophlebitis in 50% of the patients. Coagulopathy, hemolysis, transitory renal failure, and transient circulatory collapse are less frequent side effects, but have been reported.

Heme arginate (Normosang®, Leiras Oy, Turku Finland), a stabilized solution of heme, has become another agent available for heme therapy. However this treatment is not currently approved for use in the United States. Heme arginate rarely produces side effects and rapidly improves hepatic oxidative metabolism. This heme treatment, infused at 3.5 μM, is a solution of μ-oxo-dimers and high molecular weight aggregates. Heme-albumin can be used to treat acute porphyrias without the troubling side effects of aqueous hematin, while utilizing a natural protein, albumin, that binds heme.

Heme infusion as a method to reduce hepatic ALA synthase activities is the only treatment widely practiced that utilizes the biochemical knowledge about the regulation of hepatic heme biosynthesis. One of the problems with i.v. heme treatments, independent of the carrier, is the large amount of heme required in order to control the patient's heme biosynthetic pathway. Heme at these concentrations could readily induce heme oxygenase, the rate-limiting enzyme of heme degradation, thereby decreasing the concentration of administered heme.
Regulation of Heme Degradation

Introduction. Degradation of heme by heme oxygenase (E.C. 1.14.88.3) leads exclusively to the formation of biliverdin IXα.\textsuperscript{1,226,227,544-546} Mammals, but not birds, amphibians, or fish,\textsuperscript{227,547} further reduce biliverdin IXα to bilirubin IXα. The reaction is catalyzed by the enzyme biliverdin reductase (BVR) (E.C. 1.3.1.24) which is in excess so that heme oxygenase catalyzes the rate-limiting reaction in heme degradation.\textsuperscript{1,548-551} The importance of the rate of heme degradation on the control of heme synthesis has been implicated under certain conditions,\textsuperscript{251,258,552} and relates to the investigation of metalloporphyrins inhibitory for heme oxygenase as chemical modulators of ALA synthase activity.

Heme oxygenase can be induced by its substrate, heme, metal ions and oxidative stress.\textsuperscript{1,258,548,553-557} Induction of heme oxygenase activity may reflect the importance of heme degradation to prevent oxidative damage,\textsuperscript{558} and the importance of bilirubin as an anti-oxidant.\textsuperscript{559-561} Heme itself can catalyze oxidative damage of lipids, proteins, and the cellular cytoskeleton\textsuperscript{562-564} and, it potentiates damage mediated by granulocytes\textsuperscript{565}. Due to the potentially toxic effects of heme it is thought to be contained during intracellular and intercellular transport.\textsuperscript{220,566} Although some have posited that heme oxygenase exists in excess compared to its substrate heme,\textsuperscript{567} it is clear that heme oxygenase activity is rate-limiting in heme degradation and does not exist in excess as a safety feature.\textsuperscript{568}
The reaction mechanism of heme oxygenase involves a change in the oxidation state of heme from the thermodynamically favored ferric form, to the reduced ferrous form. After the iron has been reduced, molecular oxygen can be accepted as the 6th ligand and this oxygen is used to hydroxylate carbon 5. Reduction of heme oxygenase is accomplished by electrons donated from NADPH via NADPH-ferrihemoprotein reductase (E.C. 1.6.2.4). The one electron reduction of iron and the binding of oxygen are obligatory for the reaction to occur. Inhibition of heme oxygenase by some non-heme metalloporphyrins occurs because such compounds bind to heme oxygenase but do not activate oxygen.

Dysfunctions in heme degradation. Dysfunctions in heme degradation will not be directly probed in the research, however some of the results will have relevance to further understanding heme degradation. There are no known human diseases that result from a mutation of heme oxygenase. Not even a leaky mutant has been described to date suggesting an absolute requirement of heme oxygenase for survival. Most perturbations of heme catabolism result from the inability of the human body to conjugate and excrete bilirubin. Uridine diphosphoglucuronate β-D-glucuronosyltransferase (UDPGT) (E.C. 2.4.1.17) is responsible for bilirubin conjugation, and disruptions in UDPGT activity result in increases in unconjugated bilirubin in affected patients. Neonatal hyperbilirubinemia is common and is the result of increased bilirubin production and the delayed maturation of UDPGT. Crigler-Najjar syndrome, type 1, results from an autosomal recessive absence of UDPGT, while Crigler-Najjar syndrome, type II is the result of a milder defect in
UDP GT, although the inheritance has not been precisely resolved. Gilbert's syndrome causes increases in unconjugated plasma bilirubin, due to a partial UDP GT deficiency. Dubin-Johnson syndrome, Rotor syndrome, and benign recurrent intrahepatic cholestasis are all related to increases in conjugated bilirubin levels and are reviewed elsewhere.

**Treatments of dysfunctions of heme degradation.** Bilirubin disorders can be treated with metalloporphyrins that inhibit heme oxygenase, similar to the treatments that have been used in treating porphyria. Clinical trials have demonstrated the effectiveness of tin-protoporphyrin on both neonatal hyperbilirubinemia and Crigler-Najjar syndrome, since the metalloporphyrins inhibit heme oxygenase. Any studies involving inhibitors of heme oxygenase have potential clinical applications for any of the hyperbilirubinemic diseases.

**Interrelationships in heme metabolism.** The use of metalloporphyrins in treatment for hyperbilirubinemia demonstrates the effectiveness of these compounds at inhibiting human heme oxygenase. The use of tin-protoporphyrin to suppress chemically-induced porphyria in rats suggests the use of these compounds in the control of acute hepatic porphyrias. Heme compounds used to treat patients with porphyria are potent inducers of heme oxygenase, at least in culture, and probably in patients. The use of metalloporphyrins as inhibitors of heme oxygenase in the treatment of porphyria may be preferable if these compounds would be more effective than any of the heme preparations, or would enhance the effectiveness of heme so that much lower doses could be administered, and
perhaps prolong the effectiveness of heme. Metalloporphyrins, if effective, could overcome the side-effects normally seen with i.v. heme administration.\textsuperscript{529,530,534,535,606} However some metalloporphyrins produce certain side effects, most notably photosensitivity,\textsuperscript{607-610} although chemical modifications of the side chains on the pyrrole macrocycle may alleviate some of the photosensitivity.\textsuperscript{611,612} Different metals substituents may also alleviate the problem of photosensitivity.\textsuperscript{613} Porphyrins and porphyrin derivatives have even been used as photosensitizing agents in phototherapy for tumors.\textsuperscript{614-625} Although the side effects of these compounds can be significant, their usefulness as an effective treatment for porphyria has been suggested.\textsuperscript{626}

**Photosensitizing properties of metalloporphyrins.** The ideal metalloporphyrin for use in humans, for treatment of porphyria, Crigler-Najjar syndrome, and neonatal jaundice, would be potent and efficacious and would not be photoreactive. This ideal compound has not yet been described. The metalloporphyrins most frequently researched are the tin-zinc- and chromium- metalloporphyrins.\textsuperscript{582,597,620,627-633} The consensus opinion in the field is that the mesoporphyrins are a better choice for \textit{in vivo} use because of the lower reactivity of the C\textsubscript{3} and C\textsubscript{8} ethyl groups.\textsuperscript{550,611} However, there is no agreement on which metal would function best. Kappas \textit{et al.} are strong proponents for the use of tin-metalloporphyrins,\textsuperscript{577,578,580,585,596,600,604} while others suggest porphyrins containing zinc- or chromium.\textsuperscript{582,583,601,607-609,613,634}

The systems utilized to determine phototoxicity and photoreactivity have generally been \textit{in vitro},\textsuperscript{613,629,635-638} although one study has tested the
effects of tin-protoporphyrin on the survival of neonatal rats.\textsuperscript{639} The in vitro experiments have used solutions with metalloporphyrin, with various organic molecules, such as histidine, tryptophan, human serum albumin, medium-chained triglycerides, and NADPH.\textsuperscript{613,636} This type of in vitro system indicated the severe photosensitizing properties of zinc porphyrins,\textsuperscript{629} by testing the degradation of tryptophan as a measure of singlet oxygen production.\textsuperscript{640} The results demonstrate that within several minutes zinc-mesoporphyrin generated enough singlet oxygen to photodegrade tryptophan at a rate 2.5 times faster than tin-mesoporphyrin. This research tested for the ability of metalloporphyrins to inhibit heme oxygenase activity and found that zinc-mesoporphyrin was much less effective than tin-mesoporphyrin or zinc-deuteroporphyrin-2,4-bisglycol,\textsuperscript{629} a result that conflicts with other experimental data.\textsuperscript{582,634,641,642} Others have found that zinc meso- and zinc proto- porphyrins are some of the least photoactive metalloporphyrins along with manganese meso- and manganese proto- porphyrins, chromium meso- and chromium protoporphyrins, and nickel protoporphyrin.\textsuperscript{613} These results were based on the generation of carbon monoxide in the presence of NAD(P)H\textsuperscript{636} and the photodegradation of bilirubin.\textsuperscript{637} The heme oxygenase inhibition studies supported previously published data.\textsuperscript{582,583,634,643} Probably the most convincing data supporting the use of zinc-mesoporphyrin for human use was the mortality studies of metalloporphyrins in rats where LD\textsubscript{50} for tin-protoporphyrin was 11.7 \(\mu\)mol/kg body weight.\textsuperscript{639} Tin-mesoporphyrin caused 42\% mortality at 30 \(\mu\)mol/kg body weight, while zinc-mesoporphyrin caused no mortality at 45 \(\mu\)mol/kg body weight.\textsuperscript{639} While the bolus
administration of metalloporphyrin was much larger than that used in humans,\textsuperscript{626} the regimen used in patients requires the infusion of up to 1 mg/kg/day over the course of several days.\textsuperscript{597-601} Since the pharmacokinetics of metalloporphyrins in humans has not exhaustively been studied\textsuperscript{595,644} the less potentially fatal compounds deserve consideration for use.
CHAPTER II

RESEARCH OBJECTIVES

Primary chick embryo liver cells as a model for hepatic heme metabolism. Primary chick embryo liver cell cultures are a simple culture system primarily consisting of hepatocytes, although other cell types are included. This relatively inexpensive system does not require an extracellular matrix, unlike mammalian liver culture systems. These cultures have been shown to retain normal levels and inducibility of key enzymes related to heme metabolism. An initial objective was to use the chick embryo liver cell culture system to study the effects of iron on the induction of heme oxygenase, ALA synthase, cytochrome P-450's and porphyrin accumulation, since iron was known to potentiate heme synthesis under certain conditions.

Additionally, the chick embryo liver cell culture system was used to test the porphyrinogenicity of different terpenes, in relation to the disease suffered by Vincent van Gogh. The structures of the chemicals tested here and elsewhere are shown in Figure 2.1. These preliminary results were designed to demonstrate the utility of the chick embryo liver culture model for studying hepatic heme metabolism, and to test the ability of chemicals to potentiate, or cause porphyria.

Synergistic effects of iron on induction of heme oxygenase by glutethimide. Iron is known to potentiate the onset of PCT in humans and animal models, and to potentiate the induction of ALA
Figure 2.1: Structures of drugs tested in chick embryo liver cell cultures. The drugs shown are either known as porphyrinogenic or were tested for their ability to be porphyrinogenic. Glutethimide [31] was the compound used throughout the research as a prototypical cytochrome P-450 IIB inducer, while camphor [32], thujone [33], and α-pinene [34] were subsequently shown to induce cytochrome P-450 IIB and to be porphyrinogenic (Chapter IV). The structure of 20-methylcholanthrene [35], a cytochrome P-450 IA inducer, is shown to emphasize the structural differences between the chemicals that induce the IA forms of P-450 and those that induce the IIB forms of P-450, although a clear picture of the structural requirements for inducers of P-450 IIB have yet to emerge.
20-methylcholanthrene

Glutethimide

Camphor

Thujone

α-Pinene
synthase in culture, and intact rats. Iron was also described to induce heme oxygenase in animal systems, although the activity of heme oxygenase in PCT is still not known. Small amounts of iron (5μM) were known to increase uroporphyrin accumulation in cultured chick embryo liver cells and these experiments were to be extended to describing the effects of iron on hepatic ALA synthase activity, and mRNA levels, in the presence of different porphyrinogenic chemicals. Research was undertaken to elucidate the mechanism whereby the combination of glutethimide and FeNTA produced synergistic increases in heme oxygenase activities in cultured chick embryo liver cells.

Mechanism of the synergistic induction of ALA synthase by glutethimide and iron. Regulation of hepatic heme metabolism is thought to occur via a regulatory heme pool. This model was first proposed and further developed by Granick, who suggested that heme is first used for formation of cellular hemoproteins; second, any “excess” heme is used to regulate ALA synthase and effectively turn off heme biosynthesis; and third, if the cell is presented with too much heme, heme oxygenase is induced. The synergistic induction of both ALA synthase and heme oxygenase in chick embryo liver cells upon treatment of the cells with glutethimide and iron would not be predicted by the regulatory heme pool model and seems at variance with the view that regulatory heme is solely responsible for the regulation of heme metabolism. Two different hypotheses could be proposed that would explain these inductions: First, glutethimide and iron could cause a rapid uncontrolled induction of ALA synthase, exposing the cell to high levels of heme and producing a
secondary induction of heme oxygenase; or second, glutethimide and iron could cause a rapid induction of heme oxygenase, depleting the cell of heme and producing a synergistic induction of ALA synthase. Research was undertaken to describe the interrelationship that exists between ALA synthase and heme oxygenase and the role of the regulatory heme pool on both enzymes.

**Effects of metalloporphyrins on ALA synthase.** The discovery that, after treatment of chick embryo liver cells with glutethimide and iron, heme oxygenase activity was synergistically induced before ALA synthase activity, provided a rationale for the use of the metalloporphyrins to modulate ALA synthase activity. The use of a metalloporphyrin to decrease activity of ALA synthase was first described in 1984 and proposed as a therapeutic agent for inducible porphyria after studies in AIA-treated rats. The first patient studies reporting the use of tin-protoporphyrin appeared in 1991 and further studies reported the use of the combination of heme arginate and tin-protoporphyrin to lower the excretion of ALA and PBG in patients with acute porphyria. The rationale for the use of tin-protoporphyrin was to inhibit heme oxygenase and thereby prevent the heme arginate from being degraded. The report by Dover et al. appeared after the experiments describing the additive effect of tin-mesoporphyrin and heme at rapidly lowering ALA synthase activity had been completed. The final stages of the research were to describe which combinations of heme and a series of metalloporphyrins (Figure 2.2) were effective at reducing ALA synthase activity in chick embryo liver cell cultures, and to
Figure 2.2: Structures of heme-analog metalloporphyrins tested in chick embryo liver cell cultures. Chapter VII shows the screening of a number of metalloporphyrins for inhibitory effects on heme oxygenase and their ability to decrease ALA synthase activity.
H = Hydrogen
I = Iodine
Et = CHOHCH₃
E = CH₂CH₃
V = CHCH₂
G = ——CH-CH₂
    |    |    |    |    |    |    |
          OH    OH

**Porphyрин**

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provide evidence which provided some insight into the mechanism whereby metalloporphyrins exerted their effects.
CHAPTER III

MATERIALS AND METHODS

Materials. All tissue culture dishes were from Corning (Corning, NY). Chloroform, dimethylformamide, glacial acetic acid, isopropanol, methanol, perchloric acid were from Fisher (Pittsburgh, PA). Dexamethasone was from Gensia Pharmaceuticals (Irvine, CA). Methoxyresorufin, ethoxyresorufin, benzyloxyresorufin, pentoxvresorufin, 3-cyano-7-ethoxycoumarin, resorufin, and 3-cyano-7-hydroxycoumarin were from Molecular Probes (Eugene, OR). pd(N)₆, Oligo dT(12-18), PhastSystem pre-cast 8-25% gels and buffer strips, Sephadex G-25, and Sephadex G-75 were from Pharmacia (Piscataway, NJ). Bicinchoninic acid was from Pierce (Rockford, IL). δ-Aminolevulinic acid, coproporphyrin I, hemin chloride, HPLC fluorescence standards, mesoporphyrin IX, N-methylmesoporphyrin, protoporphyrin IX, Sn-2,4-diododeuteroporphyrin IX, Sn-mesoporphyrin IX, Sn-protoporphyrin IX, uroporphyrin I, Zn-deuteroporphyrin-2,4-bisglycol, Zn-mesoporphyrin IX, and Zn-protoporphyrin IX were from Porphyrin Products (Logan, UT). Cu-mesoporphyrin, Cu-protoporphyrin, Cu-hematoporphyrin, Cu-deuteroporphyrin were generous gifts from Dr. Dabney Dixon (Georgia State University, Atlanta, GA). The plasmid pALX²⁶⁵ was a generous gift from Dr. Doug Engel (Northwestern University, Evanston, IL). AMV-Reverse Transcriptase, dTTP, dGTP, dATP, dCTP, Klęnow DNA polymerase, and RNAsin were from Promega (Madison, WI). Adenosine
triphosphate, actinomycin D, ammonium phosphate, ammonium chloride, 
β-mercaptoethanol, bovine serum albumin, coenzyme A, deferoxamine, 
4,6-dioxoheptanoic acid, diethylpyrocarbonate, dimethylsulfoxide, 
$p$-dimethylaminobenzaldehyde, DNase, ficoll, Folin's phenol reagent, 
formaldehyde (37%), $\alpha$-D-glucose (tissue culture grade), glutethimide, 
glycine, hemin chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic 
acid, $p$-hydroxymercuribenzoic acid, keyhole limpet hemocyanin, 
leupeptin, magnesium acetate, magnesium chloride, m-maleimidobenzoic 
acid-N-hydroxysuccinimide ester, mercuric chloride, nicotinamide adenine 
dinucleotide phosphate (reduced), nitrilotriacetic acid, poly (A), 
polyvinylpyrrolidone, potassium carbonate, potassium flouride, potassium 
phosphate (dibasic), potassium phosphate (monobasic), pyridoxal 
5'-phosphate, salmon sperm DNA, sodium acetate, sodium bicarbonate, 
sodium chloride, sodium citrate, sodium dodecyl sulfate, sodium 
hydroxide, sodium phosphate (monobasic), sodium phosphate (dibasic), 
sodium succinate, trichloroacetic acid, 3,4,3' triiodo-L-thyronine and 
trizma base were from Sigma (St. Louis, MO). Fertilized chicken eggs were 
either Barred Rock from Carrousel Farms (Hopkinton, MA), or White 
Leghorn from Hyline Farms (Mansfield, GA) or Spafas (Norwich, CT). 
William's E medium, trypsin, and penicillin/streptomycin and blocking 
solution for immunoblotting were from Gibco (Grand Island, NY). 
Formamide (spectral grade) and levulinic acid was from Aldrich 
(Milwaukee, WI). 2,4-pentanedione was from E.M. Science (Cherry Hill, 
NJ). Succinic thiokinase was from Boeringer Mannheim (Indianapolis, 
IN). RNAzol was from Cinna/Biotexc (Friendswood, TX). The mini-
protean gel system, alkaline phosphatase conjugated goat anti-rabbit IgG, mini-protean gel system and alkaline phosphatase 5-bromo-4-chloro-3-indoyl phosphate-Nitroblue tetrazolium color development reagents were from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose 0.45 μm was from Schleicher and Schuell (Keene, NH). All 32P and 35S radionuclides were from New England Nuclear (Boston, MA).

Equipment. The SLM•Aminco DW-2000 was from SLM•Aminco Instruments (Urbana, IL). Waters Baseline 810 HPLC System equipped with a WISP 712 Autoinjector was from Waters Division of Millipore (Milford, MA). The Shimadzu model RF-551 fluorescence detector was from Shimadzu Instruments (Gaithersburg, MD). The LS-50B luminescence fluorometer was from Perkin Elmer (Newton, MA). The fluorometers were equipped with red sensitive photomultiplier tubes (R944) to increase the sensitivity to the fluorescent porphyrins. All centrifuges were from Beckman (Fullerton, CA). The dot blot apparatus was from Schleicher and Schuell (Keene, NH). The β-counter was from β-Gen (Millvale, CA). The Speed-Vac was from Savant (Farmingdale, NY). The computer equipment was from Apple, Inc. (Cupertino, CA).
Chick embryo liver cell cultures

Introduction. Primary chick embryo liver cell cultures are a simple, reproducible system for exploring hepatic function. These investigations have included testing intercellular communications, viral infections, porphyrin metabolism, and other metabolic pathways. Other culture systems, most notably primary rat hepatocyte cultures, do not retain normal heme and cytochrome P-450 metabolism. Since chick embryo liver cultures are not pure hepatocytes, but contain approximately 5-10% fibroblasts, use is essential within 3-5 days since the fibroblasts divide in culture, while the hepatocytes do not. Preparation of cells for culture is a simple process in which the liver is minced into fine pieces, and treated with a protease to digest the hepatocellular matrix. The red blood cells must be removed, and finally the small groups of cells must be separated from the larger fragments, and plated to form a monolayer culture.

Preparation of liver cells. Chick embryos were dissected 16-18 days after fertilization. The livers were removed and washed three times in Hanks physiologic buffer, minus calcium (136 mM NaCl, 5.55 mM α-D-glucose, 0.345 mM MgCl₂, 0.338 mM Na₂HPO₄, 0.441 mM KH₂PO₄, 4.17 mM NaHCO₃, pH 7.4). Ten milliliters of final inoculum can be obtained from 1 chick embryo liver. Gall bladders were removed from the livers before washing, to prevent contamination of the livers with bile, especially bile salts. Ten to twelve livers were chopped for two minutes with razor blades in Hanks buffer minus calcium with 4.5x10⁻⁵% trypsin (w/v) in 60
mm glass culture dishes. Contents of two or three chopping dishes were
combined and transferred to 125 ml sterile Erlenmeyer flasks, with an
enlarged bore pipette*, mixed thoroughly with the pipette, and incubated for
a total of 20 minutes in a 37°C water bath. Ten minutes after the start of
incubation DNase was added to a final concentration of 75μg/ml. After an
additional two minutes the mixture was again mixed thoroughly with the
large bore pipette and returned to the water bath for the remaining time.
The mixture from each Erlenmeyer flask was evenly distributed among 12
fifteen ml conical tubes containing 10 mls of Hanks buffer minus calcium
at 4°C. The tubes were centrifuged at 500 x g for 90 seconds, and the Hanks
buffer was aspirated. The cellular pellets were resuspended in 10 ml of
room temperature ammonium bicarbonate buffer (129 mM NH₄Cl, 17 mM
Tris, 10 mM K₂CO₃, pH 7.5) to lyse red blood cells, and mixed on a
rotating wheel for three minutes. The suspension was centrifuged at 500 x
g for 90 seconds and the buffer aspirated. This procedure was repeated
once, and after the second wash, the cellular pellet was resuspended in
William’s E medium containing dexamethasone, insulin, and 3,5,3’-
triiodo-thyronine. The cells were spun at 80 x g momentarily to remove the
large debris, and the supernatant was removed, the volume corrected as
necessary, and the cells were plated onto tissue culture dishes. The volume
of inoculum added per size of plate was as follows: 25 mm plate (or 1 well
of a 6 well plate), 2 ml; 60 mm plate, 6 ml; 100 mm plate, 15 ml.

* The enlarged bore pipette was made by removing the tip on a glass 25 ml pipette so that the
remaining opening was approximately 4 mm I.D. The tip was flamed to remove any sharp
edges, and the pipette sterilized prior to use.
Maintenance and treatment of cultures. The insulin was removed from the cells after 24 hours in culture by washing each dish with the William's E medium containing only dexamethasone and T3. The dishes were allowed to incubate for 1 hour after each wash in the presence of the washing medium, the dishes were washed a total of two times, and the induction volumes were dispensed into the dishes. The wash and induction volumes for each of the dishes was as follows: 25 mm plate (or 1 well of a 6 well plate), 1 ml, 2 ml; 60 mm plate, 2 ml, 5 ml; 100 mm plate, 5 ml, 10 ml, respectively. The cells were not generally left exposed to the same medium for more than 24 hours, although the volumes or times occasionally were varied slightly if these changes expedited experimentation. Appropriate controls were always done.
**ALA synthase assay**

**Theory.** δ-Aminolevulinate (ALA) synthase (E.C. 2.3.1.37) catalyzes the condensation of 1 molecule of glycine and 1 molecule of succinyl-CoA to form δ-aminolevulinic acid (Figure 3.1, reaction 1). The $K_m$ for glycine is 2.5-51 mM$^{199,680}$ and the $K_m$ for succinyl-CoA is 10-55 μM.$^{680,681}$ This enzyme is only physiologically active in mitochondria because of the subcellular distribution of succinyl-CoA. ALA synthase requires pyridoxal 5'-phosphate as a cofactor.$^{179,198}$ Assays for this enzyme require a source of succinyl-CoA, glycine, pyridoxal-5'-phosphate and, when crude tissue extracts are used, an inhibitor of ALA-dehydratase to prevent further metabolism of the product all within a suitable buffer system. ALA generated in cellular supernatants is converted to a pyrrole (Figure 3.1, reaction 2) and reacted with Ehrlich's reagent (p-dimethylaminobenzaldehyde) to form a colored Ehrlich's pyrrole salt (Figure 3.1, reaction 3).$^{682-684}$ The concentration of this resulting product is determined spectrophotometrically.$^{682-684}$

**Description.** δ-Aminolevulinic acid was produced from chick embryo liver cell sonicates in 150 μl of an appropriate assay buffer containing: 35 mM Tris, 30 mM sodium phosphate, 8 mM magnesium chloride, 5 mM EDTA, 15 mM sodium succinate, 10 mM sodium levulinate, 500 μM pyridoxal 5'-phosphate, and 66 mM glycine. The buffer system maintained the pH at 7.4 for at least 30 minutes at 37°C.$^{685,686}$ Magnesium-EDTA inhibits ALA dehydratase and prevents formation of aminoacetone pyrrole,$^{682}$ and levulinate also inhibits ALA dehydratase.$^{682}$ The reaction
**Figure 3.1:** Mechanisms of the reactions utilized in the assay for ALA synthase. Reaction 1: The reaction carried out *in vitro* and catalyzed by ALA synthase. This reaction requires glycine, pyridoxal 5’-phosphate, and succinyl CoA. The intermediate α-amino-β-ketoacid remains enzyme bound (See figure 1.3 reaction 1). Reaction 2: The formation of 2-methyl-3-acetyl-4-propionic acid pyrrole [46]. This reaction derivitizes the ALA formed by the enzyme into an Ehrlich’s reactive species. Reaction 3: Derivatization of 2-methyl-3-acetyl-4-propionic acid pyrrole to a chromophoric substance, the characteristic red Ehrlich’s ALA salt [47]. Solutions of this compound are quantified and the amount of ALA formed in the original reaction is calculated.
was started by adding glycine and incubating at 37°C for 30 minutes. Succinyl-CoA formation was occasionally supplemented by the addition of a succinyl-CoA generating system: 10 mM ATP, 100 μM Coenzyme A, 5 mM β-mercaptoethanol, 100 mM potassium fluoride, and succinic thiokinase (5 nmoles succinyl-CoA generated/hour/ml total volume). Succinic thiokinase condenses succinate and CoA to form succinyl-CoA, under the proper reducing conditions and potassium fluoride was added to inhibit ATPases that may have been released into solution after the disruption of the mitochondria. 687 The succinyl Co-A generating system supplements, by about 20-30%, formation of ALA in some cell culture systems, while having no additional effect in others (E. Cable, unpublished observations). This was dependent on the source for the liver cells used and should be determined empirically for each species. The reaction was allowed to proceed for 30 minutes at which time TCA was added to a final concentration of 4% to terminate the reaction. Spectrophotometric blanks were obtained from identically treated aliquots of cellular sonicates except that the blanks were not incubated. The assay samples and the spectrophotometric blanks were treated identically from this point forward. The TCA precipitates were removed after centrifugation for 10 minutes at 1000 x g, and a portion (150 μl) of the supernatant containing the ALA was removed. The ALA was then condensed with 4% 2,4-pentanediolone in the presence of 0.4 M sodium acetate (pH 4.6) to form an ALA-pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole) in a Knorr-type condensation reaction. This reaction was complete after incubation at 85°C for 15 minutes. The ALA-pyrrole was converted to a chromophore by the addition
of an equal volume of modified Ehrlich's reagent (2% p-dimethylaminobenzaldehyde (DMAB), 0.375% mercuric chloride in a 32:168 (v/v) solution of 70% perchloric acid : glacial acetic acid). The colored solution has a $\Delta \varepsilon_{555-650} = 68 \text{mM}^{-1}\text{cm}^{-1}$ The mono-DMAB adduct, a chromophoric Ehrlich's salt, forms in solution with a maximum absorbance obtained after 10 minutes. This timing was precise since longer incubation (>15 minutes) allowed a secondary reaction to form a colorless dipyrrylphenyl methane which led to a decrease in absorbance.

**Experimental results.** ALA synthase is easily detectable in sonicates of chick embryo liver cell cultures, even at basal levels (0.5 nmoles ALA•mg protein•1•hour•1), especially after addition of chemicals which induce ALA synthase to very high levels. Detection of reductions of ALA synthase activity from basal levels, or detection in tissue samples that contain very low levels (<250 pmoles ALA•mg protein•1•hour•1), require other assay methods, such as radiochemical methods, or HPLC separation and fluorometric detection of ALA derivatives.

The spectrophotometric method employed throughout the duration of this research was ideal since high levels of ALA synthase were present (0.5-25 nmoles ALA•mg protein•1•hour•1). This assay was linear through 60 minutes, even at a high specific activity (Figure 3.2), although the 30 minute incubation time employed was sufficient to produce ALA quantities that were easily detectable. The dependence of the assay on protein concentration was linear throughout the range used in all the studies (Figure 3.3).
Figure 3.2: Linearity of the assay for ALA synthase with respect to incubation time. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 18 hours prior to harvest. The cells were harvested and the assay completed as described in Methods, except that the cell sonicates were pooled, and the time of incubation was as indicated. The data are mean± SEM, n=3. Where no error bars are shown the error falls within the size of the symbol.
Formed of ALA
(nmol ALA/mg protein⁻¹)

Incubation Time (min)

\[ y = 0.095 + 0.47x \quad R = 1.00 \]
Formation of Ehrlich’s salt pyrrole was rapid for the first minute and continued with a rate that was first order with respect to ALA-pyrrole concentration and zero order with respect to DMAB concentration with an initial reaction rate of 15.7 µM•minute⁻¹ or a first order rate constant of 0.52 minute⁻¹ (Figure 3.4, inset). The concentration of chromophore stabilized after about 8 minutes and started to decay about 18 minutes after initiation of the reaction (Figure 3.4). The decay occurred at a constant rate of 18.5 nM•minute⁻¹ and was linear for at least two hours (Figure 3.4, inset). The disappearance of chromophore from solution was due to the formation of the dipyrrole adduct of DMAB as opposed to the formation of a di-DMAB adduct of the pyrrole (Figure 3.1, reaction 3). The decrease in absorbance did not significantly interfere since all the samples were analyzed 10-15 minutes after the addition of modified Ehrlich’s reagent.
Figure 3.3: Linearity of the assay for ALA synthase with respect to protein concentration. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 18 hours prior to harvest. The cells were harvested and the assay completed as described in Methods, except that the cell sonicates were pooled, and the amount of sonicate added to the 30 minute incubation was varied as indicated. Typical assays are done with the protein concentration between 0.8-1.0 mg·ml⁻¹. The data are single point determinations. Where no error bars are shown the error falls within the size of the symbol.
Protein (mg/assay)

Formation of ALA (nmol ALA/hour)

$y = 0.50 + 13.1x \quad R = 0.98$
**Figure 3.4:** The formation of the chromophoric Ehrlich's salt of 2-methyl-3-acetyl-4-propionic acid pyrrole. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA 18 hours prior to harvest. The cell were harvested and assays done as described in Methods, expect that the time for the final formation of the chromophoric Ehrlich’s salt of 2-methyl-3-acetyl-4-propionic acid pyrrole was varied. The scans were run at the times indicated in the graph using the split beam mode on the spectrophotometer run against the appropriate spectrophotometric blank, as described in Methods. The inset shows the dual wavelength kinetic formation of the chromophore and represent the change with time in ΔAbs between 555nm and 650nm.
Heme oxygenase assays

Theory. Heme oxygenase (E.C. 1.14.88.3) is the first and rate-limiting step in heme catabolism\textsuperscript{1,549,551} in chick liver cells.\textsuperscript{227,548,550} The microsomal heme oxygenase system contains two different enzymes; heme oxygenase which reduces the ferric iron of heme, binds molecular oxygen, and degrades the porphyrin macrocycle, and NADPH ferrihemoprotein reductase (E.C. 1.6.2.4) which transfers six moles of reducing equivalents obtained from the oxidation of NADPH.\textsuperscript{692} Three molecules of molecular oxygen are required, of which two atoms of oxygen, obtained from different oxygen molecules, remain as part of the biliverdin product (Figure 3.5).\textsuperscript{1} All mammals contain biliverdin reductase (BVR) (E.C. 1.3.1.24) which reduces biliverdin to bilirubin. Chickens can not reduce biliverdin to bilirubin due to a lack of biliverdin reductase. Bilirubin has an extinction coefficient more than 4-fold greater than that of biliverdin (bilirubin $\Delta \varepsilon_{470-540} = 66 \text{mM}^{-1}\text{cm}^{-1}$,\textsuperscript{230} biliverdin $\Delta \varepsilon_{680-750} = 15 \text{mM}^{-1}\text{cm}^{-1}$).\textsuperscript{227} Thus, in order to increase the sensitivity of the heme oxygenase assay, 1.5 units of exogenous biliverdin reductase are added to convert the biliverdin to bilirubin.

Kinetic assay. The original assay developed to detect heme oxygenase activity was a dual wavelength spectrophotometric method that followed the formation of bilirubin using the differences in absorbance at 470 nm, the peak absorbance of bilirubin, and 540 nm, an isosbestic point for bilirubin in the assay mixture (Figure 3.6).\textsuperscript{571} The assay mixture contained 70 mM potassium phosphate, pH 7.40, 5 mM deferoxamine, biliverdin reductase
Figure 3.5: Mechanisms of the reactions utilized in the assay for heme oxygenase. The microsomal heme oxygenase system catalyzes the degradation of heme (ferric protoporphyrin IX) to biliverdin [49]. The reduction of the iron is the first reaction which leads to the binding of molecular oxygen. The α-meso carbon of the tetrapyrrole macrocycle is specifically oxygenated leading to the formation of biliverdin IXα. This is reduced to bilirubin IXα [48] in order to enhance sensitivity and to increase the stability of the final product.
Figure 3.6: Change in absorbance between 470 nm and 540 nm due to the formation of bilirubin IXα catalyzed by the heme oxygenase assay mixture. Purified chick liver heme oxygenase (5 μl) was assayed and the calculated activity of the undiluted protein was 66 pmol·min⁻¹. Assays performed by John Healey.
(BVR, capable of producing 1.5 nmole bilirubin/minute in a total volume of one ml), 40 μM heme, in bovine serum albumin at a heme/albumin molar ratio of 5/3,554,571 in a final volume of 1 ml. Heme-albumin (ca 1.66 moles heme/1 mole albumin) was prepared by adding 100 μl of 10 mM heme (6.5 mg/ml), dissolved in dimethyl sulfoxide (DMSO), to 3.9 ml of a solution of BSA, 11.1 mg/ml, dissolved in 40 mM Tris*HCl (pH 7.4). The level of endogenous NADPH ferrihemoprotein reductase was unknown, but was also in excess since additional NADPH ferrihemoprotein reductase did not increase heme oxygenase activity, even in fresh cell sonicates that contained highly induced levels of heme oxygenase. The heme oxygenase reaction in this system was started with the addition of NADPH, after warming the entire mixture at 37°C for 5 minutes. Formation of the bilirubin product begins immediately and the activity was calculated from the change in absorbance with respect to time with $\Delta \varepsilon_{470-540 \text{nm}} = 66 \text{mM}^{-1}\cdot\text{cm}^{-1}$.

**Batch assay.** Heme oxygenase activity was also measured by scanning the final product after incubation of the assay mixture at 37°C for a fixed time. The reaction was stopped by adding 1 mM p-hydroxymercuribenzoic acid.554 The mercury ions do not affect the heme oxygenase protein per se but inhibit NADPH ferrihemoprotein reductase and prevent the transfer of reducing equivalents necessary to drive the reaction. Typically this reaction was run for 10 minutes, twice as long as the kinetic assay, to increase sensitivity. This assay was linear for at least ten minutes and had a variability from the kinetic assay of ≤5%. The assay mixture remained the same, but the reaction was started with the addition
of the heme:BSA mixture, after a five minute pre-incubation at 37°C. This pre-incubation warms the mixture from 4°C, and at the same time reduces any endogenous biliverdin in the samples. Spectrophotometric blanks were aliquots of cell sonicates, treated identically, including all the additions, except that the hemin was added after the addition of the p-hydroxymercuribenzoic acid. Heme oxygenase activity in cellular sonicates were calculated from the difference in absorbance from 470 nm-540 nm, scanned against appropriate spectrophotometric blanks, $\Delta \varepsilon_{470-540\text{nm}} = 66 \text{ mM}^{-1}\text{cm}^{-1}$ (Figure 3.7). Addition of the albumin to the reaction not only helped to solubilize the heme, but stabilized the bilirubin that was produced.
Figure 3.7: Spectral scan of bilirubin IXα formed in the heme oxygenase assay system. Purified chick liver heme oxygenase (5 μl) was assayed and the calculated activity of the undiluted protein was 63 pmol·min⁻¹. Assays performed by John Healey.
Protein assays

Protein concentrations from cell culture were quantitated utilizing the reduction of copper sulfate as an estimate of the protein concentration. The assay for the reduced monovalent copper was done either with the classic Folin phenol reagent,\textsuperscript{693} or the more sensitive bicinchoninic acid.\textsuperscript{694}
Porphyrin assays

Differential fluorometric procedure for estimating porphyrins

Theory. Estimates of porphyrin concentrations in cultures were done taking advantage of the differential fluorescence of uro-, copro-, and proto-porphyrins. The wavelength pairs for this assay were Ex (nm)/Em (nm), 400/595, 405/595, 410/605. These wavelengths were chosen to give the maximal difference in fluorescence between the corresponding porphyrins. Through solving a series of three equations for three unknowns, by using previously determined linear functions to describe the fluorescent properties of each porphyrin at each wavelength pair (Figure 3.8), the porphyrin concentrations were readily determined. The algebraic solution to this problem can be described as follows:

A matrix describing the correlation coefficients of the linear regression line describing the fluorescence of each porphyrin at each wavelength pair can be written:

\[
\begin{align*}
\text{Ex (nm)} & / \text{Em (nm)} & \text{Uro} & \text{Copro} & \text{Proto} \\
400 & / 595 & A & B & D \\
405 & / 595 & E & F & G \\
410 & / 605 & H & I & J \\
\end{align*}
\]

Where A, B, D, E, F, G, H, I, J were the respective coefficients of each line describing the fluorescent properties of the porphyrins (Figure 3.8).
Figure 3.8: Linearity of uroporphyrin [2], coproporphyrin [3], and protoporphyrin [4] at each of the wavelength pairs utilized in the spectrofluorometric detection of porphyrins. Solutions of each of the individual porphyrins were made in PCA:methanol:H₂O (5:45:50) and the fluorescence of each porphyrin assayed at each of the wavelength pairs utilized in the assay to characterize porphyrin mixtures in sonicates from cell culture. The amounts of porphyrins utilized encompass the amounts that can be produced by the cell culture system. The data represent individual points, and three separate solutions were read at each concentration and each wavelength pair. The lines were fit using a least squares regression and the line was not forced through the origin.
If:

\[
\begin{align*}
[Uro] &= Xu \\
[Copro] &= Xc \\
[Proto] &= Xp
\end{align*}
\]

and

Total fluorescence at

\[
\begin{align*}
400/595 &= F1 \\
405/595 &= F2 \\
410/605 &= F3
\end{align*}
\]

Then three equations can be written to describe the total fluorescence of the mixture as a sum of the fluorescence of the individual components (Figure 3.8):

\[
\begin{align*}
F1 &= AX_u + BX_c + DX_p \\
F2 &= EX_u + FX_c + GX_p \\
F3 &= HX_u + IX_c + JX_p
\end{align*}
\]

Which can then be rewritten as the matrix equation:

\[
\begin{bmatrix}
F1 \\
F2 \\
F3
\end{bmatrix} =
\begin{bmatrix}
A & B & D \\
E & F & G \\
H & I & J
\end{bmatrix}
\begin{bmatrix}
X_u \\
X_c \\
X_p
\end{bmatrix}
\]

This equation was easily solved within Excel (Microsoft) on a Macintosh. The total porphyrin concentration within the medium is the summation of each of the individual porphyrin concentrations.

The utility of the assay was demonstrated by the use of mixtures of the three porphyrins in known compositions (Figure 3.9A). The solutions
were read in the same fashion as an unknown and the porphyrin composition was calculated from the data presented in Figure 3.8. The assay was precise to within 15% (Figure 3.9B). Mixtures of two and single, pure porphyrin solutions were read as well and the same precision was obtained, but in the interest of clarity the data were not presented.

**Description.** Total porphyrins were solubilized from cells and media, by taking the cells plus media mixture from each 35 mm plate (or 1 well of a 6 well plate) by scraping with a spatula. The mixture of media and cells was sonicated for 5 seconds with a Branson microtip sonicator (25% of maximum setting). The sonicated solution (400 μl) was mixed with 400 μl of 10% PCA/methanol (v/v) in a 6 x 50 mm borosilicate glass tube. The PCA/methanol effectively solubilized porphyrins and oxidized porphyrinogens to their corresponding porphyrins. The tubes were spun at 500 x g for 5 minutes to precipitate any cellular debris and the fluorescence of the supernatant was quantitated within the same tube. The fluorescence of the medium not exposed to cells was subtracted after the medium had been treated in an identical manner.
Figure 3.9: Detection of porphyrins in known tertiary solutions containing uroporphyrin, coproporphyrin, and protoporphyrin utilizing the difference in fluorescence between three wavelength pairs. Solutions containing known amounts of uroporphyrin, coproporphyrin and protoporphyrin were made (A) and assayed (B). The solutions were mixed with 2.5, 10 and 50 ng of porphyrin and the concentration calculated as described in Methods. The calculated results agree within 15% of the known mixture composition. The data represent single determinations of individual solutions. The individual porphyrins and the binary solutions were assayed as well and similar correlation between the calculated and known composition of the solution was obtained (data not shown).
HPLC separation of porphyrins

**Theory.** High-performance liquid chromatography (HPLC) of porphyrin mixtures in solution is a highly quantitative method of detection. Absorbance measurements can be used to detect porphyrins however, fluorescence is more sensitive, and is the method of choice, if available.696

Porphyrins isomers were originally separated after derivitization to the methyl ester using adsorption chromatography or thin layer chromatography. Although porphyrin methyl esters can be formed from the free acids, complete derivatization might not be possible, especially for porphyrins with higher numbers of carboxyl groups. Reverse-phase HPLC was first used to separate isohardero and hardero-porphyrin methyl esters, but HPLC methods were developed that separated the porphyrin free acids, and obviated the need for a derivitization reaction. Free acids of porphyrins are easily obtained from almost any kind of sample, including cell culture, urine, blood and feces by extraction of the porphyrins directly into methanol, acetone/HCl, HCl alone, or other acidified solvents. These solvent systems conveniently extract the porphyrins and porphyrinogens, precipitate the proteins, and oxidize the porphyrinogens to porphyrins. Extracts may be injected directly onto the column without damage to the silica matrix. Reverse-phase HPLC allows the separation of different isomers, and can greatly facilitate the diagnosis of specific types of porphyria, and is now the method of choice. Linear gradients were originally
developed,\textsuperscript{702,705,715-717} however a convex gradient was used for porphyrin separations in this work (Figure 3.10).\textsuperscript{708}

\textbf{Description.} Cell culture samples, normally a mixture of cells scraped directly into the media, were extracted with 0.1N HCl. The mixture was centrifuged to precipitate the particulate matter, and the supernatant was injected using a WISP auto injector. The porphyrins, heme and biliverdin, were separated on a C\textsubscript{18} &bondpak column, the mobile phase consisted of a gradient of two solvents: solvent A was a 1:1 (v:v) mixture of 0.1 M ammonium phosphate, pH 4.5, and methanol; solvent B was 100\% methanol. The gradient went from 70\% solvent A and 30\% solvent B to 100\% solvent B over 7 minutes at a flow rate of 1.5 ml/min, followed by 100\% solvent B for an additional 11 minutes. (Figure 3.10).
Figure 3.10: Chromatographic separations of porphyrin free acids over a C18 µbondpak column. The separation of octa-, hepta-, hexa-, penta-, tetra-, and di-carboxylic acid porphyrins, (I isomers) were accomplished using a gradient separations of ammonium phosphate:methanol (solvent A) and methanol (solvent B). The percentage of solvent B during the chromatographic run is depicted along with the corresponding retention times of the different porphyrins. The porphyrins eluted in the order of 8,7,6,5,4,2 carboxyl porphyrins.
Cytochrome P-450 assays

Spectrophotometric. The classical assay for the quantitation of total cytochrome P-450 is based upon the binding of carbon monoxide as the 6th ligand to the reduced heme in the cytochrome P-450 protein.\(^{718,719}\) Chick embryo liver cell culture plates were harvested in 200 mM potassium phosphate, pH 7.4 and briefly (< 1 sec) sonicated. Cellular debris was removed by centrifugation in a microfuge for 10 minutes at 4°C. The supernatant (500 µl) was added to 2X Emulgen 913 buffer (400 mM potassium phosphate, pH 7.4, 40% glycerol, 0.5% Emulgen 913). The solution was gently mixed and split into two equal volumes and placed in a microcuvette, with one cuvette placed in the sample position and the other placed in the blank position. A few (3-5) grains of sodium hydrosulfite were added to each cuvette and a split beam wavelength scan was done from 400nm-540nm. Ten bubbles of carbon monoxide were bubbled through the sample cuvette and another wavelength scan was done. The spectra obtained before and after the addition of carbon monoxide were subtracted and the concentration of cytochrome P-450 was calculated from the difference in absorbance between 450 nm and 520 nm, \(\Delta \varepsilon_{450-520\text{nm}} = 91 \text{ mM}^{-1}\text{cm}^{-1}.\)^\(^{718}\)

Cytochrome P-450 dependent dealkylations. Cytochrome P-450 isozymes can be differentiated due to their activity on isozyme selective substrates. The use of a homologous series of phenoxazones (Figure 3.11) has been used to differentiated induction of different cytochrome P-450 isozymes in rats\(^{720-723}\) and mice.\(^{720}\) Rat cytochrome P-450 IA typically
**Figure 3.11:** Structures of substrates for cytochrome P-450 dependent mono-oxygenation reactions. The substrates of a homologous series of phenoxazone ethers, the resorufins, which are O-dealkylated by cytochrome P-450 IA. 3-Cyano-7-ethoxycoumarin is also a substrate for this isozyme. One of the best substrates for the IIB form of cytochrome P-450 is benzphetamine which is N-demethylated, however the turnover number for the reactions catalyzed by the IIB isozyme are much lower than the turnover numbers of the IA reactions.
Resorufins [50-53]

3-Cyano-coumarins [54-55]

Benzphetamine [56-57]

<table>
<thead>
<tr>
<th>Resorufins</th>
<th>[n]</th>
<th>R₁</th>
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<tbody>
<tr>
<td>Resorufin</td>
<td>50</td>
<td>H</td>
</tr>
<tr>
<td>Ethoxyresorufin</td>
<td>51</td>
<td>CH₂CH₃</td>
</tr>
<tr>
<td>Pentoxyresorufin</td>
<td>52</td>
<td>(CH₂)₄CH₃</td>
</tr>
<tr>
<td>Benzyloxyresorufin</td>
<td>53</td>
<td>CH₂ −</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Coumarins</th>
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<th>R₂</th>
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</thead>
<tbody>
<tr>
<td>3-Cyano-7-hydroxycoumarin</td>
<td>54</td>
<td>H</td>
</tr>
<tr>
<td>3-Cyano-7-ethoxycoumarin</td>
<td>55</td>
<td>CH₂CH₃</td>
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</table>

<table>
<thead>
<tr>
<th>Benzphetamine</th>
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<th>R₃</th>
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<tr>
<td>Benzphetamine</td>
<td>56</td>
<td>CH₃</td>
</tr>
<tr>
<td>Demethylated benzphetamine</td>
<td>57</td>
<td>H</td>
</tr>
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</table>
catalyzes the dealkylation of longer chain phenoxazone ethers better than the shortest chain phenoxazone ether; ethoxyresorufin [51] \( \rightarrow \) propoxyresorufin \( \rightarrow \) methoxyresorufin,\(^{721,724}\) whereas rat P-450 IIB preferentially catalyzes the dealkylation of benzyloxyresorufin [53]\(^{724}\) and 7-ethoxycoumarin,\(^{725}\) although at a rate much lower than that catalyzed by the 1A forms of the enzyme. There are substrate differences between different species, for example the Japanese quail P-450 1A dealkylate ethoxy- \( \rightarrow \) methoxy- \( \rightarrow \) propoxy- \( \rightarrow \) benzyloxy- \( \rightarrow \) pentoxy- resorufin while the IIB forms show minimal catalytic activity toward all of these substrates.\(^{724}\)

These kinds of P-450 dependent oxidation reactions can be helpful in characterizing the induction of different forms of P-450, but only after the catalytic activity of the species specific P-450's have been characterized.

**Alk oxyresorufin dealkylating assays.** The alkoxyresorufin dealkylating assays were done with 100 µl of cell culture harvested from 60 mm dishes in 500 µl Tris 20 mM, pH 7.8 and sonicated for 1 second. The reaction was carried out in a final volume of 150 µl containing 100 µl of sonicate, 1.7 mM NADPH, 20 mM dl-isocitrate, 0.03 U isocitrate dehydrogenase, 13 mM nicotinamide, 12.5 mM MgCl\(_2\), 20 µM alkoxyresorufin (e.g. ethoxy-, pentoxy- [52], or benzyloxy- resorufin). The mixture was incubated at 37°C for 15 min, and the reaction stopped by adding 1 ml cold methanol. The mixture was vortexed and centrifuged 1000xg for 10 minutes. The methanol supernatant was removed and the fluorescence Ex 528 nm Em 590 nM was recorded.\(^{720-724,726,727}\) The amount of resorufin [50] formed in each assay was calculated from a standard curve of identically treated resorufin.
Coumarin dealkylating assays. The coumarin dealkylating assays were done identically to the resorufin assays except that the substrate was the 3-cyano-7-ethoxycoumarin [55], the product was 3-cyano-7-hydroxycoumarin [54] and the excitation and emission wavelengths were Ex 407nm/Em 450nm.728

Benzphetamine demethylating assays. Cell cultures samples were harvested from 60 mm dishes in 450 µl of 0.1 M sodium phosphate pH 7.4 and sonicated for 1 second. The reaction was carried out in a final volume of 140 µl containing 100 µl sonicate, 1.7 mM NADP, 20 mM dl-isocitrate, 0.02U isocitrate dehydrogenase, 13 mM nicotinamide, 9 mM semicarbazide, 12.5 mM MgCl2, and 3 mM benzphetamine-HCl [56]. The reaction was incubated at 37ºC for 1 hour, and the reaction stopped by the addition of 50 µl 20% TCA. The mixture was centrifuged at 1400 x g for 10 min at 4ºC to precipitate the protein, and 150 µl of the supernatant was transferred to a 6x50 tube. Nash reagent (75 µl of 4 µl/ml 2,4-pentanediode in 30% ammonium acetate) was added to each tube and incubated at 70ºC for 15 minutes.245,729 The volume was adjusted with additional Nash reagent in order to fill the tube and the fluorescence of the formaldehyde-2,4-pentanediode adduct measure at Ex 420 nm Em 514 nm. The activity of the enzyme was calculated from a standard curve of formaldehyde reacted with the Nash reagent. The demethylation of 1 mole of benzphetamine [56] leads to the production 1 mole of formaldehyde, and the formaldehyde was easier to quantitate than the demethylated benzphetamine.300,730-737
**Immunodetection of ALA synthase and heme oxygenase**

**Antigen Preparation.** An attempt was made to raise antibodies specific to the pre-mitochondrial and mitochondrial form of ALA synthase using synthetic peptide sequences identical to those in the chick ALA synthase protein. The cDNA sequence for chicken hepatic ALA synthase was obtained from Genbank and the sequence translated using the universal codon preferences. The two sequences chosen represented, as much as possible, a hydrophilic α-helix, that had a high probability of surface exposure. One sequence was chosen to be as near to the carboxyl terminus as possible to be specific to both forms of ALA synthase, while the amino terminal sequence was to represent the immature, pre-mitochondrial enzyme. The sequences were analyzed with Prosis for the IBM using both the Robson and Chou, Fasman secondary structure prediction algorithms, the Jameson/Wolf antigenic index algorithm, and the Pustell surface probability algorithm (Figure 3.12).

The anti-chicken heme oxygenase antibody was raised against a partially purified chicken liver heme oxygenase protein. The details of the purification of the protein are presented elsewhere.

**Preparation of oligopeptides.** The oligopeptides for the anti-ALA synthase antibodies were synthesized using standard solid phase 9-fluorenlymethoxycarbonyl (FMOC) chemistry on a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin at the microsequence facility at Emory University. The first peptide
Figure 3.12: Protein analysis of the derived amino acid sequences used as antigens for raising anti chicken ALA synthase antibodies. Figure A shows the analysis of the amino terminal peptide specific for the precursor form of the enzyme and Figure B shows the analysis of the carboxyl terminal peptide specific for both forms of the enzyme.
Hydrophilicity Window Size = 7
Scale = Kyte-Doolittle

Hydrophilicity

Surface Probability

Antigenic index

Secondary Structure

CF Helix
CF Sheet
CF Turns
RG Helix
RG Sheet
RG Turns
CfRg Helix
CfRg Sheet
CfRg Turns
representing mature ALA synthase was CRRPLHFEVMSERER and was chosen as a hydrophilic, region that was probably exposed. Although the antigenic index was not very high for this region, the other indexes made this the choice for the oligopeptide. An additional consideration was that the amino terminal cysteine in this sequence was used to generate a specific bond from the cysteiny1 sulfur through m-maleimidobenzoic acid-N-hydroxysuccinimide ester (MBS) to free amino groups on the carrier protein. The second peptide representing the immature, cytosolic form of ALA synthase was LARVSQAFLQKAGPSC. The carboxyl terminal cysteine was added to facilitate coupling with MBS in a fashion identical to the first peptide.

**Coupling to KLH and separation.** Each oligopeptide was coupled to KLH using the bifunctional reagent, MBS. MBS couples the succinimide ester to free amino groups on the carrier protein, KLH, and the N-alkyl maleimide group to a free sulphydryl group on the oligopeptide. KLH was dissolved in 10 mM phosphate, pH 7.0 to a final concentration of 20 mg/ml, and a final volume of 2 ml. MBS was dissolved in DMF, final concentration 25 mg/ml. 100 μl of the MBS solution was added to 2 ml of the KLH solution. The solutions were mixed thoroughly and allowed to stand for 30 minutes at room temperature, 25°C. The excess MBS was removed from the solution by gel filtration through a 1 x 30 cm Sephadex G-25 column using 0.1 M phosphate pH 6.5 as an eluant. The fractions containing the protein peak, approximately 3.0 mg/ml protein concentration, were pooled. The oligopeptide was dissolved in 0.1M phosphate, pH 6.5, 2 mM EDTA to a final concentration of 10 mg/ml. 600 μl of the oligopeptide solution was
added to 6 mls of the pooled carrier protein and allowed to sit at room temperature for 4 hours. The unreacted oligopeptide was removed by gel filtration through a 1 x 30 cm Sephadex G-75 column.

**Raising Antibodies.** The ALA synthase carboxyl-terminal specific anti-peptide antibody was raised in New Zealand White rabbits using a regimen of Freund's complete and Freund's incomplete adjuvant. The ALA synthase amino-terminal specific anti-peptide antibody was raised in New Zealand White rabbits using Hunter's Titer-Max®.

The anti-chicken heme oxygenase antibody was raised against purified heme oxygenase in New Zealand White rabbits using a regimen of Freund's complete and Freund's incomplete adjuvant. The polyclonal IgG was prepared from serum using octanoic acid, from which was prepared IgG monospecific for heme oxygenase. This antibody has previously been shown to be specific for chick heme oxygenase from chick embryo liver cell cultures.

**Phast gel blotting.** Cultured cells were homogenized in 0.1 M potassium phosphate buffer (pH 7.5), 20% glycerol (v/v), 1 mM EDTA containing leupeptin (40 μg/ml) as a protease inhibitor. Samples were diluted 2:1 into a concentrated (3x) Laemmli sample buffer. The samples were separated by electrophoresis on a PhastSystem (Pharmacia) according to manufacturer's instructions on 8-25% gradient gels. The samples were transferred to nitrocellulose using the following procedure: Nitrocellulose, gels and 3 sheets of blotting paper were soaked in transfer buffer (25 mM Tris-HCl, 5% methanol, 192 mM glycine, pH 9.2) for 10 minutes then assembled in a petri dish containing transfer buffer in the following order.
Phast gel (with plastic backing facing the bottom of the petri dish), nitrocellulose, blotting paper, and a heavy (50g) weight. The transfer was complete after incubation for 2 hours at 70°C. The blot was blocked overnight with PBS containing 1% BSA and the incubated with rabbit anti-chicken heme oxygenase, and the secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase.

**Mini-gel blotting.** Cultured cells were sonicated in 0.1 M potassium phosphate buffer (pH 7.5), 20% glycerol (v/v), 1 mM EDTA. Samples were diluted in a concentrated (3x) Laemmli sample buffer. The samples (10 μg protein) were separated by electrophoresis on a mini-protean gel system using 8.5% acrylamide gels. The samples were transferred electrophoretically to PVDF membranes using an electroblotting apparatus from Idea Scientific. The blot was blocked for 2 hours with blocking solution from Gibco, and the primary antibody (anti-chicken ALA synthase) exposed to the blot overnight at room temperature. The second antibody, goat-anti rabbit IgG conjugated to alkaline phosphatase, was exposed for 2 hours and the blot developed using the BCIP-NBT development system.

**Results of ST search.** The protein sequences were used to search the Genbank database using a translation algorithm that allows “on the fly” translation of the codons in all six reading frames. The results of the search were used to determine the probability of cross reactivity between ALA synthase proteins from different species. The carboxyl terminal peptide corresponded closely with the sequences from mouse (erythroid), human (erythroid and hepatic), rat (hepatic) ALA synthase. These sequences are the only animal ALA synthase sequences to have been
The amino terminal peptide is only similar to human (hepatic) and rat (hepatic) forms of the enzyme (Figure 3.13).
Figure 3.13: Sequence homology of closely related proteins to the peptides used to raise anti chicken ALA synthase antibodies. The query sequences were searched against the animal subset of the Genbank database and the results show all of the retrieved sequences. The scoring cut-off for both searches was identical.
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<td><strong>larvsqaf1gkagps</strong></td>
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</tr>
<tr>
<td>Human ALA Synthase (Hepatic)</td>
</tr>
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<td>Rat ALA Synthase (Hepatic)</td>
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<table>
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<tr>
<td>Chick ALA synthase (Hepatic)</td>
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<tr>
<td>Human ALA Synthase (Hepatic)</td>
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<tr>
<td>Rat ALA Synthase (Hepatic)</td>
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<tr>
<td>Human ALA Synthase (Erythroid)</td>
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<tr>
<td>Mouse ALA Synthase (Erythroid)</td>
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<tr>
<td>Human calcium-ATPase (HK2)</td>
</tr>
<tr>
<td>Mouse mRNA for tenascin</td>
</tr>
<tr>
<td>Human Thy-1 glycoprotein gene</td>
</tr>
<tr>
<td>Mouse V-H region of late (E3)</td>
</tr>
</tbody>
</table>
RNA Isolation and Detection

Introduction. Extraction of RNA from chick embryo liver cell cultures can be carried out using guanidinium isothiocyanate and a phase separation technique\textsuperscript{754}. This procedure utilizes the variable solubilities of RNA, DNA and protein in solutions of guanidine and phenol. Using the commercial preparation of RNAzol\textsuperscript{®}, the cells are lysed and chloroform added to produce a biphasic mixture. The RNA partitions into the upper, aqueous phase while the DNA partitions into the lower, organic phase. The protein forms an interface between the phases. The RNA is precipitated out of the aqueous phase by adding isopropanol to 50\% (v/v). The RNA was washed with ethanol and was suitable for northern analysis. This isolation obviates the need for cesium chloride gradient centrifugation, or other handling techniques.\textsuperscript{755}

Protocol. Total RNA was isolated from 100 mm plates with RNAzol\textsuperscript{®}. The medium was aspirated from the plate and the cells were washed twice with room temperature PBS. Excess PBS was carefully aspirated and 500 µl of RNAzol was added directly to the dish. The cellular mixture was scraped off the plate with a spatula and transferred to a sterile Eppendorf tube. Chloroform was added to the RNAzol cellular suspension at a concentration of 1 µl CHCl\textsubscript{3} per 10 µl of RNAzol solution. The mixture was vortexed for 10 seconds, or until homogeneously opaque. The mixture was allowed to incubate on ice for 15 minutes and a crude phase separation had occurred. The mixture was centrifuged in a microfuge at 12,000 x g for 10 minutes at 4°C in a fixed angle rotor. The upper, aqueous phase was
carefully removed, transferred to a fresh, sterile Eppendorf tube and an equal volume of isopropanol was added to precipitate the RNA. The solution was incubated at -20°C for at least 1 hour or overnight. Overnight incubation decreased the yield of RNA and was not routinely used. The RNA was pelleted by centrifugation for 15 minutes in a microfuge at 12,000 x g at 4°C with a horizontal rotor. The supernatant was removed and the pellet was washed 2 times with 70% ethanol in DEPC treated water. After each wash the pellet was centrifuged for 7 minutes at 12,000 x g at 4°C in a horizontal rotor. After the final wash volume was removed, the RNA pellet was dried in a Speed-Vac (Savant). The dried pellet was resuspended in 50-100 μl of 0.5% SDS in DEPC treated water. The solution was heated to 65°C to facilitate solubilization of the RNA. Typical RNA concentrations were from 1-5 μg/μl and yields were 100-300 μg for a 100 mm dish. The RNA concentration was estimated from the absorbance at 260 nm (1 AU_{260} = 40 μg/ml) and quality, from absorbance ratios 260nm/280nm, greater than or equal to was 1.80 required.

**Northern Blotting.** Northern blots were done after separation of total RNA on formaldehyde agarose gels. The RNA was transferred to Genescreen® (NEN) membranes using capillary action. The blots were hybridized at 65°C overnight in a buffer containing an end-labeled oligonucleotide probe complementary to a portion of the heme oxygenase cDNA. The blots were washed in 0.1X SSC, without SDS, at room temperature and exposed to X-ray film at -80°C.

**Dot blotting.** The dot-blotting protocol used total RNA and a probe that had previously been shown to react with the mRNA of interest. 40 μg of
RNA in a final volume of 40 μl were treated with 24 μl of 20X SSC and 16 μl of 37% formaldehyde and incubated at 65°C for 15 minutes. The samples were diluted to 1200 μl with 10X SSC and 10 μg of RNA were loaded per dot, in duplicate, and duplicate nitrocellulose filters were prepared. The blots were crosslinked by exposure to 2.4 μjoules of UV radiation using a UV-Stratalinker and prehybridized at 42°C in 15 ml of a buffer containing 50% formamide, 5X SSC, 100 μg/ml salmon sperm DNA, 5X Denhardt's, and 1% SDS. After 2 hours the solution was removed and replaced with a smaller volume (5-7 mls) of the same buffer and a boiled radioactive probe was added to 30 x 10⁶ counts per blot. After hybridization for 18 hours at 42°C, the blots were washed with 0.1X SSC at room temperature for 30 minutes with 3 buffer changes and either exposed to X-ray film at -80°C or counted on a β-counter. If the background was too high, the blot was washed further using the same conditions or the temperature was increased to 32°C for subsequent washes.

**PCR amplification of pALX.** The plasmid pALX, a subclone of a λgt11 library isolated and characterized in the laboratory of Dr. Doug Engel, was grown using a mini-prep procedure. The plasmid DNA was then subjected to PCR amplification using 200 μM each dATP, dCTP, dGTP, dTTP, 1X PCR buffer (50 mM KCl, 10 mM Tris, 0.0001% gelatin), 200 μg each SP6 and T7 primers, 2 μg of mini-prep pALX, 7.25 mM MgCl₂, and 2.5 U of Taq polymerase. The reaction was run for 30 cycles: with the initial melt 94°C, 2 minutes; denaturing 94°C, 1 minute; annealing 40°C, 2 minutes; and extending 75°C, 2 minutes. This was essentially according to
Perkin-Elmer’s instructions with the exception of the primers and template DNA.

**End labelling.** The oligonucleotide specific for heme oxygenase was prepared from the sequence of chick heme oxygenase previously described (CGATAGAGCTGTTTGAACTTGTTGG). The probe was end-labeled using $\alpha$-$^{32}$P-ATP and T4 polynucleotide kinase. The unincorporated $^{32}$P was separated from the oligonucleotide using a NENsorb column (NEN) according to manufacturer’s instructions.

**Hexamer priming method.** The radioactive ALA synthase probe was generated using the method described by Feinberg utilizing random hexanucleotide primers to amplify a known cDNA template. Basically, 100-400 ng of DNA was boiled for 5 minutes, and a buffered solution containing 250 mM Tris, 250 mM HEPES, 21 mM MgCl$_2$, 12 $\mu$M $\beta$-mercaptoethanol, 6.75 U of pd(N)$_6$, 25 $\mu$M each dATP, dTTP, dGTP, 50 $\mu$Ci of dCTP (3000 Ci/mmol) and 1 U of Klenow DNA polymerase. The mixture was incubated for 2 hours at 37°C and the DNA was precipitated with 75 $\mu$l 10 mM Tris 0.1 mM EDTA pH 8.0, 4 $\mu$l (0.5 $\mu$g/$\mu$l) yeast tRNA, 100 $\mu$l 4M sterile ammonium acetate, and 600 $\mu$l 95% ethanol. The mixture was incubated at -20°C for 20 minutes and the DNA precipitated by centrifugation in a microfuge at 4°C for 5 minutes. The supernatant was removed from the pellet containing the radiolabelled cDNA fragments, and the pellet resuspended in 100 $\mu$l 10 mM Tris 0.1 mM EDTA pH 8.0. The probe was boiled for 5 minutes and immediately placed on ice for another 5 minutes. One microliter was counted and an appropriate amount of radioactivity was loaded onto the blot.
Normalization of mRNA content by a polythymidylate probe. Accuracy of the samples loading of dot-blots for RNA was checked by probing for the amount of poly-A mRNA using a polythymidylate probe (poly-T). This procedure normalizes the amount of poly-A mRNA within a given sample and can be performed on previously probed blots or blots loaded with samples from the same sample preparation. This procedure was used preferentially to probing for other control mRNAs since transcription levels change under certain conditions.

Poly-T probe was synthesized using 140 μg Poly-A (Sigma) 10μg/μl, 2 mM DTT, 50 mM Tris (pH 8.3), 40 mM KCl, 50 U AMV-reverse transcriptase, 100 mM dTTP, 40 U RNASin, 7 mM MgCl2, 2 U Oligo dT(12-18) (Pharmacia), and 50 μCi P32 dTTP (NEN) in a final volume of 500 μl. The mixture was incubated for 2 hours at 37°C and the probe then hydrolyzed by the addition of 50 μl 0.1 N NaOH at 65°C for 30 minutes. The probe was then used to probe the blots under the conditions previously described for hybridization of dot-blots.
Statistical Methods

Data collection and calculation. Routine assays (e.g. ALA synthase, heme oxygenase, porphyrins) were designed to facilitate entry of data into pre-programmed spreadsheets. Excel 3.0 or 4.0 (Microsoft Corporation, ) was used to calculate means and standard error of the mean for most data presented. When statistical analyses were done, the individual data were used instead of the calculated means and standard errors. Exstatix (Selected Micro Systems, Yorktown Heights, NY) or JMP 2.0 (SAS Institute Inc., Cary, NC) were the statistical analysis packages routinely used. Exstatix was used to perform Students t-test and ANOVA. JMP 2.0 was used to perform ANOVA, Tukey-Kramer test for multiple comparisons, linear least squares regression analysis. p Values less than 0.05 were considered significant for all tests.
CHAPTER IV

PRIMARY CHICK EMBRYO LIVER CELLS AS A MODEL FOR HEPATIC HEME METABOLISM

Introduction

The chick embryo liver cell culture system was used to characterize the effects of iron on the induction of ALA synthase, porphyrins, and heme oxygenase in the presence of phenobarbital-like drugs and iron. Primary chick embryo liver cell cultures are a simple, reproducible system for exploring hepatic porphyrin metabolism. Other culture systems, most notably primary rat hepatocyte cultures, do not retain normal heme and cytochrome P-450 metabolism and the conversion of ALA to heme is more like humans in chicks than in rats. The chick embryo liver cell culture yields data similar to that observed in whole animals such as chick embryos, mice, rats or man. Most of the previous work in the field has focused on describing the inductions of ALA synthase or porphyrins in the presence of various drugs, most notably AIA, DDC or hexachlorobenzene with some research focusing on the function of cytochrome P-450 IIB forms in chick. The research presented within this chapter further explores the induction of ALA synthase, porphyrins, cytochrome P-450s, and heme oxygenase in the presence of
phenobarbital-like drugs, terpenes and iron. These experiments further demonstrate the usefulness of the chick embryo liver system for testing chemicals for porphyrinogenic properties, and for characterizing the cytochrome P-450 enzymes for catalytic activity. The final section will demonstrate the utility of the culture system for testing the porphyrinogenicity of terpenes and the posthumous diagnosis of Vincent van Gogh's illness.
Results

ALA synthase induction. The initial goal was to determine the effects of iron on the induction of ALA synthase, and to determine a mechanism by which both ALA synthase and heme oxygenase were highly induced under these conditions. Many drugs were screened for their ability to highly induce the synthase in the presence of iron. Previous data showed that, in the presence of 5 μM FeNTA, treatment of chick embryo liver cells with phenobarbital-like drugs caused uroporphyrin accumulation and ALA synthase induction. The only inducers that had this effect were those that are traditionally classified as phenobarbital-type inducers (e.g., glutethimide [31], mephenytoin and phenobarbital). These chemicals were known to preferentially induce cytochrome P-450 IIB, while others, β-naphthoflavone and 20-methylcholanthrene [35], primarily induced cytochrome P-450 IA. These chemicals were screened for their ability to induce ALA synthase 5 hours after treatment (Figure 4.1). The typical doses of the chemicals were chosen as the concentrations that produced the highest amount of cytochrome P-450 previously described. Subsequent work was done with glutethimide as a prototypical phenobarbital-like inducer, since it produced a measurable induction of ALA synthase at a lower concentration than the other phenobarbital-like chemicals, possibly because it is more lipophilic than phenobarbital. Glutethimide was also more consistent at inducing ALA synthase than any of the other chemicals tested, and glutethimide was readily available.
**Figure 4.1:** Effects of selected chemicals on activity of ALA synthase in chick embryo liver cell cultures. Chick embryo liver cell cultures were treated as indicated for 5 hours prior to harvest. The cell were harvested and assay performed as described in methods. Data represent mean + SEM, n=3.
Control DMSO
Glut 50μM
FeNTA 50 μM
β-Naph 15μM
Meph 200μM
PB 400μM
20-MC 4μM

ALA Synthase Activity
(nmole ALA·mg protein⁻¹·hour⁻¹)
The solvents (DMSO, NTA alone or the combination) were tested to see if they had any inductive effects on ALA synthase. Treatment of the cells with glutethimide, or the combination of glutethimide and FeNTA increased ALA synthase activity, while treatment of the cells with the solvents alone, or in combination produced no change in ALA synthase activity (Figure 4.2). The controls for all subsequent ALA synthase assay data presented were cells that had not been treated with solvents alone. This was done because: first, the solvents alone had no effect on ALA synthase activity; second, the requirements for doing all of the appropriate solvent controls would double the size of the subsequent experiments due to the number of different solvents used; and third, that the inclusion of both the “no treatment” control and the “glutethimide and iron” treatment in all subsequent experiments allows the direct comparison of data from week to week.

Porphyrin accumulation. Porphyrin accumulation in chick embryo liver cells treated with glutethimide was highly stimulated by the addition of iron (Figure 4.3). The mechanisms for porphyrin accumulation may be either due to the cytochrome P-450-dependent oxidation of the porphyrinogen precursors or the inhibition of heme biosynthetic enzymes, and the marked increase in the production of porphyrins and porphyrin precursor by ALA synthase. The mechanism by which uroporphyrin accumulates in these chick embryo liver cell cultures is through the oxidation of uroporphyrinogen and not the inhibition of uroporphyrinogen decarboxylase (UROD). The accumulation of uroporphyrin in these cells in the presence of iron mimics the effects of
Figure 4.2: Synergistic induction of ALA synthase by glutethimide and FeNTA and lack of effect of DMSO or NTA. Chick embryo liver cell cultures were treated with 50 μM glutethimide, 50 μM FeNTA, or the combination for 18 hours prior to harvest. Equal concentrations of the solvents, DMSO and NTA, were added to other plates for an identical time. NTA was added at 100μM since the iron:NTA ratio is 1:2 and DMSO was not added at more than 1 μl per ml medium. The cells were harvested and assays performed as described in Methods. Data represent mean ± SEM, n=3. An asterisk (*) represents a significant difference from “none”, students t-test, P<0.05.
Figure 4.3: Effects of glutethimide and FeNTA on porphyrin accumulation in chick embryo liver cell cultures. Chick embryo liver cell cultures were treated as indicated for 18 hours prior to harvest. The cells and media were harvested together and the spectrofluorometric porphyrin assay was done as described in Methods. Data represent mean, n=3. Error bars were excluded for clarity, and were within 7.5% of the mean (data not shown). An asterisk (*) represents a significant increase in total porphyrin from "none", students t-test, P<0.05.
excess hepatic iron in patients suffering from porphyria cutanea tarda. The accumulation of small amounts of porphyrin in the presence of glutethimide, and the relatively equal distributions of these porphyrins (Figure 4.3), can be adequately explained by the increase of ALA synthase (Figure 4.2). However, in the presence of iron, porphyrins accumulated rapidly, with higher accumulations of uroporphyrin than any of the other porphyrins. The lack of effect of DMSO or NTA showed that the effect was dependent on the iron and glutethimide. Time course experiments showed that the majority of the porphyrins accumulated after an initial lag phase, and not immediately after the addition of the chemicals (Figure 4.4). Other porphyrins, such as protoporphyrin, can be accumulated in this system after treatment with deferoxamine or N-methylprotoporphyrin, both inhibitors of ferrochelatase, or after treatment with ALA (data not shown).

**Cytochrome P-450 induction.** Induction of holo-cytochrome P-450 by drugs and exogenous xenobiotics, requires production of nascent heme. Holo-cytochrome P-450 is required for completion of phase 1 drug metabolism (For reviews see 2,5,158,785-788). A priori, it would seem logical that heme would act as a positive regulator of cytochrome P-450 expression. Padmanaban and co-workers have presented data that show heme acts as a positive regulator of cytochrome P-450 gene expression in rats. Holo-cytochrome P-450 is more stable than apo-cytochrome P-450, further supporting the notion that heme should positively regulate cytochrome P-450 expression. The earlier data presented by Padmanaban used relatively toxic, somewhat non-specific heme biosynthesis inhibitors,
Figure 4.4: Time course of total porphyrin accumulation in chick embryo liver cell cultures treated with glutethimide and FeNTA. Chick embryo liver cell cultures were treated with 50 μM glutethimide, 50 μM FeNTA, or the combination for the times indicated prior to harvest. The cells and media were harvested and the spectrofluorometric porphyrin assay completed as described in Methods. Data represent mean ± SEM, n=3.
Glutethimide (50 μM)

FeNTA (50 μM)

Glutethimide + FeNTA

Total Porphyrin Accumulation (pmole porphyrin-mg protein⁻¹)

Time (hrs)

0  5  10  15  20  25

0  200  400  600  800
notably cobalt and 3-amino-1,2,4-triazole. Cobalt does not specifically inhibit heme biosynthesis, and it also induces heme oxygenase and other proteins, including metallothionein. 3-Amino-1,2,4-triazole inhibits catalase along with having other effects on ethanol-inducible cytochrome P-450. Later research used 4,6-dioxoheptanoic acid, a specific inhibitor of ALA dehydratase, and produced similar results. However, others have presented data indicating that cytochrome P-450 gene expression occurs in the presence or absence of heme, using both the same experimental model and in cultured rat hepatocytes. Whether heme regulates the transcription of P-450 remains to be definitively answered. However, inducibility of cytochromes P-450 is critical for a good culture model of hepatic heme metabolism and occurs in the chick embryo liver cell culture model (Table 4.1).

Glutethimide, phenobarbital, mephenytoin, 20-methylcholanthrene and β-naphthoflavone, all caused significant elevations of cytochrome P-450 in chick embryo liver cell cultures (Table 4.1). These inductions were associated with increases of specific cytochrome P-450 related activities. The MC-type drugs caused increases in ethoxyresorufin O-deethylase (EROD) activities, and benzyloxyresorufin O-dealkylase (BROD) (Figure 4.5). However, no induction of pentoxyresorufin O-dealkylase activity was observed under any condition, unlike that seen in the rat, but similar to that observed in quail. This may be due to the species specific differences in the catalytic properties of the P-450's between the chick and rat. None of the phenobarbital-like chemicals caused inductions of any of the alkoxyresorufin O-dealkylase activities, similar to the lack of
Table 4.1: Induction of total cytochrome P-450 in the presence of different chemicals. Chick embryo liver cell cultures were treated with the indicated chemicals for 18 hours. The cells were harvested and total cytochrome P-450 content was assayed as described in Methods. The values represent mean ± SEM, n=3. An asterisk (*) represents a significant increase in total porphyrin from “Control”, students t-test, P<0.05.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytochrome P-450 Concentration (pmol P-450•mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>Glutethimide (50 μM)</td>
<td>87 ± 7*</td>
</tr>
<tr>
<td>Phenobarbital (400 μM)</td>
<td>72 ± 5*</td>
</tr>
<tr>
<td>20-Methylcholanthrene (4 μM)</td>
<td>58 ± 10</td>
</tr>
<tr>
<td>β-Naphthoflavone (15 μM)</td>
<td>69 ± 6*</td>
</tr>
<tr>
<td>Mephenytoin (200 μM)</td>
<td>82 ± 2*</td>
</tr>
</tbody>
</table>
**Figure 4.5:** Fold induction in cytochrome P-450-dependent oxidase activities in chick embryo liver cell cultures. Chick embryo liver cell cultures were treated with 50 μM glutethimide (Glut), 400 μM phenobarbital (PB), 4 μM 20-methylcholanthrene (20-MC), 15 μM β-naphthoflavone (β-NF) or 200 μM mephenytoin (meph) as indicated 18 hours prior to harvest. The cells were harvested and assays performed as described in Methods. Data represent mean + SEM, n=3. An asterisk represent a significant difference from control (p < 0.05), Student’s t-test.
Fold Increase in Substrate Specific Cytochrome P-450 Activity
(product formed·mg protein⁻¹·minute⁻¹)

- Control
- Glut
- PB
- 20-MC
- β-Nf
- Meph
induction observed in rats. Other similarities include induction of ethoxycoumarin O-deethylase (ECOD) activity by the MC-type chemicals (Figure 4.5) and induction of benzphetamine demethylase (Bphet DME) activity by phenobarbital-like drugs, but not by MC-type drugs (Figure 4.5). The small induction of cytochrome P-450 IIB dependent activity is due to the lack of a specific substrate for this isoform with a high turnover rate. 

Heme oxygenase induction. Heme oxygenase can be induced to high levels by at least two disparate mechanisms. One mechanism is dependent upon nascent heme synthesis, and one mechanism is independent of nascent heme synthesis. The first mechanism requires the cell to produce heme, which is the proximate inducer of heme oxygenase. This induction is rapid, with maximum activity observed between 6 and 12 hours after initial treatment (Figure 4.6). In the presence of a heme biosynthesis inhibitor, the induction of heme oxygenase is blocked, demonstrating the dependence on nascent heme synthesis. The second mechanism utilized for the induction of heme oxygenase is observed in the presence of metals, such as cadmium or cobalt with maximum activity observed after 18 hours (Figure 4.6). This mechanism is fundamentally different since addition of heme biosynthesis inhibitors does not block induction of heme oxygenase. Induction of heme oxygenase by glutethimide and iron was shown to be due to the chemicals added, not the solvent vehicles (Figure 4.7), subsequently, unless otherwise specified, controls were untreated cells. The mechanism whereby glutethimide and iron synergistically induce heme oxygenase is the subject of chapter V.
Figure 4.6: Time course of induction of heme oxygenase in the presence of heme and cobalt. Chick embryo liver cell cultures were treated with 10 μM heme or 75 μM cobalt chloride for the times indicated prior to harvest. The cells were harvested and the batch heme oxygenase assay performed as described in Methods. Data represent mean ± SEM, n=3.
Figure 4.7: Effects of glutethimide and FeNTA on activity of heme oxygenase in chick embryo liver cell cultures. Chick embryo liver cell cultures were treated as indicated 18 hours prior to harvest. The cells were harvested and the batch heme oxygenase assay performed as described in Methods. Data represent mean + SEM, n=3. An asterisk (*) denotes a significant change from control, p<0.05, Student's t-test.
Activity of Heme Oxygenase

(pmole bilirubin formed·mg protein⁻¹·minute⁻¹)

- None
- Glut 50µM
- FeNTA 50µM
- Glut + FeNTA
- DMSO 5µl
- NTA 100µM
- DMSO + NTA
Porphyrinogenicity of terpenes and historical implications of porphyria. There have been some retrospective diagnoses of porphyria in persons of historical interest who suffered from undiagnosed diseases which had symptoms characteristic of porphyria. One of the more interesting historical figures is King George III, England.\textsuperscript{821-825} The hypothesis is as follows: The ancestors of George III, in the Royal Houses of Stuart and Hanover suffered from intermittent bouts of abdominal pain, and passed port wine colored urine. Sir Theodore Turquet de Mayerne, physician to James VI described one of the attacks as follows:

"On his return [from a hunting trip] he passed blood red urine.... He also told me that he quite frequently passed water, red like Alicante wine but without attendant pain...."

Intermittent bouts of abdominal pain were also suffered by James' mother, Mary Queen of Scots. Similar accounts were recorded in the extensive documentation associated with royalty for descendants of these houses, including George III. Skeptics of the porphyria theory have yet to propose an acceptable alternative theory.\textsuperscript{10} It is interesting to note that King George's illness may have caused excessive taxation for the colonies in the America's in the 1770's, and may have had a part in the independence of the American colonies, since it is proposed that part of the reason for King George's temper and lack of rational thought was the neurologic dysfunctions of porphyria. However, these conclusions must remain only tentative due to the lack of direct evidence, and the equivocal diagnosis of the attending physicians.

Vincent van Gogh, another historically interesting figure, may have suffered from acute intermittent porphyria.\textsuperscript{826} His torrid and brief career
was interspersed with bouts of abdominal pain, hallucinations and seizures, which occasionally required hospitalization. Van Gogh frequently exposed himself to terpenes, namely camphor [32], thujone [33], and α-pinene [34], the major constituent of oil of turpentine. These terpenes were tested for their ability to cause porphyrin accumulation in chick embryo liver cells, alone and in the presence of deferoxamine, a chemical that causes a block in heme biosynthesis similar to that observed in porphyria. The porphyrins were separated by reverse phase HPLC and show that all the chemicals tested caused a slight increase in porphyrin accumulation. However in the presence of deferoxamine, a large amount of porphyrin was accumulated (Figure 4.8), indicating that if van Gogh suffered from AIP, ingestion of any, or all, of these chemicals could certainly precipitate an acute attack. The accumulation of the porphyrins were associated with increases in ALA synthase activity, cytochrome P-450, and heme oxygenase. The data provided in this study support, but do not prove, the hypothesis of Arnold that Vincent van Gogh suffered from acute intermittent porphyria.
Figure 4.8: HPLC separation of porphyrins accumulated by chick embryo liver cell cultures treated with 50 μM glutethimide, 100 μM deferoxamine and selected terpenes (1 mM each). Chick embryo liver cell cultures were treated as indicated 18 hours prior to harvest. The cells were harvested and the porphyrins separated on HPLC as described in Methods (Assay performed by Sue Donohue). Data show representative samples from single 60 mm tissue culture dishes.
Conclusion

The utility of the chick embryo liver cell culture system for the study of porphyrin and heme metabolism, along with the study of phase 1 drug metabolism, has been demonstrated. The inductions of ALA synthase and heme oxygenase, the rate-limiting enzymes of heme anabolism and catabolism, respectively, recommend this system for use in the study of the regulatory features of heme metabolism. The regulation of chick P-450's recommend the use of this system for studying the biotransformation of different chemicals by the cytochrome P-450 dependent reactions, and the accumulation of porphyrins in this system provides a simple, and rapid test for porphyrinogenic chemicals. The consistency of data obtained among humans, rats and the chick culture system demonstrates the applicability of results obtained with it to humans. This includes humans suffering from porphyria, or exposed to chemicals which perturb heme metabolism, and the testing of possible treatments for these patients.
CHAPTER V

MECHANISM OF THE SYNERGISTIC INDUCTION OF HEME OXYGENASE BY GLUTETHIMIDE AND IRON

Introduction

Heme oxygenase can be induced by at least two different mechanisms. One mechanism utilized by cadmium, cobalt and other metals increases heme oxygenase activity 5-15 fold, dependent on the metal and experimental system, and is independent of endogenous heme synthesis. The induction mediated by metal ions is not diminished in the presence of heme biosynthesis inhibitors, such as 4,6-dioxoheptanoic acid (4,6-DHA). The time required for induction of heme oxygenase by metal ions is longer than the time required to induce heme oxygenase in the presence of heme (Figure 4.6). These observations apply for activities, mRNA levels, and transcriptional activity.

A second mechanism utilized for induction of heme oxygenase is mediated by its substrate, heme. The levels of induction are similar to those seen in the presence of metals, however the induction is much more rapid (Figure 4.6). One distinguishing characteristic of a heme-mediated induction of heme oxygenase is the interdicting effects of inhibitors of heme synthesis. For example, the induction of heme oxygenase by chemicals such as phenobarbital or glutethimide is blocked in the presence of 4,6-DHA. Induction of heme oxygenase in the presence of 4,6-DHA still occurs if the cells are treated with heme, providing evidence
that the induction produced by chemicals is mediated by the increase in intracellular heme, and not by some "heme-analog-like" or toxic effect of 4,6-DHA.

Previous work\textsuperscript{230} has demonstrated that 5 \textmu M FeNTA was capable of producing remarkable synergism of drug-mediated increases of heme oxygenase, ALA synthase, and experimental uroporphyria.\textsuperscript{669} The effects of iron on heme metabolism are of particular importance not only because the role of iron as an essential element and component of heme, but also because iron overload may be associated with exacerbations of acute\textsuperscript{832} and chronic\textsuperscript{415,833} porphyrias and with increases in activities of both heme oxygenase and ALA synthase. The work described in this chapter was undertaken to elucidate the mechanism whereby FeNTA produces synergistic increases in heme oxygenase activities in cultured chick embryo liver cells treated with glutethimide.
Results

Synergism of iron and time course of induction. Induction of heme oxygenase by iron alone was small (≤ 2 fold), if at all (Figure 5.1). This induction was probably due to the metal-mediated induction of heme oxygenase. The induction of heme oxygenase by glutethimide was slightly larger, 2-3 fold, and has been shown to be dependent on nascent heme synthesis. When the combination of glutethimide and iron were added to the cell cultures heme oxygenase was induced synergistically (Figure 4.6, Table 5.1). Furthermore, the addition of exogenous heme did not enhance the induction produced by glutethimide and iron (Table 5.1). A time course study demonstrated that a synergistic increase in activity was not observed until after 6 hours, with the activity remaining elevated through 24 hours. The increase over the expected additive effects of glutethimide and iron individually was significant after six hours (Figure 5.1). The time course of the induction by glutethimide and iron more closely resembled that produced by heme and was more rapid than that observed with metal ions, although there was a lag time of 4-6 hours. These data provide evidence supporting the concept that endogenously synthesized heme mediates the induction of heme oxygenase produced by glutethimide and iron. One possible reason for increased heme formation in the presence of added iron is that iron, combined with increased protoporphyrin accumulation stimulated by glutethimide, increased heme formation.
Figure 5.1: Time course of induction of hepatic heme oxygenase by glutethimide and ferric nitrilotriacetate. Chick embryo liver cell cultures were treated with 50 μM glutethimide, 50 μM FeNTA, or the combination of 50 μM glutethimide and 50 μM FeNTA as indicated. The expected additive values were obtained by summing the inductive effects of glutethimide alone and FeNTA alone. The inset shows a detailed time course for the induction of heme oxygenase in the presence of both glutethimide and FeNTA between 6 and 12 hours. The activities for the combination treatment are significantly greater (p<0.02) than the additive values at 12, 18 and 24 hours, Student's t-test. (The errors for the expected additive values that were used to determine the p-values were the sums of the errors for the glutethimide alone and FeNTA alone data points.) Points represent the means and standard deviations of three determinations. When no error bars are shown the standard deviation falls within the size of the symbol.
Activity of Heme Oxygenase

Time after Treatment (hours)

Activity of Heme Oxygenase (pmole bilirubin formed/mg protein/minute)

Theoretical Additive Induction

Clathrin (50 µM)

FNTA (50 µM)

Clathrin + FNTA
Table 5.1: Heme oxygenase activity in chick embryo liver cell cultures after 18 hour treatments with 50 μM glutethimide, 50 μM FeNTA, and the combination of both chemicals in the presence or absence of 2 mM 4,6-DHA. The results are means ± standard error of the mean, number of samples as indicated in parentheses. 4,6-DHA, an inhibitor of heme biosynthesis, abolished the synergistic induction of heme oxygenase by glutethimide and FeNTA, indicating that ongoing heme synthesis is important for heme oxygenase induction. The additive value was obtained by summing the inductive effects of glutethimide and FeNTA individually. An asterisk (*) indicates a significant difference from control, a cross (†) indicates a significant difference from Glutethimide + FeNTA, and a British pound (£) indicates a significant difference from Glutethimide + FeNTA + 4,6-DHA. The statistical differences were detected using the Tukey-Kramer test for multiple comparisons.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity of Heme Oxygenase (pmol BR·mg⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=11)</td>
<td>91.1 ± 10.5†‡</td>
</tr>
<tr>
<td>Glutethimide (n=12)</td>
<td>173.3 ± 20.3*†</td>
</tr>
<tr>
<td>FeNTA (n=11)</td>
<td>132.1 ± 12.0†</td>
</tr>
<tr>
<td>Glutethimide + FeNTA (n=15)</td>
<td>484.9 ± 20.7*‡</td>
</tr>
<tr>
<td>Additive effect of Glutethimide and FeNTA</td>
<td>214</td>
</tr>
<tr>
<td>Glutethimide + FeNTA + Heme (n=3)</td>
<td>448.0 ± 13.0*‡</td>
</tr>
<tr>
<td>Glutethimide + FeNTA + 4,6-DHA (n=14)</td>
<td>186.7 ± 14.0*†</td>
</tr>
</tbody>
</table>
Dose response of iron. Dose response studies showed that as little as 0.5 μM FeNTA synergistically enhanced the induction of heme oxygenase in the presence of glutethimide, as seen with earlier experiments (Figure 5.2). The minimally effective concentration of FeNTA is unclear, but the data demonstrate that nanomolar amounts of iron can produce a significant potentiation of heme oxygenase activity, while increasing amounts will increase heme oxygenase activity in a dose dependent manner.

Effects of 4,6-dioxoheptanoic acid. When 4,6-DHA was added at the same time as glutethimide and FeNTA, the activity of heme oxygenase only increased to a level that was equal to that seen with either iron or glutethimide alone. The effects of 4,6-DHA on the induction of heme oxygenase provided strong evidence that nascent heme synthesis was required to produce the synergistic increase in heme oxygenase activity in the presence of glutethimide and iron (Table 5.1). Glutethimide and FeNTA in presence of 4,6-DHA produced heme oxygenase activities that were significantly different from control, but not significantly different from those observed in the presence of either glutethimide or iron individually (Table 5.1). When the increases in heme oxygenase activity for both glutethimide and FeNTA individually were added to the control heme oxygenase value, the expected increase in heme oxygenase activity in the presence of both glutethimide and FeNTA was obtained. The difference between the additive increase and the observed increase accentuates the synergistic effect of the combination of glutethimide and iron, over the expected additive induction from both chemicals individually.
Figure 5.2: Dose response of the iron synergism for the induction of hepatic heme oxygenase by glutethimide. Chick embryo liver cell cultures were treated with 50 μM glutethimide and the indicated concentration of FeNTA 18 hours prior to harvest. Heme oxygenase activity for untreated cells was 91 pmol bilirubin formed·mg protein⁻¹·minute⁻¹ and the heme oxygenase activity for glutethimide alone was 173 pmol bilirubin formed·mg protein⁻¹·minute⁻¹. The points represent the means and standard deviations of three determinations. When no error bars are shown the error fell within the size of the symbol.
Heme Oxygenase Activity
(pmol BR/mg protein/min)

Concentration of Ferric Nitritetriacetate [μM]
Quantitation of immunoreactive protein and mRNA. Iron synergism of heme oxygenase induction was accompanied by increased levels of heme oxygenase protein and mRNA (Figure 5.3). The levels of heme oxygenase immunoreactive protein and mRNA correlated with the observed activities. In the presence of either glutethimide or FeNTA both the mRNA and immunoreactive protein were slightly increased; however, when both chemicals were added together, larger amounts of both protein and mRNA were observed. When 4,6-DHA was added concomitantly with glutethimide and FeNTA the levels of protein and mRNA were dramatically decreased (Figure 5.3). These results suggest that the synergistic induction of heme oxygenase protein by glutethimide and FeNTA depends upon an increase in the level of heme oxygenase mRNA and thus the amount of heme oxygenase synthesized, rather than an increase in the half-life of the heme oxygenase protein. The apparent disparity between the increases in mRNA and protein may be due to several factors: first, the samples were taken at a single time point and due to the long half-life of the protein the mRNA levels were not maximal; second, there may not be a one-to-one relationship between the amounts of mRNA and protein; and third, the differences may be due to an, as yet, undefined mechanism. Further research is needed to delineate between these possibilities. These data are in agreement with other data demonstrating increased heme oxygenase mRNA in the presence of heme due to an increase in heme oxygenase gene transcription.228,229,834,835
**Figure 5.3:** Western blots and northern blots showing the relative amounts of heme oxygenase protein and mRNA from chick embryo liver cell cultures treated to induce heme oxygenase with and without an inhibitor of heme synthesis. The northern blot (top) lanes are 1-1 μg hepatic mRNA from Cd+2 treated chicken, 2-6 25 μg of total RNA from cultured chick embryo liver cells 18 hours after treatment with [2, control; 3, 50 μM glutethimide; 4, 50 μM FeNTA; 5, 50 μM glutethimide and 50 μM FeNTA; 6, 50 μM glutethimide and 50 μM FeNTA and 2 mM 4,6,DHA]. The total RNA was resolved on formaldehyde/agarose gels and transferred to Genescreen® membranes and probed with an oligonucleotide identical to a portion of the chicken heme oxygenase cDNA as described in the Methods. The western blot (bottom) lanes are 1 μg of the following: 1- purified avian hepatic heme oxygenase, 2-6 cultures chick embryo liver cells 18 hours after treatment with [2, control; 3, 50 μM glutethimide; 4, 50 μM FeNTA; 5, 50 μM glutethimide and 50 μM FeNTA; 6, 50 μM glutethimide and 50 μM FeNTA and 2 mM 4,6,DHA]. The proteins were separated by electrophoresis on a PhastSystem and the material transferred to nitrocellulose and probed with anti-chicken heme oxygenase as described in the Methods. The figure shows the synergistic increase in the amount of heme oxygenase protein and mRNA in the cells treated with glutethimide and FeNTA and the marked decrease when heme biosynthesis was inhibited by 4,6-DHA.
Conclusion

In summary, these results indicate that the iron synergism of heme oxygenase induction by glutethimide was associated with increased levels of heme oxygenase mRNA and protein, in agreement with the transcriptional mechanism described by others.\textsuperscript{228,229,834,835} That this synergistic induction of heme oxygenase mRNA and protein was mediated by heme is supported by several pieces of evidence: first, the induction was rapid and analogous to the induction of heme oxygenase by heme alone; second, addition of heme did not further induce heme oxygenase; and third, the synergistic induction of heme oxygenase was abolished with the addition of 4,6-DHA, a potent inhibitor of heme biosynthesis. These results provide a biochemical explanation for the previously described increased rate of hepatic heme breakdown in rats treated with iron and AIA,\textsuperscript{836} and may help to account for the known potentiation by iron of drug-mediated induction of ALA synthase\textsuperscript{832,837} and for the propensity of iron to exacerbate or precipitate porphyrias.\textsuperscript{415,832,833} To my knowledge, these studies were the first to characterize changes in levels of heme oxygenase mRNA and protein produced by chemicals or iron in a primary cell culture model that retains the same expression and inducibility of heme metabolic enzymes and hemoproteins as the intact animal.\textsuperscript{838,839}
CHAPTER VI

INDUCTION OF ALA SYNTHASE: RELATIONSHIP TO INDUCTION OF HEME OXYGENASE

Introduction

Regulation of hepatic heme metabolism is thought to occur via a regulatory heme pool. In this model heme oxygenase was assigned the role of primarily degrading excess amounts of heme presented to, or produced de novo, within the cell. In contrast, others proposed that changes in activity of heme oxygenase, presumably acting by reciprocal alterations in the size of the regulatory heme pool, were capable of exerting major effects on activity of ALA synthase and on levels of cytochrome P-450. Indeed an inverse relationship between activity of heme oxygenase and levels of cytochrome P-450 is frequently observed, although no known obligatory inverse relationship exists between these enzymes. The synergistic induction of both ALA synthase (Figure 4.2) and heme oxygenase (Figure 4.6) in chick embryo liver cells upon treatment of the cells with glutethimide and iron would not be predicted by the regulatory heme pool model and seems at variance with the view that regulatory heme is solely responsible for the regulation of heme metabolism. Two different hypotheses could be proposed that would explain these inductions: First, glutethimide and iron could cause a rapid uncontrolled induction of ALA synthase, exposing the cell to transient high
levels of heme and producing a secondary induction of heme oxygenase; or second, glutethimide and iron could cause a rapid induction of heme oxygenase, depleting the cell of heme and producing a synergistic induction of ALA synthase. The following results support the latter view.
Results

Effects of tin-mesoporphyrin on induction of ALA synthase and heme oxygenase activity produced by glutethimide and iron. In agreement with results of previous experiments in chick embryo liver cell cultures (Figure 4.2, Figure 4.7, Table 5.1), treatment of the cultures with 50 μM glutethimide and 50 μM FeNTA led to synergistic induction of ALA synthase and heme oxygenase (Figure 6.1). To modulate heme oxygenase induction, 50 nM tin-mesoporphyrin [41], a potent inhibitor of heme oxygenase was used to inhibit heme oxygenase activity. Tin-mesoporphyrin, when added with glutethimide and iron 18 hours prior to harvest, inhibited heme oxygenase activity and suppressed the synergistic induction of ALA synthase (Figure 6.1). Tin-mesoporphyrin inhibited heme oxygenase activity under all conditions, but only suppressed ALA synthase induction in the group treated with glutethimide and iron. ALA synthase induction by glutethimide alone was not affected by tin-mesoporphyrin nor did tin-mesoporphyrin significantly reduce ALA synthase activity in the presence of FeNTA alone.

Time course of induction of ALA synthase and heme oxygenase. Further understanding of the synergistic inductions of heme oxygenase and ALA synthase by glutethimide and iron was gained from detailed time course experiments (Figure 6.2). The results show a small increase in ALA synthase as soon as 3 hours and that both ALA synthase and heme oxygenase are highly (>2 fold) induced after 6 hours and before twelve
Figure 6.1: Effects of 50 nM Tin-mesoporphyrin on ALA synthase and heme oxygenase induction by 50 μM glutethimide and 50 μM FeNTA. Tin-mesoporphyrin (Sn-meso) was added to chick embryo liver cell cultures together with glutethimide and FeNTA. After 18 hours the cells were harvested and assays were performed as described in Methods. The results demonstrate the effectiveness of 50 nM tin-mesoporphyrin at inhibiting heme oxygenase activity, while only exerting an effect on ALA synthase activity in the presence of both glutethimide and FeNTA but not in the presence of either one individually. Results are mean + SEM, n=3.
Activity of ALA Synthase
(nmol ALA·mg protein⁻¹·hr⁻¹)

Control
Gt
Gt + SnM
Fe
Fe + SnM
Gt + Fe
Gt + Fe + SnM

Activity of Heme Oxigenase
Figure 6.2: Time course of induction of ALA synthase and heme oxygenase by glutethimide and FeNTA. Chick embryo liver cell cultures were treated with 50 µM glutethimide and 50 µM FeNTA and harvested at the times indicated. Assays were performed as described in Methods. The inset shows a more detailed time course during the critical time from six to twelve hours after the start of treatment. Heme oxygenase reached maximum activity by nine hours while ALA synthase started to increase dramatically after 10 hours and continued to rise through 24 hours. Results are mean ± SEM, n=3. When no error bars are present the standard error of the mean falls within the size of the symbol.
hours. An hourly time course of the critical period between 6 and 12 hours indicated that the synergistic induction of heme oxygenase activity preceded the synergistic induction of ALA synthase activity (Figure 6.2, inset). Specifically, heme oxygenase reached maximum activity by nine hours, while the increase in ALA synthase activity above that observed at six hours first became significant at 11-12 hours and continued to increase through 24 hours. Since heme oxygenase was synergistically induced first, these results suggest that synergistic induction of heme oxygenase activity may produce the synergistic induction of ALA synthase by glutethimide and iron.

**Repression of ALA synthase activity by tin-mesoporphyrin and heme.** The next experiment tested whether tin-mesoporphyrin could terminate or blunt the synergistic induction of ALA synthase that had already commenced upon prior treatment with glutethimide and iron. This was done to test the hypothesis that early inductions of heme oxygenase would deplete a regulatory heme pool allowing uncontrolled induction of ALA synthase. When tin-mesoporphyrin was added 14 hours after the treatment of the cultures with glutethimide and iron, heme oxygenase activity was decreased within two hours and no further increases in ALA synthase activity were observed (Figure 6.3). Since tin-mesoporphyrin probably affects induction of ALA synthase by virtue of its ability to increase a regulatory heme pool, heme (10 µM) was added to the cultures to determine if it would have an effect similar to that of tin-mesoporphyrin. This concentration of heme was used since it gave maximal induction of heme oxygenase. Heme not only prevented further increases in ALA
Figure 6.3: Effects of 10 μM heme and 100 nM Sn-mesoporphyrin added to chick embryo liver cell cultures treated with 50 μM glutethimide and 50 μM FeNTA. Heme, Sn-mesoporphyrin, or a combination of both, were added after cultures had been treated for 14 hours with glutethimide and FeNTA. The cells were harvested at the indicated times and assays were performed as described in Methods. The results show the dramatic decrease in ALA synthase activity in the presence of the combination of heme and tin-mesoporphyrin, while either metalloporphyrin produced significant, but less rapid decreases in ALA synthase activity. The inhibitory effects of 100 nM tin-mesoporphyrin are potent even in the presence of 10 μM heme. Results are mean ± SEM, n=3. When no error bar is present the standard error of the mean falls within the size of the symbol.
Glutethimide and Iron

+ Sn-Meso @ 14 hours

- Heme @ 14 hours

+ Sn-Meso and Heme @ 14 hours

Activity of ALA Synthase

(nmol ALA/mg protein/hr)

Time after Original Treatment (h)

Activity of Heme Oxygenase

(pmol BR/mg protein/min)

Time after Original Treatment (h)
synthase activity, but caused a reduction in ALA synthase activity within 4 hours after heme addition (Figure 6.3A). The combination of tin-mesoporphyrin and heme, when added at 14 hours after treatment with glutethimide and iron, reduced ALA synthase activity by 70% within two hours after treatment, more rapidly than addition of heme alone. These data further support the concept that heme oxygenase activity can influence activity of ALA synthase by modulating levels of a regulatory heme pool.

Effects of heme synthesis inhibitors on induction of ALA synthase. In an effort to deplete the regulatory heme pool by a different means, inhibitors of heme synthesis, either 4,6-DHA, an inhibitor of ALA dehydratase, or N-methylmesoporphyrin, an inhibitor of ferrochelatase, were added to the cultures. Addition of either inhibitor in combination with both glutethimide and iron suppressed the synergistic induction of heme oxygenase (Table 6.1), indicating that this induction requires intracellular heme synthesis, in accordance with previous findings (Table 5.1). When 10 μM exogenous heme was added along with 4,6 DHA, glutethimide and iron, heme oxygenase activity was induced to a level coinciding with heme oxygenase activities following treatment with glutethimide and iron alone. When 10 μM heme was added with N-methylmesoporphyrin, glutethimide and iron, heme oxygenase activity increased but did not return to fully induced levels. ALA synthase activity was further increased when either 4,6-DHA or N-methylmesoporphyrin was added to the culture along with glutethimide and iron (Table 6.1), indicating that if a regulatory heme pool is depleted, very marked induction of ALA synthase can occur. As expected, 10 μM exogenous heme
Table 6.1: Effects of inhibitors of heme biosynthesis on activities of ALA synthase and heme oxygenase. Chick embryo liver cell cultures were treated for 18 hours with 50 μM glutethimide and 50 μM FeNTA and with 2mM 4,6-dioxoheptanoic acid (4,6-DHA), 100 nM N-methylmesoporphyrin or 10 μM heme as indicated for 18 hours prior to harvest. Cells were harvested and assays performed as described in Methods. Results are mean ± SEM, n=3. An asterisk (*) represents values that are significantly different than “Glutethimide + FeNTA”, p<0.05, Student’s t-test.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity of ALA Synthase (nmol AI formed mg-1 phenylalanine)</th>
<th>Activity of ALA Oxigenase (nmol Heme formed mg-1 phenylalanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutethimide + FenNTA + N-methylmesoporphyrin + Heme</td>
<td>1.49 ± 2.9*</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + 4.6-DHA + Heme</td>
<td>4.26 ± 4.2</td>
<td>7.1 ± 0.2*</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + N-methylmesoporphyrin</td>
<td>8.4 ± 18</td>
<td>8.9 ± 0.4*</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + 4.6-DHA</td>
<td>9.8 ± 12</td>
<td>10.9 ± 1.4</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + N-methylmesoporphyrin + Heme</td>
<td>98 ± 38</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + 4.6-DHA + Heme</td>
<td>413 ± 38</td>
<td>0.8 ± 0.1*</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + N-methylmesoporphyrin</td>
<td>43 ± 62</td>
<td>4.3 ± 62</td>
</tr>
<tr>
<td>Glutethimide + FenNTA + 4.6-DHA + Heme</td>
<td>43 ± 62</td>
<td>4.3 ± 62</td>
</tr>
<tr>
<td>Control (and Glutethimide + Phenylalanine)</td>
<td>1.49 ± 2.9*</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
</table>

*Significant difference compared to control.
diminished the induction of ALA synthase by glutethimide, iron and 4,6-DHA or N-methylmesoporphyrin.
Conclusion

The ability of iron in combination with phenobarbital-like drugs to produce synergistic induction of hepatic ALA synthase has been known for many years, although the precise mechanisms underlying this synergism have remained obscure.\textsuperscript{415,548,653,669,838} The observation that the combination of iron and glutethimide produced synergistic induction of heme oxygenase (Figure 5.1, 6.1),\textsuperscript{669} to a level that is produced by the most potent inducers, prompted us to investigate the hypothesis that induction of heme oxygenase could sufficiently deplete the regulatory heme pool permitting a synergistic induction of ALA synthase. The detailed time course experiment (Figure 6.2, inset) shows that marked synergistic induction of heme oxygenase activity by glutethimide and iron did, in fact, precede by 4-8 hours the synergistic induction of ALA synthase. A small (<3 fold) increase in ALA synthase activity was observed within 6 hours of treatment, in agreement with previous results (Figure 4.1 and 253).

Addition of tin-mesoporphyrin, a potent inhibitor of heme oxygenase, prevented (Figure 6.1) or promptly blunted (Figure 6.3) the synergistic induction of ALA synthase by glutethimide and iron. When heme was added during the period that ALA synthase activity was increasing, not only were further increases prevented, but reduction of ALA synthase activity occurred within 4 hours. When tin-mesoporphyrin and heme were added concurrently, ALA synthase activity was reduced within two hours, even more rapidly than the reduction that occurred with heme alone (Figure 6.3). This suppressive effect of tin-mesoporphyrin on the
synergistic induction of ALA synthase activity, by glutethimide and iron, is unlikely to be due to a direct or “heme-like” effect on ALA synthase by tin-mesoporphyrin because: 1) low concentrations of tin-mesoporphyrin (50 or 100 nM) were used, 2) tin-mesoporphyrin had no effect on the induction of ALA synthase by glutethimide alone, and 3) 5 μM tin-mesoporphyrin had no effect on ALA synthase activity in vitro (data not shown).

Attempts to deplete the regulatory heme pool by impeding heme synthesis within the cell using 4,6-DHA or N-methylmesoporphyrin also suggested that heme oxygenase induction by glutethimide and iron was dependent upon heme formation (Table 6.1). Thus, inhibitors of heme synthesis suppressed induction of heme oxygenase. They also led to even greater inductions of ALA synthase, demonstrating that, when heme synthesis was blocked in the presence of glutethimide, marked induction of ALA synthase occurs. These data are in agreement with previous reports. When the deficiency of regulatory heme, produced by the inhibition of heme synthesis, was corrected by the addition of exogenous heme, the effects on induction of ALA synthase were blunted or abolished (Table 6.1). As shown in Figure 6.3, effects of heme to reduce ALA synthase were rapid and impressive. The lesser effects observed in Table 6.1 are due to the long incubation time (18 hours). N-methylmesoporphyrin is not a direct inhibitor of heme oxygenase in vitro; thus, the reason that heme in the presence of N-methylmesoporphyrin failed to restore heme oxygenase activity to levels comparable to those obtained with the glutethimide and iron treatment is not clearly understood. These results support the hypothesis that alterations in activity of heme oxygenase can modulate the
size of a regulatory heme pool and thereby affect the activity of ALA synthase.
CHAPTER VII

REPRESSION OF ALA SYNTHASE BY METALLOPORPHYRINS

Introduction

ALA synthase is the first and rate-controlling enzyme of heme biosynthesis in liver and can be induced to high levels by a variety of compounds. Activity of this enzyme is elevated in humans with acute porphyrias, in whom occurrence of clinical illness is associated with increased excretions of porphyrins and porphyrin precursors, the biochemical hallmarks of the acute porphyrias. The treatment of choice, for all but mild attacks of acute porphyria, is intravenous heme, first described twenty-two years ago. Heme reduces hepatic ALA synthase levels in various experimental models of acute porphyria and undoubtedly has the same effect in patients suffering from acute porphyria. High doses of heme, 10μM in culture models and 3-4 mg/kg/day in patients, are required to decrease ALA synthase activities.

Reasons for the high required doses of heme include its ability to induce heme oxygenase, the first and rate-controlling enzyme in heme degradation. Certain non-heme metalloporphyrins, notably cobalt-, tin-, and zinc-porphyrins, inhibit heme oxygenase in a competitive manner. Tin-protoporphyrin [42], tested for its effect in hyperbilirubinemic patients, has also been shown to modestly
decrease urinary excretion of ALA and porphobilinogen in patients suffering from acute porphyria.\textsuperscript{595} Recently a combination of tin-protoporphyrin and heme has been considered effective in treating a patient with severe, recurrent attacks of acute porphyria.\textsuperscript{626} Nevertheless, concerns about the therapeutic use of tin-protoporphyrin, or other tin-porphyrins, persist because of their photosensitizing properties,\textsuperscript{597,607,609,610,613,634,639,851,852} long biological half-life,\textsuperscript{626} inhibition of steroidogenesis,\textsuperscript{628,850} and the ability of tin-porphyrins to cross the blood-brain barrier.\textsuperscript{853} The use of tin-diiododeuteroporphyrin [40], in place of tin-proto- or tin-meso-porphyrin, may allay some of the concerns, since the iodinated porphyrin has been suggested to be less photoactive.\textsuperscript{851}

Theoretically, a combination of relatively low concentrations of heme and another metalloporphyrin, inhibitory for heme oxygenase activity, should markedly repress hepatic ALA synthase activity. Accordingly, the potencies and efficacies of several non-heme metalloporphyrins (with and without heme) were compared with respect to inhibition of heme oxygenase and reduction of ALA synthase activity. The results showed that there was a direct relationship between inhibition of heme oxygenase activity and repression of ALA synthase activity, in chick embryo cultures induced with glutethimide and iron, and that zinc-mesoporphyrin or -protoporphyrin was as potent and efficacious as the corresponding tin-porphyrins in exerting these effects. In contrast, zinc-deuteroporphyrin-2,4-bisglycol or copper-porphyrins were ineffective at the concentrations tested. Furthermore, the combination of tin- or zinc-porphyrin plus heme additionally reduced ALA synthase activity, with maximal reduction of
activity obtained at concentrations of metalloporphyrins orders of magnitude lower than those previously used.
Results

Correlation of reduction of ALA synthase activity with heme oxygenase inhibition. Previously, ALA synthase activity was markedly increased after treatment of chick embryo liver cell cultures with 50 μM glutethimide and 50 μM iron. This increase in activity followed, by several hours, a synergistic induction of heme oxygenase, and could be halted, or reversed using either tin-mesoporphyrin (100 nM) or heme (10 μM) alone, or a combination of tin-mesoporphyrin (100 nM) and heme (10 μM). Tin-mesoporphyrin was previously shown to be more potent than tin-protoporphyrin for inhibiting heme oxygenase. Consequently, in the present work tin-mesoporphyrin was used as a benchmark for comparison of the effectiveness of low concentrations (50 nM) of selected metalloporphyrins, alone or in the presence of heme, in decreasing ALA synthase activity (Table 7.1). The ability to decrease ALA synthase activity was determined after induction of ALA synthase with 50 μM glutethimide and 50 μM iron. Tin-diiododeuteroporphyrin and zinc-mesoporphyrin [44] were the most effective metalloporphyrins at reducing ALA synthase activity. Similarly, in the presence of 200 nM heme, zinc-mesoporphyrin and tin-diiododeuteroporphyrin again caused the greatest reduction of ALA synthase activity. The results also showed that heme enhanced the effectiveness of metalloporphyrins in reducing ALA synthase activities with only nanomolar concentrations of each required to obtain a significant reduction. The metalloporphyrins that were not effective at reducing ALA synthase activity of intact cells were zinc-
Table 7.1: Effects of selected metalloporphyrins to inhibit heme oxygenase and decrease induction of ALA synthase in chick embryo liver cells. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA 18 hours prior to harvest, except for the uninduced group which received no treatment. Non-heme metalloporphyrins were added at a concentration of 50 nM with heme added at the indicated concentrations 4 hours prior to harvest. Cultures were harvested, sonicated and enzymatic activities measured as described in Methods. The unchelated porphyrins or the ionic metals alone (all added at 50nM concentration), had no significant effects (results not shown). An asterisk (*) denotes significant reduction of activity versus glutethimide and FeNTA, and a cross (†) denotes significant reduction of activity versus zinc-mesoporphyrin alone (p < 0.05), Student’s t-test. Data are mean ± SEM with the numbers of samples (n) in parentheses.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity of ALA Synthase $^*$</th>
<th>Activity of Heme Oxygenase $^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n mole ALA $\cdot$ mg protein$^{-1} \cdot$ hour$^{-1}$)</td>
<td>(p mole bilirubin $\cdot$ mg protein$^{-1} \cdot$ minute$^{-1}$)</td>
</tr>
<tr>
<td>Uninduced</td>
<td>$0.9 \pm 0.2$ (17)$^{*}$</td>
<td>$136 \pm 7$ (19)$^{*}$</td>
</tr>
<tr>
<td>Induced (Glutethimide + FeNTA)</td>
<td>$12.8 \pm 0.7$ (23)</td>
<td>$448 \pm 12$ (22)</td>
</tr>
<tr>
<td><strong>Induced cells treated with Heme</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 200 nM Heme</td>
<td>$9.9 \pm 0.7$ (14)$^{*}$</td>
<td>$488 \pm 9$ (23)</td>
</tr>
<tr>
<td>+ 1 $\mu$M Heme</td>
<td>$8.3 \pm 1.1$ (5)$^{*}$</td>
<td>$428 \pm 10$ (3)</td>
</tr>
<tr>
<td><strong>Induced cells treated with Tin porphyrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sn-Mesoporphyrin [41]</td>
<td>$9.3 \pm 0.8$ (3)</td>
<td>$237 \pm 2$ (3)$^{*}$</td>
</tr>
<tr>
<td>Sn-Mesoporphyrin + 1 $\mu$M Heme</td>
<td>$5.9 \pm 0.7$ (8)$^{*}$</td>
<td>$155 \pm 31$ (8)$^{*}$</td>
</tr>
<tr>
<td>Sn-Diiododeuteroporphyrin [40]</td>
<td>$6.3 \pm 0.4$ (3)$^{*}$</td>
<td>$190 \pm 19$ (3)$^{*}$</td>
</tr>
<tr>
<td>Sn-Diiododeuteroporphyrin + 200 nM Heme</td>
<td>$4.8 \pm 0.2$ (3)$^{*}$</td>
<td>$216 \pm 7$ (3)$^{*}$</td>
</tr>
<tr>
<td><strong>Induced cells treated with Zinc porphyrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn-Mesoporphyrin [44]</td>
<td>$6.7 \pm 0.4$ (26)$^{*}$</td>
<td>$191 \pm 14$ (14)$^{*}$</td>
</tr>
<tr>
<td>Zn-Mesoporphyrin + 200 nM Heme</td>
<td>$4.7 \pm 0.5$ (14)$^{*}$</td>
<td>$181 \pm 15$ (14)$^{*}$</td>
</tr>
<tr>
<td>Zn-Mesoporphyrin + 1 $\mu$M Heme</td>
<td>$4.3 \pm 0.5$ (3)$^{*}$</td>
<td>$260 \pm 0$ (3)$^{*}$</td>
</tr>
<tr>
<td>Zn-Protoporphyrin [42]</td>
<td>$8.6 \pm 0.4$ (3)</td>
<td>$326 \pm 19$ (3)</td>
</tr>
<tr>
<td>Zn-Protoporphyrin + 1 $\mu$M Heme</td>
<td>$7.3 \pm 0.6$ (3)$^{*}$</td>
<td>$379 \pm 12$ (3)</td>
</tr>
<tr>
<td>Zn-Deuteroporphyrin-2,4-bisglycol [43]</td>
<td>$11.6 \pm 1.4$ (3)</td>
<td>$544 \pm 21$ (3)</td>
</tr>
<tr>
<td>Zn-Deuteroporphyrin-2,4-bisglycol + 1 $\mu$M Heme</td>
<td>$9.3 \pm 0.3$ (3)</td>
<td>$542 \pm 57$ (3)</td>
</tr>
<tr>
<td><strong>Induced cells treated with Copper porphyrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu-Mesoporphyrin [38]</td>
<td>$15.1 \pm 1.9$ (5)</td>
<td>$441 \pm 35$ (5)</td>
</tr>
<tr>
<td>Cu-Mesoporphyrin + 200 nM Heme</td>
<td>$13.6 \pm 1.3$ (5)</td>
<td>$433 \pm 46$ (5)</td>
</tr>
<tr>
<td>Cu-Protoporphyrin [39]</td>
<td>$14.2 \pm 1.5$ (5)</td>
<td>$455 \pm 31$ (5)</td>
</tr>
<tr>
<td>Cu-Protoporphyrin + 200 nM Heme</td>
<td>$13.1 \pm 1.5$ (5)</td>
<td>$404 \pm 40$ (5)</td>
</tr>
<tr>
<td>Cu-Deuteroporphyrin [36]</td>
<td>$14.5 \pm 1.3$ (5)</td>
<td>$416 \pm 37$ (5)</td>
</tr>
<tr>
<td>Cu-Deuteroporphyrin + 200 nM Heme</td>
<td>$13.0 \pm 1.0$ (5)</td>
<td>$412 \pm 46$ (5)</td>
</tr>
<tr>
<td>Cu-Hematoporphyrin [37]</td>
<td>$13.3 \pm 1.2$ (5)</td>
<td>$401 \pm 36$ (5)</td>
</tr>
<tr>
<td>Cu-Hematoporphyrin + 200 nM Heme</td>
<td>$13.4 \pm 1.4$ (5)</td>
<td>$418 \pm 27$ (5)</td>
</tr>
</tbody>
</table>
**Figure 7.1:** Correlation of effects of selected metalloporphyrins on activities of heme oxygenase and ALA synthase. Chick embryo liver cell cultures were treated with 50 µM glutethimide and 50 µM FeNTA for 18 hours prior to harvest. Selected metalloporphyrins were added 4 hours prior to harvest at the concentrations described in Table 7.1. The cultures were then harvested and the assays done as described in Methods. In all cases the greater the inhibition of heme oxygenase, the larger the reduction of ALA synthase activity. As shown in the legend, the metal ions are denoted by the large open symbols, the different porphyrin macrocycles denoted as the smaller cross-like inset symbols, and the additions of heme as the smallest round (white or black) symbols. The error bars represent the standard errors for either ALA synthase (vertical) or heme oxygenase (horizontal) (Table 7.1). Where no error bars are shown, the error falls within the size of the symbol. The correlation is highly significant (p<0.00002), linear regression analysis.
Additions

Tin Porphyrins

1000 nM Heme

Protoporphyrins

200 nM Heme

Mesoporphyrins

Copper Porphyrins

Zinc Porphyrins

Heme Oxygenase Activity (pmol bilirubin mg protein\(^{-1}\) min\(^{-1}\))

Activity of ALA Synthase (mole ALA mg protein\(^{-1}\) hr\(^{-1}\))

\[ Y = 1.1 + 0.02x \quad R^2 = 0.77 \]
deuteroporphyrin-2,4-bisglycol [43] and all the copper metalloporphyrins tested [36-39].

*In vitro* effects of metalloporphyrins on ALA synthase and heme oxygenase activities. To characterize the effects of metalloporphyrins further, they were added *in vitro* to cell homogenates (Table 7.2). At concentrations of 50 nM, all the tin- and the zinc- metalloporphyrins (including zinc-deuteroporphyrin-2,4-bisglycol) were effective heme oxygenase inhibitors but had no effect on ALA synthase activity. The copper porphyrins were ineffective at inhibiting heme oxygenase and had no effect on ALA synthase. In intact cells, the ability of metalloporphyrins to decrease ALA synthase activity was directly correlated to their ability to inhibit heme oxygenase (Table 7.1, Figure 7.1). The metalloporphyrins that strongly inhibited heme oxygenase, e.g., zinc- and tin- porphyrins, also produced the large reduction of ALA synthase activity. In contrast, the metalloporphyrins that produced little or no heme oxygenase inhibition, e.g., copper-porphyrins and zinc-deuteroporphyrin-2,4-bisglycol, had little or no effect on ALA synthase activities.

Dose-dependent reduction of ALA synthase activity by zinc-mesoporphyrin and heme. The effects of increased concentrations of zinc-mesoporphyrin, in the presence of different concentrations of heme, were studied further (Figure 7.2). As shown, there was a dose-dependent decrease in ALA synthase activity with increasing concentrations of zinc-mesoporphyrin, and addition of heme (200 nM or 1 µM) enhanced this effect.
Table 7.2: Effects of selected metalloporphyrins on activities of ALA synthase and heme oxygenase in vitro. Cells were treated for 18 hours with 50 μM glutethimide and 50 μM FeNTA, after which they were harvested, sonicated and pooled. Metalloporphyrins (50 nM) were added to portions of the sonicates and enzyme activities assayed as described in Methods. Results shown are the means ± SEM, n=3, expressed as a percent of the control to which the carrier (BSA) had been added. The absolute activity of the control ALA synthase was 20.1 ± 0.2 n mole ALA·mg protein⁻¹·hr⁻¹ and the control heme oxygenase value was 357 ± 5 pmole bilirubin·mg protein⁻¹·minute⁻¹. Asterisks (*) denote a significant different from glutethimide and ferric nitrilotriacetate alone (p<0.05), Dunnet’s.
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50 nm Zinc Porphyrins

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50 nm Tin Porphyrins

Addition of Porphyrins to Assay

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Percent of Control
Figure 7.2: Effects of zinc-mesoporphyrin and heme on activity of ALA synthase. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 18 hours prior to harvest. The indicated concentrations of heme and zinc mesoporphyrin were added 4 hours prior to harvest. Cells were harvested, sonicated, and the activity of ALA synthase measured as described in Methods. The data represent means ± SEM, n=3. Where no error bars are shown, the error falls within the size of the symbol.
Activity of ALA Synthase (mole ALA/mg protein⁻¹·hr⁻¹) vs. Concentration of Zinc-mesoporphyrin (nM)

- 0 nM heme
- 200 nM heme
- 1 μM heme
Effects of zinc-mesoporphyrin and heme on ALA synthase induced by different chemicals. In addition to the combination of glutethimide and iron, marked induction of hepatic ALA synthase can be produced by treatment with glutethimide, or other phenobarbital-like drugs, and inhibitors of heme biosynthesis such as 4,6-DHA\textsuperscript{258,554} or deferoxamine (Des).\textsuperscript{245} The induction is thought to be due to a direct effect of the drug on the apparatus for enzyme synthesis, amplified by marked depletion of the regulatory heme pool due to inhibition of heme synthesis. If the effect of zinc- or tin- porphyrins to decrease the activity of ALA synthase is indeed due to the ability of these compounds to inhibit heme oxygenase, they should have little, if any, effect when heme synthesis has been blocked by prior treatment within the time frame studied (4 hours). The results shown in Figure 7.3 confirm this prediction. Unlike the ability of zinc-mesoporphyrin to significantly reduce ALA synthase induced by glutethimide and iron (Figure 7.3, Table 7.3), zinc-mesoporphyrin had no significant effect on ALA synthase activity induced by either glutethimide and 4,6-dioxoheptanoic acid or glutethimide and deferoxamine (Table 7.3). In contrast, 200 nM heme had a large effect on ALA synthase activity, probably because heme oxygenase activities found under these conditions are not significantly different from basal heme oxygenase activity (data not shown). Since heme oxygenase activity was low under these conditions, a relatively low concentration of heme (200 nM) significantly reduced the activity of ALA synthase (Figure 7.3, Table 7.3). At the time chosen for study, the combination of heme and zinc-mesoporphyrin was significantly
**Figure 7.3:** Effects of zinc-mesoporphyrin and heme on ALA synthase activity induced by glutethimide and inhibitors of heme synthesis. Chick embryo liver cell cultures were exposed to 50 μM glutethimide and 50 μM FeNTA or an inhibitor of heme biosynthesis, either 250 μM 4,6-dioxoheptanoic acid (4,6-DHA) or 1 mM deferoxamine (Des), 18 hours prior to harvest. Zinc-mesoporphyrin (50 nM) and/or heme (200 nM) was added as indicated 4 hours prior to harvest. Cells were harvested, sonicated, and the activity of ALA synthase measured as described in Methods. The results are mean ± SEM, n≥8.
Table 7.3: Tukey-Kramer statistics of the data presented in Figure 6.3 using JMP software. The positive (bold) values show pairs of treatments that are significantly different. As shown, the results for cells pre-treated with glutethimide and iron and then with heme, zinc-mesoporphyrin, or the combination were all significantly different from one another (α<0.05). For the other pre-treatment regimens, zinc-mesoporphyrin alone had no effect, whereas addition of heme led to significant decreases in activity, compared with no heme addition. For simplicity, the value for each comparison is listed once, the duplicate values being replaced by . For each condition n ≥ 8.
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more effective than the individual treatments only in the model where both ALA synthase and heme oxygenase had been induced (Table 7.3).

**Effects of zinc-mesoporphyrin and heme on the half-life of ALA synthase activity.** There was no change in the half-life of ALA synthase activity, after treatment with glutethimide and iron, in the presence of 10 μM heme, or 200 nM heme and 50 nM Zn-mesoporphyrin (Figure 7.4, apparent half-life =1.9-2.1 hours).

**Appearance of precursor ALA synthase protein.** The appearance of the precursor, cytosolic form of ALA synthase was observed in western blots using the antibodies raised against the carboxyl terminal peptide. These antipeptide antibodies should detect both the mature and immature forms of the enzyme. An early time course (0-2 hours after addition of both zinc-mesoporphyrin and heme) showed the rapid appearance of a higher molecular weight immunoreactive protein with the maximum observed 1 hour after metalloporphyrin treatment (Figure 7.5). The premature form of the enzyme was no longer observed 2.5 hours after metalloporphyrin treatment. These data are consistent with the concept that both heme and the combination treatment of zinc-mesoporphyrin and heme decreases mitochondrial uptake of the precursor ALA synthase protein via the same mechanism, involving the heme regulatory motif. The data correlate with the recent identification of this motif as a regulatory element for ALA synthase\(^2\) and other data describing an inhibitory effect of heme on the mitochondrial translocation of ALA synthase.\(^2\)\(^1\)\(^5\)-\(^2\)\(^1\)\(^6\)

**Effects of Zinc-mesoporphyrin and heme on the half-life of ALA synthase mRNA.** The combination of zinc-mesoporphyrin and heme
**Figure 7.4:** Effects of zinc-mesoporphyrin and heme on the half-life of ALA synthase activity. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 14 hours. Cultures were also exposed to a combination of 50 nM zinc-mesoporphyrin (ZnM) and 200 nM heme or 10 μM heme as indicated after 14 hours and 4 μM cycloheximide was added 15 hours after the original treatment. Cells were harvested at the indicated times, sonicated, and the activity of ALA synthase measured as described in Methods. The results are mean ± SEM, n=3.
Figure 7.5: Appearance of the cytosolic ALA synthase protein after treatment with heme or the combination of zinc-mesoporphyrin and heme. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA 14 hours prior to addition of the metalloporphyrins. Then either 10 μM heme or 50 nM zinc-mesoporphyrin and 200 nM heme were added and the cells harvested at the times indicated after the addition of the metalloporphyrins. The cell culture samples were harvested in a buffer containing potassium phosphate, 20% glycerol, and EDTA. The samples were sonicated and immediately placed into SDS-PAGE sample buffer as described in Methods. The samples were separated by PAGE and electrophoretically blotted onto PVDF, exposed to rabbit anti-chick ALA synthase IgG as described in Methods. The image was digitized at 300 dpi on a Hewlett Packard ScanJet Plus, and transferred into Image 1.41 (available from NIH). The image was digitally enhanced to highlight the appearance of precursor ALA synthase, and the densitometric analysis completed as described in the Image manual.
A

Glutethimide + FeNTA
+ 50 nM Zinc Mesoporphyrin
+ 200 nM Heme

Pre-ALAS + 10 μM Heme

B

Arbitrary Density Units

0 30 60 90 120 150

Time (min)

100

80

60

40

20

0

0 30 60 90 120 150

Time (min)
decreased the half-life of ALA synthase mRNA in a manner similar to that observed with heme (Figure 7.6). This is in agreement with previously published data describing heme decreasing the half-life of ALA synthase mRNA.189,190 These data suggest that the combination of zinc-mesoporphyrin and heme in sub-micromolar concentrations regulate ALA synthase using the same cellular mechanisms that lead to the decrease in ALA synthase activity in the presence of 10 μM heme.
Figure 7.6: Effect of heme and zinc-mesoporphyrin on the half-life of hepatic ALA synthase mRNA after pretreatment with glutethimide and FeNTA. Chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA 14 hours prior to addition of the metalloporphyrins. Then either 10 μM heme or 50 nM zinc-mesoporphyrin and 200 nM heme were added and the cells harvested at the times indicated after the addition of the metalloporphyrins. A) Time course of the disappearance of ALA synthase mRNA with the time on the abscissa shown after the addition of glutethimide and FeNTA. B) Apparent half-lives after treatment with 10 μM heme or 50 nM zinc-mesoporphyrin and 200 nM heme. Actinomycin D (0.4 μM) was added 15 hours after original treatment. The values on the abscissa represent the time after the addition of the metalloporphyrins.
**Graph A**

- Glutethimide + FeNTA
- + 10 μM Heme
- + 50 nM Zn-meso + 200 nM Heme

**Graph B**

- $y = 2100 \cdot e^{(-0.13x)} \quad R = 0.95$ (Glut + Fe)
- $y = 2500 \cdot e^{(-0.32x)} \quad R = 0.99$ (+ Heme (10 μM))
- $y = 3000 \cdot e^{(-0.28x)} \quad R = 0.96$ (+ ZnM (50 nM) + Heme (10 μM))

**Equations**

- $T_{1/2} = 5.2 \text{ h}$
- $T_{1/2} = 2.5 \text{ h}$
- $T_{1/2} = 2.2 \text{ h}$
Conclusion

In other systems, zinc-porphyrins have been effective inhibitors of heme oxygenase. In chick embryo liver cell cultures, low concentrations (50 nM) of zinc-mesoporphyrin and tin-mesoporphyrin were the only metalloporphyrins that significantly reduced ALA synthase, while tin-mesoporphyrin did not (Table 7.1). Heme oxygenase inhibition and reduction of ALA synthase activity were closely correlated, supporting the notion that heme is the proximate mediator of ALA synthase reduction (Figure 7.1). As shown in this chapter, chapter VI, and elsewhere, heme decreases ALA synthase activity, and metalloporphyrins inhibit heme oxygenase (Tables 7.1 and 7.2). Therefore, it is reasonable to conclude that a reduction of heme oxygenase will lead to an increase in heme within the cell, which in turn, will repress ALA synthase. The marked induction of ALA synthase by glutethimide and iron is related to synergistic induction of heme oxygenase after 6-12 hours of treatment (Figure 6.2).

The ability of metalloporphyrins to inhibit heme oxygenase in vivo parallels the ability of these compounds to inhibit heme oxygenase in vitro (Table 7.1, 7.2), except that zinc-deuteroporphyrin-2,4-bisglycol had a higher inhibitory effect in vitro than in vivo. This is probably due to the higher hydrophilicity of this compound, which reduces its ability to cross biological membranes. The lack of inhibitory effects of the copper-chelated porphyrins on heme oxygenase may be due to the low formation constants of axially ligated copper metalloporphyrins. The lower formation
constants, as much as 2-3 orders of magnitude when compared to zinc-metalloporphyrins, would lead to much lower levels of copper-metalloporphyrin:heme oxygenase complexes and therefore, at these low concentrations, to virtually no heme oxygenase inhibition.

The significant differences in ALA synthase activity produced by heme, zinc-mesoporphyrin, or the combination of zinc-mesoporphyrin and heme, in the cultures treated with glutethimide and iron unequivocally demonstrate the effectiveness of the combination of heme and metalloporphyrin. Reduction of ALA synthase by a relatively high concentration of heme (10 μM) and tin-mesoporphyrin (100 nM) had previously been shown to occur (Figure 6.3), although, at these concentrations, no further reduction of ALA synthase activity was produced by 100 nM tin-mesoporphyrin, compared to those produced by 10 μM heme alone. These preliminary studies provided the framework for the present study, which establishes that, individually, relatively low concentrations of heme (200 nM) and zinc-mesoporphyrin (50 nM), not only significantly reduced ALA synthase activity, induced by glutethimide and iron, but produced a further, significant and additive reduction when used together.

In contrast, at the times and concentrations studied thus far, additive effects are not observed in cultures treated with glutethimide and either 4,6-DHA or deferoxamine, although all of the induction regimens produced a marked induction of ALA synthase (Figure 7.3). The most likely explanation for the different effects of zinc-mesoporphyrin (± heme) on ALA synthase activity following glutethimide and iron, glutethimide
and 4,6-DHA, or glutethimide and deferoxamine treatment, lies in the fact that glutethimide and iron produce a synergistic induction of heme oxygenase while glutethimide and either of the heme biosynthesis inhibitors do not.\textsuperscript{258,554} Thus, following treatment with glutethimide and iron, zinc-mesoporphyrin, a potent inhibitor of heme oxygenase, is able to increase cellular heme, which represses ALA synthase. In contrast, following treatment with glutethimide and either 4,6-DHA or deferoxamine, the models where heme oxygenase activity remains low, zinc-mesoporphyrin (50 nM) had no effect on ALA synthase, at the times studied. This supports the conclusion that the effect of zinc-mesoporphyrin on ALA synthase (Figures 7.1,7.2) is secondary to inhibition of heme oxygenase, rather than a direct "heme-like" effect on ALA synthase. Probably even lower concentrations of zinc-mesoporphyrin, or other metalloporphyrin, would be required to prevent heme breakdown when heme oxygenase activities are at a basal level, like those observed in the presence of 4,6-DHA or deferoxamine.\textsuperscript{258,554} However addition of exogenous heme will induce heme oxygenase after 4-8 hours,\textsuperscript{230} even in the presence of heme biosynthesis inhibitors. Thus, in the presence of heme biosynthesis inhibitors, addition of zinc-mesoporphyrin would most likely prolong the heme suppression of ALA synthase, similar to that recently described clinically for tin-protoporphyrin.\textsuperscript{626,862}

In the acute hepatic porphyrias in relapse, there is marked induction of hepatic ALA synthase, often related to patients' ingestion of porphyrinogenic chemicals (Table 1.1).\textsuperscript{161,415,416} Activities of hepatic heme oxygenase activity have not been reported for patients with acute
porphyrias. Thus, the experimental model of ALA synthase induction that most closely recapitulates acute porphyria remains uncertain. Probably, the most comprehensive understanding will be gained by further study of several such models. Regardless of the method used to achieve induction of ALA synthase, low (nanomolar) concentrations of a combination of zinc-mesoporphyrin and heme were highly effective in reducing ALA synthase activity.

The short half-life of ALA synthase activity facilitates rapid regulation of this activity in response to heme and other chemicals (Table 7.1, Figures 7.1, 7.2 and 7.4).258 The lack of a change in the half-life of the activity following exposure of cells to heme or heme and zinc-mesoporphyrin (Figure 7.4) demonstrates that regulation of this activity by heme occurs by a mechanism other than a change in half-life of the protein. The transient appearance of the premature ALA synthase provides evidence consistent with the concept that in vivo the heme regulatory motif211 plays a role in controlling ALA synthase activity by inhibiting the mitochondrial processing of pre-ALA synthase.205-208,210,211,215,216 The decrease in the half-life of ALA synthase mRNA after treatment with 10 μM heme or the combination of much lower concentrations of zinc-mesoporphyrin and heme supports the observation that heme decreases the half-life of the mRNA.189,190 The mechanisms whereby heme decreases the half-life of ALA synthase mRNA remain to be elucidated. The principal focus of the work presented was to demonstrate that a combination of metalloporphyrins, inhibitory for heme oxygenase activity, and heme, both at nanomolar concentrations, is just as effective as
each individual treatment at micromolar concentrations and to present evidence that supports the regulatory mechanisms recently reported for high concentrations of heme alone.¹⁸⁹,¹⁹⁰,²¹¹
CHAPTER VIII

CONCLUSION

Introduction

The primary chick embryo liver cell culture system was used for exploring the regulation of hepatic porphyrin and heme metabolism. Results shown here (Chapter IV) show that the cultures retain normal inducibility of ALA synthase, cytochrome P-450's, heme oxygenase, and overproduction of porphyrins. Other culture systems, most notably primary rat hepatocyte cultures, do not retain normal heme and cytochrome P-450 metabolism. The rate of conversion of ALA to heme in chicks liver homogenates is similar to that observed in human liver homogenates, although lower than that observed in rat liver homogenates. In the absence of additional iron human and chick liver homogenates accumulate similar amounts of protoporphyrin and heme, while the addition of iron dramatically reduces the amount of protoporphyrin and increases the accumulation of heme in human liver homogenates.

The primary chick embryo liver cell culture system has yielded data similar to that observed in whole animals such as chick embryos, mice, rats or man. The ability of iron to increase ALA synthase, porphyrins and heme oxygenase were described in chick liver cell cultures. The results served to initiate further research investigating
the reason for the induction of both the rate controlling enzymes for heme synthesis and degradation in the presence of iron. The utility of the system was further demonstrated as a tool for testing the porphyrinogenicity of terpenes, with a focus on supporting the posthumous diagnosis of Vincent van Gogh as suffering from AIP.826

The synergistic increase in heme oxygenase activity, in the presence of glutethimide and iron, confirmed earlier observations that iron increased heme degradation by phenobarbital-like drugs.548 Subsequent research showed that the mechanism of the effect of iron was dependent upon nascent heme synthesis (Chapter V),554 and was not related to the metal-dependent induction previously described.230 The increases of heme oxygenase activity were correlated with increased immunoreactive protein and mRNA (Figure 5.3). These results were the first to provide a biochemical explanation for the effects of iron on hepatic heme degradation, and posed the possibility that the increased heme degradation could deplete a regulatory heme pool159,161,415 causing increases in ALA synthase.415,548,653,669,838

The hypothesis that induction of heme oxygenase could deplete the regulatory heme pool sufficiently to contribute to synergistic induction of ALA synthase was investigated (Chapter VI). This hypothesis would be considered unlikely using the model proposed by Granick168,181,222 for control of ALA synthase, because the concentrations of heme needed to saturate or induce heme oxygenase (≥ 2 μM) are orders of magnitude greater than those thought to be required to repress ALA synthase (~ 0.1 μM).168,181 However, data to support the hypothesis that induction of
heme oxygenase can lead to induction of ALA synthase were later presented by others.\textsuperscript{251} The time course experiments (Figure 6.2) and the additions of tin-mesoporphyrin or heme (Figure 6.3) supported the hypothesis that depletion of the regulatory heme pool played a role in causing the induction of ALA synthase. When tin-mesoporphyrin and heme were added concurrently, ALA synthase was repressed within two hours, even more rapidly than the repression that occurred with heme alone (Figure 6.3). These data thus provide further evidence that ALA synthase activity is modulated via a regulatory heme pool and that a sufficient amount of heme in the regulatory pool can repress ALA synthase activity. They strongly suggest that, under at least some conditions, increases in heme oxygenase activity can lead to increases in activity of ALA synthase (Figure 8.1).

Heme oxygenase inhibition and reduction of ALA synthase activity were closely correlated, supporting the notion that heme is the proximate metabolite reducing ALA synthase activity (Figure 7.1). Heme decreases ALA synthase activity,\textsuperscript{3,215,258,856,857} and metalloporphyrins inhibit heme oxygenase (Tables 7.1 and 7.2).\textsuperscript{576,580,858} Therefore, it is reasonable to conclude that a reduction of heme oxygenase will lead to an increase in heme within the cell, which in turn, will repress ALA synthase. The significant differences in ALA synthase activity produced by heme, zinc-mesoporphyrin, or the combination of zinc-mesoporphyrin and heme, in the cultures treated with glutethimide and iron unequivocally demonstrate the effectiveness of this combination treatment. Reduction of ALA synthase by relatively high concentrations of heme (10 \( \mu \text{M} \)) and tin-mesoporphyrin
Figure 8.1: Model for the synergistic induction of heme oxygenase and ALA synthase in the presence of glutethimide and iron. A) Glutethimide immediately caused a small induction of ALA synthase by a direct interaction with the gene, and the added iron permitted greater amounts of protoporphyrin to be converted to heme. B) After 6 hours the regulatory heme pool was large enough to exert an inductive effect on the heme oxygenase gene thereby increasing heme oxygenase activities to maximal levels. C) After 14-18 hours the heme originally produced de novo was degraded by heme oxygenase and there was no longer any regulatory effect on ALA synthase, thereby allowing ALA synthase activities to increase dramatically. The numbers in parentheses after the enzyme names represent enzyme activities typically found within the specified time frame. Units of measurement for ALA synthase activity are nanomoles ALA formed•mg protein⁻¹•hour⁻¹ and for heme oxygenase are picomoles bilirubin formed•mg protein⁻¹•minute⁻¹.
A (Addition of Glutethimide and FeNTA)

- Glycine + Succinyl-CoA
- Glutethimide

**ALA Synthase (0.7)**
- ALA
- Proto

**Heme Oxygenase (100)**
- Biliverdin, CO, Fe

**Iron**

**B (6-9 hours after original treatment)**

- Glycine + Succinyl-CoA
- ALA Synthase (2.0)
- ALAS mRNA

**Heme Oxygenase (500)**
- Biliverdin, CO, Fe

**Heme**

**C (14-18 hours after original treatment)**

- Glycine + Succinyl-CoA
- ALA Synthase (12)
- ALAS mRNA

**Heme Oxygenase (450)**
- Biliverdin, CO, Fe

**Heme**
(100 nM) had previously been shown to occur (Figure 6.3), although, at these concentrations, no further reduction of ALA synthase activity was produced by 100 nM tin-mesoporphyrin, compared to those produced by 10 μM heme alone. These results provided the framework for the present study, which establishes that, individually, relatively low concentrations of heme (200 nM) and zinc-mesoporphyrin (50 nM), not only significantly reduced ALA synthase activity, induced by glutethimide and iron, but produced a further, significant and additive reduction when used together.

Tin-chelated porphyrins can cause cutaneous photosensitivity,597,609,610,639,863,864 and are not readily catabolized in vivo 595,602,605,865 raising concerns about possible long-term adverse effects of these compounds.597,609,620,628,639,864 Previous results show that tin-mesoporphyrin is a potent inhibitor of heme oxygenase,578,611 that iodination of the tetrapyrole can reduce the photosensitizing properties of tin-protoporphyrin612 and that zinc-protoporphyrin is also effective at inhibiting heme oxygenase.582,583,627,628,634,866 Most of these studies were carried out in rats. Zinc, unlike tin, is an essential trace metal, and increased amounts of zinc-protoporphyrin occur naturally in red blood cells and perhaps other tissues under some conditions.138,140,141,867-871 Thus, if zinc-mesoporphyrin or another zinc-porphyrin can be shown to be effective in animals with little toxicity, its use would seem preferable to that of tin-porphyrins. Other zinc porphyrins, with less catalytic activity for triplet state reactions,872 and other non-photoactive inhibitors of heme oxygenase require further development and study. The results obtained here with zinc-mesoporphyrin recommend its further investigation for possible
clinical use, in conjunction with low doses of heme. A recent brief report of one patient with acute intermittent porphyria suggested that a combination of intravenous heme arginate and tin-protoporphyrin led to a more prolonged repression of hepatic ALA synthase than intravenous heme alone. This report and the data presented in this thesis suggest that the combination of low doses of heme and a metalloporphyrin that is a potent inhibitor of heme oxygenase may prove to be the most effective treatment for the acute or inducible porphyrias in relapse.

In summary, the data presented provide a biochemical explanation for the increases in heme oxygenase activity in the presence of a phenobarbital-like drug and iron, and establishes that increases in heme oxygenase activity can deplete the regulatory heme pool and lead to increases in ALA synthase activity. The model of chick embryo liver cell cultures treated with glutethimide and iron, was used to characterize a series of metalloporphyrins with respect to their ability to inhibit heme oxygenase and decrease ALA synthase, alone and in the presence of heme. These studies provide a rationale for the future clinical testing of the combination of a metalloporphyrin and heme for patients suffering from acute inducible porphyrias, or for other patients in whom heme may be a therapeutic agent.

Note added in proof. Further studies carried out in the laboratory under the direct supervision of Dr. Shirley Russo, using the theories developed here, recently demonstrated that the combined effect of zinc-mesoporphyrin and heme on ALA synthase in cultures treated with glutethimide and 4,6-dioxoheptanoic acid, were greater than either alone,
and that the combination produced a longer effect than heme alone (Figure 8.2).
Figure 8.2: Effects of zinc-mesoporphyrin and heme on ALA synthase activity after pretreatment with glutethimide and 4,6-dioxoheptanoic acid. Chick embryo livers cell cultures were treated with 50 μM glutethimide and 250 μM 4,6-dioxoheptanoic acid for 12 hours prior to treatment with metalloporphyrins. Then 200 nM zinc-mesoporphyrin and/or 1 μM heme were added and the cells harvested at the indicated times. The assays were performed as described in Methods. Data represent means ± SEM, n≥6. The assays were performed by Dr. Shirley Russo.
**Graph Description:**

The graph represents the activity of ALA synthase over time for different treatments.

- **Heme (1μM)**
- **ZnMP (200nM)**
- **Heme (1μM)+ZnMP (200nM)**

The graph shows:

- **X-axis:** Time (hr) ranging from 0 to 16
- **Y-axis:** ALA Synthase Activity (nmole ALA/mg protein · hr⁻¹)

**Legend:**
- Uninduced, untreated
- Induced, untreated

**Observations:**
- Heme treatment significantly reduces ALA synthase activity over time.
- ZnMP treatment also reduces activity, but less than Heme.
- Heme plus ZnMP treatment shows the lowest activity, indicating a synergistic effect.
- Uninduced and untreated samples show the highest activity.

**Data Points:**
- Each treatment has a set of data points indicating activity at different time points.

**Conclusion:**
- Heme and ZnMP treatments lead to decreased ALA synthase activity, with Heme plus ZnMP being the most effective.
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APPENDIX A: ENZYME COMMISSION NUMBERS

Aconitase 4.1.2.3
δ-Aminolevulinate dehydratase 4.2.1.24
δ-Aminolevulinate synthase 2.3.1.37
Biliverdin reductase 1.3.1.24
Coproporphyrinogen oxidase 1.3.3.3
Ferrochelatase 4.99.1.1
Heme oxygenase 1.14.88.3
L-alanine:4,5-dioxovaleric acid aminotransferase 2.6.1.43
L-alanine:glyoxylate aminotransferase 2.6.1.44
NADPH ferrihemoprotein reductase 1.6.2.4
Porphobilinogen deaminase (hydroxymethylbilane synthase) 4.3.1.8
Protoporphyrinogen oxidase 1.3.3.4
Uridine diphosphoglucuronate β-D-glucuronosyltransferase 2.4.1.17
Uroporphyrinogen III decarboxylase 4.1.1.37
Uroporphyrinogen III synthase 4.2.1.75
APPENDIX B: CONCISE LABORATORY PROTOCOLS

Chick Embryo Liver Cell Culture

I. Theory:
A. Remove livers, place in physiological buffer.
B. Chop liver in presence of trypsin to degrade extracellular matrix and release cells.
C. Lyse red cells and wash debris from cell suspension.
D. Suspend cells in media and plate onto culture dishes.
E. Treat cells with substances and measure response (enzyme levels, etc.).

II. Materials:
A. Plastic bags (double layer) in ice buckets for carcasses (30 eggs/bag).
B. Sterile instruments: scissors; small, fine-toothed curved forceps; toothed, straight end forceps; surgical, narrow end forceps for transfers, mosquito clamp and single edged razor blade (for chopping).
C. Sterile, 5 cm glass petri dishes for chopping (need one dish per ten eggs).
D. Stopwatch.
E. Sterile 25 ml glass pipette with large-bore end.
F. Sterile 125 ml flasks (need one flask per two chopping dishes).
G. Disposable glass 10 ml and 25 ml serological pipettes.
H. New, sterile, 15 ml plastic culture tubes (8 tubes/125 ml flask).
I. New, sterile culture dishes (labeled top and bottom).

III. Solutions:
A. Hank's minus Ca-sterile filtered same day.
B. Sassa's Ammonium bicarbonate Tris buffer-sterile filtered same day.
C. William's E Medium without glutamine (one packet/liter, additions detailed below).
D. 5% trypsin, stored in 0.6 ml aliquots.
E. DNase
F. Penicillin (10,000 U/ml)/Streptomycin (10,000 U/ml)-commercial stock.
G. Insulin
H. Dexamethasone phosphate
I. T3 (Triiodo-thyronine)

IV. Procedure:
1. Start with 16-17 day embryo, assume 8-10 ml innoculum/egg.
2. Cut blunt end of egg off with scissors, and remove embryo by holding neck between scissors blades.
3. Hold neck with forceps, cutting off head with scissors.
4. Holding skin of abdomen with forceps, cut up the middle; avoid cutting liver.
5. Dissect out liver, taking care not to rupture gall bladder.
6. Place livers in 10 cm dish containing 20 ml Hank's.
7. Using surgical forceps, transfer livers to new 10 cm culture dish containing 20 ml Hank's. Repeat transfers to fresh dish of Hank's two times.
8. Add 90 µl 5% trypsin to each glass petri dish containing 10 ml Hank's. (trypsin must be vortexed to resuspend any particulate matter).
9. Evenly distribute the livers among the glass dishes, using surgical forceps, 10-13 livers per dish.

10. Using sterilized razor blade in clamp, chop livers in dish for two minutes, timed with stopwatch.

11. Transfer livers to 125 ml sterile flask. (put 2-3 chopping dishes per flask)

12. Pipette liver up and down 2X using 25 ml wide bore pipette.

13. Place flask in 37°C water bath for a total of 20 min.

14. After 10 min in bath, add to flask 75 μl 1mg/ml DNase per chopping dish.

15. After 12 min in bath, remove flask and pipette up and down 4X using 25 ml wide bore pipette.

16. After 20 min in bath, remove flask and pipette up and down 2X using same pipette.

17. Evenly distribute liver suspension into 15 ml conical tubes containing 10 ml cold Hank’s (refrigerated prior to dissection) Note: Use one rack of 12 tubes for 2-3 chopping dishes.

18. Centrifuge tubes 90 sec at 1200 rpm (~500xg) in tabletop centrifuge.

19. Aspirate supernatant and resuspend pellet in 10 ml Sassa’s buffer, inverting and flicking to suspend pellet.

20. Place tubes on rotating wheel (wheel of fortune) for three min.


22. Repeat steps 19-21, then aspirate supernatant taking care to remove as much as possible.

23. Suspend pellet in 10 ml WEIH, inverting and flicking tube.

24. Centrifuge tubes to 200 rpm, turning off centrifuge as soon as speed is reached (no need to balance tubes).

25. Remove supernatant with 10 ml pipette, and place in innoculum bottle, DO NOT ASPIRATE SUPERNATANT INTO VACUUM FLASK.

26. If desired, resuspend cells in more media and repeat 23-25.

27. Plate cells as follows:

<table>
<thead>
<tr>
<th>Plate size</th>
<th>Innoculum vol.</th>
<th>Rinse</th>
<th>Treatment vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 cm</td>
<td>2 ml</td>
<td>1 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>6.0 cm</td>
<td>6 ml</td>
<td>2 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>10.0 cm</td>
<td>15 ml</td>
<td>5 ml</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

28. Shake tray at right angles to distribute innoculum evenly.

29. Next day, wash cells with WEH 2X at 37°C (add Dex and T3).

30. Remove all media with a vacuum system and add appropriate rinse volume of WEH, warmed to 37 C in water bath.

31. Allow incubation for 45-60 min, and repeat steps 30-31.

32. Remove second rinse and add appropriate volume of treatment.
SOLUTONS

I. William's E without glutamine

1 envelope prepackaged powder
2.2 g sodium bicarbonate (NaH₂CO₃)
1 liter Milli Q water
2.0 g glucose
10 ml glutamine (200mM)
10 ml Pen/Strep (commercial stock)
10 mg/liter phenol red
Filter sterilize.
Filter Dex, T3, and insulin into sterile media. (Add Dex and T3 for WEH on the day of use. Add insulin for WEIH first day only!)

Dexamethasone: stock is 10 mg/ml, stored at room temp. Make up dilution 30 µl/10 ml water; use 10 ml/liter media. Make fresh daily!

T₃ (Tri-iodo-L-thyronine): make up 1 mg/ml stock in .05 N NaOH; Dilute 1:10 in water and use 10 ml/liter media. Can be stored in refrigerator for use on day 2 only!

Insulin: make up 10 mg/ml in .05 N NaOH. Dilute 1:10 in water. Use 1 ml/liter media. Used only on day 1!

DNase: 1 mg/ml in water.

II. Hank's minus Ca (1 liter)

Add ingredients to water, otherwise precipitates occur.

8.0 g NaCl
1.0 g Dextrose (glucose)
.345 ml 1M Magnesium chloride (20.3g MgCl₂ * 6 H₂O/100 ml)
.676 ml .5M Sodium phosphate dibasic (7.1 g/100 ml)
.441 ml 1M Potassium phosphate monobasic (13.6 g/100 ml)
4.17 ml 1M Sodium bicarbonate (8.4 g/100 ml)

Adjust pH to 7.4 with 1 N HCl, make up to 1 liter, and store in non-sterile bottle at 4°C. Sterilize through Millipore filter the day of use. *Stable for 2 weeks.

III. Sassa's Ammonium bicarbonate Tris buffer (1 liter)

6.91 g Ammonium chloride
2.06 g Tris base
1.38 g Potassium carbonate

Add about 800 ml water, pH to 7.5 using (~10 ml) 6 N and 1 N HCl, make up to 1 liter and store in non-sterile bottle at 4°C. Filter sterilize the day of use. *Stable for 2 weeks. *
IV. Trypsin

Gibco (lyophilized): add 10 ml sterile water to achieve 5% (w/v). Store at -20 C in 0.6 ml aliquots. Discard after thawing.

CATALOG NUMBERS

Gibco: tel # 899-828-6686

Williams' Medium E without L-glutamine, without NaHCO₃: formula #785133

Trypsin, 25 mg lyophilized. Cat. #610-5095
ALA Synthase Assay

Purpose:

Measure the ALA-S (aminolevulinic acid synthase) activity in CELC (chick embryo liver culture) sonicates.

Buffers and Solutions:

Homogenization/Assay Buffer (pH 7.4 with HCl) for 250 mls

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 mM Tris</td>
<td>8.75 mls</td>
</tr>
<tr>
<td>30 mM NaP</td>
<td>15 mls</td>
</tr>
<tr>
<td>8 mM MgCl₂</td>
<td>4 mls</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>2.5 mls</td>
</tr>
<tr>
<td>15 mM succinate</td>
<td>3.75 ml of 1 M solution</td>
</tr>
<tr>
<td>10 mM Na levulinate</td>
<td>2.5 ml of 1 M solution</td>
</tr>
<tr>
<td>0.5 mM pyridoxal-5'-phosphate</td>
<td>0.03089g (Note: add after adjusting the pH of the buffer)</td>
</tr>
</tbody>
</table>

This buffer made be made as a stock solution and kept at 4°C for at least one month.

1M Glycine, pH 7.0 (7.5g/100mls pH with NaOH)

Note: freeze in 1 ml aliquots at -20°C

10% TCA

1:10 dilution of 100% TCA (Trichloroacetic Acid) stock solution in H2O

100% TCA Stock Solution

Add 500 mls H2O to a 500 g bottle of TCA; let dissolve

1M Sodium Acetate (13.61 g/100 mls)

10% 2,4 pentanedione in 1M sodium acetate (1ml added to 9 mls NaAC)

a) warm quickly in 85°C bath to get pentanedione into solution
b) Also the source of this is important - use from E.M. Science only) Another name for this chemical is acetylacetone
d.) The solution should be pale yellow; if it is dark yellow it is no good!

Modified Erlich Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>glacial acetic acid</td>
<td>200 mls</td>
</tr>
<tr>
<td>70% Perchloric acid</td>
<td>168 mls</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>32 mls</td>
</tr>
<tr>
<td></td>
<td>0.75 g</td>
</tr>
</tbody>
</table>

NOTE: a) the day to be used add 200 mg DMAB (p-dimethylamino-benzaldehyde) per 10 mls of the above solution
b) This solution will turn a yellow color.
1x PBS

Dilute from 10x PBS stock
Procedure:

1. Wash the cells on a 6 cm plate 2 times with PBS at room temperature.
2. Add 700 μl of complete homogenization buffer to the cells & scrape cells into buffer.
   Transfer suspension to a 12 x 75 cm glass culture tube. Place on ice.
3. Sonicate for 2 seconds at output setting 1.5, constant output.
4. Split each sonicate into 2 tubes (150 μl per tube), one labeled reference & the other labeled sample.
   (Note: there is enough sonicate to do 2 sample tubes & this may be necessary sometimes).
5. To the tubes labeled reference, add 10 μl 1 M glycine & 100 μl 10% TCA. Place on ice.
6. To the tubes marked sample, add 10μl 1M glycine. Incubate at 37°C for 30 minutes.

6a. Optional Also setup a blank and two standard tubes for each assay. This will show that the ALA, 2,4 pentanedione and the Ehrlich’s reagent really worked. The blank should consist of 150 μl homogenization buffer + 10 μl of 1 M glycine. The standard should consist of 140 μl homogenization buffer, 10 μl of 2μM ALA, & 10 μl 1M glycine. Incubate at 37°C for 30 minutes. Stop reaction with 100 μl 10% TCA. Continue processing tubes just like the reference tubes in the assay.

8. Add 100 μl 10% TCA to the sample tubes.
9. Spin all tubes at 3,600 rpms (3,000 x g) in the Beckman Tabletop centrifuge at 4°C, for 10 minutes.
10. Remove 150 μl of supernatant from each tube, being careful not to disturb the pellet, and place in a fresh tube.
11. Add 100 μl of 10% 2,4 pentanedione in 1M sodium acetate to each tube.
12. Incubate tubes at 85°C for 15 minutes.
13. Remove and chill on ice, for ~5 minutes, Remove and let come to room temperature.
14. Add 250 μl of complete modified Ehrlich’s reagent to each tube. Let sit at room temperature for 15 minutes for color development.
15. Read on the Amico with the following setup:
   -split beam
   -lower wavelength = 530 nm
   -upper wavelength = 650 nm
   -do wavelength acquisition
   -set x axis as the wavelength & y axis as absorbance (0 to 0.2)
   -use short skinny slits and rubber stoppers to raise cuvette up in order to read these small samples-put reference tube into the R cuvette position & the sample tube into the S position
   Note: use plastic mini cuvettes (1 ml capacity) to read the sample.
16. Calculate the nmole ALA/mg protein/hr for each sample using the ALA-S program in Excel. The information necessary to do this includes:
   -Absorbance at both O.D.=555 & O.D.= 650
   -the results of the protein determination on standards & samples or the protein concentrations of each sample
   -Note: the program is designed to calculate the average & standard error for a set of triplicates for each treatment or assay group; be careful of where you enter the data.
17. How the calculations are actually done by hand:
\[ \Delta OD(550-650) \times 177.78 / mg \text{ protein/ml} = \text{nmoles ALA/mg protein/hr} \]

\[
\frac{\text{nmoles ALA}}{mg \text{ protein/hr}} = \frac{\Delta OD_{555-650} \times 1 \text{ cm}}{0.065 \mu \text{moles/ml/cm}} \times 0.25 \text{ ml Ehrlich's salt} \times 0.25 \text{ ml ALA Pyrrole} \times 0.26 \text{ ml final assay} \times 60 \text{ min/hour} \times 1 \text{ ml}
\]

18. Typical Results:
Untreated CELC: 0.2 - 0.8 nmoles ALA/mg protein/Hr
Glut + Fe treated CELC: 6.5 - 8.5 nmoles ALA/mg protein/Hr.

Heme Oxygenase Assay (Batch)\textsuperscript{554}

1. Wash plates twice with 1.5ml room temperature PBS (pipetting the PBS down side of plate to prevent disturbing the cells).
2. Harvest cells from 6cm cell culture plates in .7-1.0 ml \textit{cold} Harvest Buffer with a rubber policeman or spatula.
3. Transfer each into a labeled 12 x 75mm culture tube in a rack on ice.
4. Sonicate each for 3 seconds using a microtip sonicator.
5. Remove aliquot for protein assay. (~50 \textmu l)
6. Label 16 x 100mm culture tubes 1,1A,2,2A....
7. Put tubes in rack on ice. To each tube add,

\[
\begin{align*}
\text{KPi } & 0.1M,pH 7.40 & 530 \mu l \\
\text{*Desferal} & 50 \mu l (65mg/ml water) \\
\text{BVR (rat liver 105,000g sup.)} & 20 \mu l \\
\text{Sonicate} & 200 \mu l (0.2-0.8 mg protein) \\
\text{*NADPH (Sigma N6505)**} & 50 \mu l (4mM -3.96 mg/ml in .1M KPi)
\end{align*}
\]

8. Run one control and one test per sample.
9. Place rack of tubes in 37\textdegree C shaking water bath for 5 min.
10. Add 150\mu l *Methemalbumin to sample (“A”) tubes.
11. After 10 min., place tubes in ice bath.
12. Add 20 \mu l p-hydroxymercuribenzoic acid (50mM stock stored in dark bottle at room temperature) to each tube to completely stop the reaction.
13. Shake well.
14. Add Methemalbumin to Blank tubes (150\mu l).
15. Scan 450-550 nm. (Sample v. Blank-Split beam)
16. Use \textit{EMM}=66 for 470-540 to calculate nmole bilirubin/ml.

\[
\text{O.D. (470-540) } \times \text{1.010 ml final volume } \times \text{1000 pmole}
\]

\[
0.066 \text{ O.D/nmol bilirubin/ml } \times \text{0.2ml initial vol } \times \text{10 min } \times \text{mg prot/ml } \times \text{1nmol}
\]

\[
= \text{pmol bilirubin/min } \times \text{mg prot}
\]

*Make fresh before use; keep on ice except for hemin/DMSO prior to adding to albumin.

Reagents:

<table>
<thead>
<tr>
<th>PBS</th>
<th>Harvest Buffer</th>
<th>Methemalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM KPi</td>
<td>0.1M KPi</td>
<td>Hemin 6.5 mg/ml in DMSO</td>
</tr>
<tr>
<td>.15 M KCl</td>
<td>20% Glycerol</td>
<td>BSA 1.1 mg/ml in 40mM Tris 7.6</td>
</tr>
<tr>
<td>pH to 7.4</td>
<td>1.0mM EDTA</td>
<td>Add 100 \mu l heme stock to 3.9 ml BSA</td>
</tr>
<tr>
<td></td>
<td>pH to 7.4</td>
<td></td>
</tr>
</tbody>
</table>

Chemicals Used:

- Desferal (deferoxamine mesylate) \textsuperscript{Sigma D-9533}
- NADPH \textsuperscript{Sigma N-6505}
- Hemin \textsuperscript{Sigma H-2375}
- BSA (Bovine serum albumin) \textsuperscript{Sigma A-7906}
- P-hydroxymercuribenzoic acid \textsuperscript{Sigma H-0642}
**12/7/92 Change:**
To save cost of NADPH, we decided to use .2 μmoles per reaction rather than the 1 μmole per reaction that we had been using. Testing shows no more than a 5% decrease in Heme Oxygenase activity.
Heme Oxygenase Assay (Kinetic)\textsuperscript{571}

1. Wash plates twice with 1.5ml room temperature PBS (pipetting the PBS down side of plate to prevent disturbing the cells)
2. Harvest cells from 6cm cell culture plates in .7-1.0 ml cold Harvest Buffer with a rubber policemen or spatula.
3. Transfer each into a labeled 12 x 75mm culture tube in a rack on ice.
4. Sonicate each for 3 seconds using a microtip sonicator.
5. Remove aliquot for protein assay. (~50 µl)
6. Mix in a cuvette:
   - KPi 0.1M, pH 7.40
   - *Desferal
   - BVR (rat liver 105,000g sup.)
   - Sonicate
   - Methemealbumin
   - 530 µl
   - 50 µl (65mg/ml water)
   - 20 µl
   - 200 µl (0.2-0.8 mg protein)
   - 150 µl

8. Let cuvette come to 37°C in heated cuvette holder.
9. Add 50µl *NADPH (Sigma N6505)**
10. Shake well
11. Record dA/dT one the Aminco, Dual wavelength assay, Mono 1 = 470, Mono 2 = 540 for 5 minutes.
12. Use Erm=66 for 470-540 to calculate nmole bilirubin/ml.

\[
\text{A.O.D.} (t_2-t_1) \times \frac{1000 \text{ ml final volume} \times 1000 \text{ pmole}}{0.066 \text{ O.D./nmol bilirubin/ml} \times 0.2 \text{ml initial vol} \times 10 \text{ min} \times X \text{ mg prot/ml} \times 1 \text{nmol} \times (t_2-t_1)} = \text{pmol bilirubin/min} \times \text{mg prot}
\]

*Make fresh before use; keep on ice except for hemin/DMSO prior to adding to albumin.

**Reagents:**

<table>
<thead>
<tr>
<th>PBS</th>
<th>Harvest Buffer</th>
<th>Methemealbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM KPi</td>
<td>0.1M KPi</td>
<td>Hemin 6.5 mg/ml in DMSO</td>
</tr>
<tr>
<td>.15 M KCl</td>
<td>20% Glycerol</td>
<td>BSA 1.1 mg/ml in 40mM Tris 7.6</td>
</tr>
<tr>
<td>pH to 7.4</td>
<td>1.0mM EDTA</td>
<td>Add 100 µl heme stock to 3.9 ml BSA</td>
</tr>
<tr>
<td>pH to 7.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*
HPLC Porphyrins

University of Massachusetts Protocol Developed by Sue Donohue

Reagents

Concentrated Phosphoric Acid
10% Phosphoric acid: measure 10 ml of phosphoric acid and dilute to 100 ml with Milli-Q water. Store at room temperature. Stable indefinitely.

Methanol: HPLC grade Fisher. The solvent is degassed by filtration using a 0.45 micron filter. On each day of use stir under vacuum for 5 minutes. Stable indefinitely at room temperature.

0.1M ammonium phosphate: measure 11.5 grams of ammonium phosphate (Fisher Scientific A-685) and dissolve in 950 ml of Milli-Q water.

Solvent A: Mix 0.1M ammonium phosphate with an equal volume of HPLC grade methanol. The pH of the final mixture is adjusted to 3.5 with concentrated phosphoric acid. The solvent is then degassed by filtration using a 0.45 micron filter. On each day of use, stir under vacuum for 5 minutes. Stable indefinitely at room temperature.

Porphyrin acid chromatographic marker kit (Porphyrin Products, Logan Utah): Dissolve contents of one tube in 2 ml 1M HCl. Final concentration of each porphyrin is 5 nM. Protect from light. Immediately before injection, dilute 100 μl of porphyrin standard with 2.0 ml of 0.1M HCl and inject 50 μl. Each peak will contain 12.5 pmoles of porphyrin.

Other Equipment and Supplies

Waters Baseline 810 HPLC System
Shimadzu RF-551 Fluorometer with a R-928 (red-sensitive) PM tube
C18 Reverse Phase Radialpak #85721

Calibration and Quality Control

A calibration standard curve was developed using the Porphyrin Acid Chromatographic marker kit which contains 5 nmole/ml of porphyrins of the Type 1 series: Uroporphyrin I, 7-COOH, 6-COOH, 5-COOH, Coproporphyrin I, and Mesoporphyrin IX.

Two point calibration curves are calculated by the Baseline System and printed as needed. The calibration standard is run with each run and at repeated intervals during the run. Using a 50 μl injection, the concentration is 12.5 pmoles. The 810 Baseline System will calculate concentrations and print the result on the custom report.

The porphyrin mixture, dissolved in 0.1 m HCl had a optimum peak fluorescence excitation wavelength of 398 nm and peak emission wavelength of 612nm. The excitation was scanned from 398-408nm and the emission scanned from 608-624nm.

Cell Culture Sample Preparation

Cell culture samples are obtained by scraping the cells into the media with a rubber policeman or spatula and sonicating the cell:medium mixture for 5 seconds, sonicator setting 2.5. Remove 400 μl and add an equal volume of 0.2 M HCl and centrifuge at 12,000 x g for 10 minutes to precipitate cellular debris. The supernatant is removed and 500 μl placed in a WISP micro sample tube and 200 μl of each sample is injected. Place the samples in the WISP starting at position 3.
A calibration standard is prepared each day of use: measure 2.0 ml 0.1M HCl into a WISP sample vial and add 100 µl of the porphyrin acid chromatographic marker. Cap the vial using the screw top cap with the septum and place in position 1 on the sample wheel.

Place 2.0 ml of 0.1 m HCl in a vial and load into sample position 2.

Procedure for turning on Baseline 810 System

Turn on the HPLC and all peripherals. Follow the prompts which appear on the screen:

Baseline, enter, n, enter until the Baseline 810 logo appears on the screen. Click on mouse.

Move the mouse to Run and Monitor Control Events. The screen will show the pumps, percentage of solvents and the flow per minute. Set the B pump to 30% and the flow to 1.5 ml per minute. Move the cursor to GO and click the left mouse button, the screen should display Active. The system will take at least 15 minutes to equilibrate.

Set the detectors:

**Waters 484:**
- 405nm wavelength
- Sensitivity: 0.01
- Data Display:
- Auto Zero
- AUFS: 0.01

**Shimadzu RF-551:**
- Excitation: 398nm:
- Emission: 620nm
- Range: x 16
- Response: 1.5

The absorbance detector only needs to by used for bilirubin, heme or biliverdin analysis.

Next click the cursor on the Sample Queue. Click on Edit and clear the queue. Next fill the sample queue with the appropriate information for the day's run including: a HCL blank as the first sample, three calibration standards at the beginning and one set the end of the run and one after every five samples.

Check under the queue wide parameters and make sure the queue processing options and report options are selected. If you want to process and print automatically, then the “review chromatogram” option should be de-selected; if you want to process and delete extraneous peaks from the integration, then the “review chromatogram” option should be selected.

When the WISP sample wheel has been loaded, click on RUN, Execute Methods, move the cursor to the M column, and click on the first sample and type F. Next go to the last sample to be run and click on this sample and type L. All the samples in between will fill with X. Any you don't want to run, click on the sample and hit the space bar to de-select.

Click on START and F5 to display acquisition.

Return to the execute methods screen(F3) and click on resume to resume processing and printing of reports.

To manipulate the chromatograms edit the queue wide parameters selecting "Review chromatogram". Now each chromatogram will be displayed and many parameters can be set, changed, verified and printed.
RNAzol

TOTAL RNA ISOLATION FROM CELL CULTURES
Eppendorf Tube Protocol

1. **Harvesting and Lysis of Cultures**
   Rinse the cells twice with room temperature PBS. (Aspirate well to remove any excess PBS as this will add enough volume so that the preparation will not fit in a bullet tube.) Cells grown in monolayer are lysed directly in the culture dish by the addition of 500 µl RNAzol to one 10 cm plate. Scrape the cells with a rubber policeman or spatula, and transfer the solution to the other plate. Scrape again and remove the suspension and place in a sterile Eppendorf tube.

2. **RNA Extraction**
   Add 100 µl chloroform to the lysate in RNAzol. Cover the samples tightly; vortex vigorously for 5 seconds and let them stay on ice for 15 min. Centrifuge the suspension at high speed (~12,000g, 4°C) for 15 min. After addition of chloroform and centrifugation, the mixture forms two phases: the lower phenol-chloroform phase and the upper aqueous phase. RNA remains exclusively in the aqueous phase, whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 50% of the initial volume of RNAzol plus the volume of cells used.

3. **RNA Precipitation**
   Transfer the aqueous phase to a fresh Eppendorf tube, add an equal volume of isopropanol and store the sample for at least 1 h (or overnight if this fits the time schedule) at -20°C to precipitate RNA. Centrifuge the samples for 15 min. at high speed. The RNA precipitate forms a white pellet at the bottom of the tube.

4. **RNA Wash**
   Remove the supernatant and wash the RNA pellet twice with 1 ml of 75% ETOH by vortexing and subsequent centrifugation for 8 min at high speed. Briefly dry (1-2 min) the pellet under vacuum, using a Speed-Vac. It is important not to let the RNA pellet dry completely, as it will greatly decrease its solubility.

5. **Dissolution of RNA**
   Dissolve the RNA pellet in 50 µl of 0.5% (w/v) SDS solution by vortexing or by passing a few times through a pipette tip. Heating for 15-20 min to 65°C may be required to dissolve a large scale preparation of RNA.

6. **Check of Purity and Quantity of RNA**
   Dilute the above prep 1/200 (e.g. 5 µl + 995 µl) with DEPC treated water, add to a semi-micro quartz cuvette and read absorbance at 260 and 280 nm. If the ratio A260/A280 is 1.8 or above, the nucleic acid is pure. To calculate the concentration of RNA, use the formula:

   \[
   \text{conc. RNA (µg/ml)} = \frac{A_{260} \times 40 \ µg/ml}{\text{per 1 Abs unit} \times \text{Dilution factor}}
   \]
RNA Dot Blot
Protocol for 10 μg RNA/dot and 4 dots total

1. In a sterile bullet tube place 40 μg RNA.
2. Add DEPC H2O to 50 μl.
3. Add 50 μl 20X SSC.
4. Add 40 μl 37% formaldehyde.
5. Incubate at 65°C for 15 minutes.
6. Remove and add 1100 μl of 10X SSC and mix thoroughly.
7. Add 300 μl of RNA solution per well to previously prepared nitrocellulose (NC).
8. Wash each well with 400 μl 10X SSC.
9. When wells are dry, remove the NC from the apparatus and immobilize the RNA by UV irradiation 2400 μJoules exposure.
10. Prehybridize blot for 2-4 hours in 20 mls of Hybridization buffer 42°C.
11. Remove 13 mls of the hybridization buffer and add boiled probe.
12. Hybridize blot overnight (>12 hours) at 42°C in blotting oven.
13. Wash blot 2X, in bottle, with 0.1X SSC at 25°C for 15 minutes.
14. Allow to air dry and quantitate radioactivity of the βgen counter or expose to X-ray film at -80°C.

NOTE: If more samples are to be prepared, increase the H2O, SSC and formaldehyde proportionally. These samples can also be prepared in sterile cryovials or snap cap tubes.

Hybridization Buffer (50 ml)
25 ml Formamide
12.5 ml 20X SSC
5 ml 50X Denhardt’s
2 ml DEPC H2O
500 μl Boiled salmon sperm DNA (10 mg/ml)

50X Denhardt’s
1% Ficoll
1% Polyvinylpyrrolidone (PVP)
1% BSA
Filter sterilize, store aliquotted at -20°C

20X SSC
3.0 M NaCl
0.3 M Sodium citrate
pH 7.0 with 10 N NaOH
Autoclave 45 minutes liquid cycle.

Nitrocellulose Preparation (Schleicher and Schuell, BA85 0.45 μm)
1. Wet the NC in DEPC H2O for 5 minutes (If there are still white spots on the NC, heat the water to facilitate wetting. This will occur with older NC.)
2. Rinse the NC in 10X SSC
3. Assembly the Dot/Slot Apparatus with 2 sheets of 3MM paper underneath the NC.
4. Rinse wells with 400 μl of 10X SSC.
5. Load samples as described above.
Hexamer Primer Labelling

1. Boil 3 μl (100-400 ng) DNA in 10.5 μl H₂O for 5 minutes.
2. Incubate on ice for 5 minutes.
3. Add 11.4 μl LS + 5 μl ³²P dCTP (3000 Ci/mmol, or 50 μCi final).
4. Add 1 μl of Klenow DNA polymerase.
5. Vortex briefly and microfuge.
6. Incubate 1-2 hours 37°C.
7. Add the following in order:
   - 75 μl T₁E₀.₁
   - 4 μl (0.5 μg/μl) yeast tRNA
   - 100 μl 4M Ammonium Acetate (Sterile)
   - 600 μl 95% ETOH
8. Incubate -20°C for 30 minutes.
9. Microfuge 10 minutes to precipitate DNA, and aspirate the supernatant (Supernatant MUST be discarded in the liquid radioactive waste).
10. Add 100 μl of T₁E₀.₁.
11. Boil probe for 5 minutes.
12. Place on ice immediately for another 5 minutes.
13. Count a 1 μl aliquot.
14. Add 5x10⁶ cpm per blot to either fresh hybridization solution, or to the prehybridization solution, depending on whether the solution is changed or not.

LS

- 37.1 μl total
- 15.6 μl 1M HEPES (pH 6.6)
- 8.0 μl 0.2 mM dATP, dGTP, dTTP
- 8.0 μl 2X TM
- 5.5 μl pd(N)₆ Pharmacia 27-2166-01 (90 U in 1 mM Tris pH 7.5, 1 mM EDTA)

2X TM

- 100 ml total
- 50 ml 1M Tris pH 8.0
- 5 ml 1M MgCl₂
- 620 μl β-mecaptoethanol
- 45 ml H₂O
### Poly Thymidylate Probe

**Aim:** To find out relative level of RNA in the samples

**Synthesis:** Add following in the order

<table>
<thead>
<tr>
<th>STOCK</th>
<th>FINAL</th>
<th>FOR 500 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLY- A (sigma) 10 µg/µl</td>
<td>140 µg</td>
<td>14 µl</td>
</tr>
<tr>
<td>DTT 1M</td>
<td>2 mM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Tris pH 8.3 1M</td>
<td>50 mM</td>
<td>25 µl</td>
</tr>
<tr>
<td>KCl 1M</td>
<td>40 mM</td>
<td>20 µl</td>
</tr>
<tr>
<td>AMV-RT (promega) 8 U/µl</td>
<td>50 units</td>
<td>6 µl</td>
</tr>
<tr>
<td>dTTP (promega) 100 mM</td>
<td>1 mM</td>
<td>5 µl</td>
</tr>
<tr>
<td>RNAsin (promega) 40 U/µl</td>
<td>40 units</td>
<td>1 µl</td>
</tr>
<tr>
<td>MgCl₂ 1M</td>
<td>7 mM</td>
<td>3.5 µl</td>
</tr>
<tr>
<td>Oligo dT(12-18)(pharmacia) 0.25 u/µl</td>
<td>2 units</td>
<td>8 µl</td>
</tr>
<tr>
<td>P³² dTTP (NEN) 5 µCi/µl</td>
<td>50 µCi</td>
<td>10 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>406.5 µl</td>
</tr>
</tbody>
</table>

Mix gently by vortexing very slowly

Incubate 2 hrs at 37°C shaking

Hydrolyse the probe adding 50 µl 1 N NaOH (0.1 N) 65°C, 30 min.

Boil the probe before probing membrane

Strip previously probed nitrocellulose blots by boiling in DEPC treated water for 5 minutes.

Prehyb. NC blots at 42°C for 1-3 hrs

Hyb. blots for 30 hrs at 42°C

Wash blots at room temperature two times 0.1% SSC

**Prehybridization & Hybridization Buffer**

- 50% Formamide
- 5X SSC
- 5X Denhardt’s
- 1% SDS
- 0.10 mg/ml Salmon sperm DNA
<table>
<thead>
<tr>
<th>Abbreviation or synonym</th>
<th>Drug</th>
<th>Dose in culture</th>
<th>Abbreviation or synonym</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminolevulinate</td>
<td>2 ml per ml media, 1 ml final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthoflavone</td>
<td>0.4 ml per ml media, 1 ml final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadmium Chloride</td>
<td>1 ml per ml media, 1 ml final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>0.5 ml per ml media, 5 ml final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mephenytoin</td>
<td>40.8 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metalloporphyrin</td>
<td>0.8 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-Methylcholanthrene</td>
<td>15.8 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1 ml of a 10% stock in DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX-Pinene</td>
<td>1 ml of a 10% stock in DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sn-mesoporphyrin IX</td>
<td>1 ml of a 10% stock in DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thujone</td>
<td>1 ml of a 10% stock in DMSO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**APPENDIX C: PREPARATIONS OF CHEMICALS FOR CULTURE**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Stock Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL (freezer) in water</td>
<td>0.8 mg/ml</td>
</tr>
<tr>
<td>NF (frig) in DMSO</td>
<td>22.5 mg/dm</td>
</tr>
<tr>
<td>CdCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2 M in water</td>
</tr>
<tr>
<td>COCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.79 mg/ml</td>
</tr>
<tr>
<td>Succinyl acetone</td>
<td>5 ml/ml in water</td>
</tr>
<tr>
<td>SA (Desicator) in water</td>
<td>15.8 mg/ml</td>
</tr>
<tr>
<td>FeNTA in water (Desicator)</td>
<td>1 ml of a 10 mM stock in DMSO</td>
</tr>
<tr>
<td>Meph in DMSO</td>
<td>1.79 mg/ml</td>
</tr>
<tr>
<td>Mephenytoin</td>
<td>0.8 mg/ml</td>
</tr>
<tr>
<td>Metalloporphyrin</td>
<td>20-Methylcholanthrene</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>IX-Pinene</td>
<td>1 ml of a 10% stock in DMSO</td>
</tr>
<tr>
<td>Sn-meso</td>
<td>5 M Stock (DMSO)</td>
</tr>
<tr>
<td>Thujone</td>
<td>1 ml of a 10% stock in DMSO</td>
</tr>
</tbody>
</table>

**INSTRUCTIONS ON PREPARATION**

- Dose in culture: 2 ml per ml media, 1 ml final
- Abbreviation or synonym: Aminolevulinate
APPENDIX D: FORMULATION OF WILLIAM'S E MEDIUM

### Component

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganic Salts</strong></td>
<td></td>
</tr>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>200.0</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fe(NO₃)₃·9H₂O</td>
<td>0.0001</td>
</tr>
<tr>
<td>KCl</td>
<td>400.0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>97.67</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.0001</td>
</tr>
<tr>
<td>NaCl</td>
<td>6,800.0</td>
</tr>
<tr>
<td>NaHCO₃*</td>
<td>2,200.0</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>140.0</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>Other Components</strong></td>
<td></td>
</tr>
<tr>
<td>α-D-Glucose*</td>
<td>2,000.0</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.05</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>0.03</td>
</tr>
<tr>
<td>Phenol Red*</td>
<td>10.00</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>25.00</td>
</tr>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>90.0</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>50.0</td>
</tr>
<tr>
<td>L-Asparagine·H₂O</td>
<td>20.0</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>30.0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>40.0</td>
</tr>
<tr>
<td>L-Cystine·2HCl</td>
<td>26.07</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>50.0</td>
</tr>
<tr>
<td>L-Glutamine*</td>
<td>292.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>50.0</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>15.0</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>50.0</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>75.0</td>
</tr>
<tr>
<td>L-Lysine·HCl</td>
<td>87.46</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>15.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>25.0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>30.0</td>
</tr>
<tr>
<td>L-Serine</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* These chemicals were added the day the medium was reconstituted.
<table>
<thead>
<tr>
<th>Component</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acids cont.</strong></td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>40.0</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>10.0</td>
</tr>
<tr>
<td>L-Tyrosine•2Na•2H₂O</td>
<td>50.65</td>
</tr>
<tr>
<td>L-Valine</td>
<td>50.0</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>2.0</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.5</td>
</tr>
<tr>
<td>D-Ca Pantothenate</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>1.5</td>
</tr>
<tr>
<td>Ergocalciferol</td>
<td>0.1</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>2.0</td>
</tr>
<tr>
<td>Menadione sodium bisulfate</td>
<td>0.01</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyridoxal•HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.1</td>
</tr>
<tr>
<td>α-Tocopherol phosphate, sodium salt</td>
<td>0.01</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin A acetate</td>
<td>0.1</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.2</td>
</tr>
</tbody>
</table>