MECHANISM OF ACTION OF THE
PORPHYROGENIC AGENT ATMP AND THE
IDENTIFICATION OF GRISEOFULVIN INDUCED
GREEN PIGMENTS IN THE MOUSE

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A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy to the Council for National Academic Awards

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May, 1991
ABSTRACT

Hepatic protoporphyria is a disorder of liver haem metabolism characterised by inhibition of the enzyme ferrochelatase (FK) and consequent accumulation of protoporphyrin. Two compounds that induce such a disorder are ATMP \([1\,\text{–}\,[4-(acetyl-2,4,6-trimethylphenyl)-2,6-cyclohexanedionyl]-O-ethyl propionaldehyde oxime]\) and griseofulvin. These drugs are thought to be metabolised by cytochrome P450 as suicide substrates so that a fragment of the drug (a methyl group) becomes attached to the haem moiety of the enzyme, producing a powerful inhibitor of FK. The present work was undertaken to elucidate the mechanism of action of ATMP and further characterise the N-alkylated porphyrins (green pigments) produced by feeding mice griseofulvin.

ATMP causes hepatic protoporphyria in mice but not in similarly treated rats, guinea-pigs, hamsters and chick embryos. A green pigment was isolated from the liver of mice treated with ATMP and identified by its electronic absorption spectrum and HPLC chromatographic properties as N-methyl protoporphyrin (N-MePP). The ATMP pigment markedly inhibited the enzyme FK in vitro, thus supporting its identification as N-MePP. The possible involvement of cytochrome P450 in the production of N-MePP after treatment with ATMP was also investigated. Loss in cytochrome P450 was not demonstrable in mice treated with ATMP. However, a role for cytochrome P450 was suggested by the findings that two inhibitors of cytochrome P450, SKF525-A and piperonyl butoxide, both afforded protection against ATMP-induced porphyria and production of N-MePP.

In the mouse griseofulvin causes suicidal destruction of cytochrome P450 giving rise to green pigments in the liver. Two such pigments have been characterised here, using fast atom bombardment (FAB) mass spectrometry. One of them has been conclusively identified as N-MePP; the second is also an N-alkylated protoporphyrin and has been tentatively identified as the adduct of griseofulvin to protoporphyrin (N-griseofulvin protoporphyrin).
ACKNOWLEDGEMENTS

I wish to express my deepest thanks and gratitude to Dr. F. De Matteis for his kindness, help and support throughout this project. I would also like to thank Dr. E. A. Lock for his advice and helpful comments made during this work. Sincere thanks are also due to Mr. A. H. Gibbs, Dr. S. Naylor, Dr. D. Wilmot, Dr. G. Biset and Dr. A. Brady who offered assistance and participated in many constructive discussions during this project. In addition, I would like to thank Mr. D.J. Judah and Dr. R. Dover who helped prepare this manuscript.

Finally, I would like to thank ICI for their financial support and the Medical Research Council for a postgraduate scholarship.
ABBREVIATIONS

In addition to those in general usage, the following abbreviations may be found in the text:

\[
\begin{array}{ll}
\alpha\text{-NF} & \alpha\text{-naphthoflavone} \\
BNF & \beta\text{-naphthoflavone} \\
CFA & \text{clofibric acid} \\
N\text{-MePP} & N\text{-methyl protoporphyrin} \\
SKF525\text{-A} & 2\text{-diethylaminoethyl-3,3\text{-diphenylpropylacetate}} \\
DDC & \text{Diethoxycarbonyl-1,4\text{-dihydro-2,4,6\text{-trimethylpyridine}}} \\
DMSO & \text{Dimethyl sulphoxide} \\
FK & \text{Ferrochelatase} \\
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\]
To my parents and family
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Chapter 1

INTRODUCTION
1-[4-(3-Acetyl-2,4,6-trimethylphenyl)-2,6-cyclohexanedionyl]-O-ethyl propionaldehyde oxime (ATMP) (fig 1.1a) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) (Fig 1.1b) are two chemicals which can be metabolised in the liver of a number of species including dog and mouse. Similarly, 7-chloro-2,4,6-trimethoxy-6-methylspiro[benzofuran-2(3H),1-[2]cyclohexene]-3,4-dione (Griseofulvin) (Fig 1.1c) is metabolised in the liver of rodents and man. These xenobiotics are being considered here because of their ability to cause the disease Protoporphyria. Fundamental to the development of this disease is a block in the enzyme ferrochelatase (FK) by these compounds and a concomitant increase in haem biosynthesis. The enzyme inhibitor which is formed is a green pigment, N-methyl protoporphyrin (N-MePP) (Fig 1.2) in the case of DDC. The present work shows that a similar mechanism occurs for ATMP and griseofulvin. Interestingly, griseofulvin is currently used clinically as an antifungal agent and may cause porphyria in individuals with the appropriate genetic defect. Also, ATMP, which has herbicidal activity, is known to cause accumulation of protoporphyrin in dogs and rodents. It may be postulated that all three compounds share a common mechanism in the formation of protoporphyrin (Fig 1.3) and consequently the development of protoporphyria which will be discussed later.

Porphyria is a disorder in the body, where there is a disruption of haem synthesis, leading to an over-production of porphyrins and their precursors. Before discussing the types of porphyria which may occur, the normal events of haem synthesis and the control of this pathway will be discussed.
Fig 1.1 Chemical structure of the porphyrogenic agents (a) ATMP, (b) DDC and (c) Griseofulvin

(a) 
\[ \text{Chemical structure of ATMP} \]

(b) 
\[ \text{Chemical structure of DDC} \]

(c) 
\[ \text{Chemical structure of Griseofulvin} \]
Fig 1.2 Chemical structure of N-MePP showing:
(a) side view with the methyl group attached to ring A and the ring tilted out of the porphyrin plane
(b) a front view with the methyl group attached to ring B
Fig 1.3 Major biochemical changes in the liver giving rise to protoporphyria

Drug causes suicidal inactivation of cytochrome P450

↓

Formation of inhibitory green pigment

↓

Inhibition of FK

↓

Increase in porphyrin synthesis by induction of ALA-S

↓

Accumulation of protoporphyrin

↓

PROTOPORPHYRIA
Cytochrome P450 is the key enzyme linking haem synthesis to drug-induced protoporphyria. Cytochrome P450 is responsible for activating and precipitating a suicidal attack by DDC, griseofulvin and ATMP which results in formation of \( \text{N-MePP} \) which in turn inhibits FK. In addition, haem is required to form more cytochrome P450 which results in a depletion of the regulatory haem pool. A compensatory mechanism is set into motion, whereby more haem is produced. The block in FK limits the amount of protoporphyrin utilized by the enzyme, causing a build-up of the porphyrin and hence protoporphyria occurs. For these reasons, a detailed discussion of cytochrome P450 and its suicidal inactivation by drugs will also be given in this Introduction; in addition, the properties of FK will be discussed. This will facilitate an understanding of the rationale behind the various experiments which are presented in this thesis.

1.1 The structure of haem

The demand of the cell for haemoprotein biosynthesis is such that haem is continually being formed. Haem is ubiquitous and is required by all cells for normal cellular functions. However, under normal physiological conditions the required amount of haem is produced, with little waste of the intermediary metabolites along the biosynthetic pathway. Haem is a tetrapyrrolic structure which comprises protoporphyrin with an atom of iron positioned in the centre. The basic structure of the porphyrin is related to porphin, which consists of 4 pyrrole groups linked together by 4 methene bridges \((\alpha, \beta, \gamma, \delta)\) to produce a tetrapyrrole (Fig 1.4). The nomenclature most commonly adopted for a porphyrin involves numbering side chains on the \( \beta \)-positions of the pyrrole rings 1–8 (Moore 1990). The 4 pyrrole rings are labelled A–D, and each pyrrolic nitrogen is labelled to the corresponding ring e.g. \( N_A \) refers to pyrrolic nitrogen in ring A of the tetrapyrrole. In the case of protoporphyrin, the substituents
Fig 1.4 Chemical structure of porphin based on the Hans Fisher nomenclature
attached to the tetrapyrrole include four methyl, two vinyl and two propionate. There are 15 different ways in which these substituents may be arranged around the protoporphyrin molecule; however only one isomeric type (isomer IX) of protoporphyrin is present in biological systems. The iron atom in haem is covalently bound to all four pyrrole nitrogens and exists in one of the two oxidation states, either Fe$^{3+}$ or Fe$^{2+}$. The four pyrrole rings can be distinguished from each other, and one side of the porphyrin plane can be distinguished from the opposite side by virtue of the asymmetrical arrangement of the $\beta$-substituents of protoporphyrin IX.

The haem group binds to an apoprotein moiety to form a haemoprotein. Several types of haemoproteins can be distinguished according to the valency of the iron or their prosthetic group. In haemoglobin and myoglobin, the iron must be in a ferrous state for a functional haemoprotein; in catalase and peroxidases the iron is in a ferric state while in the mitochondrial cytochromes, the valency of the central iron oscillates between the divalent and trivalent states, thus allowing these haemoproteins to act as electron carriers. Although the iron complex of protoporphyrin IX (protohaem) is present as the prosthetic group of several haemoproteins, including haemoglobin, catalase cytochrome b$_5$ and cytochrome P450; in other haemoproteins, haem may be chemically modified (for example haem a in cytochrome a) or the haem moiety is covalently bound to the apoprotein as in cytochrome c.

1.2 The biosynthesis of haem

Elegant studies performed by Shemin and Rittenberg made pioneering contributions towards elucidating the haem biosynthetic pathway (for recent reviews see Moore 1990; Rimington 1989; Battersby et al. 1980). Most of the
enzymatic steps involved have since been elucidated. A schematic diagram of the haem pathway is shown in Fig 1.5. The first step is the formation of 5-aminolaevulinc acid (ALA). The substrates involved in this reaction are succinyl Co-A, which is generated predominantly by the tricarboxylic acid cycle, and glycine. This first step in the haem biosynthetic pathway is the rate-limiting step and is catalysed by 5-aminolaevulinate synthetase, [EC2.3.1.37] ALA-S, which is located in the matrix compartment of the mitochondrion. Pyridoxal phosphate, and also magnesium, are required for normal activity.

Two molecules of ALA then combine to form the monopyrrole porphobilinogen (PBG) (Barnard et al. 1977). ALA-dehydratase (ALA-D) [EC4.2.1.24], which is involved in the formation of PBG, is a cytosolic enzyme made up of 8 subunits; the protein requires zinc for normal activity (Bevan et al. 1980; Hasnain et al. 1985; Jaffe et al. 1984). ALA-D binds one ALA molecule to a lysine residue at its active site to generate a Schiff base (Jaffe and Hanes 1986). The ALA molecule aligns itself such that it gives rise to a propionic acid side chain in the molecule PBG, while its amino nitrogen forms part of the pyrrole ring. A second molecule of ALA forms the acetic acid side chain in the PBG molecule (Jordan and Seehra 1980; Jordan and Gibbs 1985).

The cytosolic enzyme, porphobilinogen deaminase [EC4.3.1.8], catalyses the polymerization of 4 molecules of PBG to yield hydroxymethylbilane (HMB). Condensation of the four monopyrroles to a linear tetrapyrrole HMB occurs in a step-wise head-to-tail fashion (Anderson et al. 1981; Battersby et al. 1978). HMB has a half-life of 4.5 mins and is rapidly metabolised by uroporphyrinogen cosynthetase [EC4.2.1.75] to uroporphyrinogen III. (Jordan 1990; Rimington 1989). The initial step is the rearrangement of pyrrole ring D through a spiro
Fig 1.5 The haem biosynthetic pathway

Mitochondrion

Cytoplasm

Succinyl-CoA

CH₃

C

H

C

H

CoA

S

CH₃

C

H

C

H

CoASH

CO₂

ALA-synthetase

ALA-dehydratase

COO⁻

C

H

C

H

O

4NH₃

Porphobilinogen deaminase

Glycine

δ-Aminolevulinic acid

Hydroxymethylbilane

Uroporphyrinogen III oxidase

Coproporphyrinogen III oxidase

Protoporphyrinogen IX

Protoporphyrin IX

Coproporphyrinogen III

B₆ = pyridoxal-5'-phosphate; Ac = acetate; Pr = propionate; Vi = vinyl
intermediate which is then followed by ring closure (Jordan et al. 1979; Battersby et al. 1983). The significance of a defect of this enzyme is seen in congenital erythropoietic porphyria which will be discussed in section 1.7.1.

Uroporphyrinogen decarboxylase [EC4.1.1.37], the next enzyme in the haem pathway, randomly decarboxylates uroporphyrinogen III to form coproporphyrinogen III (Luo and Lim 1990; De Verneuil et al. 1983). In the mitochondrion, coproporphyrinogen III is converted by coproporphyrinogen oxidase [EC1.3.3.3] to protoporphyrinogen IX. Coproporphyrinogen oxidase has an absolute requirement for molecular oxygen, possibly forming a \( \beta \)-hydroxypropionate as an intermediate (Sano 1966). It has been demonstrated that one tyrosine residue is involved in the active site and that the stereoselectivity of the enzyme allows an oxidative decarboxylation to form two vinyl groups in positions 2 and 4 in the tetrapyrrole molecule (Yoshinaga and Sano 1980).

The penultimate step in haem biosynthesis is the conversion of protoporphyrinogen IX into protoporphyrin IX. This is catalysed by protoporphyrinogen oxidase [EC1.3.3.4] (Poulson 1976). Protoporphyrinogen oxidase utilizes flavin adenine dinucleotide (FAD) as a cofactor transferring electrons to molecular oxygen (Siepker et al. 1987). Immunological cross-reactivity studies have shown that protoporphyrinogen oxidase and FK [EC4.99.1.1], the last enzyme in the haem pathway, exhibit a high degree of homology (Siepker et al. 1987). The final enzyme in haem biosynthesis is FK which is responsible for the insertion of ferrous iron (FeII) into protoporphyrin IX to produce haem (Jones and Jones 1969). Unlike other enzymes of the biosynthetic sequence which utilize the fully reduced porphyrinogens or
hexahydroporphyrinogens (uroporphyrinogen, coproporphyrinogen and protoporphyrinogen), FK utilizes a porphyrin (protoporphyrin IX) as a substrate. Other dicarboxylic porphyrins, for example deuteroporphyrin and mesoporphyrin, serve as good substrates for the enzyme as do other divalent metals, such as zinc and cobalt, a point which will be discussed further in section 1.5.1.

1.3 Control of haem synthesis

5-Aminolaevulinate synthetase (EC2.3.1.37; Succinyl CoA:glycine C-Succinyi transferase; ALA-S) is the rate limiting enzyme in haem synthesis. The level of activity of ALA-S is tightly controlled by haem through a negative feedback mechanism. Pioneering work carried out by Granick demonstrated that many xenobiotics and endogenous compounds cause an induction of ALA-S (Granick 1966). The current concept is that a regulatory haem pool exists where haem is removed and is utilized by the cell, for example cytochrome P450 and tryptophan pyrrolase, or for haem degradation (Yannoni and Robinson 1975; Grandchamp et al. 1981). The regulatory haem pool in hepatic cytosol is of the order 0.05–0.1 μM (Granick and Beale 1978). When the level of haem falls, the inhibitory effect (exerted by haem) upon ALA-S is removed, resulting in an increase in the enzyme's activity (Srivastava et al. 1988). Xenobiotics induce ALA-S by lowering the free haem pool. Porphyrogenic agents, such as DDC and 2- Allyl-2-isopropylacetamide (AIA), both deplete the regulatory haem pool causing an increase in ALA-S through transcription of the ALA-S gene: with the use of c.DNA probes firm evidence of this has been provided employing chick embryo and rat liver (Borthwick 1985; Srivastava et al. 1988). However, the mechanism by which depletion of haem is obtained is different with AIA as compared to DDC, and will be discussed later.
under suicide substrate inhibitors (section 1.6.3). Another agent which governs ALA–S activity is glucose, shown by the so called glucose effect. In experimental animals it has been demonstrated that glucose causes a block in drug–induced ALA–S (Kim and Kikuchi 1974; Giger and Meyer 1981). Yamamoto et al. (1982) have tentatively suggested that glucose blocks ALA–S induction by preventing the translocation of the enzyme into mitochondria, thereby enhancing free haem levels (Yamamoto et al. 1982).

1.4 ALA–S

A wealth of information concerning ALA–S has been derived primarily from rodent and chick embryo studies. There are two forms of ALA–S that exists in the cell, (i) cytoplasmic ALA–S, (pre–ALA–S) with a molecular weight of 75 KDa and (ii) mitochondrial ALA–S having a molecular weight between 63–68 KDa. Both of these proteins have been determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Borthwick et al. 1984; Ades and Harpe 1981). ALA–S is a mitochondrial enzyme synthesized on free cytoplasmic polyribosomes (Borthwick et al. 1985). This precursor form of ALA–S migrates into the mitochondrion and is processed to form the mature ALA–S (May et al. 1986). In rat liver mitochondria, it has been shown using electron microscopy and gold antibody–labelling technique that ALA–S is attached to the matrix side of the cristae (May et al. 1986).

The mechanism whereby haem regulates ALA–S activity occurs in one of three possible ways (Fig 1.6). These are by:
(i) inhibition at the transcription level;
(ii) inhibition at the translational level; and
(iii) prevention of translocation of pre-ALA-S (the precursor form of ALA-S) into the mitochondrion.

Haem acts to repress ALA-S mRNA production (Whiting 1976; Yamamoto et al. 1982). In the nucleus, haem acts at the transcriptional level by binding to an operon codon on the ALA-S gene (Granick 1966; Srivastava et al. 1980). The second site for haem action is at the translational stage where haem represses pre-ALA-S formation (Tyrrel and Marks 1972). The third site of haem action is in the uptake of pre-ALA-S into the mitochondrion. Pulse-labelling studies in chick embryo livers have shown that haem prevents the uptake of pre-ALA-S into the mitochondria, resulting in accumulation of the precursor form of the enzyme in the cytosol (Srivastava et al. 1983). Using $[^{35}S]$-methionine to label pre-ALA-S it was found that the cytosolic fraction contained the most radioactivity following haem treatment. Haem specifically blocks the uptake of pre-ALA-S since other mitochondrial enzymes, for example pyruvate carboxylase, remain unaffected (Srivastava et al. 1983).

The effect of haem on ALA-S activity, which has been reported, is different depending on whether the enzyme has been purified, or studied in the intact cell. Using the purified ALA-S, isolated from drug-induced chick embryo liver, attempts to inhibit the enzyme with concentrations of 100 $\mu$M haem proved unsuccessful (Piroia et al. 1984). However, in isolated chick embryo hepatocytes ALA-S was inhibited with 10 nM haem (Pirola et al. 1984). A possible explanation for the differences observed is that, in the intact cell transcription, translation and translocation may be contributing factors involved in suppressing ALA-S activity. It is generally considered that haem at physiological concentrations does not inhibit ALA-S activity. In the cell the
Fig 1.6 Regulation of ALA-S production by haem in the liver

Nucleus

ALA-S gene

mRNA

mRNA

Pre-ALA-S

Mitochondrion

ALA-S

Succinyl CoA + glycine

ALA

FREE HAEM POOL

TRANSCRIPTION

TRANSLATION

TRANSLOCATION

Haem

ALA
half-life of ALA–S is approximately 1–2 hrs (Tschudy et al. 1965; Sassa and Granick 1970; Ades 1983; Ades et al. 1983). This allows for a rapid change in activity through modifications of the rate of synthesis of the enzyme.

1.4.1 ALA–S in non-erythroid and non-hepatic tissues

Apart from in the liver, ALA–S may be found in other non-erythroid tissues such as steroid-synthesizing tissues (adrenal cortex) and the kidney. AIA causes an increase in ALA–S activity in the kidney and this suggests that renal ALA–S is under the same control as that found in the liver, namely haem repression (Schwartz et al. 1976). However, in testes and ovaries ALA–S is not induced by porphyrogenic agents such as AIA or DDC. The main function of these tissues is to synthesize steroids, as compared to the liver which is primarily involved in metabolism of drugs (Tofilon and Piper 1980; Anderson et al. 1981). However, hormones arising from the pituitary can increase levels of ALA–S in these tissues (Tofilon and Piper 1980). Similarly, in the brain ALA–S may be repressed by injecting haem into rat cerebellum (De Mattels and Ray 1982).

1.4.2 ALA–S in the erythropoietic system

In erythroid cells, the mechanism of ALA–S in regulating haem biosynthesis is poorly understood as compared with the liver. This is partly due to the generalised activation of the haem pathway one observes in erythroid differentiation, but also on account of conflicting data regarding the exact timing of ALA–S induction (for recent reviews see Dierks 1990; Andrew et al. 1990; May et al. 1986). In differentiating erythroid cells ALA–S acts as a rate-limiting enzyme in the haem biosynthetic pathway (Levere and Granick 1967; Gardner and Cox 1988). In the whole animal, the erythroid cell is required primarily for
the synthesis of haemoglobin. The activity of most, if not all, the enzymes in the haem pathway is induced when there is an increased demand on haem synthesis (Sassa 1976; Rutherford et al. 1979). Two further rate-limiting steps have been proposed to explain haem regulation in erythroid cells: the formation of haem by FK (Sassa 1976; Rutherford et al. 1979) and the transport of iron (Ponka and Neuwirt 1970; Laskey et al. 1986).

Recently, it has been shown that ALA–S is encoded for by at least two distinct genes: a house-keeping gene, which is expressed in all tissues (Dierks 1990), and an erythroid-specific gene responsible for the production of ALA in the erythroid cell (Riddle et al. 1989).

The present studies focus on the variety of porphyria where FK is inhibited and where the porphyrin-inducing chemicals generate an inhibitor of FK on interaction with cytochrome P450. Therefore, the properties of FK and of the cytochrome P450 system will now be discussed in some detail as well as the phenomenon of suicidal inhibition of cytochrome P450, as these are directly relevant to the experimental work which forms the main part of this thesis.

1.5 FK

FK [protoheme ferrolyase, heme synthase, EC4.99.1.1] is the terminal enzyme in the haem biosynthetic pathway and, like ALA–S, is located in the mitochondrion. FK is of particular interest since certain chemicals, for example DDC and griseofulvin, can inhibit this enzyme resulting in a porphyria with a distinctive biochemical profile. The role of FK is to insert ferrous ion into protoporphyrin to form haem (Jones and Jones 1969; Meyer and Schmid 1979).
FK is bound to the mitochondrial inner membrane with its active site facing the matrix (Harbin and Dailey 1985; Jones and Jones 1969).

FK is encoded by nuclear genes. The immature form of FK is synthesized on cytoplasmic ribosomes and has a molecular weight of 43 KDa (Karr and Dailey 1988). Pre-FK is translocated into the mitochondrion in an energy requiring step and processed by a metalloproteinase to yield a mature-sized protein (Karr and Dailey 1988). The molecular weight of the functionally active enzyme is 40 KDa as determined by SDS polyacrylamide gel electrophoresis (Dailey et al. 1986; Taketani and Tokunaga 1981).

1.5.1 Properties of FK

FK has been purified from rat, mouse, bovine and chicken liver using Sepharose CL-6B chromatography and SDS gel electrophoresis (Dailey et al. 1986; Bloomer et al. 1987; Taketani and Tokunaga 1981). In the rat and mouse purified FK contains a high proportion of fatty acids, for example oleic and myristic acid, and phospholipids such as phosphatidylcholine (Taketani and Tokunaga 1981). Lipids are important for normal FK activity since their removal leads to a loss in enzyme activity (Mazanowska et al. 1966). FK is situated in a lipid-rich environment; lipids are thought to have two roles: (i) serve as a reaction medium for the substrates by increasing porphyrin solubility and/or (ii) they participate in the transfer of iron from aqueous to non-aqueous environment (Simpson and Poulson 1977). As yet no co-factor has been demonstrated to be involved in FK function (Dailey and Fleming 1983; Dailey 1982). FK contains sulfhydryl groups that are essential for enzyme activity, as shown in particular by the observation that the sulfhydryl reagents N-ethylmaleimide and monobromobimane both inactivate the enzyme in a
pseudo-first order fashion (Taketani and Tokunaga 1981; Dailey 1984; 1982). Another class of reagents, the divalent cations, mercury, arsenite and lead, inhibit FK by binding with free sulphydryl groups (Dailey 1982; Dailey 1984). Although iron and protoporphyrin are the physiological substrates for FK, the enzyme can also accept dicarboxylic porphyrins and divalent metals other than Fe$^{2+}$. Thus mesoporphyrin and deuteroporphyrin are good substrates with Vmax and Km values of 1.0 nmol/min/mg and 1.9 μM respectively for mesoporphyrin, and 1.4 nmol/min/mg and 4.0 μM for deuteroporphyrin (Honeybourne et al. 1979). The two vinyls in positions 2 and 4 of protoporphyrin are substituted by ethyl groups in mesoporphyrin and by hydrogen in deuteroporphyrin. It is clear that the nature of the substituents in these two positions influences the affinity of a porphyrin substrate for FK. The implication of this for the properties of the active site will be referred to later.

1.5.2 Model for FK active site

Ferrochelatase is viewed as a porphyrin/haem binding site which consists of a distinct porphyrin (Dailey 1985; De Matteis et al. 1980a; Honeybourne et al. 1979) and an iron binding site (Dailey 1985). It has been demonstrated that the porphyrin binding sites possess arginine and tryptophan amino acid residues that facilitate porphyrin binding (Dailey 1984; Dailey and Fleming 1986). The iron binding site contains one or more cysteine residues (Dailey 1985; 1984).

A model for the active site has been proposed, in which the porphyrin binds to the enzyme, in a region analogous to the haem binding pocket of globins and cytochromes (Dailey 1985; Dailey et al. 1986). A different model has been put forward by Ortiz De Montellano et al. (1986) to explain the binding of protoporphyrin at the enzyme's active site. They have proposed that the
porphyrin is drawn into the active site by hydrogen bonding or electrostatic interaction between the propionate carboxylic groups from the porphyrin and residues in the active site. The two pyrrole rings C and D are bound in a sterically constrained region, preventing a porphyrin bearing a large N-substituent on the C or D pyrrole rings from interacting with the active site. The pyrrole rings A and B of the molecule remain in a relatively open region of the active site which can accommodate larger sized N-alkyl groups (this point will be referred to in more detail later).

In the alternative model proposed by Dailey, the FK active site is viewed as a hydrophobic pocket into which the porphyrin fits with the two propionate side chains extending outwards into the aqueous exterior (Dailey 1985; Dailey et al. 1989). The main differences between the two models are the position of the two propionate side chains in the active site, and the degree of exposure of the enzyme bound porphyrin to the aqueous environment (Fig 1.7). Dailey argues that the model proposed by Ortiz De Montellano does not explain the side chain specificity in the porphyrin substrate for catalysis (that is different Km values for protoporphyrin, mesoporphyrin and deuteroporphyrin), nor why N-alkyl porphyrins bind FK more strongly than non-alkylated porphyrins (Dailey et al. 1989). In Dailey's view, his own model gives support to previous findings concerning the role of the 2,4, substituents (Dailey 1986; Honeybourne et al. 1979) and is consistent with the observations derived from N-alkylporphyrins (De Matteis et al. 1980a; Cole and Marks 1984; McCluskey et al. 1988; De Mattels et al. 1985). From the literature and from what is known about FK and its active site, the model proposed independently by both Dailey's and De Mattels's groups probably accommodates most of the data.
Fig 1.7 Two models proposed for FK active site:
(a) Dailey's model
(b) Ortiz De Monteilano's model
1.5.3 Formation of haem by FK

The biological substrates protoporphyrin and iron are utilized by FK. It has been proposed that haem biosynthesis occurs through an ordered Bi–Bi mechanism at the active site with iron binding first then protoporphyrin followed by the release of haem then the hydrogen ions (Dailey and Fleming 1983; Dailey et al. 1989) (Fig 1.8). FK has an absolute requirement for ferrous ions; recent findings demonstrated that ferric ions are first reduced by an oxido–reductant system, for example NADH dehydrogenase, before being utilized by the enzyme (Taketani et al. 1985).

1.5.4 FK and N–alkylated porphyrins

N–alkyl porphyrins are important metabolites generated in the liver of animals given drugs such as AIA, DDC and griseofulvin. The relevance of N–alkyl porphyrins here is that some of these modified porphyrins have the ability to inhibit FK. In all cases studied, the green pigment produced by unsaturated substrates such as AIA does not cause inhibition of FK (De Matteis et al. 1980a). The reason for this is that the N–alkylated porphyrin cannot be accommodated at FK’s active site.

The term N–alkylated porphyrin refers to a porphyrin bearing an alkyl (or aryl) grouping attached to a pyrrolic nitrogen in the porphyrin molecule. In some cases, a simple carbon unit such as a methyl group is attached. However, unsaturated grouping such as vinyl derivatives, oxygen–containing groups (for example hydroxyethyl group) and aromatic structures such as the benzyl grouping can also be bound to one of the pyrrolic nitrogens (Ortiz De Montellano and Grab 1986; Grab et al. 1988; De Matteis et al. 1980b).
Fig 1.8 Bi–Bi mechanism for the formation of haem by FK

\[
\begin{align*}
\text{Fe}^{3+} & \rightleftharpoons \text{Fe}^{2+} & \text{PP} \\
E & \rightleftharpoons E: \text{Fe}^{2+} & \rightleftharpoons \\
\left[ E: \text{Fe}^{2+}: \text{PP} \right] & \rightleftharpoons \\
E: \text{Haem}:2\text{H}^+ & \rightleftharpoons \\
\text{Haem} & \\
E:2\text{H}^+ & \rightleftharpoons \\
E + 2\text{H}^+ & 
\end{align*}
\]

Key:
- E: Enzyme
- PP: Protoporphyrin free acid
In order to inhibit FK the following structural requirement must be met by an N-monosubstituted porphyrin:

a) Two propionate side chains must be present in position 6 and 7 in a free carboxylate form. This is shown by the finding that N-MePP is a powerful inhibitor as the free acid, while the ester derivative is not, unless preliminary hydrolysis of the ester to the free acid takes place, for example by the action of carboxyl esterase (De Matteis et al. 1985). Inhibitory studies have shown that other dicarboxylic porphyrins, such as mesoporphyrin and deuteroporphyrin are inhibitory towards FK when N-methylated (De Matteis et al. 1985).

b) The nature of the 2 and 4 side chains present in a dicarboxylic porphyrin is important for the inhibitory activity, as shown by the finding that N-MePP (containing vinyl grouping in these positions) is a more powerful inhibitor than N-methyl mesoporphyrin (containing ethyl groups) and N-methyl deuteroporphyrin (where hydrogen is present in these positions) (De Matteis et al. 1985).

c) More than two carboxylic acid side chains lead to a loss of inhibitory activity, as for example in N-methyl uroporphyrin and N-methyl coproporphyrin.

d) The size of the N-alkyl group is also important for the inhibitory activity of a dicarboxylic porphyrin, particularly if it is present on either ring C or D. The structural requirements described so far under (a) and (b) above closely mirror the requirements for a porphyrin to be accepted by FK as a substrate, strengthening the view that the N-alkylated porphyrin inhibitor and the porphyrin substrate compete with each other for the same binding site.

The essential features of FK inhibition by N-MePP (or N-alkyl porphyrins) are that: (i) there is competitive inhibition between the N-alkyl porphyrin and the
endogenous substrate, protoporphyrin (Dailey and Fleming 1983); (ii) if protoporphyrin is added before the N-MePP then protection is seen (De Matteis et al. 1985); and (iii) inhibition of FK by N-methyl porphyrins is reversible, by addition of excess substrate (De Matteis et al. 1985).

As already mentioned, two different models for the active site of FK have been proposed, and attempts to accommodate these findings in either model have been made. Thus Ortiz De Montellano et al. (1986) suggest that the two propionate groups are important in the binding of the N-alkyl porphyrin at FK's active site; whereas Dailey and also De Matteis emphasize the significance of the distortion of the N-alkylated ring, and have also proposed that the N-alkyl porphyrin may be a transition-state analogue, hence its great affinity for FK.

1.6 The role of cytochrome P450 in drug metabolism

1.6.1 Nomenclature of cytochrome P450

Cytochrome P450 constitutes a superfamily of haemoproteins proficient in metabolising a diverse number of chemicals. The gene nomenclature, recommended by the Committee on Standardized Nomenclature of the cytochrome P450 Genes, identifies gene families by Roman numerals, subfamilies by capital letters and individual genes by Arabic numbers (see Table 1.1) (Nebert et al. 1987; Gonzalez 1988). The nomenclature adopted for the different types of cytochrome P450 is based on the work by Nebert et al. (1987), who categorised the cytochrome P450 into gene families and subfamilies. Members of different families are less than 36% homologous, while members of the same subfamily are 70% or more homologous. Therefore, within a gene family of a given species, two proteins will share a 40–65% homology, regardless of the subfamily to which the proteins may belong (Nebert
et al., 1987). It has been shown that at least 8 cytochrome P450 gene families exist and 5 subfamilies within the cytochrome P450 II family (Nebert et al., 1987). The versatility of cytochrome P450 is due to the different isoenzyme forms which have been shown to exist (Thomas et al., 1976; Waxman 1986; Black and Coon 1986). The criteria employed for identifying the various types of isoenzymes of cytochrome P450 include SDS gel electrophoresis, spectral properties, catalytic activity, immunological properties, peptide mapping and amino acid sequencing. A detailed account of the various types of techniques employed to identify the different isoenzymes is outside the scope of this thesis. However, there are comprehensive reviews by several workers in this field (Gonzalez 1988; Waxman 1986; Black and Coon 1986; Lu and West 1980).
Table 1.1 Classification of cytochrome P450 based on cytochrome P450 gene superfamily

<table>
<thead>
<tr>
<th>Cytochrome P450 gene superfamily family, subfamily and gene designation</th>
<th>Some of the other names commonly used</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450I (Polycyclic Aromatic compound-inducible)</td>
<td></td>
</tr>
<tr>
<td>P450IA1 (the only subfamily)</td>
<td>Rat c, mouse P1</td>
</tr>
<tr>
<td>P450II (Major)</td>
<td></td>
</tr>
<tr>
<td>P450IIA1</td>
<td>Rat a</td>
</tr>
<tr>
<td>P450IIB Subfamily (Phenobarbital-inducible)</td>
<td></td>
</tr>
<tr>
<td>P450IIB1</td>
<td>Rat b</td>
</tr>
<tr>
<td>P450IIC Subfamily</td>
<td></td>
</tr>
<tr>
<td>P450IIC6</td>
<td>Rat PB1 (k)</td>
</tr>
<tr>
<td>P450IIC11</td>
<td>Rat h</td>
</tr>
<tr>
<td>P450IV (Peroxisome proliferator-inducible)</td>
<td></td>
</tr>
<tr>
<td>P450IV A1 (the only subfamily)</td>
<td>Rat LAw</td>
</tr>
</tbody>
</table>

The table represents a few examples of families and subfamilies of the cytochrome P450 superfamily.
1.6.2 Catalytic cycle of cytochrome P450

The liver is the major organ in the body responsible for the metabolism of xenobiotics; other organs and tissues including lung, kidney and olfactory epithelium do play a role in metabolism but to a much lesser extent (Reed et al. 1988; Schwartz et al. 1976; Riddick et al. 1990). The most important role in biotransformation is made by a group of enzymes, collectively called cytochrome P450. This drug metabolizing enzyme forms part of an electron transport system and is recognised as the terminal oxidase. Cytochrome P450 is ubiquitous in cells and is located in the smooth endoplasmic reticulum. In addition to the metabolism of xenobiotics cytochrome P450 plays a vital role in steroid, hormone and bile acid synthesis.

In the biotransformation of drugs, the essential role of cytochrome P450 is to metabolise a lipophilic substrate to a more polar substrate, thereby facilitating the excretion of metabolites. In order to achieve this, cytochrome P450 can operate either as (i) a monooxygenase (ii) an oxidase or (iii) a reductase. cytochrome P450 behaves as a monooxygenase when it inserts a single oxygen atom into a substrate while the other oxygen combines with hydrogen ions to form water. The name "mixed function oxidase" has also been used to describe this behaviour of cytochrome P450. The overall reaction of cytochrome P450 interacting with substrate is:

\[
\text{NADPH} + \text{O}_2 + \text{DRUG} + \text{H}^+ \rightarrow \text{NADP}^+ + \text{MONOOXYGENATED DRUG} + \text{H}_2\text{O}
\]

The reaction takes place by two sequential one-electron steps and it has been suggested that a carbon radical of the drug undergoing hydroxylation is formed as an intermediate (Ortiz De Monteilano 1989). An example using the
compound Pulegone demonstrates the formation of a radical intermediate (McCianaham et al. 1988).

Where cytochrome P450 behaves as an oxidase, oxygen is involved as the terminal electron acceptor, but does not become inserted into the substrate; rather in this reaction there is a transfer of electrons from the drug to the enzyme, for example DDC and 3-[2-(2,4,6-trimethylphenyl-thioethyl]-4-methyl sydnone (TTMS) (Ortiz De Montellano et al. 1981c; Augusto et al. 1982; Ortiz De Monteliano and Grab 1986). The role of cytochrome P450 acting as a reductase involves transferring electrons from the enzyme to the drug, as in the case of carbon tetrachloride (Manno et al. 1988; De Groot and Haas 1981)

In the resting state two forms of cytochrome P450 exist in equilibrium with each other, namely hexacoordinate low-spin iron (III) complex and pentacoordinate high-spin iron (III) complex. Upon binding a substrate there is a shift in the equilibrium towards a pentacoordinate state. The high-spin enzyme-substrate complex is reduced by a single electron, and oxygen binds forming a hexacoordinate low-spin complex, SFe(III)O_2^-. A second electron reduces the complex to yield iron-peroxo species, SFeO_2^2-. Oxygen undergoes heterolytic cleavage of O−O bond to form a high valent iron-oxo complex [FeO]^3+; an oxygen atom is then transferred from the iron-oxo complex to the substrate while, concomitantly, a molecule of water is produced. The oxidised substrate is released from the active site to regenerate an intact cytochrome P450 (Mansuy et al. 1989; Ortiz De Montelliano 1986; White and Coon 1980). Two electrons are required per catalytic cycle, and these are donated by NADPH−P450 reductase and cytochrome b₅. Normal events occurring at cytochrome P450 active site were discussed above: events and consequences
of a suicide substrate inhibitor on cytochrome P450 in relation to porphyria will now be discussed.

1.6.3 Suicide substrates of cytochrome P450

Suicide substrates are also known as mechanism–based enzyme inhibitors or enzyme activated inhibitors. That is, they cause autocatalytic destruction of cytochrome P450: the enzyme is unable to participate in subsequent catalytic reactions (Walsh 1977; 1984; Ortiz De Montellano and Correia 1983). Characteristic properties of a suicide substrate are the following:

(i) covalent binding (or tight binding inhibition) occurs
(ii) a stoichiometric ratio is seen, that is, a 1:1 molar ratio between inactivator and enzyme
(iii) inhibition of the enzyme is a saturable, time– and dose–dependent process which follows pseudo first–order kinetics (Walsh 1977; 1984).

Suicide inactivation may be described as a competition between turnover and inactivation (Walsh 1984;1982) and is defined as a partition ratio, that is, the number of product molecules per inactivation event.
Consider the schematic diagram below:

\[ E + S \xrightarrow{k_1} E.S \xrightarrow{k_2} E.X \xrightarrow{k_3} E + P \]
\[ E - I \xleftarrow{k_4} \]

**PARTITION RATIO**

\[ \frac{k_3}{k_4} \]

\[ i.e. \text{PARTITION RATIO} = \frac{\text{number of product molecules}}{\text{inactivation events}} \]

Where

\[ E = \text{Enzyme} \]
\[ S = \text{Substrate} \]
\[ E.S = \text{Enzyme-substrate complex} \]
\[ E.X = \text{Enzyme-substrate intermediate} \]
\[ P = \text{Product} \]
\[ E-I = \text{Enzyme-inhibitor complex} \]

The partition ratio (P.R.) is directly related to the destructive efficiency of the substrate (Waley 1980), for example, suicide substrates such as AIA and carbon tetrachloride have a partition ratio of 200:1 and 26:1 respectively (Manno et al. 1988; Ortiz De Montellano and Mico 1981; Loosemore et al. 1981).
Inactivation of cytochrome P450 may involve covalent modification of either the apoprotein or the prosthetic group. These two mechanisms of cytochrome P450 destruction are now discussed in turn.

1.6.3.1 Covalent modification of the apoprotein

A number of compounds such as carbon disulphide, chloramphenicol and parathion inactivate the cytochrome P450 system by covalently binding to the apoprotein (Halpert and Neal 1980; Halpert et al., 1980; De Matteis and Seawright 1973). Compounds which modify the apoprotein do so by preventing either substrate interaction, for example carbon disulphide or the flow of electrons to the active site as in the case of chloramphenicol. Carbon disulphide forms a reactive sulphur which binds to the active site leading to a loss of microsomal haem (Halpert et al., 1980; Bond and De Matteis 1969; Dalvi et al., 1974; De Matteis 1974). In the case of chloramphenicol, cytochrome P450 destruction is due to the drug binding to a lysine residue at the active site. As a direct consequence the flow of electrons from NADPH–cytochrome P450 reductase to haem is inhibited (Halpert et al., 1985).

1.6.3.2 Covalent modification of the haem moiety

Many porphyrogenic agents are able to modify the haem moiety at the active site of cytochrome P450 by haem alkylation. This property is shared by griseofulvin and ATMP alike, both of which cause haem alkylation and result in the inhibition of FK.

Drugs alkylating the haem moiety do so in one of two ways:
(i) the whole drug becoming covalently bound to the haem, or
(ii) a small chemical grouping is transferred from the drug to the tetrapyrrole.
Drugs which fall into the first category include acetylenes and olefins.
Substrates containing a sterically unhindered double or triple bond cause
destruction of cytochrome P450 resulting in the formation of a green pigment
(N-alkylated porphyrin). Oral contraceptives (norethisterone), anaesthetics
(secobarbital, fluoroxene) and sedatives (novonal) all cause enzyme
inactivation and green pigment formation (Ortiz De Monteliano and Correia
1983). The unsaturated chemical AIA, a well documented chemical, causes a
70% reduction in cytochrome P450 content in the liver. Selective isoenzymes of
cytochrome P450 namely cytochrome P450 a,b,h and PB-1, are catalytically
inactivated by this drug (Bornheim et al. 1987; Waxman 1986). Cytochrome
P450 inactivation may be demonstrated in vitro which results in the destruction
of pre-existing haem (Loosemore et al. 1981; De Matteis 1971; Levin et al.
1972).

It should be recognised that the presence of a double or triple bond in a
chemical does not always convey suicidal inactivation on cytochrome P450 nor
give rise to a green pigment. In the case of styrene and 3,3 dimethyl-1-hexene,
due to steric hindrance the enzyme activity remains intact (Ortiz De Montellano
and Mico 1980). Similarly, an unsaturated substrate, for example 2-heptyne,
which effectively destroys cytochrome P450, does not give rise to a green
pigment (Ortiz De Montellano and Kunze 1980).

In the second class of compounds which involve fragmentation of the drug
a small chemical grouping is attached to the haem. Agents such as DDC,
griseofulvin, sydnones, and dihydroquinolines fall into this category (De Matteis
et al. 1980b; De Matteis and Gibbs 1980; Lukton and Ortiz De Montellano 1985;
Ortiz De Montellano et al. 1986). DDC undergoes oxidative metabolism leading
to aromatization and simultaneous ejection of the 4-methyl substituent (De Matteis et al. 1982b; Lee et al. 1988; Augusto et al. 1982). A radical alkyl cation is formed when a dihydropyridine undergoes a one-electron oxidation (Augusto et al. 1982; Ortiz De Montellano 1989; 1986). The methyl substituent covalently binds to one of the pyrrolic nitrogens in haem to form N-MePP, the antimetabolite of FK. The 4-methyl group from DDC is transferred intact to the haem moiety of cytochrome P450 since deuterated 4-CD$_3$-DDC when metabolised in vivo produces N-CD$_3$-protoporphyrin identified by mass spectrometry (De Matteis et al. 1981). The 4-methyl substituent of DDC if replaced with ethyl, propyl or butyl grouping the corresponding N-alkyl porphyrin is generated in mouse liver in vivo (McCluskey et al. 1986; De Matteis et al. 1982b). A good correlation has been shown to exist between loss of cytochrome P450 and formation of the N-MePP in vitro (De Matteis et al. 1982b; Marks et al. 1985).

Recently studies have shown that apart from haem alkylation, haem-derived protein adducts may be formed which contribute to cytochrome P450 destruction (Riddick et al. 1990; Davies 1986a; 1986b). This concludes the discussion of how different suicide substrate inhibitors destroy cytochrome P450. The next part is concerned with the formation of the N-alkyl porphyrin at the cytochrome P450's active site.

1.6.4 Production of N-alkylated porphyrin by cytochrome P450

The active site of cytochrome P450 consists of haem with the iron linked to an apoprotein by a cysteine residue. Evidence for this structure comes from spectroscopic studies and X-ray analysis (White and Coon 1980; Miwa and Lu 1986). The exact topology of mammalian liver cytochrome P450 is unknown but
Fig 1.9 Hypothetical model for drug at cytochrome P450 active site

Drug approaches cytochrome P450’s active site from the oxygen-bearing side of the haem.
a hypothetical model figure 1.9 taken from De Matteis (1987) and De Matteis et al. (1982a) can be used to discuss its salient features, which are as follows:

i) The haem plane in cytochrome P450 has an absolute configuration with respect to each of its two axial ligands, oxygen and the mercaptide anion. This is because the two vinyl substituents attached to pyrrole ring A and B, and two propionate substituents attached to ring C and D, confer an asymmetric structure on haem; that is, the two faces of the haem plane are not superimposable and therefore are distinguishable from each other.

ii) When a drug approaches the active site, the oxygen-bearing side of the haem plane is exposed to the drug, making this side more likely to suffer attack from any possible electrophilic metabolite. A drug at the active site of cytochrome P450 lies in close proximity to the iron-bound oxygen to ensure oxygen insertion or electron transfer.

Further support for the view, that only one side of the haem plane (the oxygen-bearing side) is exposed to the drug ((ii) above) comes from studies examining N-alkylated porphyrins generated in vivo. Several workers have demonstrated that green pigments isolated from the liver of animals treated with DDC, ethylene, AIA, secobarbitone, and propyne all exhibit circular dichroism spectra (De Matteis et al. 1982a; Ortiz De Montellano and Kunze 1981). These findings indicate that alkylation of the tetrapyrrrole takes place on an enzymic template, and from one side of the porphyrin face (De Matteis et al. 1982a; Ortiz De Montellano and Kunze 1981; Kunze et al. 1983). In contrast, chemically synthesized N-MePP remains optically inactive due to the racemic mixtures of the two enantiomorphs formed (De Matteis et al. 1982a).
1.6.5 Reconstitution of cytochrome P450 by haem and its effect on the cell

Reconstitution of the apoprotein with haem to form a functionally intact holoenzyme has important effects on the haem biosynthesis in the cell. Cytochrome P450 draws haem from the regulatory haem pool and eventually the haem pool is depleted. Depletion of the regulatory haem pool leads to stimulation of ALA–S (De Matteis 1978; De Matteis and Gibbs 1972). An increase in the output of porphyrins and their precursors leads to porphyria. AIA causes destruction of cytochrome P450 and pre-existing haem (De Matteis 1973; 1978). The apoprotein remaining reconstitutes with fresh haem and the suicidal cycle is continued causing a "haem stripping" effect which, in turn, leads to a gradual depletion in the haem regulatory pool (Lunetta et al. 1989; De Matteis 1973; Bornheim et al. 1987; Davies et al. 1986a; 1986b). This contrasts most clearly with the drugs which are also suicide substrates of cytochrome P450, but give rise to an N–alkylated porphyrin which powerfully inhibits the enzyme FK. In the case of DDC, following cytochrome P450 destruction the apoprotein remaining reconstitutes with fresh haem to continue the cycle of cytochrome P450 inactivation.

1.6.6 Griseofulvin and protoporphyria

Griseofulvin is an antifungal agent used in the treatment of dermatophytes by preventing the growth of trichophyton, epidermophytes and microsporum. Interest in griseofulvin developed when it was noted that patients receiving this antibiotic accumulated and excreted protoporphyrin in the urine (Berman and Franklin 1965). The disruptive effect griseofulvin exerted on the liver resulted in hypercholesterolaemia, liver enlargement and increased activity of microsomal enzymes (De Matteis 1966a; 1966b). In rodents, griseofulvin would cause a development of photosensitivity, alteration of liver structure, intrahepatic
deposition of solid porphyrin pigment and biliary cirrhosis (De Matteis 1967). Griseofulvin is used as an experimental tool to understand porphyrin accumulation, since the biochemical changes observed in rodents, namely photosensitivity, liver derangement and biliary cirrhosis, all bear the hallmarks of erythropoietic protoporphyria, a disorder seen in man (De Matteis 1967; De Matteis and Stonard 1977). In experimental animals griseofulvin causes a decrease in FK activity, accumulation of protoporphyrin and an increase in ALA-S activity (Nakoa et al. 1967; De Matteis 1966a; De Matteis and Gibbs 1975).

1.7 Human and experimental porphyrias
1.7.1 Hereditary porphyrias and their classification

In living organisms the cell utilizes haem for a vast number of normal cellular functions. Perturbation in the production of haem synthesis results in a condition known as porphyria. Porphyria is best described as a disorder of porphyrin metabolism resulting in increased levels of porphyrins and its precursors. In man porphyria may be inherited or acquired, but both conditions are attributable to a defect in one or more enzymes in the haem biosynthetic pathway (Table 1.2). Clinically porphyria may be classified in one of three ways. First, whether the disease shows acute presentation, that is, abdominal pains and neurological disorder. Second, the principal site of porphyrin biosynthesis, such as involvement of the liver (hepatic porphyria), or arising from the bone marrow (erythropoietic porphyria). Finally, the pattern of porphyrin accumulation describes which of the 8 enzymes in the haem pathway is blocked (Rimington 1989; Bottomley and Muller-Eberhard 1988; Moore et al. 1987). As shown in figure 1.10, there are seven potential sites where a partial block in one of the enzymes may give rise to porphyria. For example, Doss porphyria or
### Table 1.2 Summary of the different types of inherited human porphyrias

<table>
<thead>
<tr>
<th>Classification</th>
<th>Inheritance</th>
<th>Deficient Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Hepatic porphyrias</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A) Acute Porphyrias</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute intermittent porphyria</td>
<td>Autosomal dominant</td>
<td>PBG-Deaminase</td>
</tr>
<tr>
<td>Hereditary coproporphyria</td>
<td>Autosomal dominant</td>
<td>Coproporphyrinogen oxidase</td>
</tr>
<tr>
<td>Variegate porphyria</td>
<td>Autosomal dominant</td>
<td>Protoporphyrinogen oxidase</td>
</tr>
<tr>
<td>Doss porphyria</td>
<td>Autosomal recessive</td>
<td>ALA-dehydratase</td>
</tr>
<tr>
<td>B) Nonacute porphyria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porphyria cutanea tarda (familial type)</td>
<td>Autosomal dominant</td>
<td>Uroporphyrinogen decarboxylase</td>
</tr>
<tr>
<td>II Erythropoietic porphyria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congenital erythropoietic porphyria (Gunthers disease)</td>
<td>Autosomal recessive</td>
<td>Uroporphyrinogen cosynthetase</td>
</tr>
<tr>
<td>Erythropoietic protoporphyria</td>
<td>Autosomal recessive</td>
<td>Ferrochelatase</td>
</tr>
</tbody>
</table>
### Fig 1.10 Enzyme blocks leading to different porphyrias

<table>
<thead>
<tr>
<th>Porphyria</th>
<th>Substrate</th>
<th>Enzyme</th>
<th>E.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plumbo porphyria</td>
<td>Glycine + Succinyl CoA</td>
<td>5-Aminolaevulinate synthase</td>
<td>2.3.1.37</td>
</tr>
<tr>
<td></td>
<td>5-Aminolaevulinic acid</td>
<td>Aminolaevulinate dehydratase</td>
<td>4.2.1.24</td>
</tr>
<tr>
<td>Acute intermittent porphyria</td>
<td>Porphobilinogen</td>
<td>Porphobilinogen deaminase</td>
<td>4.3.1.8</td>
</tr>
<tr>
<td>Congenital porphyria</td>
<td>Hydroxymethylbilane</td>
<td>Uroporphyrinogen cosynthase</td>
<td>4.2.1.75</td>
</tr>
<tr>
<td>Porphyria cutane tarda</td>
<td>Uroporphyrinogen</td>
<td>Uroporphyrinogen decarboxylase</td>
<td>4.1.1.37</td>
</tr>
<tr>
<td>Hereditary coproporphyria</td>
<td>Coproporphyrinogen</td>
<td>Coproporphyrinogen oxidase</td>
<td>1.3.3.3</td>
</tr>
<tr>
<td>Variegate porphyria</td>
<td>Protoporphyrinogen</td>
<td>Protoporphyrinogen oxidase</td>
<td>1.3.3.4</td>
</tr>
<tr>
<td>Erythropoietic protoporphyria</td>
<td>Protoporphyrin</td>
<td>Ferrochelatase</td>
<td>4.99.1.1</td>
</tr>
</tbody>
</table>
Plumboporphyría, arises due to a defect in ALA–dehydratase; in that example the biochemical presentation is similar to that of lead poisoning with increased excretion of both ALA and coproporphyrin (Labbe and Bird 1985; Thunnell et al. 1987).

In acute intermittent porphyria (AIP) the defective enzyme is PBG–deaminase, in which there is a 50% reduction in the enzyme activity (Bottomley and Muller–Eberhard 1988; Meyer et al. 1972). The biochemical features of this disease are an increased urinary excretion of ALA and PBG together with an increase in uroporphyrin (Meyer and Schmid 1979; Kappas et al. 1985). Factors precipitating the attack of AIP include hormones, drugs and the nutritional status of an individual (Goldberg 1985; Kappas et al. 1985). AIP is inherited as an autosomal recessive trait and different investigators have documented the existence of cross reacting immunological material (CRIM), CRIM–negative and CRIM–positive mutations, both of which are used as a basis for the different subtypes of AIP (Grandchamp and Nordmann 1988).

In AIP two groups may be identified based on the amount of CRIM, that is, an immunoreactive enzyme protein in the red blood cells. In one group the amount of CRIM is directly proportional to the enzyme activity; this is referred to as CRIM–negative. In the second group, the immunoreactive enzyme protein is present but there is no corresponding enzyme activity; this is termed CRIM–positive (Anderson et al. 1981).

In congenital erythropoietic porphyria (CEP) or Gunther's disease, there is a defect in the enzyme uroporphyrinogen III cosynthetase; the defective gene is inherited as an autosomal recessive trait (Moore et al. 1978). Under normal
conditions uroporphyrinogen III cosynthetase catalyses the conversion of hydroxymethylbilane (HMB) (preuroporphyrinogen) to uroporphyrinogen III. This conversion gives rise to the normal isomeric type of coproporphyrinogen III, protoporphyrinogen IX, and finally to protoporphyrin IX, the porphyrin present in haem. The function of uroporphyrinogen cosynthetase is to catalyse the rearrangement of pyrrole ring D and ring closure of HMB to produce uroporphyrinogen III (Jordan 1990; Moore et al. 1987). In CEP–sufferers uroporphyrinogen cosynthetase activity is less than 20% of normal activity; as a consequence of this, uroporphyrinogen I and coproporphyrinogen I accumulate and are then oxidised to the corresponding porphyrins. In the absence of uroporphyrinogen cosynthetase the unstable HMB spontaneously cyclizes without rearrangement to form uroporphyrinogen I Fig 1.11. Both isomers III or I of uroporphyrinogen serve as substrate for uroporphyrinogen decarboxylase; however, uroporphyrinogen I is metabolised less effectively than the series III isomer. Also coproporphyrinogen III oxidase, the next enzyme in the haem pathway, cannot utilize coproporphyrinogen I (Jordan 1990; Moore et al. 1987). As uroporphyrin and coproporphyrin are powerful photodynamic agents, people with this disease who accumulate large amount of uroporphyrin I in their tissues become sensitive to sunlight, hence the skin lesion may lead to mutilations and severe scarring.

Porphyria cutanea tarda (PCT) is the most common form of porphyria. The basic biochemical defect is reduced activity of uroporphyrinogen decarboxylase (in the liver) (Fig 1.10), which results in over–production and increased excretion of uroporphyrin and other polycarboxylated porphyrins (Doss et al. 1971; Felsher et al. 1982). PCT is classified into two types, familial or sporadic (acquired) PCT. In the familial variety there is a 50% reduction of
Fig 1.11 Fate of hydroxymethylbilane in the presence of normal or reduced uroporphyrinogen III cosynthetase activity

Hydroxymethylbilane

Uroporphyrinogen I  \[\rightarrow\]  Coproporphyrinogen I

Uroporphyrinogen III  \[\rightarrow\]  Coproporphyrinogen III

\[\rightarrow\]  \[\rightarrow\]  \[\rightarrow\]  Haem
uroporphyrinogen decarboxylase activity in erythrocytes and this is inherited in an autosomal dominant fashion (Moore 1990; Grandchamp and Nordmann 1988). In sporadic PCT there is reduced uroporphyrinogen decarboxylase in the liver although enzyme activity is normal in erythrocytes. Factors precipitating sporadic PCT include oestrogens and alcohol ingestion (Moore 1990; Moore et al. 1987).

In hereditary coproporphyria there is a partial deficiency of the enzyme coproporphyrinogen oxidase Fig 1.10 (Brodie et al. 1977; Grandchamp and Nordmann 1977). This disorder is characterised by high levels of coproporphyrin, ALA and PBG in the individuals and is due to a defective gene inherited as an autosomal dominant trait (Moore et al. 1987; Meyer and Schmid 1979). A variant of hereditary coproporphyria, called harderoporphyria, has been identified, the major finding being an overproduction and excretion of harderoporphyrin (Nordmann et al. 1983).

A partial block in the penultimate enzyme protoporphyrinogen oxidase leads to the disorder variegate porphyria (Fig 1.10) where an overproduction of protoporphyrin is seen accompanied by ALA and PBG during acute attacks (Boyle et al. 1986; Brenner and Bloomer 1980). The defective gene is inherited as an autosomal trait (Nordmann and Deybach 1990).

The final enzyme in the haem pathway, FK, if partially defective causes the development of erythropoietic protoporphyria (EPP) (Bonkowsky et al. 1975; Sassa et al. 1982) (Fig 1.10). Individuals suffering from the disorder inherit the gene in an autosomal dominant manner and the major porphyrin accumulating is protoporphyrin (Nordmann and Deybach 1990; Moore et al. 1987). A clinical
feature in these individuals is the extreme sensitivity to sunlight; the accumulated protoporphyrin can absorb light energy and become phototoxic to the cell. (Two disorders which have high levels of protoporphyrin are lead intoxication and iron deficiency anaemia that differ from EPP in that there is no photosensitivity).

Individuals who suffer from any variety of acute porphyria or have a genetic defect in one of the enzymes in haem synthesis are susceptible to drugs. Many lipophilic drugs containing unrelated chemical groupings exacerbate or precipitate acute attacks of porphyria in people with the appropriate genetic defect. These drugs include barbiturates, sedatives, and phenylbutazone, and a comprehensive list of drugs which are thought to be potentially dangerous is given by Hift et al. (1989). Attacks may also be precipitated by steroids, infections, fasting or by a combination of these factors. In contrast with acute porphyrias, the nonacute varieties are thought to be insensitive to precipitating factors with the exception of PCT which is made worse by iron-containing preparations and oestrogens (Sweeney 1986).

1.7.2 Acquired porphyria

In acquired porphyria an individual inherits normal genes. Under these conditions porphyria is due to either a lesion in one of the enzymes in the haem biosynthetic pathway or depletion in the regulatory haem pool by a chemical agent. In experimental porphyria drugs may be given which produce similar effects to those seen in human porphyrias. However only two types of porphyria will be discussed here, namely uroporphyrria and protoporphyria.
1.7.2.1 Drug-induced uroporphyria

This particular type of drug-induced porphyria resembles the porphyria cutanea tarda (PCT) seen in man. Hexachlorobenzene (HCB), a polyhalogenated aromatic hydrocarbon, first drew attention in 1955 when an outbreak of cutaneous porphyria occurred in Turkey. Wheat seed that had been contaminated with HCB was used for human consumption (Schmid 1960). In rats given HCB the major biochemical changes are that uroporphyrin accumulates and uroporphyrinogen III decarboxylase becomes inhibited (Elder et al. 1978; Smith and De Matteis 1980). Although uroporphyrin is the major porphyrin accumulating, 5,6 and 7 carboxylated porphyrins are also elevated (De Matteis et al. 1961).

The mechanism of drug-induced uroporphyria has not been fully elucidated. However, it is recognised that non-haem iron precipitates a worse attack of uroporphyria in experimental animals given HCB (Smith and Francis 1983; Louw et al. 1977). Non-haem iron may exacerbate uroporphyria in two ways. Firstly, a reactive species such as peroxides and free radicals are thought to be generated which inactivate uroporphyrinogen decarboxylase (De Matteis and Stonard 1977); or secondly, the substrate uroporphyrinogen is oxidised to its corresponding porphyrin by the reactive species and is unable to interact with uroporphyrinogen III decarboxylase (Ferioli et al. 1984).

1.7.2.2 Drug-induced protoporphyria

In the case of DDC the biochemical changes observed are a decrease in FK activity and accumulation of protoporphyrin (De Matteis et al. 1973; Onisawa and Labbe 1963). N–MePP, a potent antimetabolite of FK, is generated in the livers of mice and rats given DDC (De Matteis et al. 1980c; Tephly et al. 1981;
Ortiz De Montellano et al. (1981a). DDC causes a loss in cytochrome P450 (Wada et al. 1968; Waterfield et al. 1969). Due to a block in FK activity there is a decrease in haem synthesis. The inhibitory influence of haem normally exerted on ALA–S is removed and ALA–S formation becomes stimulated (Bissel and Hammaker 1976; Abbritti and De Matteis 1973). A compensatory mechanism is set in motion and the dual effect, namely inhibition of FK and stimulation of ALA–S, results in protoporphyrin accumulation (Abbritti and De Matteis 1973).

1.7.2.3 Role of cytochrome P450 in drug-induced protoporphyria

The cytochrome P450 system is the key enzyme in the liver responsible for metabolising xenobiotics. A property of this enzyme is its ability to be induced by chemicals thereby increasing the metabolism of a drug. Cytochrome P450 is often the site of attack when xenobiotics undergo biotransformation for two main reasons. Firstly, when a compound is oxidised by cytochrome P450, a reactive intermediate may be formed either at or nearby the active site making the enzyme the first site of attack. Secondly, the lipid rich environment which envelops the enzyme may undergo lipid peroxidation due to the generation of radical species (De Matteis et al. 1977; Schacter et al. 1973; Levin et al. 1972). Cytochrome P450 loss on its own is not responsible for porphyria. Aminobenzotriazole is known to cause a significant loss in hepatic cytochrome P450 without porphyrins accumulating (Costa and Ortiz De Montellano 1985). However, when the loss of cytochrome P450 is due to selective destruction of the prosthetic group with the apoprotein still capable of utilizing haem from the regulatory pool, then the regulatory haem will be depleted and the ALA–S will become stimulated. This stimulation leads to accumulation of porphyrins and their precursors (De Matteis 1973; Abbritti and De Matteis 1973) (Fig 1.12).
The aim of this present work has been (i) to elucidate the mechanism of action of ATMP; (ii) to isolate, purify and identify the griseofulvin green pigment responsible for FK inhibition; and (iii) to isolate, purify and identify the major griseofulvin pigment. Aims (ii) and (iii) involved improving existing techniques in order to produce enough green pigment to be analysed by mass spectrometry. This work is described in the chapters that follow.
Chapter 2

MATERIALS AND METHODS
2 Materials

2.1 Chemicals and reagents

Griseofulvin was a gift from the Pharmaceutical Division of ICI or was obtained from Sigma Chemical Company, Poole, Dorset, U.K.;
1-[4-(3-Acetyl-2,4,6-trimethylphenyl)-2,6-cyclohexane dionyl]-O-ethyl propionaldehyde oxime (ATMP), propyl analogue and oxazole analogue of ATMP were gifts from ICI, Central Toxicology Laboratory; $^{14}$C-labelled ATMP (site of labelling shown in Fig 3.5) with a specific activity of $127 \times 10^3$ dpm/nmol, was a gift from ICI, Central Toxicology Laboratory;
Methanol and chloroform (distilled) were from Fisons Scientific Apparatus (FSA) Bishop Meadow Road, Leics, U.K.;
Methanol and dichloromethane (Hipersolv) were from The British Drug Houses (BDH) Chemicals Company, Broom Road, Poole, U.K.;
Hexane (Hipersolv) was from BDH;
Chromatographic grade silica (Kieselgel 60, 70 – 230 mesh) was from Merck, also silica Gel 60 precoated TLC plates (without fluorescent indicator);
Carbon monoxide and oxygen–free nitrogen were from British Oxygen Company (BOC), Priestley Road, Guildford, Surrey, U.K.;
Protoporphyrin IX dimethylester was from Sigma;
Mesoporphyrin IX dimethylester was from Koch–Light laboratories, Haverhill, Suffolk, U.K.;
3,5–Diethoxycarbonyl–1,4–dihydro–2,4,6–trimethylpyridine (DDC) was a gift from A.Gibbs;
5–Aminolaevulinc acid (ALA) was obtained from Sigma;
Boron trifluoride was from BDH;
β-naphthoflavone was from Aldrich Chemical Company, Gillingham, Dorset, England;
3-Nitrobenzyl alcohol was obtained from Aldrich Chemical company;
Di-n-butylamine was from BDH;
Deuterated chloroform was from Aldrich Chemical company;
All other reagents used were of Analar grade and were obtained from BDH or FSA, unless otherwise stated.

2.2 Methods
2.2.1 ATMP treatment
(a) Mice

ATMP (300 mg/kg) was given by intraperitoneal (i.p.) injection (dissolved in arachis oil 5 ml/kg body weight) to fed male MF1 mice (MRC or Alderley park strain), or to mice pretreated with inducers or inhibitors of the cytochrome P450 system as described below. For the inducer studies mice were pretreated for 3 consecutive days with either sodium phenobarbitone (80 mg/kg/daily, dissolved in saline), β-naphthoflavone (40 mg/kg daily, dissolved in corn oil and sonicated) both i.p. or with clofibric acid (300 mg/kg daily, dissolved in corn oil and sonicated) given orally. Twenty-four hours after dosing with an inducer the mice were given either 20 or 75 mg/kg ATMP and 24 hrs later the animals sacrificed. The livers were removed and hepatic porphyrins measured.

In a second study, the inducers phenobarbitone (0.05% (w/v)) and β-naphthoflavone (40 mg/kg dissolved in corn oil) were given in the drinking water and i.p., respectively for 3 days. Twenty-four hours later ATMP (150 mg/kg) was given and 4 hrs later the livers were removed. Hepatic porphyrin levels and FK activity were estimated.
In a third study mice were given the inhibitors SKF525–A (40 mg/kg dissolved in saline), piperonyl butoxide (300 mg/kg dissolved in corn oil), \( \alpha \)-naphthoflavone (80 mg/kg dissolved in corn oil), or metyrapone (50 mg/kg dissolved in saline) i.p. 1 hr before dosing with ATMP (either 75 mg/kg, 150 mg/kg or 300 mg/kg each dissolved in arachis oil); then, 4 hrs later, animals were sacrificed and livers removed. Measurement of hepatic porphyrins and FK were made.

The structural analogues propyl– and oxazole–derived ATMP were treated similarly to ATMP. Each analogue was dissolved in arachis oil and given intraperitoneally to mice. Animals were sacrificed 24 hrs later and total porphyrins and FK activity from the liver were determined. In another study radiolabelled ATMP (Fig 3.5), was given to mice. The aim of this study was to show that ATMP uptake was not prevented even in the presence of the inhibitor SKF525–A. The radiolabelled chemical was diluted with unlabelled chemical to a specific activity of \( 8.26 \times 10^6 \) dpm/mmol and 150 mg/kg of \( ^{14} \)C ATMP. Mice were given a single injection i.p. and 1, 2, and 4 hrs later the livers were removed and the amount of radioactivity present determined.

(b) Rat, hamster and guinea–pig

Male Porton rats (MRC), Hartley guinea–pigs and Syrian hamster (from Mill Hill, London) aged 6–8 weeks were given a single dose of ATMP (dissolved in arachis oil, i.p injection). Livers were removed 24 hrs later and total porphyrins and FK activity determined.
(c) Experiments with Chick Embryo

(i) Chick embryo hepatocytes

The method of Ferioli et al. (1984) was used. Embryos, 16 days old of Rhode Island Red x White Rock chickens were obtained from Orchards Farm, (P.O. Box 19, Pinner, Middlesex.), and stored for approximately 19 hrs in a humidified incubator at 37°C before preparing hepatocytes.

Ice-cold Hanks balanced buffered saline (HBBS) (5 ml), was injected through the heart in order to perfuse the liver. The liver cells were separated by treating with collagenase (0.5 mg/ml) in HBBS without Ca²⁺ and Mg²⁺ as described by Ferioli et al. (1984). Hepatocyte viability was determined by the trypan blue exclusion method (Seglen 1972). The hepatocytes were suspended at a density of 0.9 x 10⁵ cells/ml in Williams E medium (containing glutamine (2 mM), gentamycin (50 µg/ml), insulin (5.7 µg/ml), cortisol (4.8 µg/ml) and foetal calf serum (5% (v/v)). Using eight-well multiplates (26 mm x 3 mm) a 2 ml suspension of hepatocytes were placed in each well and left in a humidified atmosphere of CO₂:air 1:19 at 37°C. Following a 19 hrs incubation period, the medium was changed with fresh medium identical to that described above but containing no foetal calf serum. Drugs either alone or with exogenous ALA were also added at this time and the cells were then exposed to the drugs for 19 hrs before measuring porphyrin levels.

Porphyrin levels were measured in the medium and the cells. A sample of medium (100 µl) was added to 800 µl 0.1 M perchloric acid/ methanol (1:1 (v/v)) and mixed thoroughly, the fluorescence of the sample was then measured as described in section 2.2.5c. The remaining medium was then aspirated and 1.5 ml 0.1 M perchloric acid: methanol (1:1 (v/v)) was added to the cells and
mixed gently to extract cellular porphyrins from the monolayer. Samples were left in the dark for 15 – 20 mins and then 800 μl of the mixture was taken and porphyrin fluorescence measured in an L.S. 5 fluorescence spectrophotometer. When necessary, samples were diluted with perchloric acid/methanol mixture before measurement of fluorescence.

(ii) Chick embryos in ovo

Dosing chick embryos in ovo entailed injecting the drugs or chemicals dissolved in 0.1 ml DMSO into the fluids surrounding the embryo through a small hole in the shell and the air sac. Sellotape was then used to cover the small hole in the shell so as to avoid excessive water loss through evaporation. The embryos were then returned to the incubator for a further 24 hrs before removing their livers for porphyrin determination.

The following were the doses of drugs given to chick embryos in ovo.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume of DMSO/egg (ml)</th>
<th>Dose of drug (mg)/egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-DMSO</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>DDC</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Low dose ATMP</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>High dose ATMP</td>
<td>0.1</td>
<td>6.0</td>
</tr>
</tbody>
</table>

2.2.2 Griseofulvin treatment

Male MF1 mice aged 6–8 weeks old, were bred at the MRC Toxicology Unit, Carshalton, Surrey. Mice were housed in cages in a controlled environment with a 12 hr daylight : 12 hr dark cycle, at a temperature of 22°C. They were fed a powdered diet (Rat and Mouse diet No.3) containing 2% (v/w)
arachis oil for 3 days followed by a similar diet containing griseofulvin at 1% (w/w) for a further 3 days. Mice were permitted drinking water ad libitum. Following the treatment mice were sacrificed by cervical dislocation, and their livers were quickly removed, rinsed, blotted and then transferred to a suitable homogenisation medium and kept cold on ice.

2.2.3 Isolation and purification of green pigments
(a) Isolation and purification of the ATMP green pigment

Mice dosed i.p. with ATMP (300 mg/kg) were sacrificed 24 hrs later and their livers removed. ATMP green pigment was isolated from liver homogenate using the method of De Matteis et al. (1980a).

Aliquots (15 ml) of a 50% (w/v) liver homogenate were stored overnight at -20°C. An equal volume of acetone (15 ml) was added to the homogenate shaken well and centrifuged in a MSE 4L centrifuge at 1,200 rpm for 5 mins. This step was repeated a second time in order to help remove any lipids. The porphyrins and haem were then extracted into acidified acetone (1.3% (v/v) conc. HCl in acetone); the extract (30 ml) was reduced in volume under nitrogen down to approximately 0.5 ml. Acetone (1 ml) was then added and the solution was placed onto a freshly packed column (30 cm x 1 cm) of Sephadex LH20 swollen in acetone. The pigment was eluted off the column using acetone. ATMP green pigment moved down the column slower than haem but faster than protoporphyrin. Eluates (3 ml) were collected and each was scanned in a Varian 2200 spectrophotometer to detect a Soret peak. Fractions containing a Soret peak between 410nm - 420nm were pooled into an extraction tube and stored at -70°C overnight.
The crude ATMP pigment was defatted further by centrifuging in a MSE 6L centrifuge at 1500 rpm at -30°C for 1 – 2 mins and immediately transferred to a round-bottom flask. The fat-free pigment present as the free acid, was dried under nitrogen, dissolved in washed chloroform and applied to a TLC silica plate before developing in the system chloroform:methanol:acetic acid 40:10:3 (v/v/v). A band corresponding to the green pigment (approximate $R_f$ value 0.25) was eluted off the silica and converted to the dimethylester by heating with boron trifluoride for 30 mins at 70°C (Smith and Francis 1979). The pigment was extracted into 3 ml chloroform and washed as described under pigment washing (see section (iv), isolation of griseofulvin green pigment). The sample was dried under nitrogen then dissolved in the mobile phase (dichloromethane:methanol:ammonia 50:50:0.1 v/v/v) before purifying further by HPLC using a Nucleosil 5 column, isocratic elution (2 ml/min) and monitoring the eluate at 417 nm. The main steps in the isolation and purification of ATMP green pigment are given in Figure 2.1.

(b) Isolation and purification of griseofulvin green pigments

During the isolation and purification procedure acid-washed glassware and distilled or HPLC grade reagents were used throughout. Essentially six steps were involved in the isolation and purification of griseofulvin green pigments. These are:

(i) Methylation process

Methylation of the griseofulvin green pigments was a modified method of Ortiz De Montellano et al. (1981c). Mice livers were rapidly removed and the gall bladder excised. Livers from 10 – 12 mice were pooled and, after rinsing thoroughly with distilled water, the livers were blotted dry and weighed. The
Fig 2.1 A summary of the procedure involved in the extraction and purification of ATMP green pigment

50% (w/v) Liver homogenate

↓

Wash with acetone twice

↓

Extract porphyrs into acidified acetone, twice. Evaporate acidified acetone off under nitrogen until porphyrins are moist

↓

Dissolve in acetone and place on Sephadex LH20 column. Elute with acetone

↓

Scan fractions for green pigment and pool green pigment fractions. Leave overnight at -70 °C

↓

Defat and dry combined green pigment fractions

↓

Dissolve residue in chloroform; run on a TLC plate

↓

Elute green pigment off silica and methylate using boron trifluoride

↓

Extract pigment into chloroform and wash

↓

Dissolve in mobile phase and purify on HPLC
livers were cut in ice-cold 0.25 mM sucrose and homogenised at 1425 rpm using a Potter-type homogeniser.

Aliquots (15 ml) of a 50% (w/v) liver homogenate were added drop-wise to 250 ml 5% (v/v) conc. sulphuric acid in methanol while maintaining a rapid agitation on a magnetic stirrer. A fine white suspension of liver tissue was formed as the methylating mixture changed to a ruby red colour. The flask was stoppered and left in the dark at 4°C overnight to allow conversion of the free acid of any porphyrin present to the dimethylester.

(ii) Extraction and washing of crude green pigment

A glass buchner funnel attached to a filter flask was used to filter the methanolic sulphuric acid mixture. The white tissue residue was washed with 25 ml of methylating agent. The red filtrate was transferred to a 1 L separating funnel and mixed with 50 ml of chloroform, distilled water (500 ml) was then added and the components mixed well. The organic and aqueous phase partitioned with the green pigment moving into the organic layer. The aqueous phase was then washed twice with chloroform (25 ml) before combining the three organic extractions. The pooled pigment extract was transferred to the separating funnel and gently washed twice with distilled water (200 ml). The chloroform solution of the pigment was then dried by passing down a column 1.5 cm internal diameter (i.d) x 5 cm long of anhydrous sodium sulphate; chloroform (10 ml) was also added to the column to wash off any remaining green pigment. The eluate was collected into a 500 ml round-bottom flask and dried under vacuum using a rotary evaporator at 37°C.
(iii) Separation of a green pigment from protoporphyrin by a silica column

The porphyrin methylesters were dissolved in chloroform (2 ml) and applied to a 1.5 cm (i.d) x 7 cm long dry silica column. Lipids were removed from the green pigment by washing the sample on the column with hexane (100 ml). The green pigment was further washed by passing chloroform (65 ml) down the column, this step also eluted protoporphyrin from the column. The protoporphyrin-free green pigment was eluted off the silica column using chloroform:methanol 50:50 (v/v). The eluate was collected (approximately 25 ml) and after adding chloroform (5 ml) it was divided equally between two 50 ml extraction tubes. The pigment was washed as described below.

(iv) Pigment washing following separation from protoporphyrin

To each tube containing the pigment distilled water (35 ml) was added and after several mixing by inversion, the mixtures were centrifuged in an MSE 4L instrument for 5 mins at 1500 rpm. The aqueous phase was discarded and the pigment washed again as above. Water was removed from the sample by careful pipetting and the last traces by passing over anhydrous sodium sulphate as described previously (see (ii) extraction and washing of crude green pigment).

(v) Separation of minor and major griseofulvin pigment and haem by TLC

There is a considerable difference in the chromatographic properties of haem, minor and major griseofulvin pigment which allowed a good separation on TLC. The pigment obtained from a maximum of 7.5 g of liver was loaded on to a TLC silica plate. The crude pigment was dissolved in chloroform (2 ml), applied to a TLC plate using a Hamilton syringe and developed in a TLC tank containing chloroform:methanol 20:3 (v/v) for 45 mins. The developed
chromatogram was allowed to dry at room temperature then examined under UV-light. The bands corresponding to minor and major griseofulvin pigment fluoresced red and dark red respectively. The separated pigments were scraped off the TLC plate using a glass hoover, then eluted off silica using chloroform:methanol 50:50 (v/v). Chloroform (2 ml) was added to each pigment and washed as described in section (iv). Finally both pigments were dried under nitrogen and stored at -20°C until further required.

(vi) Purification of minor griseofulvin pigment by HPLC

A Nucleosil 5 column 4.6 mm x 250 mm with a guard column was attached to a HPLC apparatus. A Cecil spectrophotometer linked to a deuterium lamp was used to monitor the absorbance of the eluate at a wavelength of 417 nm. Also linked to the HPLC was a Milton Roy CI-10B integrator and plotter. The mobile phase used was dichloromethane:methanol:ammonia (sp.gr. 0.88), 50:50:0.1 (v/v/v). A 20 μl injection loop was used to inject the green pigment onto the column. The chromatographic properties of the green pigment was compared with chemically-synthesized N-MePP and protoporphyrin. The above steps were used to isolate and purify two griseofulvin green pigments known as minor and major griseofulvin pigment. A summary of the method is shown in Fig 2.2.

2.2.4 Spectral analysis

(a) Electronic absorption spectrum of the neutral porphyrin

The porphyrin neutral spectrum was obtained in one of two ways. Either the sample was exposed to ammonia fumes for 15–20 mins to neutralise the green pigment before dissolving it in an aliquot of chloroform (2.5 ml) and scanning from 700 nm to 360 nm, or, in the second method the
Fig 2.2 Schematic diagram summarising the main steps in the extraction and purification of griseofulvin–green pigments

50% (w/v) liver homogenate methylated in 5% (v/v) conc. sulphuric/methanol mixture.

↓

Crude pigment extracted into chloroform washed and applied onto a dry silica column

↓

100 ml hexane followed by 65 ml chloroform passed down silica column.

↓

Green pigment eluted off silica column using chloroform/methanol 50:50 (v/v)

↓

Major and minor griseofulvin pigment separated on TLC

↓

Major and minor griseofulvin pigment purified by HPLC
pigment was dissolved in an aliquot (2.5 ml) of DMSO:Tris 1 M pH 8.2 (10:1 v/v) and scanned. Either the Varian Cary 2200 or the Uvikon 860, each with appropriate plotter was used. Both instruments gave identical results, as measured against synthetic N-MePP and protoporphyrin standards.

(b) Copper dealkylation reaction

The copper catalysed dealkylation process was a modified method of De Matteis et al. (1985). Copper acetate (0.1 g) was dissolved in methanol (100 ml) and 10 μl added to the sample cuvette cell containing the green pigment (with an absorbance reading between 0.5 and 1.0) in chloroform (2.5 ml). The reference cell contained chloroform and copper acetate in methanol. The reaction was followed by scanning every 2 mins in a Uvikon 860 spectrophotometer between 460 nm and 360 nm. The copper reaction was repeated using N-MePP or protoporphyrin standards: in all cases porphyrins were exposed to ammonia fumes before scanning.

2.2.5 Special analytical techniques
(a) Mass spectrometry

Mass spectra were obtained on a tandem mass spectrometer (VG70-SEQ) of EBQ₁Q₂ geometry, where Q₁ is a quadrupole which acts as a collision cell. Each sample was first ionized by positive ion fast atom bombardment. Xenon atoms from an Ion Tech (Model B11N, Teddington, UK) saddle-field fast atom gun, was used as the primary ionizing beam and impacted the sample at 8.5 KeV. The secondary ions produced by the xenon atoms were accelerated to 8 KeV from the source region and the magnet scanned at 5 secs/decade over the mass range m/z 1350 – 50. Each sample
was dissolved in dichloromethane and added to 2 µl matrix, which was 3-nitrobenzyl alcohol and placed on the probe tip.

In the tandem mass spectrometry studies parent ions (MH)+ were selected with a resolution of approximately 1000 using EB (equivalent to the first mass spectrometer MS1) and subjected to collision activated dissociation using argon as the collision gas. Collision energies were varied from 5 – 460 eV, and the gas pressures in the collision cell were approximately 10⁻⁶ mbar. Daughter ion spectra were acquired by scanning Q₂ (equivalent to the second mass spectrometer MS₂) over mass range m/z 1000 – 40 and 15 scans were acquired under data system control in the multi-channel analysis (MCA) mode.

(b) Nuclear magnetic resonance (NMR)

The major griseofulvin pigment (200 µg) was dissolved in deuterated chloroform and transferred to a glass NMR sample tube (5 mm diameter). The sample was scanned in a Bruker AC 250 NMR spectrometer at 5.9 MHz overnight.

(c) Determination of total porphyrins in liver homogenate

The method of Grandchamp et al. (1981) was usually used. Porphyrins were extracted from 10% (w/v) liver homogenate (0.5 ml) into 0.1 M perchloric acid/methanol, 1:1 (v/v), (10 ml) and the mixture left in the dark for 15 mins. Samples were centrifuged in a bench centrifuge at 2,500 rpm for 3.5 mins to remove the precipitated protein. The fluorescence of the supernatant was measured on a Perkin–Elmer LS–5 luminescence spectrometer with three excitation/emission wavelength pairs set at 400 nm / 595 nm, 405 nm / 595 nm and 410 nm / 605 nm to obtain approximate concentrations of uroporphyrin,
coproporphyrin and protoporphyrin. A 100 nm coproporphyrin standard was used along side the samples to be measured. The concentration of each sample was calculated using a computer program (run on an Apple microcomputer) calibrated with different concentrations of the three porphyrins present together in different proportions. The same method was followed to determine total porphyrins and porphyrin profile in the chick embryo hepatocytes experiments.

In some experiments the method described by Abbritti and De Matteis (1971) was used. Porphyrins were extracted from liver homogenate into methanolic perchloric acid, as described above. Supernatant (1 ml) was transferred to a cuvette and its fluorescence was recorded in a Perkin-Elmer fluorescence spectrophotometer. Following the addition of 10 μl of 10 μM protoporphyrin, the fluorescence was again recorded and the protoporphyrin concentration was calculated as follows:

\[
\frac{(100^*)}{(F_{\text{stand}} - F_{\text{samp}})} \times F_{\text{samp}} \times 10.5 \times 2 \times 10 = \text{pmol/g liver}
\]

*100 pmol of protoporphyrin standard used in the assay.

F_{\text{stand}} = 10 \mu l of 10 \mu M protoporphyrin added to the sample and fluorescence recorded.
F_{\text{samp}} = fluorescence of 1 ml sample.

2.2.6 Enzymic assays

(a) Preparation of a crude mitochondrial FK

Mitochondria were prepared by a slight modification of the method of Jones and Jones (1969). A 10% (w/v) liver homogenate was centrifuged at 800 g for 10 mins at 4°C. The supernatant was centrifuged at 17,000 g in a
Beckman instrument for 4 mins at 4°C. The mitochondrial pellet was then resuspended in 0.25 M sucrose and centrifuged at 17,000 g for 4 mins. Finally, the pellet was resuspended in 0.25 mM sucrose and kept at -70°C until further use.

(b) Measurement of mitochondrial FK activity

The method of De Matteis and Gibbs (1972) was followed. The following reagents were placed in a 1 cm cuvette:

0.3 ml (5–8 mg protein) of mitochondrial preparation
1.1 ml 0.5 M Tris–HCl pH 8.2
1.2 ml deionized water
100 μl 1 mM Mesoporphyrin IX containing 1% (v/v) Tween 80

The above mixture was allowed to incubate for 10 mins at 30°C in a waterbath before running a baseline. 2 mM Cobalt chloride (CoCl₂·6H₂O, 50 μl) was added and the absorbance difference between 498 nm and 511 nm wavelength determined. The rate of formation of cobalt–mesoporphyrin was determined in a dual wavelength spectrophotometer.

(c) Determination of FK activity using total liver homogenate

FK was also measured in total homogenate by the method of Cole et al. (1981) as follows:

A 2% (w/v) homogenate (0.02 M Tris buffer pH 8.2) was prepared using an ultra–turrax polytron. Thunberg tubes were lightly greased at the joints and kept on ice. The following reagents were placed into the tube; mesoporphyrin (1 mM, 120 μl), Tween 80 (1% (v/v) solution, 0.3 ml), ethanol (0.3 ml), dithioerythritol (0.2 M, 60 μl), and assay buffer (0.2 M Tris buffer pH 8.2, 1.5 ml).
The tubes were flushed with nitrogen so as to carry out the reaction under anaerobic conditions. All tubes were pre-incubated for 5 min in a 37°C waterbath. The reaction was started by the addition of the enzyme into the main compartment of the tube. Termination of the reaction was by the addition of 0.4 M iodoacetamide (0.5 ml) and by placing tubes in ice-water. Pyridine (1.5 ml) and 0.5 N sodium hydroxide (1.16 ml) were then added to the incubate (3 ml) before dividing it equally between two cuvettes. To the reference cell a 0.1% (w/v) solution of potassium ferricyanide (50 μl) was added and the cell left unstoppered. Meanwhile to the sample cell sodium dithionite was added and the cell stoppered. Both cuvettes were scanned in a spectrophotometer between wavelengths 600 nm and 500 nm.

(d) Measurement of cytochrome P450

Cytochrome P450 was measured in microsomes obtained by a modification of the method of Kamath and Rubin (1972). Between 10 ml and 15 ml of a 20% (w/v) liver homogenate was centrifuged at 10,000 rpm in a MSE HS18 for 20 min. 30 ml of 12.5 mM sucrose containing 8 mM calcium chloride (CaCl₂) was added to 6 ml of the supernatant and the mixture centrifuged at 2,500 rpm in 6L MSE for 10 min. The microsomal pellet was resuspended and washed with 20 ml of 12.5 mM sucrose containing 8 mM CaCl₂. The pellet was finally resuspended in 15 ml 0.1 M phosphate buffer pH 7.4 containing 1 mM ethylenediamine tetraacetic acid (EDTA).

Cytochrome P450 content was determined by the method of Omura and Sato (1964). Carbon monoxide was bubbled through the microsomes for approximately 30 seconds and the sample cuvette reduced by adding a few grains of sodium dithionite. Difference spectra were recorded between 400 nm
and 500 nm using a Cary 2200 spectrophotometer, and the concentration of cytochrome P450 calculated from the optical density, $\epsilon_{450-490}$, using a millimolar extinction coefficient of 91 cm$^{-1}$.

2.2.7 Additional methods

(a) Synthesis of N-methyl protoporphyrin dimethylester

The synthesis of N-MePP was a modified method of De Matteis et al. (1980c). Protoporphyrin dimethylester (2 mg) was dissolved in 1 ml methyl iodide and heated at 100°C for 3 hrs in a test tube with a polytetrafluoroethylene lined screw cap. The N-MePP dimethylester ($R_f$ value 0.5) was purified on TLC with a developing system of chloroform:methanol 20:3 (v/v) and identified by its electronic absorption spectrum.

The free acid was prepared by hydrolysing the dimethylester. The porphyrin was dissolved in 0.1 ml 6 N HCl and left for 3 hrs in the dark at room temperature. The free acid was dried in a dessicator over potassium hydroxide under vacuum overnight.

(b) Protein estimation

Proteins were determined using the method of Lowry et al. (1951). A standard curve was prepared using Bovine serum albumin and the protein content of each sample was measured alongside 5 concentrations of standard.

(c) Statistics

The probability, $P$, of the significance of the difference between the control and treated group was determined using the student's t-test. Where $P < 0.05$ was seen the two groups were considered to be significantly different.
Chapter 3

ATMP-INDUCED PROTOPORPHYRIA: A NOVEL PORPHYROGENIC AGENT
Introduction

ATMP is a synthetic chemical which possess herbicide activity. During routine toxicity testing ATMP was shown to cause porphyrin accumulation and cholestasis in the liver of mice. The work presented here examines the biochemical changes observed in the liver of mice given ATMP.

Little is known regarding the potential toxicity to man of ATMP, hence particular care was taken when handling the chemical at all times.

The aim of these studies was to determine the following:

(i) Does ATMP induce hepatic porphyria?
(ii) If so, what is the major porphyrin that accumulates in the liver?
(iii) Does ATMP behave in a dose- and time-dependent manner?
(iv) Is FK inhibited in ATMP-treated mice in vivo?
(v) Can the presence of a green pigment be demonstrated in ATMP-treated mouse liver and can this be shown to inhibit FK in vitro?
(vi) Does chemical modification, to the propyl or oxazole derivative, impair the porphyrogenic activity of ATMP in mouse liver?
(vii) Is there a species difference in response to ATMP treatment?
(viii) Can ATMP-induced porphyria be modified by induction or inhibition of the cytochrome P450 system?

Several parameters, total liver porphyrins and porphyrin profile, FK activity and cytochrome P450 content were measured in this study. The biochemical
changes produced by ATMP were compared with the mechanism of action of DDC and griseofulvin, both of which cause hepatic porphyria and cholestasis.

3.1 Biochemical features of ATMP–induced hepatic protoporphyría

The effects of increasing amounts of ATMP on hepatic porphyrin levels is shown in Fig 3.1. With the minimum dose given, 20 mg/kg, a 4–fold increase was seen 24 hrs after dosing; whereas, at the top dose of 300 mg/kg, porphyrin levels were increased 200 times above the control value. The major porphyrin was protoporphyrin (Table 3.1). When the time course of porphyrin accumulation was studied, after a single dose of 300 mg/kg ATMP, a significant rise corresponding to a 10–fold increase, in protoporphyrin levels was already demonstrable in mouse liver as early as 4 hrs after treatment (Table 3.2).

ATMP caused a loss in FK activity and this effect was also found to be dose–dependent although not as clearly as for the accumulation of liver porphyrins (Fig 3.2). The low dose of 20mg/kg produced a marked inhibition of the enzyme which fell to 40% of control value. At the top dose of 300 mg/kg, ATMP inhibited FK activity to approximately 30% of control value.

When FK activity was measured in liver homogenate at different times after dosing, a significant inhibition was seen after 2 hrs (Table 3.3). In a separate study, 150 mg/kg ATMP produced a less marked inhibition of FK activity than 300 mg/kg ATMP 4 hrs after dosing (Table 3.4).
Fig 3.1 The effect of increasing dose of ATMP on the liver porphyrin concentration in mice
Fig 3.2 The effect of increasing dose of ATMP on mitochondrial FK activity in mice

![Graph showing the effect of ATMP dose on mitochondrial FK activity.](image)

- Y-axis: Mitochondrial FK (nmol mesohaem/min/mg protein)
- X-axis: Dose of ATMP or vehicle (mg/kg or ml/kg)
Table 3.1 The major porphyrin present at several dose of ATMP in mouse liver

<table>
<thead>
<tr>
<th>Treatment and dose (mg/kg or ml/kg)</th>
<th>Percentage of total porphyrin present as protoporphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachis oil 5</td>
<td>64</td>
</tr>
<tr>
<td>ATMP 20</td>
<td>87</td>
</tr>
<tr>
<td>ATMP 75</td>
<td>97</td>
</tr>
<tr>
<td>ATMP 150</td>
<td>99</td>
</tr>
<tr>
<td>ATMP 300</td>
<td>99</td>
</tr>
</tbody>
</table>

A single i.p injection of either arachis oil or ATMP (dissolved in arachis oil), at the doses indicated above, was given to mice and animals sacrificed 24 hrs later.
Table 3.2 Determination of porphyrin levels in mouse liver at different times after a single dose of ATMP

<table>
<thead>
<tr>
<th>Time of killing after ATMP (hrs)</th>
<th>Total porphyrins (nmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>0.5</td>
<td>1.49 ± 0.85</td>
</tr>
<tr>
<td>1.0</td>
<td>1.58 ± 0.38</td>
</tr>
<tr>
<td>4.0</td>
<td>1.14 ± 0.18</td>
</tr>
<tr>
<td>24.0</td>
<td>1.46 ± 0.63</td>
</tr>
</tbody>
</table>

A single i.p. injection of either arachis oil or ATMP (300 mg/kg) was given to mice and animals sacrificed at different times after dosing (as indicated in the table above).

Data represents the mean ± s.d.

Each value obtained from at least 3 observations.
Table 3.3 Effect of ATMP on FK activity at different times after dosing

<table>
<thead>
<tr>
<th>Times of killing After ATMP (hrs)</th>
<th>FK activity in liver homogenate (μmol Mesohaem/hr/g liver)</th>
<th>Enzyme activity as a percentage control value</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>ATMP</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>4.8 (4.8,4.8)</td>
<td>4.8 (4.8, 4.7)</td>
</tr>
<tr>
<td>1</td>
<td>4.05 ± 0.41</td>
<td>*3.25 ± 0.67</td>
</tr>
<tr>
<td>2</td>
<td>5.83 ± 1.9</td>
<td>*3.07 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>6.1 ± 0.7</td>
<td>**2.63 ± 0.43</td>
</tr>
<tr>
<td>24</td>
<td>4.83 ± 0.41</td>
<td>1.46 ± 0.1</td>
</tr>
</tbody>
</table>

*Not statistically different from corresponding control group at p < 0.05 level.
**Statistically different from corresponding control group at P < 0.05 level.

Mice were given a single i.p. injection of either arachis oil or ATMP (300 mg/kg) and animals sacrificed at different times after dosing (as indicated in the table above).

Data represents mean ± s.d.

Each value represents at least 3 observations. Where the value is the mean of 2 observations individual values are indicated in parenthesis.
Table 3.4 Changes observed in FK activity 4 hrs after dosing with 150 mg/kg and 300 mg/kg ATMP

<table>
<thead>
<tr>
<th>Time of killing after ATMP (hrs)</th>
<th>FK activity in liver homogenate (μmol Mesohaem/hr/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control ATMP</td>
</tr>
<tr>
<td></td>
<td>150 mg/kg 300 mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>6.45 ± 0.5 3.65 ± 0.6 2.61 ± 0.2 (57) (40)</td>
</tr>
</tbody>
</table>

Mice were given a single i.p. injection of either arachis oil or ATMP, dissolved in arachis oil, at the doses specified in the table above.

Each value obtained from 3 observations.

Data represents mean ± s.d.

Percentage of control value is shown in parenthesis.
3.2 Isolation of an ATMP–induced green pigment

The findings of increased protoporphyrin concentration and inhibition of FK, in the liver of ATMP–treated mice, are characteristic biochemical features of drug–induced protoporphyria. Drugs with a similar mechanism of action include griseofulvin and DDC. With these latter two drugs it has been reported that N–MePP, an inhibitor of FK, accumulates in the liver, thus providing an explanation for the inhibition of FK, and the accumulation of protoporphyrin seen with these drugs. N–MePP has been shown to arise from the alkylation of the haem moiety of cytochrome P450, as a result of the metabolic transfer of the 4–methyl group of DDC to one pyrrolic nitrogen of the haem, in a suicidal type of inactivation reaction (De Matteis et al. 1981; Tephly et al. 1981; Ortiz De Montellano et al. 1981a). Several experiments, were therefore carried out to investigate whether a FK inhibitory pigment (N–alkyl protoporphyrin) was present in the liver of ATMP–treated mice.

Loss of cytochrome P450 content was not demonstrable in mice treated with ATMP, whether the cytochrome was measured after different doses of ATMP (Table 3.5) or at different times following a single dose (Table 3.6).
Table 3.5 A dose response study examining the effect of ATMP on Cytochrome P450 content 24 hrs after treatment

<table>
<thead>
<tr>
<th>Treatment and dose (mg/kg and ml/kg)</th>
<th>Total liver g/100g body weight</th>
<th>Cytochrome P450 Content (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachis Oil 5</td>
<td>3.3 (3.4, 3.2)</td>
<td>1.29 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.4 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>3.6 ± 0.08</td>
</tr>
<tr>
<td>ATMP 150</td>
<td>3.1 ± 0.14</td>
<td>*1.24 ± 0.06</td>
</tr>
<tr>
<td>300</td>
<td>3.2 ± 0.17</td>
<td>*1.29 ± 0.09</td>
</tr>
</tbody>
</table>

*Not statistically different from the control group at p < 0.05 level.

Mice were given a single i.p. injection of either arachis oil or ATMP, dissolved in arachis oil, at the doses specified in the table above.

Data represent mean ± s.d.

Each value was obtained from at least 3 observations.

For each observation 2 mouse livers were used.
Table 3.6 Measurement of cytochrome P450 content in mice sacrificed at different times following a single dose of ATMP

<table>
<thead>
<tr>
<th>Time of killing After ATMP (hrs)</th>
<th>Cytochrome P450 Content (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>1.32 ± 0.25</td>
</tr>
<tr>
<td>4</td>
<td>1.53 ± 0.19</td>
</tr>
<tr>
<td>**24</td>
<td>1.29 ± 0.09</td>
</tr>
</tbody>
</table>

*Not statistically different from corresponding control group at p < 0.05 level.

Mice were given a single i.p. injection of either arachis oil or ATMP (300 mg/kg), dissolved in arachis oil and animals sacrificed 24 hrs later.

Each value was obtained from at least 3 observations.

Data presented as mean ± s.d.

Each observation obtained from 2 mouse livers pooled.

**Taken from table 3.5.
3.2.1 Characterisation of the ATMP-induced green pigment

As described previously, ATMP causes the formation and accumulation of an inhibitory pigment. The absorption spectrum and chromatographic properties of the pigment will now be considered.

A crude green pigment extract, from the liver, was applied to a Sephadex LH column. Fractions (3 ml) containing haem-free green pigment were collected off the Sephadex LH20 then scanned between 460 nm and 360 nm. The green pigment (scanned in acidified acetone) had a Soret band between 410 nm and 420 nm. Twenty-four hours after a single dose of ATMP (300 mg/kg), the amount of this green pigment, extracted from the liver and collected from the LH20 column, was 4.95 ± 2.1 nmol/g wet liver (mean ± s.d. of 3 observations each obtained from the livers of 4 mice). The pigment was further purified on TLC using a developing system chloroform:methanol:acetic acid 40:10:3 (v/v/v). The green pigment, having a mean R_f value of 0.67 ± 0.07 (n=6) could be separated from haem and protoporphyrin both of which had R_f values of 0.72 ± 0.01 and 0.76 ± 0.04, respectively. The electronic absorption spectrum of the green pigment was measured, after converting the free acid to its dimethylester and exhibited a bathochromic shift of all absorption maxima, when compared with those of protoporphyrin (Table 3.7). The absorption maxima shown by the ATMP green pigment were very similar to those of authentic N-MePP.

The green pigment was separated into 2 fractions (F1 and F2) by isocratic HPLC elution, using dichloromethane:methanol:ammonia (sp.gr. 0.88) 50:50:0.1 (v/v/v). These had a retention time of 3.72 mins and 6.11 mins, respectively,
Table 3.7 Comparison of ATMP green pigment electronic absorption spectrum with N-MePP and protoporphyrin standards

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td>407</td>
</tr>
<tr>
<td>N-MePP</td>
<td>418</td>
</tr>
<tr>
<td>ATMP green pigment</td>
<td>420</td>
</tr>
</tbody>
</table>

Each porphyrin present as the dimethylester was exposed to ammonia fumes before dissolving in chloroform.
Fig 3.3 HPLC analysis on Nucleosil 5 of (a) green pigment isolated from the liver of ATMP-treated mice (b) N–MePP standard.

A Nucleosil 5 silica column eluted with dichloromethane:methanol:ammonia (sp.gr. 0.88) 50:50:0.1 (v/v/v) was used for the analysis of the dimethylesters of (a) ATMP–green pigments, (b) authentic N–MePP. The majority of ATMP green pigment is present in $F_1$ ($N_A$ and $N_B$).
The ATMP-derived green porphyrin was shown to be markedly inhibitory towards FK in vitro, a 10 nM final concentration caused a 20% inhibition in enzyme activity and a top dose of 100 nM final concentration showed over 70% loss in enzyme activity (Fig 3.4). N-MePP standard showed a similar profile when added to FK (Fig 3.4).

### 3.3 Structural analogues of ATMP

A study of certain analogues of ATMP was carried out to examine which part of the molecule was required to produce the porphyrogenicity. Two analogues of ATMP, the propyl and oxazole derivative illustrated in Figure 3.5 show modification at the oxime end of the molecule.

The aim was to understand further the behaviour of ATMP by asking the following question: is the oxime part of the molecule essential for metabolic activation leading to protoporphyria?

Tables 3.8 and 3.9 show that both ATMP analogues are inactive, over the dose range 20–300 mg/kg studied. This range caused no porphyrin accumulation. At the top dose of 300 mg/kg mitochondrial FK activity was not affected by treatment with either of the analogues (Tables 3.8 and 3.9). Although no loss in FK was detected it was still possible that a green pigment could be formed. Extracted samples from mouse liver, previously treated with propyl–ATMP) were collected off Sephadex LH20 column and scanned to obtain an absorption spectrum, but no N-alkyl porphyrin could be detected.
Fig 3.4 Comparison between ATMP-green pigment and N-MePP standard on FK activity measured in vitro

Key:
- ■ N-MePP standard
- ● ATMP-green pigment

FK activity (% of control)

Inhibitor concentrations (nM)
Fig 3.5 Chemical structures of (a) ATMP (b) Propyl-ATMP and (c) oxazole derivative of ATMP

Radiolabelled ATMP contained $^{14}$C in the side chain COCH$_3$ as shown by the asterisk.
Table 3.8 Measurement of hepatic porphyrins and mitochondrial FK in mice given propyl-ATMP or arachis oil 24 hrs after treatment

<table>
<thead>
<tr>
<th>Treatment and Dose (mg/kg or ml/kg)</th>
<th>Total Porphyrins (nmol/g wet liver)</th>
<th>Mitochondrial FK (nmol Mesohaem/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachis oil</td>
<td>5</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>Propyl-ATMP 20</td>
<td>1.09 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>75</td>
<td>1.14 ± 0.05</td>
<td>-</td>
</tr>
<tr>
<td>150</td>
<td>1.15 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>300</td>
<td>*1.42 ± 0.5</td>
<td>*0.70 ± 0.19</td>
</tr>
</tbody>
</table>

*Not statistically different from corresponding control value at p < 0.05 level.

Mice were given an i.p. injection of arachis oil, ATMP or the propyl-ATMP (Propyl-ATMP was dissolved in arachis oil)

Data represent mean ± s.d.

Each value was obtained from at least 3 observations.
Table 3.9 Measurement of hepatic porphyrins and mitochondrial FK in mice given oxazole derivative of ATMP or arachis oil 24 hrs after treatment

<table>
<thead>
<tr>
<th>Treatment and dose (mg/kg or ml/kg)</th>
<th>Total porphyrins (nmol/g wet liver)</th>
<th>Mitochondrial FK (nmol Mesohaem/ min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachis Oil 5</td>
<td>0.76 ± 0.06</td>
<td>1.2 ± 0.14</td>
</tr>
<tr>
<td>Oxazole derivative of ATMP 20</td>
<td>0.41 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.38 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0.78 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>*0.90 ± 0.14</td>
<td>*0.94 ± 0.04</td>
</tr>
</tbody>
</table>

*Not statistically different from corresponding control value at p < 0.05 level.

Mice were given an i.p. injection of arachis oil, or the oxazole analogue of ATMP (the analogue was dissolved in arachis oil).

Data represent mean ± s.d.

Each value was obtained from at least 3 observations.
3.4 Species differences in response to ATMP

In the experiments described above, ATMP has been shown to be porphyrogenic in the mouse. Further studies of this chemical have been carried out to investigate its potency in other species including chick embryo, rat, hamster and guinea-pig.

3.4.1 Chick embryo hepatocytes in vitro

Granick (1966) demonstrated that chick embryo hepatocytes in vitro, when exposed to certain chemicals such as AIA, resulted in an accumulation of porphyrins several hours after exposure to the drug. Determining the pattern of porphyrin accumulation in chick embryo liver cells would prove a useful tool in elucidating the mechanism of action of ATMP. Also, in vitro the conditions are well defined with no physiological influence being exerted from outside the cell. ATMP was found to be unresponsive in chick embryo hepatocytes (Table 3.10). DDC which causes a marked accumulation of protoporphyrin in chick embryo was used as a positive control. Each chemical was dissolved in dimethyl sulphoxide (DMSO) and 2.5 μl aliquots administered to the cell.

3.4.2 Chick Embryo in ovo

17 day old chick embryos were also dosed in ovo with a low and a high dose of ATMP and livers were examined 24 hrs later for porphyrin accumulation. ATMP at a concentration 10 times higher than that used for DDC, produced a very weak response (Table 3.11). DDC was used as a positive control. In both systems, in vivo and in vitro, ATMP was shown to be inactive.
Table 3.10 Changes observed in porphyrin levels in chick embryo hepatocytes given ATMP or DDC 24 hrs after exposure

<table>
<thead>
<tr>
<th>Treatment and dose (μg/well or μl/well)</th>
<th>Porphyrins (pmol/mg protein)</th>
<th>Increase as compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Cells</td>
<td>39</td>
<td>/</td>
</tr>
<tr>
<td>DMSO alone 2.5</td>
<td>45</td>
<td>/</td>
</tr>
<tr>
<td>ATMP 100</td>
<td>114</td>
<td>2.5</td>
</tr>
<tr>
<td>40</td>
<td>43</td>
<td>N.C.</td>
</tr>
<tr>
<td>20</td>
<td>47</td>
<td>N.C.</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>N.C.</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>N.C.</td>
</tr>
<tr>
<td>DDC 10</td>
<td>280</td>
<td>6</td>
</tr>
</tbody>
</table>

ATMP and DDC were dissolved in DMSO.

Mean of 2 observations for each value.

Control is DMSO treated cells.

N.C. is No change.
3.4.3 Comparison of mouse with rat, hamster and guinea-pig models

ATMP was also given to rats, hamster and guinea-pigs to investigate the effect of the drug on the liver porphyrin concentrations and FK activity in these species. Rat, hamster and guinea-pig were unresponsive, with porphyrin levels remaining the same as control (Table 3.12). FK activity showed a certain degree of inhibition (Table 3.13) in all species. However, in the mouse FK was reduced to 27% of control whereas in the three other species FK activity remained between 60% and 80% of control value.
Table 3.11 Effect of dosing chick embryo in ovo with either ATMP or DDC and measurement of hepatic porphyrin levels 24 hrs later

<table>
<thead>
<tr>
<th>Treatment and Dose</th>
<th>Total Porphyrins (nmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO alone</td>
<td>0.99 ± 0.3</td>
</tr>
<tr>
<td>DDC 0.3 mg/egg</td>
<td>53.7 (79.4, 27.9)</td>
</tr>
<tr>
<td>ATMP 0.3 mg/egg</td>
<td>0.99 ± 0.46</td>
</tr>
<tr>
<td>ATMP 6 mg/egg</td>
<td>1.30 ± 0.12</td>
</tr>
</tbody>
</table>

Data presented as mean ± s.d.
Each value was obtained from 3 or more observations.

* Mean of 2 observations.
** Not statistically different from control at p < 0.05 level.
Table 3.12  Changes in porphyrin levels from mouse, rat, hamster and guinea-pig 24 hrs after dosing

<table>
<thead>
<tr>
<th>Species</th>
<th>Total porphyrins (nmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td>Rat</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>Hamster</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>0.91 ± 0.2</td>
</tr>
</tbody>
</table>

All animals were given a single i.p. injection of arachis oil or ATMP (300 mg/kg), dissolved in arachis oil.

Results are presented as mean ± s.d.

Each value is obtained from 3 or more observations.

*Not statistically different from the corresponding control at p < 0.05 level.
Table 3.13 Changes observed in mitochondrial FK from mouse, rat, hamster and guinea-pig 24 hrs after dosing

<table>
<thead>
<tr>
<th>Species</th>
<th>FK activity (nmol Mesohaem/min/mg protein)</th>
<th>FK Activity expressed as a percentage of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ATMP-treated</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.2 ± 0.14</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Rat</td>
<td>1.4 ± 0.16</td>
<td>*1.03 ± 0.07</td>
</tr>
<tr>
<td>Hamster</td>
<td>1.2 ± 0.3</td>
<td>0.97 ± 0.1</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>0.45 ± 0.07</td>
<td>*0.30 ± 0.09</td>
</tr>
</tbody>
</table>

All animals were given a single i.p. injection of arachis oil or ATMP (300 mg/kg), dissolved in arachis oil.

Data presented as mean ± s.d.

Each value obtained from 3 or more observations.

*Not statistically different from corresponding control at p < 0.05 level.
3.5 Cytochrome P450 inhibitors on ATMP–induced protoporphyria

With the use of cytochrome P450 inhibitors it may be possible to modify cytochrome P450 and thus influence ATMP metabolism. There are two ways in which an alteration in the metabolism of ATMP could affect the induction of porphyria. One is by increasing the production of the postulated reactive metabolite responsible for the formation of N–MePP; the other is by channelling ATMP into an alternative pathway of metabolism, so that less is available for formation of N–MePP. The main questions raised were:

(i) Can treatment with cytochrome P450 inhibitors protect mice against ATMP–induced protoporphyria?

(ii) Can cytochrome P450 inhibitors prevent the formation of N–alkyl porphyrin?

The work carried out in this section examined the role of the cytochrome P450 inhibitors SKF525–A, piperonyl butoxide, metyrapone and α–naphthoflavone (α–NF) on ATMP–induced porphyria. Compounds SKF525–A and piperonyl butoxide are general inhibitors of the cytochrome P450 system. Metyrapone and α–NF are both differential inhibitors with α–NF inhibiting aromatic hydrocarbon hydroxylase.

(i) Effect of cytochrome P450 inhibitors on porphyrin levels

Saline pretreatment followed by a dose of ATMP at 300 mg/kg resulted in protoporphyrin accumulation with an 11–fold rise above control values. SKF525–A pretreatment by itself showed no significant elevation when compared with the appropriate control (Table 3.14). Piperonyl butoxide
pretreatment likewise showed no porphyrin accumulation. Both inhibitors markedly reduced the increase in liver porphyrin content due to ATMP. By contrast, mice pretreated with metyrapone or α-NF still showed a significant rise in porphyrin levels when challenged with ATMP. In metyrapone pretreated mice porphyrin accumulation was approximately 40 times above control value (Table 3.15).

(ii) Effect of cytochrome P450 inhibitors on FK activity

Mice pretreated with SKF525-A showed no decrease in FK activity after ATMP, compared to 50% decrease in enzyme activity in saline pretreated mice (Table 3.16). Metyrapone pretreatment caused a decrease in FK activity to similar levels seen in mice given saline pretreatment followed by ATMP treatment.

(iii) Effect of cytochrome P450 inhibitors on N-alkyl porphyrin formation

a) Electronic absorption spectra

Spectral analysis was carried out as described in section 2.2.4a. ATMP-treated mice produced a green pigment with a soret at 420 nm (Fig 3.6). The effect of the two inhibitors, SKF525-A and metyrapone on the amount of N-alkyl porphyrin formed after ATMP was investigated by comparing the amount of pigment formed in mice treated with ATMP alone and in the presence of one of these inhibitors. An important finding was that SKF525-A pretreatment, followed by ATMP completely suppressed the production of the N-alkyl porphyrin (Fig 3.7); while metyrapone enhanced the formation of the N-alkyl porphyrin (Fig 3.8).
Table 3.14 Effect of SKF525–A and piperonyl butoxide pretreatment on protoporphyrin accumulation

<table>
<thead>
<tr>
<th>Dose of ATMP or vehicle (mg/kg or ml/kg)</th>
<th>Total Porphyrins (nmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>Arachis oil</td>
<td>0.81 ± 0.3</td>
</tr>
<tr>
<td>ATMP 75</td>
<td>8.4 ± 1.4</td>
</tr>
<tr>
<td>150</td>
<td>14.6 ± 3.1</td>
</tr>
<tr>
<td>300</td>
<td>9.1 ± 4.7</td>
</tr>
</tbody>
</table>

Mice were pretreated with either an i.p. dose of saline (5 ml/kg), SKF525–A (40 mg/kg), corn oil (5 ml/kg) or piperonyl butoxide (300 mg/kg) followed by an i.p. injection of either arachis oil (5ml/kg) or ATMP (at the dose indicated above). Mice were killed 4 hrs after receiving ATMP. Results are mean ± s.d. Each value was obtained from 3 observations.

*Statistically different from corresponding control at p < 0.05 level.
Table 3.15 Effect of inhibiting cytochrome P450 enzyme with α-NF or metyrapone on protoporphyrin accumulation

<table>
<thead>
<tr>
<th>Dose of ATMP or vehicle (mg/kg or ml/kg)</th>
<th>Total Porphyrins (nmol/g wet liver)</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn oil</td>
<td>α-NF</td>
</tr>
<tr>
<td>Arachis oil</td>
<td>1.0 ± 0.2</td>
<td>0.69 ± 0.4</td>
</tr>
<tr>
<td>ATMP 75</td>
<td>10.1 ± 0.4</td>
<td>*13.4 ±10.7</td>
</tr>
<tr>
<td>ATMP 150</td>
<td>17.9 ± 6.6</td>
<td>*15.2 ± 4.8</td>
</tr>
<tr>
<td>ATMP 300</td>
<td>16.7 ± 8.8</td>
<td>/</td>
</tr>
</tbody>
</table>

Mice were pretreated with a dose of either corn oil (5 ml/kg), α-NF (80 mg/kg), saline (5 ml/kg) or metyrapone (50 mg/kg) followed by a single i.p. injection of either arachis oil (5 ml/kg) or ATMP (at the doses indicated above). Mice were sacrificed 4 hrs after receiving ATMP. Results are mean ± s.d. Each value was obtained from 3 observations. *Not statistically different from corresponding control group at p < 0.05 level.
Table 3.16 Biochemical changes in mouse liver in the presence and absence of SKF525-A or metyrapone

<table>
<thead>
<tr>
<th>Pretreatment and treatment (mg/kg or ml/kg)</th>
<th>Total liver porphyrins (nmol/g wet liver)</th>
<th>Mitochondrial FK activity (nmol Mesohaem/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline ATMP</td>
<td>17.5 ± 1.8</td>
<td>0.73 ± 0.15</td>
</tr>
<tr>
<td>SKF525-A ATMP</td>
<td>1.3 ± 0.4</td>
<td>*1.44 ± 0.04</td>
</tr>
<tr>
<td>Saline ATMP</td>
<td>15.4 ± 5.1</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>Metyrapone ATMP</td>
<td>8.7 ± 2.9</td>
<td>0.33 ± 0.05</td>
</tr>
</tbody>
</table>

*Statistically different from the corresponding group not pretreated with an inhibitor at p < 0.05 level.

Mice were pretreated with a dose of saline (5 ml/kg), SKF525-A (40 mg/kg), or metyrapone (50 mg/kg). A single i.p. injection of either arachis oil or ATMP (300 mg/kg) 1 hr later and mice were sacrificed 4 hrs later. Results are mean ± s.d. Each value was obtained from 3 observations.
b) HPLC analysis of \( \text{N-MePP dimethylester} \)

In SKF525–A pretreated mice given ATMP no green pigment was detected on HPLC, although saline pretreated mice given an equivalent dose of the chemical in their liver generated approximately 90 pmol/g wet liver. These results therefore indicate that SKF525–A not only prevents porphyria and the decrease in FK activity caused by ATMP, but it also abolished the production of \( \text{N-MePP} \). The following experiment was carried out to exclude that SKF525–A produces all these effects by preventing ATMP uptake by the liver.

(iv) Effect of SKF525–A on ATMP uptake

SKF525–A prevents the formation of ATMP-derived \( \text{N-MePP} \) as shown in section (iii b) above. The working hypothesis put forward was that SKF525–A blocked the formation of \( \text{N-MePP} \) by preventing the uptake of ATMP into the liver. To test this hypothesis, radiolabelled ATMP was injected into mice and the amount of radiolabelled ATMP in the liver measured. In SKF525–A pretreated mice a higher level of radioactivity was noted in the liver compared with the saline pretreated group and the rate of disappearance of the drug (as measured by loss of radioactivity between a limited number of time points) was not greatly affected (Table 3.17). A summary of the biochemical changes observed in the presence of cytochrome P450 inhibitors is given in figure 3.9.
Fig 3.6 Absorption spectrum of green pigment extracted from ATMP-treated mice

Green pigment in acidified acetone collected off Sephadex LH20 column and scanned between 450 nm and 350 nm. A peak showing at approximately 370 nm indicates the presence of haem.
Fig 3.7 Absorption spectrum of sample extracted from SKF525-A-pretreated mice given ATMP

No green pigment between 410 nm and 420 nm could be detected. A peak seen at approximately 370 nm corresponds to haem.
Fig 3.8 Absorption spectrum of green pigment from metyrapone-pretreated mice given ATMP

Green pigment in acidified acetone is collected off Sephadex LH20 column and scanned between 450 nm and 350 nm. A peak showing at approximately 370 nm indicates the presence of haem.
Table 3.17 Recovery of radioactivity in the liver of mice given \(^{14}\text{C}\)-labelled ATMP after pretreatment with either saline or SKF525–A

<table>
<thead>
<tr>
<th>Period of exposure (hr after ATMP)</th>
<th>Liver weight g/100g body wt</th>
<th>Saline pretreatment</th>
<th>SKF525–A pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Radioactivity (dpmx10(^3))</td>
<td>Recovery in whole liver</td>
</tr>
<tr>
<td>1</td>
<td>4.73 ± 0.3</td>
<td>24.5 ± 5.8</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>4.69 ± 0.2</td>
<td>14.5 ± 2.2</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>4.89 ± 0.11</td>
<td>0.43 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

Mice were pretreated with either saline (5 mg/kg) or SKF525–A (40 mg/kg) then given a single dose i.p. injection of \(^{14}\text{C}\)-labelled ATMP and mice killed at different times after dosing (as indicated above). Results are mean ± s.d. Each value obtained from 3 mice.

*Statistically different as compared with the corresponding control P < 0.05.
Fig 3.9 The effect of cytochrome P450 inhibitors on hepatic porphyrins, FK activity and N-alkyl porphyrin formation in ATMP-treated mice.

<table>
<thead>
<tr>
<th>Cytochrome P450 inhibitor</th>
<th>Porphyrin accumulation</th>
<th>FK inhibition</th>
<th>N-alkyl porphyrin formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SKF525-A</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>α-NF</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: + = An effect of ATMP was observed.

- = The relevant effect of ATMP was diminished or abolished

/ = Not measured
3.6 Cytochrome P450 inducers on ATMP–induced protoporphyrria

In the liver cytochrome P450 enzymes have been shown to play an important role in initiating the formation of ATMP–induced protoporphyrria. Indirect evidence to support this statement is the studies with inhibitors of the drug metabolising system (section 3.5).

The aim of the work in this section, was to investigate what effects cytochrome P450 inducers had upon ATMP metabolism. Three inducers of the cytochrome P450 system, namely, PB, BNF and CFA were studied. The major gene induced by the inducers are PB; P450IIB1 and B2, for BNF; P450IA1 and A2 and for CFA P450IVA1.

The working hypothesis being tested was as follows
(i) does cytochrome P450 induction enhance the porphyrogenic effect of ATMP or
(ii) do cytochrome P450 inducers serve to protect the liver against ATMP–induced protoporphyrria.

PB caused an increase in FK activity as reported previously by Tephly et al. (1971). Mice pretreated with PB showed no elevation in hepatic porphyrins when given ATMP (Table 3.18) even though the inhibition of FK was still demonstrable (Table 3.19). Pretreatment with either BNF or CFA did not abolish the porphyria caused by ATMP (Table 3.18).
Table 3.18 Effect of pretreating mice with PB, BNF (i.p.) or CFA orally for 3 days followed by ATMP 20 mg/kg or 75 mg/kg and 24 hrs later measuring total porphyrin levels

<table>
<thead>
<tr>
<th>Pretreatment, Treatment and Dose (ml/kg or mg/kg)</th>
<th>Total Porphyrins (nmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Arachis Oil</td>
<td>0.45 ± 0.18</td>
</tr>
<tr>
<td>Water ATMP (20)</td>
<td>1.17 ± 0.09</td>
</tr>
<tr>
<td>Water ATMP (75)</td>
<td>28.9 ± 6.7</td>
</tr>
<tr>
<td>PB Arachis Oil</td>
<td>0.79 ± 0.58</td>
</tr>
<tr>
<td>PB ATMP (20)</td>
<td>0.84 ± 0.59</td>
</tr>
<tr>
<td>PB ATMP (75)</td>
<td>*0.6 ± 0.45</td>
</tr>
<tr>
<td>Corn oil Arachis oil</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>Corn oil ATMP (20)</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>Corn oil ATMP (75)</td>
<td>34.6 (17.6, 51.7)</td>
</tr>
<tr>
<td>BNF Arachis oil</td>
<td>2.2 (0.9, 3.5)</td>
</tr>
<tr>
<td>BNF ATMP (20)</td>
<td>4.5 ± 1.7</td>
</tr>
<tr>
<td>BNF ATMP (75)</td>
<td>32.5 ± 7.8</td>
</tr>
<tr>
<td>Saline Arachis oil</td>
<td>0.56 ± 0.1</td>
</tr>
<tr>
<td>Saline ATMP (20)</td>
<td>1.8 ± 1.0</td>
</tr>
<tr>
<td>Saline ATMP (75)</td>
<td>20.7 ± 12</td>
</tr>
<tr>
<td>CFA Arachis oil</td>
<td>0.45 (0.49, 0.4)</td>
</tr>
<tr>
<td>CFA ATMP (20)</td>
<td>2.0 (1.9, 2.0)</td>
</tr>
<tr>
<td>CFA ATMP (75)</td>
<td>14.1 (13.2, 14.9)</td>
</tr>
</tbody>
</table>

Mice were pretreated with either PB (80 mg/kg daily for 3 days), BNF (40 mg/kg daily for 3 days) or CFA (300 mg/kg daily for 3 days) or given the corresponding control vehicle (Water ad libitum, corn oil (5 ml/kg) or saline (5 ml/kg)). A single dose of arachis oil (5 mg/kg) or of ATMP (at the doses specified above) then 24 hrs later mice (3 per group) were sacrificed. Where there is a mean of 2 results the individual values are given in parenthesis.

* Statistically different from corresponding group pretreated with water alone at p < 0.05.
Table 3.19 Changes of FK activity observed in PB-pretreated mice given ATMP in liver homogenate at two different time intervals

<table>
<thead>
<tr>
<th>Time of killing after ATMP (hrs)</th>
<th>Pretreatment</th>
<th>Treatment</th>
<th>FK Activity (μmol Mesohaem/hr/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td></td>
<td>Arachis oil</td>
<td>6.68 ± 0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATMP</td>
<td>2.26 ± 0.18</td>
</tr>
<tr>
<td>4</td>
<td>PB</td>
<td>Arachis oil</td>
<td>7.51 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>ATMP</td>
<td>2.76 ± 0.54</td>
</tr>
<tr>
<td>24</td>
<td>PB</td>
<td>Arachis oil</td>
<td>15.89 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>ATMP</td>
<td>2.64 ± 0.98</td>
</tr>
</tbody>
</table>

Mice were pretreated with PB (05% (w/v)) or given drinking water for 3 days then either a single dose of arachis oil (5 mg/kg) or of ATMP (300 mg/kg) in oil by i.p. injection and were killed 4 hrs or 24 hrs later. Results are mean ± s.d. of 3 or more observations.
In tables 3.20 and 3.21 results are given on both total porphyrins and mitochondrial FK which had been determined in the same mouse. (Tables 3.20 and 3.21 differ from table 3.18 in that a higher dose of ATMP was used and mice killed 4 hrs later). In the PB-pretreated group porphyrins accumulated (but to a much smaller extent than in the absence of PB pretreatment) and at the same time FK was decreased to 34% of control value (Table 3.20). In the BNF-ATMP treated group porphyrin levels were elevated and FK activity was decreased to 33% of control value (Table 3.21). A table summarising the effect of PB, BNF and CFA in ATMP-treated mice is given below (Table 3.22).
Table 3.20 Effect of pretreating mice with PB or distilled water followed by ATMP or arachis oil on hepatic porphyrin levels and mitochondrial FK activity

<table>
<thead>
<tr>
<th>Pretreatment and Treatment (mg/kg or ml/kg)</th>
<th>Total liver porphyrins (mg/kg or ml/kg)</th>
<th>Mitochondrial FK Activity (nmol Mesohaem/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None None /</td>
<td>*1.45 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Water ATMP</td>
<td>20.6 ± 7.6</td>
<td>0.31 ± 0.009</td>
</tr>
<tr>
<td>PB Arachis oil</td>
<td>1.4 (0.9, 1.9)</td>
<td>1.69 (1.8, 1.58)</td>
</tr>
<tr>
<td>PB ATMP</td>
<td>5.2 ± 3.9</td>
<td>0.57 ± 0.15</td>
</tr>
</tbody>
</table>

Mice were pretreated with either PB (0.05% (w/v)) or given drinking water for 3 days then either a single dose of arachis oil (5 mg/kg) or of ATMP (150 mg/kg) in oil by i.p. injection and were killed 4 hrs later. Results are mean ± s.d. of 3 observations.

Mean of 2 results individual values shown in parenthesis.

*Each observation obtained from 5 pooled mouse livers, n=4.
Table 3.21 Effect of pretreating mice with BNF or corn oil followed by ATMP or arachis oil on hepatic porphyrins and mitochondrial FK

<table>
<thead>
<tr>
<th>Pretreatment and Treatment (mg/kg or ml/kg)</th>
<th>Total liver porphyrins (nmol/g wet liver)</th>
<th>Mitochondrial FK Activity (nmol Mesohaem/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNF Arachis oil</td>
<td>1.5 ± 0.12</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>BNF ATMP</td>
<td>*11.0 ± 4</td>
<td>*0.53 ± 0.1</td>
</tr>
<tr>
<td>Corn oil ATMP</td>
<td>8.9 ± 2.8</td>
<td>0.65 ± 0.1</td>
</tr>
</tbody>
</table>

Mice were pretreated with either corn oil or BNF (40 mg/kg, dissolved in corn oil) then dosed with either a single dose of arachis oil (5 mg/kg) or ATMP (150 mg/kg) in oil by i.p. injection and were killed 4 hrs later. Results are mean ± s.d. of 3 observations.

*Not statistically different from ATMP treated mouse group at p < 0.05 level.
Table 3.22 Effect of PB, BNF and CFA on ATMP toxicity in mouse liver

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Porphyrin Accumulation</th>
<th>FK Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PB</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BNF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CFA</td>
<td>+</td>
<td>/</td>
</tr>
</tbody>
</table>

KEY: + = Positive response to ATMP
- = Negative response to ATMP
/ = Not measured
Chapter 4

GRISEOFULVIN
GREEN PIGMENTS
4.1 Introduction

In previous work (Holley 1987) two different green pigments were isolated from the liver of mice given griseofulvin and both of them were partially characterised as N-alkylated protoporphyrins. A minor pigment was shown to inhibit FK and to exhibit chromatographic properties identical to those of synthetic N-MePP. A second green pigment was also isolated in those studies, in quantities approximately 10-times greater than the minor pigment and this green pigment (or major pigment) did not inhibit FK. In the discussion below these two griseofulvin-derived N-alkyl porphyrins will be referred to as the griseofulvin major and minor pigments, respectively.

In this present work both pigments have been isolated and purified in order to identify their structure. This has involved a combination of chromatographic, and spectral techniques as described below.

4.2 Griseofulvin major pigment: chromatographic properties on TLC

Griseofulvin major pigment was developed on a TLC plate in the developing system chloroform:methanol 20:3 (v/v). The band corresponding to the griseofulvin major pigment had an $R_f$ value of $0.83 \pm 0.02$ (n=14) and an intensely red fluorescing band was seen when viewed under a UV-lamp. In contrast, protoporphyrin which had an $R_f$ value of 0.7 showed only a slight and transient fluorescence when viewed under a UV-lamp. The griseofulvin major pigment, on a silica column, did not move when either, hexane alone or chloroform alone, developing systems were used.
4.2.1 Griseofulvin major pigment: electronic absorption spectra

An aliquot (2 μl) of griseofulvin major pigment was dissolved in DMSO:Tris 1M pH 8.2 (10:1 v/v, 2.5 ml) and the sample scanned in the Uvikon 860 between 700 nm and 360 nm. A similar concentration was chosen for N–MePP and protoporphyrin standards. The spectra of all three porphyrins were compared and the bathochromic shifts of the various absorption bands of the major griseofulvin pigment (related to wavelength maxima shown by protoporphyrin were calculated and compared to the bathochromic shifts shown by synthetic N–MePP wavelengths of maximum absorption (Table 4.1).

Griseofulvin major pigment produced a bathochromic shift when compared with its parent porphyrin, protoporphyrin. The bathochromic shifts are in agreement with those reported by De Matteis and Canton! (1979) who studied the spectral characteristics of other N–alkyl porphyrins. Interestingly, N–MePP standard produced a greater Soret shift than did griseofulvin major pigment (Table 4.1).

4.2.2 Griseofulvin major pigment: mass spectrometry studies

FAB–mass spectrometry, in the positive mode, detected a monoprotonated molecular ion of the griseofulvin major pigment. This has been tentatively identified as N–griseofulvin protoporphyrin. The pigment was dissolved in dichloromethane and placed on the probe tip, in the matrix nitrobenzyl alcohol. The pigment was bombarded with high energy Xenon particles as described under special analytical techniques (section 2.2.5). A molecular ion (MH) at m/z 941 was produced (Fig 4.1). The molecular ion (MH) at m/z 941 can be accounted for by the sum of (protoporphyrin dimethylester – 1 pyrrole proton, 589) + (griseofulvin – 1 proton, 351) + (1 additional proton).
Table 4.1 Electronic Absorption Spectrum of Griseofulvin Major Pigment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret</td>
</tr>
<tr>
<td>Griseofulvin major pigment (A)</td>
<td>415</td>
</tr>
<tr>
<td>Protoporphyrin standard (B)</td>
<td>407</td>
</tr>
<tr>
<td>Bathochromic shift (A–B)</td>
<td>8</td>
</tr>
<tr>
<td>N-MePP standard (C)</td>
<td>418</td>
</tr>
<tr>
<td>Difference in spectra (C–B)</td>
<td>11</td>
</tr>
</tbody>
</table>

All porphyrins were read as the dimethylester derivative, dissolved in DMSO:Tris 1 M pH 8.2 10:1 (v/v) and exhibited a typical aetiotype spectrum.
Fig 4.1 Positive ion FAB–mass spectrum of major griseofulvin pigment

Signal Peak at 941 corresponds to the N–griseofulvin protoporphyrin adduct.

The signal peak at 603 is unknown.
The molecular ion (MH)^+ ion at m/z 941, N-griseofulvin protoporphyrin was subjected to tandem mass spectrometry, and the pattern of the daughter ions generated were analysed in a second mass spectrometer. A dealkylation process occurred and two daughter ions were seen: a molecular ion (MH)^+ at m/z 591 corresponding to protoporphyrin dimethylester and a second molecular ion (MH)^+ at m/z 353 representing griseofulvin were detected (Fig 4.2). An additional molecular ion (MH)^+ was detected at m/z 605 which is considered to be the N-MePP.

4.2.3 Major griseofulvin pigment: proton NMR

A proton NMR spectrum of the major griseofulvin pigment gave rise to partial information concerning the pigment's structure. The methene bridges produced a chemical shift of 610 parts per million (ppm) and the vinyl chains produced a chemical shift of 58.5 – 8.0 ppm. The main finding was a chemical shift found at -63.0 ppm indicating the presence of an alkyl group on one of the pyrrole nitrogens.
Parent molecular ion fragments giving rise to the daughter ions at 605, 591 and 353, which corresponds to N-MePP, protoporphyrin and griseofulvin.
4.3 Copper dealkylation process

4.3.1 Spectral analysis

A property of N-alkylated porphyrins is their ability to incorporate copper (or other divalent metal ions) into their molecule (Lavallee 1976; Shears and Hambright 1970). Although copper may be inserted into the protoporphyrin molecule, this occurs at a much reduced rate than with N-alkyl porphyrins since the latter are more basic than their corresponding non-alkylated porphyrin, as will be shown later. The process of copper dealkylation occurs in two steps. Firstly, copper is inserted into the N-alkyl porphyrin resulting in a bathochromic shift, reduction of Soret intensity and the disappearance of band IV. Secondly, the ejection of the N-alkyl group generates the copper–protoporphyrin complex which is shown by the appearance of a new peak at a shorter wavelength. These spectral changes readily take place in chloroform (De Matteis et al. 1985). A comparison between the rates of insertion of copper into the N-griseofulvin protoporphyrin and the standards N-MePP and protoporphyrin was carried out. Also, a comparison was made between the rate of loss of the alkyl substituent from N-griseofulvin protoporphyrin and N-MePP.

Griseofulvin major pigment (1.9 nmol) was exposed to ammonia fumes for 15 mins then dissolved in chloroform (2.5 ml) and scanned, using a pair of quartz cuvettes, in a Varian 2200 spectrophotometer between 700 nm and 360 nm (the absorption maxima are given in Table 4.1). The first cuvette was used as the sample cell. The second cuvette was used as the reference cell and contained chloroform (2.5 ml) and copper acetate dissolved in methanol 0.1% (w/v), (5 μl) but no N-alkyl porphyrin. The reaction was started by adding to the sample cell copper acetate dissolved in methanol (0.1% (w/v), 5 μl) then
scanned between 460 nm and 360 nm repeatedly until the reaction was complete.

The N-MePP dimethylester standard containing the unresolved isomers was treated in a similar manner to N-griseofulvin protoporphyrin. Copper acetate dissolved in methanol (0.1% (w/v), 5 μl) was added to a cuvette cell containing N-MePP standard dissolved in chloroform (2.5 ml). The reaction was followed using repeated scans. Once the reaction was completed a scan of the reaction product (copper–protoporphyrin complex) was made (Fig 4.3).

Protoporphyrin dimethylester standard was treated as described above, for the griseofulvin major pigment. The protoporphyrin and copper reaction was monitored using repeated scans, however, the reaction proceeded at a very slow rate. A sample containing protoporphyrin and a saturated solution (5 μl) of copper acetate (dissolved in methanol) was left standing for 3 days at room temperature which led to complete insertion of copper into protoporphyrin. The absorption spectra for final products obtained by copper insertion from griseofulvin major pigment, N-MePP and protoporphyrin were compared with each other and found to be identical in spectral properties (Table 4.2).

The final product of copper incorporation into griseofulvin major pigment, and N-MePP were pink when dissolved in chloroform. Each sample was then developed on a TLC plate in the developing system chloroform:acetone 6:1 (v/v). A non–fluorescent spot was detected with identical $R_f$ values (approximately 0.7) as the synthetic copper–protoporphyrin.
Fig 4.3 Spectral analysis following the addition of copper acetate to authentic N-MePP, giving rise to copper–protoporphyrin complex
Table 4.2 Spectral analysis of copper chelate complex of major griseofulvin pigment, N-MePP and protoporphyrin standards

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Absorption maxima (nm)</th>
<th>Copper-porphyrin complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret</td>
<td>α</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>standard</td>
<td>(i) 406</td>
<td>532</td>
</tr>
<tr>
<td></td>
<td>(ii) 406</td>
<td>533.5</td>
</tr>
<tr>
<td>N-MePP standard</td>
<td>(i) 407</td>
<td>532</td>
</tr>
<tr>
<td></td>
<td>(ii) 405.5</td>
<td>533</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>major pigment</td>
<td>(i) 405</td>
<td>532</td>
</tr>
<tr>
<td></td>
<td>(ii) 406</td>
<td>533</td>
</tr>
</tbody>
</table>

Spectral reading obtained from 2 observations.

All porphyrins present as the dimethylester and dissolved in chloroform (2.5 ml); N-MePP and major griseofulvin pigment present as the unresolved isomers.
The results strongly suggest, that on copper-catalysed dealkylation of N-MePP and major griseofulvin pigment the copper complex of protoporphyrin is formed indicating that protoporphyrin is the porphyrin moiety of the griseofulvin major pigment. The rate of insertion of copper (divalent ions) into the porphyrin, occurs in the order:

**Major griseofulvin pigment > N-MePP > protoporphyrin**

**4.3.2 Mass spectrometry analysis**

It has been also shown by previous workers (Lavallee 1976) that when the reaction of copper incorporation is carried out in acetonitrile, incorporation of copper into the N-alkyl porphyrin takes place, but subsequent dealkylation only follows if a suitable acceptor molecule (i.e. an amine) is also added. This provides a convenient approach for monitoring the copper-catalysed dealkylation reaction by mass spectrometry, as both the copper chelate of the porphyrin and the amine alkyl adduct should be detectable. A technique has been developed for the identification of the N-alkyl group of a N-alkylated porphyrin which is based on this experimental approach (Gibbs *et al.*, 1990), as shown below by the use of N-MePP. Once copper has been incorporated into a N-alkyl porphyrin, dealkylation of the copper complex takes place, provided that a suitable molecule, like an amine is also present (for example di-n-butylamine).

Synthetic N-MePP (10 nmol) dissolved in acetonitrile (25 μl) and copper acetate in methanol (0.1% (w/v), 5 μl) were mixed with conc. di-n-butylamine \([\text{Bu}_2\text{NH} + 1 \text{ proton}]^+ (2 \mu l)\) in an eppendorf tube and left at room temperature for 10 mins. The sample mixture was applied to the probe tip of the mass
spectrometer using nitrobenzyl alcohol as the matrix. A molecular ion (MH)$^+$ at m/z 666 indicating the copper complex of N-MePP and a molecular ion at m/z 651 corresponding to dealkylated porphyrin, that is, copper–protoporphyrin were both detected (Fig 4.4a, b). It was also possible to detect two molecular ions at m/z 130 and 144 which corresponded to di-n-butylamine and methylated di-n-butylamine. A second amine dodecylamine was used to detect the N–methyl group. Dodecylamine was chosen as the nucleophilic amine because dodecylamine and its alkylated derivative both produced a strong signal peak when observed in FAB–mass spectrometry (Naylor et al. 1986).

In an attempt to trap the N–methyl group from the N–alkylated porphyrin, synthetic N-MePP (10 nM) was dissolved in acetonitrile (25 μl) in the presence of dodecylamine (porphyrin : amine, 1:5 v/v) with copper acetate and heated at 50°C for 10 mins. The molecular ions (MH)$^+$ at m/z 186 and 200 corresponding to dodecylamine:

$$[\text{CH}_3(\text{CH}_2)_{11}\text{NH}_2 + 1 \text{ proton}]$$

and methylated dodecylamine:

$$[\text{CH}_3(\text{CH}_2)_{11}\text{NHCH}_3 + 1 \text{ proton}]$$

were detected.
Fig 4.4a An early scan in FAB mass–spectrometry monitoring the insertion of copper into authentic N–MePP and the formation of the dealkylated porphyrin.

The signal peak at 666 corresponds to N–MePP–copper complex and the signal at 651 corresponds to the dealkylated porphyrin (see results for details).
Fig 4.4b A late scan in FAB mass-spectrometry monitoring the insertion of copper into authentic N-MePP and the formation of the dealkylated porphyrin.

Note that with time the amount of N-MePP–copper complex (molecular ion at m/z 666) present diminishes as it is converted to its dealkylated derivative (molecular ion at m/z 651–652).
The process of copper dealkylation involves copper binding to three pyrrole nitrogens of the N-MePP. At this stage the N-methyl substituent is still bound to the porphyrin structure. Finally in the presence of a suitable amine acceptor for example di-n-butylamine or dodecylamine, the N-methyl group is ejected from the porphyrin and binds to the amine molecule (Fig 4.5). This approach proved to be useful in confirming the identity of the minor griseofulvin pigment as will be discussed below in section 4.4.1. Attempts were also made to trap the griseofulvin released from N-griseofulvin protoporphyrin, but this proved unsuccessful.

4.4 Minor griseofulvin pigment

The final method used in the extraction of the griseofulvin minor pigment was aimed at eliminating the fatty impurities normally seen on TLC. The handling of minor griseofulvin pigment offered some technical problems since the yield of this pigment was small. In total approximately 50 mouse livers were required in order to purify sufficient minor griseofulvin pigment for mass spectrometry studies. During the extraction and purification procedure approximately 80% of the original minor griseofulvin pigment was lost. When griseofulvin-treated mouse livers were spiked with synthetic N-MePP the amount recovered, of the spiked standard, was 20% of the starting material. In the case of the minor pigment it is thought that a low recovery was due in part to endogenous substances such as proteins, or lipids tightly binding to griseofulvin minor pigment thus causing substantial loss during the isolation procedure. Also loss of green pigment occurred due to TLC metal chelation and at the HPLC stage. The amount of minor griseofulvin pigment, purified by HPLC from mouse
Fig 4.5 Schematic diagram showing the insertion of copper into an N-alkylated porphyrin and the N-alkyl substituent released becomes bound to the amine acceptor

Me = —CH₃; Vi = —CH═CH₂; PrMe = —(CH₂)CO₂CH₃; R = alkyl substituent; Nu = amine acceptor (e.g. di-n-butylamine or dodecylamine)
The minor griseofulvin pigment was isolated and purified from mouse livers – see methods for details. The minor griseofulvin pigment has a molecular ion at m/z identical to that of authentic N–MePP.
Fig 4.7 FAB mass–spectrum of authentic N–MePP

N–MePP standard present as the unresolved isomers.
Parent molecular ion at m/z 605 fragments, giving rise to daughter ions at m/z 590 and 518. Both of these latter ions correspond to protoporphyrin and protoporphyrin minus a methyl ester side chain.

N-MePP produced an identical spectrum.
livers was approximately 1 nmol/wet g liver. A clean sample was finally obtained ready to be analysed by mass spectrometry.

Using positive mode FAB–mass spectrometry a monoprotonated molecular ion (MH)_+ was obtained for minor griseofulvin pigment. A peak signal at m/z 605 was observed corresponding to (N–MePP dimethylester + 1 additional proton) (Fig 4.6). Synthetic N–MePP (Fig 4.7) and protoporphyrin dimethylesters formed molecular ions m/z at 605 and 591 respectively; these values corresponded to (N–MePP dimethylester + 1 additional proton) and (protoporphyrin dimethylester + 1 additional proton) respectively. In positive mode tandem mass spectrometry the griseofulvin minor pigment produced identical daughter ions to N–MePP standard with ions at m/z 590 and 518 in both cases (Fig 4.8).

4.4.1 Minor griseofulvin pigment: copper dealkylation process

Further confirmation that the minor griseofulvin pigment is N–MePP was obtained by copper–catalysed dealkylation studies as follows. When the minor pigment was incubated with copper chloride in acetonitrile in the presence of dodecylamine, under similar conditions as those described for authentic N–MePP in section 4.3.2, molecular ions at m/z 186 and 200 were detected representing the dodecylamine itself and the adduct of the amine with the methyl group released from the minor griseofulvin pigment (Fig 4.9). Further studies involved incubating the minor griseofulvin pigment with copper chloride in acetonitrile and using di–n–butylamine as the amine acceptor (conditions same as those described above for authentic N–MePP in section 4.3.2). Two molecular ions at m/z 130 and 144 corresponding to di–n–butylamine and methylated di–n–butylamine, respectively were both detected (Fig 4.10).
Fig 4.9 FAB mass–spectrum of dodecylamine and methylated dodecylamine following the dealkylation of the minor griseofulvin pigment with copper chloride

The reaction was carried out in the presence of acetonitrile and the matrix nitrobenzyl alcohol.
Fig 4.10 FAB mass-spectrum of di-n-butylamine and methylated di-n-butylamine following the dealkylation of the minor griseofulvin pigment with copper chloride.

The reaction was carried out in the presence of acetonitrile and the matrix nitrobenzyl alcohol.

M denotes the matrix signal peak.
Chapter 5

DISCUSSION
5.1 Biochemical changes in ATMP treated mice

So far four classes of chemicals are known to cause experimental protoporphyria, DDC and related dihydropyridines, griseofulvin, TTMS and 2,4 diethyl-2-methyl-1,2-dihydroquinoline (DMDQ) (Onisawa and Labbe 1963; De Matteis and Gibbs 1975; Sutherland et al. 1986; Poules et al. 1988). The work presented in this thesis shows that a new chemical, ATMP, which does not belong to any of the classes mentioned above, can also cause hepatic protoporphyria in mice. ATMP–induced hepatic protoporphyria has three main characteristics. These are: (1) increased accumulation of protoporphyrin, (2) inhibition of FK and (3) formation and accumulation of a green pigment which is inhibitory towards FK in vitro. These biochemical changes are in agreement with those seen after DDC and griseofulvin, two protoporphyric agents which have been well documented (Onisawa and Labbe 1963; De Matteis and Gibbs 1975; Tephly et al. 1979; De Matteis and Gibbs 1980).

ATMP causes the accumulation of protoporphyrin in the liver of mice. A single dose of ATMP (300 mg/kg) resulted in a 200–fold increase in porphyrins above control values of which 99% was present as protoporphyrin (Table 3.1, Fig 3.1). Marks et al. (1987) have demonstrated that the pattern of porphyrin accumulation in the liver is predictive of which enzyme in the haem biosynthetic pathway is inhibited. For example, accumulation of uroporphyrin by hexachlorobenzene is consistent with the inhibition of uroporphyrinogen decarboxylase (Elder et al. 1976). An over–production of protoporphyrin therefore, suggests a partial block in FK activity. In ATMP treated mice, FK activity was significantly inhibited to 33% of control value at 24 hrs after dosing.
In a similar manner the chemicals griseofulvin, DDC and TTMS were also found to cause marked loss in FK activity (Onisawa and Labbe 1963; De Matteis and Gibbs 1975; McCluskey et al. 1986; Sutherland et al. 1986). The finding that protoporphyrin accumulation is accompanied by a decrease in FK activity is in keeping with the concept that protoporphyrin accumulation arises due to a block in its conversion to haem (De Matteis et al. 1973; De Matteis and Gibbs 1972; Onisawa and Labbe 1963).

In the present work, a significant inhibition of FK activity was seen as early as 1 hr after administration of ATMP, when protoporphyrin level was still normal or just starting to rise (Table 3.2, 3.3). By analogy, in DDC–induced protoporphyria there is a decrease in FK activity before there is an increase in protoporphyrin concentration (De Matteis et al. 1973; De Matteis and Gibbs 1972). In this study it has been shown that a loss in FK activity is important in the accumulation of protoporphyrin in the liver. Tables 3.2 and 3.3 illustrate this point, demonstrating that an increase in protoporphyrin levels occurs at a later time when the decrease in FK activity is even greater. These findings are in close agreement with those of DDC and griseofulvin–treated mice, where a good correlation was noted between inhibition of FK activity and concentration of porphyrins in mice (De Matteis et al. 1973; De Matteis and Gibbs 1975). It is possible therefore to conclude that in ATMP–induced protoporphyria the primary lesion is the inhibition of FK activity and as a secondary response there is an accumulation of protoporphyrin.
5.2 Isolation of ATMP–green pigment

Tephly et al. (1979) first isolated a green porphyrin–like pigment, with inhibitory properties towards FK activity, from livers of DDC–treated mice. This green pigment was rapidly formed and accumulated in the liver. The isolated DDC–green pigment in vitro, was found to be a potent inhibitor of FK from untreated mitochondria (De Matteis et al. 1980a). In a similar manner a green pigment was also isolated from ATMP–treated mice and was shown to be an inhibitor of untreated FK activity in vitro (Fig 3.4). The isolation of the green pigment from mouse livers involved acidified acetone extraction. The amount of ATMP–green pigment collected off the Sephadex LH20 column was 4.95 ± 2.1 nmol/g wet liver (mean ± s.d. (n=3)). As the free acid, the green pigment had the same Rf value as synthetic N–MePP in the developing system chloroform:methanol:acetic acid 40:10:3 (v/v/v) (section 3.2.1). The importance of the TLC step was to help remove any protoporphyrin. Following methylation the pigment was finally purified by HPLC to eliminate any further protoporphyrin which may be present.

From the literature it has been shown that DDC–green pigment interacts with the porphyrin binding site of FK (De Matteis et al. 1980a; De Matteis et al. 1980c). The green pigment has a Ki value between 8.5 nM and 10 nM indicating it to be a high–affinity, tight–binding inhibitor of the FK (De Matteis et al. 1980a; Dailey et al. 1986a). It is postulated here, that the ATMP–green pigment binds to FK active site in a similar fashion.

The green pigment isolated from DDC–treated mice has been shown to be an N–monoalkylated protoporphyrin, based on its electronic absorption
spectrum and titration properties with acid (De Matteis and Cantoni 1979). Furthermore, DDC–green pigment has been identified as N–MePP by its chromatographic and mass spectral characteristics (De Matteis et al. 1980b), as well as by its NMR spectrum (Ortiz De Montellano et al. 1981a). In agreement with this, the ATMP–green pigment produced a characteristic bathochromic shift of the absorption maxima when compared with authentic protoporphyrin and an aetiotype spectrum (Table 3.7).

There is strong evidence to suggest that the ATMP–green pigment is N–MePP. The chromatographic properties of the green pigment, having a retention time of 3.72 mins and 6.11 mins are almost identical to those of authentic N–MePP with retention times of 3.69 mins and 6.02 mins (Fig 3.3). Another interesting feature of the ATMP–green pigment is that over 95% of the fraction collected off the HPLC resides in fraction 1 (this contains the isomeric pairs N_A and N_B). This suggests the ATMP is metabolised by a specific isoenzyme of the cytochrome P450 and that the ATMP–alkylated protoporphyrin is synthesised on an enzymic template. It has been shown by previous workers that the isomeric composition of the N–methyl protoporphyrin (that is, the amount of N_A, N_B, N_C and N_D present) favours the N_A, that is one of the vinyl substituted ring isomers. In the case of DDC and griseofulvin, it has been shown that the major isomer is present as N_A (Ortiz De Montellano et al. 1981a; Holley et al. 1991).

It has been postulated that ring distortion is a normal process which occurs when a metal ion is inserted into the porphyrin molecule (Lavallee 1988; Bain–Ackerman and Lavallee 1979). In crystallography studies it has been
shown that N-alkyl porphyrins have a pyrrole ring (the ring bearing the alkyl substituent) tilted out of the porphyrin plane by approximally 30° (Lavallee and Anderson 1982). Dailey (1990) has postulated that an N-alkyl porphyrin binds to FK as a stable transition-state analogue. The ring distortion which occurs with N-alkyl porphyrins is thought to enhance the affinity of the modified porphyrin for the enzyme's active site, which may in part explain the high affinity and tight binding property displayed by the drug-induced green pigment. FK interacting with N-MePP occurs through a tight-binding, competitive inhibition (Dailey and Fleming 1983). By analogy, it is thought that the ATMP-green pigment also inhibits mouse FK in a competitive manner.

In summary, the action of the ATMP-green pigment is identical to that of N-MePP, in as much there is, inhibition of FK both in vitro and in vivo in mouse liver. The source of the green pigment and the possible role of cytochrome P450 in producing the green pigment will be discussed below under cytochrome P450 inhibitors.

5.3 Cytochrome P450 inhibitors

Certain drugs which cause porphyria are also classed as suicide substrate inhibitors of cytochrome P450. For example, AIA has been shown to cause porphyria when given in vivo to rodents (Abbritti and De Matteis 1971) and is directly responsible for the loss of cytochrome P450 (De Matteis 1971) occurring as a suicidal process (Ortiz De Montellano and Mico 1981). The significant loss in cytochrome P450 seen after AIA treatment gives rise to a green pigment (De Matteis 1971). Several other drugs such as DDC and its analogues cause significant loss in cytochrome P450 and a corresponding
increase in the formation of a green pigment (Tephly et al. 1979; De Matteis et al. 1982b; Marks et al. 1985). A correlation exists between the loss of cytochrome P450 and green pigment formation (De Matteis et al. 1982b; Marks et al. 1985).

In the present work (section 3.2) it has been shown that a single dose of ATMP can give rise to a green pigment even though there was no detectable loss of cytochrome P450 (Table 3.5). At earlier time points, 2 hrs and 4 hrs after dosing with ATMP, still no significant loss of cytochrome P450 could be detected (Table 3.6). The experiments described in section 3.5 were aimed at addressing this problem, and try to give a clearer indication for the possible involvement of cytochrome P450 in the production of N-MePP, after ATMP treatment in mice.

Indirect evidence for the role of cytochrome P450 in ATMP activation has arisen from inhibitory studies with SKF525–A, piperonyl butoxide, metyrapone, and α–NF. These inhibitors may be classified into two groups depending on whether they protect against ATMP–induced protoporphyria or offer no protection against ATMP–induced protoporphyria.

SKF525–A and piperonyl butoxide both offered protection against ATMP–induced protoporphyria. SKF525–A and piperonyl butoxide have been shown to prevent protoporphyrin accumulation (Table 3.14). In addition no FK inhibition was found when either of these compounds were given 1 hr before treating mice with ATMP (Table 3.14). When the green pigment was searched for, in mice pretreated with SKF525–A followed by ATMP, no pigment could be
detected (Fig 3.7). Three different conclusions are possible from the SKF525–A study. Firstly, the parent compound, ATMP is not responsible for inducing hepatic protoporphyria in the mouse, but rather requires metabolic activation to a reactive species. Secondly, cytochrome P450 plays an important role in the metabolic activation of ATMP, since no porphyric response could be detected following cytochrome P450 inhibition (Table 3.14). Thirdly, no pigment was formed in SKF525–A pretreated mice given ATMP, which suggests that the haem moiety of cytochrome P450 may be the source of the protoporphyrin present in the green pigment. To test this latter point, further experiments are required, such as incubating ATMP in vitro with liver microsomes. In addition, in vivo labelling experiments, whereby $^{14}$C–ALA is given to mice before treating with ATMP; if the green pigment was also labelled it would demonstrate at least that the green pigment was derived from a haem pool.

Radiolabelled ATMP was taken up by the liver in SKF525–A pretreated mice (Table 3.17). The presence of radiolabelled ATMP in the liver demonstrates that the protection by SKF525–A against ATMP–induced porphyria cannot be due to interference with the ATMP uptake by the liver.

In the second class of cytochrome P450 inhibitors, metyrapone and α–NF lacked the ability to protect mice against ATMP–induced protoporphyria. As a consequence, protoporphyrin accumulated in the liver and there was an accompanying block in FK activity. A green pigment could also be detected. Metyrapone may exacerbate the disorder since a slightly greater degree of FK inhibition was seen (Table 3.16) and an apparent increase in the amount of green pigment was detected (Fig 3.8).
No direct evidence has been presented in this work showing that ATMP causes cytochrome P450 loss. From the work presented here it is postulated that an isoenzyme is involved, which is present in only very small levels. By way of analogy, in DDC metabolism only 1% of the chemical metabolised leads to suicidal inactivation of cytochrome P450, thereby making it difficult to detect appreciable loss in cytochrome P450 levels (Lee et al. 1988). Furthermore, an analogue of DDC, namely ethyl-DDC, preferentially destroys cytochrome P450h, k and p isoenzymes (Correia et al. 1987). In a similar way, it is postulated that ATMP may selectively destroy one particular isoenzyme of the cytochrome P450 system. One can therefore conclude from the above indirect findings that cytochrome P450 is involved in ATMP-induced protoporphyria. The following experiments were designed to show that by inducing the cytochrome P450 system, the degree of green pigment formation, FK inhibition and protoporphyrin accumulation could be increased.

5.4 Cytochrome P450 inducers

In PB-pretreated mice, treatment was anticipated to produce more ATMP-green pigment and a greater porphyrogenic response. The work presented in this study demonstrates on the contrary that PB offered partial protection against protoporphyria. A low and middle-range dose of ATMP were used in order to produce a submaximal effect. Any changes by the inducer would then be detected. The results obtained were unexpected, as PB-pretreated mice given ATMP showed no protoporphyrin accumulation (Table 3.18). However, there was marked inhibition of FK activity (Table 3.19), and under two different conditions, it was shown that PB-pretreatment did not protect against FK inhibition (Table 3.19 and 3.20).
The data presented in tables 3.18, 3.19, and 3.20 demonstrate that a marked decrease in FK activity is not the only criterion necessary to cause porphyrin accumulation. As seen here inhibition of FK does not lead to an increase in protoporphyrin levels. This view is also supported by work carried out by De Matteis et al. (1973), who demonstrated cycloheximide pretreatment followed by DDC gives a similar inhibition of FK (as compared with in the absence of cycloheximide), but with no corresponding increase in protoporphyrin levels. It is possible that the activity of ALA-S, which is important for porphyrin synthesis, may be depressed by PB pretreatment. The rate of porphyrin utilization compared with the rate of porphyrin formation is an important factor governing protoporphyrin accumulation (De Matteis et al. 1973). On the contrary, at twenty four hours after ATMP treatment the degree of FK inhibition (17% of control value) appeared to be greater in PB-pretreated mice given ATMP (Table 3.19) than in ATMP-treated mice receiving no PB (33% of control value, Table 3.3). An alternative possibility is that PB-pretreatment may prevent the accumulation of protoporphyrin by enhancing the clearance of the porphyrins out of the liver into the bile canaliculi (Erlinger 1982).

The two remaining inducers, BNF and CFA showed no protection against ATMP-induced protoporphyria, as FK activity was inhibited and protoporphyrin levels raised. Taken together, these results suggest that BNF and CFA inducible isoenzymes are not involved in ATMP-induced porphyria, whereas the PB-inducible isoenzyme may be important for the formation of the ATMP inhibitory green pigment.
5.5 Structural analogues of ATMP

Modification of the structure of ATMP at the oxime bearing end of the molecule

\[-C=N-O C_2H_5\]
\[\backslash C_2H_5\]

to either the propyl-ATMP

\[-C=N-O C_2H_5\]
\[\backslash C_3H_7\]

or to the oxazole derivative (see Figure 3.5) resulted in loss of porphyrogenic activity. Mice dosed with large amounts of either of these analogues (300 mg/kg) showed no inhibition of FK and no accumulation of protoporphyrin (Tables 3.8 and 3.9). Two possible explanations for these findings are:

(i) neither the propyl nor the oxazole analogues of ATMP are capable of forming a green pigment, or,

(ii) a green pigment can be formed, but possesses no inhibitory properties towards FK.

The rationale for searching for a green pigment even though FK was not inhibited, is that several suicide substrates of cytochrome P450, among these AIA and isobutyl-DDC, produce green pigments which do not inhibit FK (De Matteis et al. 1980a; Marks et al. 1988; McCluskey et al. 1986). To test which theory was correct, liver extracts taken from propyl-ATMP treated mouse livers were applied to a Sephadex LH20 column. The eluate was collected and scanned. No green pigment could be detected with a Soret between 410 nm and 420 nm. From these results it appears, therefore, that an inactive structural
analogue of ATMP, propyl-ATMP, lacks the ability to produce a green pigment, either due to (i) the propyl (or the oxazole) part of the molecule sterically hinders the interaction of the xenobiotic with cytochrome P450's active site and its metabolism by the cytochrome P450 or (ii) because even though the analogues are metabolised by cytochrome P450, their metabolism does not lead to alkylation of the haem prosthetic group, at least not to a N-alkyl protoporphyrin product. As a direct consequence there is no inhibition in FK activity and no protoporphyrin accumulation.

5.6 Species differences in response to ATMP

ATMP-induced protoporphyria is species selective. In the mouse (a responsive species) a single dose of ATMP (300 mg/kg) caused a marked accumulation of protoporphyrin 24 hrs later (Table 3.12). This change was not seen in similarly treated rat, guinea-pig, or hamster (Table 3.12). However, in the rat and hamster FK activity was inhibited to approximately 70% and 80% of their control values respectively (Table 3.13). Based on FK measurements the degree of enzyme inhibition seen is in decreasing order:

Mouse > Guinea-pig > Rat > Hamster

This suggests that a green pigment, an inhibitor of liver FK may still be formed in rat and hamster. When chick embryo hepatocytes were exposed to ATMP, protoporphyrin accumulation could not be detected (Table 3.10) 24 hrs later. Similarly, chick embryo dosed in ovo with ATMP showed no accumulation of protoporphyrin (Table 3.11).
Fig 5.1 Schematic diagram postulating the mechanisms whereby unresponsive species are insensitive to ATMP treatment

RAT       HAMSTER       GUINEA–PIG       CHICK EMBRYO

↓

A single dose of ATMP

↓

No change in hepatic porphyrin levels

↓   ↓   ↓   ↓   ↓   ↓

ATMP metabolised through an innocuous pathway
Compensatory stimulation of ALA–S prevented
Increased clearance of porphyrin from the liver
Green inhibitory pigment not formed
Constitutive isoenzyme of CytochromeP450, responsible for ATMP activation, not expressed
These differences seen between the mouse (a responsive species) and rat, hamster, guinea-pig and chick (a unresponsive species) for ATMP-induced protoporphyria, may in part, be explained by species differences in liver cytochrome P450 enzymes. It is possible that the mouse possesses a constitutive isoenzyme of cytochrome P450 responsible for activating ATMP along a suicidal pathway, whereas the remaining species, rat, hamster, guinea-pig and chick do not possess this particular isoenzyme. A species difference also exists in response to the porphyrogenic agents, DDC and griseofulvin (Marks et al. 1988; De Matteis and Gibbs 1975; De Matteis et al. 1973) and this too may depend on species differences in liver cytochrome P450. A schematic diagram postulating the underlying mechanisms involved in the unresponsive species is given below in Figure 5.1.

From the work presented here, a postulated mechanism of action for ATMP–induced protoporphyria is put forward. ATMP is metabolised to a reactive species, which in turn alkylates the haem moiety of liver cytochrome P450, thus producing N–MePP. This inhibits the enzyme FK and, as a secondary response, protoporphyrin accumulates. In conclusion, ATMP has the ability to induce hepatic protoporphyria in the mouse. Further work is required to identify conclusively by mass spectrometry the ATMP–derived green pigment. Also work studying the loss of cytochrome P450 during ATMP metabolism, using liver microsomes, should prove invaluable in understanding the role that cytochrome P450 plays. An overall mechanism for the action of ATMP is given in Fig 5.2
Fig 5.2 A postulated mechanism for ATMP giving rise to protoporphyria in mice

ATMP → cytochrome P450 → N-MePP → Inhibition of FK → Induction of ALA-S → Accumulation of protoporphyrin → Protoporphyria
5.7 Griseofulvin discussion

The finding by Holley (1987) of two green pigments after griseofulvin–feeding has been confirmed here. In this present work identity of each of these two pigments has been conclusively established and has allowed a discussion of their possible inter–relationship.

Until now it was thought that griseofulvin might produce an N–alkylated protoporphyrin by transferring an alkyl group to the prosthetic haem of cytochrome P450, as has been shown with DDC (De Matteis et al. 1980b; Ortiz De Montellano et al. 1981c). Work presented here, has identified by mass spectrometry two different N–alkylated protoporphyrins, the major and the minor griseofulvin pigments, as N–griseofulvin protoporphyrin and N–MePP, respectively. Each will be discussed in turn below.

5.7.1 Major griseofulvin pigment

Previous work has shown that in drug–induced porphyria, N–alkyl porphyrins can be produced in the liver, through two different mechanisms. Either:

a) the N–alkylating moiety is derived from the whole drug, plus an oxygen atom – this occurs with unsaturated compounds such as ethylene and propyne (De Matteis et al. 1980c; Ortiz De Montellano and Kunze 1981)

or:

b) a small alkyl group leaves the drug, for example, DDC and TTMS and then covalently binds to the porphyrin moiety (De Matteis et al. 1980c; Grab et al. 1986).
By contrast, the present study shows a new mechanism for haem alkylation, whereby the whole drug, griseofulvin, acts as the alkylating species which binds directly to the porphyrin molecule, with no oxygen insertion. The evidence for this will be discussed in detail.

a) Mass spectral analysis

A green pigment can be extracted from mouse livers following ethylene or propyne treatment (De Matteis et al. 1980b; Ortiz De Montellano and Kunze 1981) which contains an oxygen in its molecule. In the case of the major griseofulvin pigment an oxygen does not appear to be present. The molecular ion was seen in FAB-mass spectrometry at m/z 941 (Fig 4.1). This is equivalent to griseofulvin plus protoporphyrin (dimethylester) and can be accounted for as follows: [protoporphyrin dimethylester – 1 pyrrole hydrogen (589) + griseofulvin – 1 hydrogen (351) + 1 additional proton].

In the present study, the molecular ion at m/z 941 was also subjected to tandem mass spectrometry and two daughter ions were formed at m/z 591 and 353 which corresponds to the protoporphyrin dimethyl ester and griseofulvin, respectively (Fig 4.2). The signal peak at m/z 591 corresponding to protoporphyrin dimethylester, is in agreement with other workers who found evidence for N-dealkylation leading to the corresponding unsubstituted porphyrin, in this case protoporphyrin (Jackson and Dearden 1973; Smith and Farmer 1982). An unexpected daughter ion at m/z 605 could also be detected in the present study. It may be postulated that there is a facile loss of a portion of the griseofulvin substituent leaving behind, attached to the nitrogen of
The major griseofulvin pigment isolated from mouse livers has also been identified as \( \text{N-} \)griseofulvin protoporphyrin by using absorption spectral analysis and NMR, as indicated below.

b) NMR study of major griseofulvin pigment

A brief study was carried out in which the major griseofulvin pigment was subjected to NMR scanning. It was found that a small but significant signal was produced a chemical shift at \(-53.0\) ppm which further supports the existence of an \( \text{N-} \)alkylated porphyrin.

c) Absorption spectral analysis

Further evidence for \( \text{N-} \)substitution has been obtained from absorption spectral analysis of the major griseofulvin pigment. The latter showed a shift towards a longer wavelength, that is, a bathochromic shift of all the absorption maxima, when compared to those of protoporphyrin, while retaining the aetiotype spectrum (415, 510, 543, 596, 653 nm) (Table 4.1). These wavelengths and the corresponding bathochromic shifts, compared to protoporphyrin are, diagnostic for an \( \text{N-} \)alkylated porphyrin, which lends support to the structure of the major griseofulvin pigment which has been identified by the present work.

When copper was added to major griseofulvin pigment a reaction occurred leading to the formation of the copper–protoporphyrin complex (Table 4.2). A
similar complex was formed when starting with either N-MePP or protoporphyrin standards (Table 4.2). All three porphyrins produced similar adsorption spectra, suggesting that the porphyrin moiety from the major griseofulvin pigment is the same as the protoporphyrin standard.

5.7.2 Minor griseofulvin pigment

The minor griseofulvin pigment isolated from mouse livers made porphyric with griseofulvin, has been shown conclusively to be N-MePP by the present work. This particular green pigment is responsible for the inhibition of the enzyme FK, as first demonstrated by De Matteis and Gibbs 1980, and more recently by Holley (1987) and Holley et al. (1991). The identification has involved the following different approaches:

a) Copper dealkylation studies

N-alkyl porphyrins can readily accept zinc or copper and under the appropriate conditions release the N-alkyl substituent (Lavallee 1976; De Matteis et al. 1985). A recently developed technique by the present author and other members of the unit makes use of the release of the N-alkyl substituent during the copper dealkylation process. The N-alkyl group is trapped by dodecylamine and the amine adduct identified by FAB mass spectrometry (Gibbs et al. 1990).

Essentially, on addition of copper, the dealkylation of copper by the N-alkyl protoporphyrin and the subsequent dealkylation reaction can be followed in the spectrophotometer: a transient spectrum corresponding to a copper chelate of N-MePP is first seen, followed by ejection of the N-methyl
group (in the presence of dodecylamine) to afford the copper chelate of protoporphyrin. The resulting mixture was analysed by positive ion FAB–mass spectrometry using 3–nitrobenzyl alcohol as matrix.

The above technique was employed in the identification of the N–methyl from the minor griseofulvin pigment. Insertion of copper into the minor griseofulvin pigment (molecular ion at m/z 666) was followed by dealkylation of the N–alkyl group, which was seen as a loss of 15 mass units (this corresponds to a –CH₃ grouping) to give a molecular ion at m/z 652–651 (Fig 4.4 a and b). A molecular ion at m/z 200 corresponding to the methylated dodecylamine was also detected in the mass spectrometer (Fig 4.9).

Other workers in this field have postulated that the process of dealkylation occurs via a nucleophilic attack of the amine on the alkyl group (Lavallee 1976), leading to the ejection of the alkyl moiety. A copper protoporphyrin complex is left remaining.

b) Mass spectrometer analysis of minor griseofulvin pigment

A FAB mass spectrum obtained in the positive ion mode for the minor griseofulvin pigment produced a molecular ion at m/z 605 (Fig 4.6). This is compatible with an ion corresponding to N–MePP [protoporphyrin dimethyl ester – 1 pyrrole hydrogen (589) + the methyl grouping (15) + 1 additional proton]. Under similar conditions, authentic N–MePP dimethyl ester produced a molecular ion at m/z 605 (Fig 4.7). Furthermore in tandem mass spectrometry studies the molecular ions of the authentic N–MePP and the minor griseofulvin pigment both produced identical fragmentation patterns, with daughter ions at
m/z 590 and 518. In figure 4.8 the tandem mass spectrum for minor griseofulvin pigment only is shown. The daughter ion at m/z 590 is thought to be the radical cation \((M^+)\) of protoporphyrin following the loss of the methyl group; whereas the daughter ion at m/z 518 is believed to correspond to a loss of a side chain ester \(-\text{CH}_2\text{-CH}_2\text{-COOCH}_3\) from the minor griseofulvin pigment.

### 5.7.3 General discussion

To date, there is no direct evidence that major and minor griseofulvin pigments originate from cytochrome P450. Indirect evidence supporting the involvement of cytochrome P450 is from the findings that there is a loss of cytochrome P450 and microsomal haem in the liver, after feeding griseofulvin in the diet to mice (Wada et al. 1968; Denk et al. 1977; Williams and Simonet 1986). Griseofulvin is primarily metabolised through O-demethylation at one of its three methoxy groups (Lin et al. 1973; Chang et al. 1973). It may be that a free radical is formed which is sufficiently long-lived to diffuse away from iron-bound oxygen but reacts and binds with a pyrrole nitrogen to give rise to N-griseofulvin protoporphyrin (the major griseofulvin pigment).

As yet the relationship between N-griseofulvin protoporphyrin and N-MePP is unknown. However, the finding that – as discussed above – the major griseofulvin pigment gave rise on tandem mass spectrometry to an ion with m/z 605, suggests that N-MePP may arise as a secondary fragmentation product from the N-griseofulvin protoporphyrin. This finding suggests that griseofulvin is also bound to one of the pyrrole nitrogens through a carbon linkage, possibly one of the methoxy groups (Holley et al. 1991). Also, it is possible that N-griseofulvin protoporphyrin may undergo a secondary metabolic
step to form N-MePP in vivo (Holley et al. 1991), but this has not yet been demonstrated.

In vivo, rodents require a longer exposure time to griseofulvin (3 days) as compared with DDC (1.5 hrs), in order to observe a significant decrease in FK activity and increase in porphyrin levels leading to protoporphyria (De Matteis and Gibbs 1975; Cantoni et al. 1983). It is postulated that the delayed onset of griseofulvin–induced protoporphyria is because N–griseofulvin protoporphyrin must first be formed before N–MePP can be generated; the latter being a potent inhibitor of FK.
5.7.4 Conclusion

The overall postulated mechanism for protoporphyria would therefore be as follows.
Griseofulvin is metabolised by cytochrome P450 in the liver which gives rise to N-griseofulvin protoporphyrin (which is the major griseofulvin pigment). By a secondary pathway via cytochrome P450 or some other mechanism, the N-griseofulvin protoporphyrin then give rise to N-MePP. This would then inhibit FK with diminution of haem synthesis. The drop in concentration of the haem pool is postulated to remove the negative feedback response on ALA-S, resulting in a compensatory stimulation of ALA-S, such that porphyrin synthesis is increased along the haem pathway. The concomitant increase in porphyrin synthesis and block of FK, results in an overproduction of protoporphyrin, giving rise to the classical picture of protoporphyria.

Although griseofulvin has been in clinical use for over 30 years it still attracts much attention from a toxicologist's point of view. Success in identifying conclusively, by FAB-mass spectrometry, the minor pigment as N-MePP has essentially come from developing a technique to isolate and purify in sufficient amounts the minor pigment. In addition, the major pigment has also been identified, by FAB-mass spectrometry as, N-griseofulvin protoporphyrin. The mechanism whereby these two biological pigments are formed have opened further new areas to be explored.
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Coproporphyrinogen oxidase: reaction mechanism and role of tyrosine residues
Copper-induced dealkylation studies of biologically \textit{N}-alkylated porphyrins by fast atom bombardment mass spectrometry

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(Received 19th April 1990)

Abstract

Copper has been reported to promote the demethylation of \textit{N}-methylporphyrins. Copper-induced dealkylation is observed at room temperature in acetonitrile in the presence of a suitable nucleophilic acceptor molecule, or in chloroform alone. Electronic absorption spectra of \textit{N}-methylprotoporphyrin IX (in the form of its dimethyl ester) in the presence of copper (with either chloroform alone or acetonitrile–dibutylamine as solvent) exhibited a transient spectrum corresponding to a copper chelate of the \textit{N}-methylprotoporphyrin followed by ejection of the \textit{N}-methyl group to afford the copper chelate of protoporphyrin.

The use of fast atom bombardment mass spectrometry (FAB-MS) to identify the \textit{N}-alkyl group of a series of \textit{N}-substituted porphyrins was investigated. The alkyl group released on reaction with copper ions was trapped by an amine (e.g., dodecylamine). The resulting mixture was analysed by positive-ion FAB-MS using 3-nitrobenzyl alcohol as matrix. Molecular ions (MH$^+$) were detected at $m/z$ 200, 214 and 228, corresponding to [CH$_3$(CH$_2$)$_n$NHCH$_3$ – H$^+$, [CH$_3$(CH$_2$)$_n$NHC$_2$H$_5$ + H$^+$ and [CH$_3$(CH$_2$)$_n$NHC$_3$H$_7$ + H$^+$, respectively.

The analysis was extended to \textit{N}-alkylporphyrins isolated from liver homogenates of mice treated with the porphyria-inducing drug griseofulvin. Using a combination of UV and copper-induced dealkylation studies in FAB-MS, the porphyria-producing pigment was identified as \textit{N}-methylprotoporphyrin IX.

Keywords: Porphyrins; Alkylporphyrins

\textit{N}-Substituted porphyrins [1] are produced by the reaction of numerous xenobiotics, such as olefins [2] and acetylenes [3], with the prosthetic haeme group [iron(III) protoporphyrin IX] of the detoxifying cytochrome P-450 enzymes. A wide range of compounds including anaesthetics and prescription drugs are known to produce such \textit{N}-substituted porphyrins [1,4], and some of these porphyrins (where the \textit{N}-substituent is small) have been shown to inhibit the activity of the enzyme ferrochelatase [5–7]. This enzyme is required in the biosynthesis of haeme, which is the prosthetic group found in a series of important functional proteins including the cytochromes, myoglobin and haemoglobin. Inhibition of ferrochelatase activity can lead to such drug-induced diseases as protoporphyria [8].

Identification of \textit{N}-alkylated porphyrins is usually achieved by a combined approach involving UV–visible spectrophotometry and $^1$H NMR studies [1]. However, with biologically derived \textit{N}-alkylporphyrins, the amounts available are normally limited so $^1$H NMR analysis is usually precluded. Analysis for \textit{N}-alkylporphyrins by mass spectrometry (MS) has received limited attention and then only using electron-impact MS [9]. This latter technique is limited to low-molecular-weight, thermally stable compounds in which the \textit{N}-alkyl group is small.

In this study the copper-induced dealkylation
of a series of synthetic and biologically derived N-alkylated protoporphyrins was investigated using a combination of UV–visible spectrophotometry and fast atom bombardment (FAB)-MS. In order to provide additional structural information, the alkyl group released during the copper-induced dealkylation was trapped by a suitable nucleophile and the resulting adduct was detected by FAB-MS.

EXPERIMENTAL

Chemicals

All synthetic N-alkylporphyrins, as their dimethyl esters (DME), were prepared and purified as described previously [10]. The synthetic and biologically derived N-alkylporphyrins were all analysed as the unresolved mixture of four stereoisomers. The copper chelate of protoporphyrin IX DME was prepared by the method of Falk [11]. The biologically derived N-alkylporphyrin was isolated from mouse liver homogenate, from mice fed with griseofulvin (1% in the diet for 3 days) as described previously [10]. Purification of the porphyrin was effected by liquid chromatography on a Nucleosil-5 analytical column [isocratic elution with dichloromethane–methanol–ammonia solution (50 + 50 + 0.1, v/v/v) at a flow-rate of 2 ml min⁻¹]. Dibutylamine and dodecylamine were purchased from Aldrich and used without further purification.

UV–visible spectra

Absorption spectra were obtained using either a Varian Cary 2200 or a Kontron Unikon 860 spectrophotometer. The porphyrin was dissolved in the appropriate solvent (either chloroform or acetonitrile) and, after mixing, scanned from 700 to 350 nm. A methanolic solution of either copper(II) acetate or chloride was added (followed, in the case of acetonitrile solutions, by addition of either dibutylamine or dodecylamine) and the change in the absorption spectrum was recorded.

Mass spectra

Mass spectra were obtained on a VG 70-SEQ instrument of EBQQ geometry. All samples were ionized by positive-ion FAB-MS using xenon as the source of fast atoms. The primary beam impacted on the sample at 8.5 keV with a beam flux equivalent to 1 μA. The secondary ions produced were accelerated to 8 keV from the source region and analysed in the first mass spectrometer (EB) using a scan speed of 5 s decade⁻¹ at a resolution of 1500.

Solution mixtures containing the N-alkyl porphyrin were analysed directly without purification by dissolving the mixture in 2 μl of 3-nitrobenzyl alcohol (NBA), which served as the FAB-MS matrix for all these studies. Typically, 20 nmol of N-alkylporphyrin were dissolved in 10 μl of acetonitrile–methanol (9 + 1, v/v) in the presence 100 nmol of either dibutylamine or dodecylamine (amine : porphyrin ratio = 5 : 1) and 50 nmol of copper(II) chloride were added. The solution mixture was heated for 10 min at 50 °C and a 1-μl aliquot was added to 2 μl of NBA matrix and analysed by FAB MS.

RESULTS

Addition of copper(II) acetate to a solution of N-methylprotoporphyrin IX DME (1) in chloroform results in a marked bathochromic shift of the Soret band, followed by the appearance of a new Soret band (λmax = 406 nm) characteristic of copper protoporphyrin IX DME (2), where R = Me, as detailed in Fig. 1. The transient intermediate has a UV–visible spectrum indicative of a copper chelate of N-methylprotoporphyrin IX DME (2), where R = Me.
Compound 1 (concentration 2.5 μM) was dissolved in chloroform (3.0 ml) and its absorption spectrum (dashed curve) determined. A 10-μl volume of a methanolic solution of 0.1% (v/v) copper(II) acetate monohydrate was added and the absorption spectrum was determined after various time periods (solid curves). Note the rapid change in the absorption spectrum indicative of copper incorporation, followed by the gradual appearance of a characteristic Soret λ max (406 nm) of copper protoporphyrin IX DME, indicating loss of the N-methyl group.

A rapid change in the UV–visible spectrum of 1 in acetonitrile on addition only of Cu^{2+} was also observed, corresponding to the copper chelate of 2, but no further spectral change occurred, even on heating, to indicate the formation of 3. However, addition of a suitable nucleophile such as dibutylamine to 2, followed by gentle heating, gave a UV spectrum corresponding to 3, indicating loss of the N-methyl group (results not shown).

The existence of the two distinct copper chelate derivatives observed in the UV–visible spectrum was confirmed by the analysis of aliquots of the reaction mixture at specified time points using positive-ion FAB-MS. The solution mixture containing 1 plus Cu^{2+} afforded an abundant ion at m/z = 666 corresponding to 2 (MCu^+, where M corresponds to N-methylprotoporphyrin DME). However, on addition of dibutylamine, ions at m/z 652 and 651 were observed, corresponding to dealkylated copper protoporphyrin (3). (The ion at m/z 652 corresponds to [copper protoporphyrin IX DME + H]^+ = MH^+). However, the
ion at \( m/z \) 651 is assignable to the radical cation \( M^+ \) of copper protoporphyrin IX DME. A synthetic sample of copper protoporphyrin IX DME gave an identical FAB mass spectrum as described above. The occurrence of radical cations in the positive-ion FAB mass spectra of metalloporphyrins is well documented \([12,13]\).

FAB-MS has found increasing use in studies of reaction mechanisms occurring in solution chemistry \([13-15]\). In this instance we used the technique to investigate further the dealkylation mechanism of \( N \)-alkylporphyrins suggested by Lavallee \([1]\).

FAB-MS analysis of a solution mixture containing 1 plus copper(II) chloride and dibutylamine, after heating, afforded molecular ion clusters at \( m/z \) 666, 652–651, 144 and 130, as shown in Fig. 2. The high-mass ions at \( m/z \) 666 and 652–651 correspond to 1 and 3, respectively. The ion at \( m/z \) 130 is attributable to \( [\text{Bu}_2\text{NH} + \text{H}]^+ \) and the ion at 14 daltons higher (\( m/z = 144 \)) corresponds to methylated dibutylamine.

![Fig. 2.](image-url)
Fig. 2 (continued). Positive-ion FAB mass spectra of a solution mixture containing 1 plus copper(II) chloride and dibutylamine in acetonitrile, using NBA as the FAB matrix. (a) m/z range 150–120; (b) m/z range 680–640.

[But$_2$NCH$_3$ + H]$^+$ and confirms that the amine does serve to trap the alkyl group after dealkylation of the porphyrin has occurred. To investigate the feasibility of using FAB-MS to detect alkyl groups that have been trapped by suitable nucleophiles, in order to ascertain the structure of an unknown alkyl group, a series of N-alkylporphyrins were dealkylated in the presence of dodecylamine and analysed by FAB-MS. Dodecylamine was chosen as the nucleophilic amine as its considerable hydrophobic character and that of its alkylated derivatives would enhance the signal ion abundance observed in FAB-MS [16].

The synthetic N-methyl-, N-ethyl- and N-propylprotoporphyrin IX DME in acetonitrile were dealkylated by addition of copper(II) chloride and dodecylamine and the resulting individual solution mixtures were subjected to positive-ion FAB-MS. Ions at m/z 200, 214 and 228 corresponding to [CH$_3$(CH$_2$)$_{11}$NHCH$_3$ + H]$^+$, [CH$_3$(CH$_2$)$_{11}$NHCH$_2$H$_5$ + H]$^+$ and [CH$_3$(CH$_2$)$_{11}$NHCH$_3$H$_7$ + H]$^+$, respectively, were detected. Figure 3 shows the results obtained for N-methylprotoporphyrin IX DME.

The alkyl transfer trapping technique using dodecylamine was used to identify a ferrochelatase-inhibiting biologically derived pigment isolated from mouse liver. FAB-MS of the biological
porphyrin in the presence of Cu$^{2+}$ gave a molecular ion at $m/z = 666$ and, on addition of dodecylamine and gentle heating, afforded ions at $m/z$ 652–651 and 200, corresponding to 3 and $[\text{CH}_3(\text{CH}_2)_{11}\text{NHCH}_3 + \text{H}]^+$, respectively. This evidence confirmed the identity of the inhibitory biological pigment as N-methylprotoporphyrin IX.

**DISCUSSION**

Numerous metals incorporated into N-alkylporphyrins have been reported to promote dealkylation of the N-alkylporphyrins in solution [1]. However, copper-dependent dealkylation was observed at room temperature in acetonitrile in the presence of a suitable nucleophilic acceptor molecule [17] or in chloroform [18]. The extensive work of Lavallee [1] has suggested that the nature of the metal ion incorporated into the porphyrin and also the character of both the solvent and nucleophile acceptor molecules are important in determining the rate of dealkylation. In this work the copper-dependent dealkylation was investigated in more detail and the study of this reaction was extended to a series of synthetic and a biologically occurring N-alkylprotoporphyrin, the products being identified by FAB-MS as sum-
Fig. 4. Reaction of Cu$^{2+}$ with N-alkylprotoporphyrin in the presence of a nucleophilic amine RNH$_2$. Me = CH$_3$; Vi = CH = CH$_2$; PrMe = (CH$_2$)$_4$CO$_2$CH$_3$.

marized in Fig. 4. After insertion of Cu$^{2+}$ into the N-alkylporphyrin to give 2, dealkylation of the porphyrin occurs via nucleophilic attack of the proximate amine on the alkyl group, with subsequent loss of 3.

This method represents a useful diagnostic tool in the study of biologically N-alkylated porphyrins. By demonstrating that the two copper complexes, described earlier in the initial UV-visible studies, could be characterized by FAB-MS, it allowed the characterization of an unknown porphyrin as the N-monosubstituted protoporphyrin IX and the definite determination of the nature of the alkyl group by trapping and determining the dodecylamine adduct.

The authors thank the MRC for financial support of this work.

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Isolation of two \( N \)-monosubstituted protoporphyrins, bearing either the whole drug or a methyl group on the pyrrole nitrogen atom, from liver of mice given griseofulvin*

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INTRODUCTION

The porphyrogenic compounds 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), griseofulvin and isogriseofulvin all promote the accumulation in rodent liver of an abnormal porphyrin (or green pigment) which has strong inhibitory activity towards mitochondrial ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) both \textit{in vivo} and \textit{in vitro} (Tephly \textit{et al}, 1979, 1980; De Matteis \& Gibbs, 1980). The increased production in the liver of this inhibitory porphyrin and the consequent inhibition of mitochondrial ferrochelatase represent the primary mechanism by which these drugs induce hepatic protoporphyrina [see De Matteis \textit{et al} (1987) and Marks \textit{et al} (1988) for two recent reviews].

The DDC-derived pigment has been conclusively identified as \( N \)-methylprotoporphyrin by comparing its absorption spectrum, chromatographic behaviour and n.m.r. and m.s. data with those of authentic \( N \)-methylprotoporphyrin and by showing that the authentic porphyrin was a powerful inhibitor \textit{in vitro} of mitochondrial ferrochelatase (De Matteis \textit{et al}, 1980a; Tephly \textit{et al}, 1981). Subsequent work has shown that \( N \)-methylprotoporphyrin is produced during oxidative metabolism of DDC by cytochrome P-450, resulting in the transfer of the intact 4-methyl group of the drug to a pyrrole nitrogen of the haem prosthetic group of cytochrome P-450 (De Matteis \textit{et al}, 1981; Ortiz de Montellano \textit{et al}, 1981a; Tephly \textit{et al}, 1981; Marks \textit{et al}, 1985). All four possible structural isomers of \( N \)-methylprotoporphyrin are formed biologically after DDC treatment, with isomer \( N_0 \), predominating (Ortiz de Montellano \textit{et al}, 1981b).

The ferrochelatase-inhibitory pigments obtained after treatment with griseofulvin or isogriseofulvin have been studied less extensively. Although they have previously been reported (De Matteis \& Gibbs, 1980) to exhibit electronic spectra very similar to those of authentic \( N \)-methylprotoporphyrin, they have not yet been conclusively identified, neither has the mechanism of their production been elucidated. In the present paper we report evidence that the inhibitory pigment produced by griseofulvin treatment is in fact \( N \)-methylprotoporphyrin. In addition, we report the isolation and characterization of a second green pigment obtained from the liver of griseofulvin-treated mice: this second pigment is also a \( N \)-monosubstituted protoporphyrin, but in this case the intact griseofulvin moiety appears to be present as a substituent on the pyrrole nitrogen atom.

MATERIALS AND METHODS

Source of special chemicals

Griseofulvin was either a gift from ICI Pharmaceuticals, Macclesfield, Cheshire, U.K., or was purchased from Sigma Chemical Co., Poole, Dorset, U.K. DDC and \( N \)-methylprotoporphyrin IX dimethyl ester were both prepared as described (De Matteis \textit{et al}, 1981). The copper chelate of protoporphyrin IX was prepared from protoporphyrin dimethyl ester and cupric acetate, as described by Falk (1964) for the synthesis of Co\textsuperscript{2+}-protoporphyrin. The product was purified by silica-column chromatography, using chloroform as the developing solvent.

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals. The Nucleosil 5 analytical h.p.l.c. column (4.6 mm \times 250 mm) and the Partisil 10-PAC semi-preparative h.p.l.c. column (4.6 mm \times 250 mm) were obtained from Macherey–Nagel, Düren, Germany, and Whatman respectively. Methanol and dichloromethane (Rathburn Chemicals), hexane (Fisons Scientific Apparatus) and tetrahydrofuran (Chromatographic Services) were all h.p.l.c. grade. Tetrahydrofuran was made peroxide-free and dried immediately before use by filtration through aluminium oxide (type UG; Koch–Light Laboratories), previously activated by heating at 100 °C overnight.

Abbreviations used: DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; NBA, 3-nitrobenzyl alcohol; f.a.b., fast atom bombardment.

* This paper is dedicated to the memory of the late Professor A. H. Jackson (deceased 12 September 1990) in appreciation of his contributions in the field of \( N \)-alkylated porphyrins.

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Isolation and characterization of hepatic green pigments

Green pigments were extracted from liver homogenates, isolated by column chromatography on Sephadex LH-20 and purified by t.l.c. on silica gel as described by De Matteis et al. (1980). The dimethyl esters were prepared by reaction with BF₃ in methanol (Smith & Francis, 1979) and further purified by t.l.c. with a developing system of chloroform/methanol (20:3, v/v). After elution from silica with methanol/11.6 % HCl (40:1, v/v), the dimethyl ester derivatives were chromatographed by isocratic h.p.l.c. on a Nucleosil 5 column using a mobile phase consisting of dichloromethane/methanol/conc. aq. NH₄ (relative density 1.03, 1.53, 4.76 and 8.00 min respectively. Note that the retention time of the third and fourth peaks are identical with those of the isomeric fractions of authentic A-methylprotoporphyrin. These pigments were resolved into four components with retention times (in order of elution) of 1.03, 1.53, 4.76 and 8.00 min respectively. Food consumption was measured and found to be similar in control and treated groups. Mice were killed by cervical dislocation, and their livers were quickly removed and homogenized in ice-cold 0.25 M sucrose.

M.s. studies

Porphyrin methyl esters for m.s. studies were purified by Nucleosil 5 h.p.l.c. immediately before use. A developing system consisting of dichloromethane/methanol (9:1, v/v) and a flow rate of 2 ml/min was also used in some cases, as this was found to achieve better separation of the major griseofulvin pigment from traces of contaminating protoporphyrin.

All mass spectra were obtained on a tandem mass spectrometer (VG70-SEQ) of EBQQ geometry, where E is an electrostatic analyser, B is the magnet and Q₁ is a radio frequency-only quadrupole which acts as a collision cell. All samples were ionized by positive-ion fast atom bombardment (f.a.b.). Xenon atoms from a model B11N (Ion Tech, Teddington, Middx., U.K.) saddle-field fast-atom gun were used as the primary ionizing beam and impacted the sample at 8.5 keV. The secondary ions produced by the xenon atoms were accelerated to 8 keV from the source region and the magnet scanned at 5 s/decade over the mass range m/z 1350-50. All samples were dissolved in dichloromethane and added to 2 µl of matrix, which was usually 3-nitrobenzyl alcohol (NBA) or thiodiglycol on the stainless-steel probe tip.

In the tandem-m.s. studies, parent ions (MH⁺) were selected with a resolution of approx. 1000 using EB (equivalent to the first mass-spectrometer MS₁) and subjected to collision-activated dissociation using argon as the collision gas. Collision energies (laboratory frame-of-reference) were varied from 5 to 460 eV, and target gas pressures in the collision cell were typically ~10 mPa (~10⁻⁴ mbar). Daughter-ion spectra were acquired by scanning Q₂ (equivalent to the second mass spectrometer MS₂) usually over mass range m/z 1000-40, and 15 scans were acquired under data system control in the multi-channel analysis mode.

RESULTS AND DISCUSSION

Chromatographic properties, electronic absorption spectra and ferrochelatase-inhibitory activity of the griseofulvin pigments

The hepatic green pigments accumulating in the liver of mice treated with griseofulvin were isolated by Sephadex LH-20...
A Partisil 10-PAC column eluted with hexane/tetrahydrofuran/methanol (97:9:2 by vol.) was used for the analysis of the dimethyl esters of (a) the minor and (b) the major pigment, both isolated by Nucleosil 5 h.p.l.c. (see Fig. 1) from the liver of griseofulvin-treated mice. The isomers of authentic N-methylprotoporphyrin were eluted in the following order [retention times (min) in parentheses]: $N_a$ (5.75), $N_g$ (6.30), $N_a$ (11.56) and $N_g$ (12.93). All four isomers were present in griseofulvin-derived N-methylprotoporphyrin (a), with isomer $N_a$ predominating. The major griseofulvin pigment was also resolved into four components (b) with retention times of 2.61, 3.30, 4.61 and 5.54 min respectively. Note that retention times of all these peaks are shorter than those of authentic N-methylprotoporphyrin.

chromatography and purified by t.l.c. After methylation, the pigments were resolved by h.p.l.c. on Nucleosil 5 and their elution profiles compared with that of authentic A-methylprotoporphyrin. In this system N-methylprotoporphyrin was resolved into two isomeric fractions (F$_1$ and F$_2$) with retention times of 4.69 and 8 min, each containing two structural isomers, ($N_a$ + $N_g$) and ($N_g$ + $N_a$) respectively (Fig. 1a). In this notation, the suffixes A–D indicate the pyrrole ring that is A-methylated in the pigments.

The spectra of the various porphyrins were also determined in chloroform and those of the DDC pigment, and of the minor griseofulvin pigment (as compared with protoporphyrin) and similarity to those exhibited by authentic A-methylprotoporphyrin. The third peak accounted for most (approx. 90%) of the total injected material, and its neutral and zinc complex spectra were very similar to those discussed below for the unresolved major pigment (that is prior to Partisil 10-PAC chromatography).

Both griseofulvin pigments showed a neutral spectrum of the aetio-type with all absorption maxima shifted to longer wavelengths, as compared with the parent porphyrin, protoporphyrin (Table 1). Their zinc-complex derivatives also showed a bathochromic shift of all absorption maxima (as compared with the zinc complex of protoporphyrin) and exhibited, in addition to the $\alpha$ and $\beta$ bands typical of a metalloporphyrin, a minor band (band $\alpha'$ in Table 1), approx. 200 nm towards the red from the Soret maximum. The absorption maxima of the two griseofulvin pigments were in fact quite close to the maxima of authentic N-methylprotoporphyrin, with the minor pigment showing a closer similarity. The presence of an $\alpha'$ band in the zinc-complex spectrum, as well as the relative intensity of the bathochromic shifts of the various absorption bands, are diagnostic for N-monosubstitution, and the absolute values of these shifts allow the identification of the particular porphyrin which bears the N-substituent, in this case.

Table 1. Absorption spectra of the green pigments obtained after treatment with griseofulvin: comparison with protoporphyrin and with chemically synthesized or biologically produced N-methylprotoporphyrin

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Neutral Absorption maxima (nm)</th>
<th>Zinc complex Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret IV III II Ia I</td>
<td>Soret $\beta$ $\alpha$ $\alpha'$</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methylprotoporphyrin IX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Obtained chemically</td>
<td>407 505 541 575 603 529.5</td>
<td>410 539 578 –</td>
</tr>
<tr>
<td>(b) Produced by treatment</td>
<td>419 513 545 594 626 652</td>
<td>431 545 594 633</td>
</tr>
<tr>
<td>with DDC</td>
<td>419 513 545 594 626 653</td>
<td>431 547 596 633</td>
</tr>
<tr>
<td>Griseofulvin pigments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor</td>
<td>419 512 545 594 628 652</td>
<td>431 547 596 634</td>
</tr>
<tr>
<td>Major</td>
<td>417 510 544 597 626 656</td>
<td>434 545 596 635</td>
</tr>
</tbody>
</table>
Table 2. Absorption maxima of the Soret, \( \alpha \) and \( \beta \) bands of authentic Cu\(^{2+}\)-protoporphyrin and of the products of copper-catalysed dealkylation obtained from authentic N-methylprotoporphyrin and from minor and major griseofulvin pigments

For copper-catalysed dealkylation, 10 \( \mu l \) of a 0.1 % (w/v) methanolic solution of cupric acetate monohydrate were added to 0.5 ml of a chloroform solution of each pigment (containing 0.3-0.9 nmol of N-methylprotoporphyrin equivalents). The reaction was allowed to proceed at 36 °C for at least 3 h, when the absorption maxima of the products were determined. The electronic absorption spectra of the various pigments listed were all obtained with the dimethyl ester derivatives dissolved in chloroform.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret</td>
</tr>
<tr>
<td>Authentic Cu(^{2+}) protoporphyrin</td>
<td>406</td>
</tr>
<tr>
<td>Products of dealkylation obtained from:</td>
<td></td>
</tr>
<tr>
<td>(a) Authentic N-methylprotoporphyrin</td>
<td>405.5</td>
</tr>
<tr>
<td>(b) Minor griseofulvin pigment</td>
<td>405.8</td>
</tr>
<tr>
<td>(c) Major griseofulvin pigment</td>
<td>406</td>
</tr>
</tbody>
</table>
to one pyrrole nitrogen of protoporphyrin, as the value of 941 can be accounted for by the sum: [protoporphyrin dimethyl ester - 1 pyrrole hydrogen (589)] + [griseofulvin - 1 hydrogen (351)] + one additional proton.

When the molecular ion (MH)+ at m/z 941 was subjected to tandem m.s. (see Fig. 4c), the two daughter ions predicted from N-dealkylation were seen, namely a peak at m/z 591, representing the protoporphyrin dimethyl ester moiety (see also Jackson & Dearden, 1973; Smith & Farmer, 1982), and an ion at m/z 353, representing griseofulvin, the putative N-alkyl group. An unexpected daughter ion was also detected, however, at m/z 605. One possible interpretation for this finding is that, in the gas-collision cell of the tandem mass spectrometer, facile loss of a portion of griseofulvin occurs, with concomitant proton transfer to the porphyrin moiety. This process affords an ion which is isobaric with protonated N-methylprotoporphyrin.

A similar fragmentation pattern was obtained with the zinc complex of the major griseofulvin pigment. In positive-ion f.a.b. m.s. this gave a molecular ion at m/z 1003, as expected [major griseofulvin pigment - 2 pyrrole hydrogen atoms (938) + Zn (65) = 1003]. When the molecular ion at m/z 1003 was subjected to tandem m.s., an ion at m/z 653 was seen, corresponding to the zinc complex of protoporphyrin dimethyl ester, predicted from N-dealkylation [protoporphyrin dimethyl ester - 2 pyrrole hydrogen atoms (588) + Zn (65) = 653]; in addition, a prominent ion was observed at m/z 667, a value corresponding to the zinc complex of N-methylprotoporphyrin [N-methylprotoporphyrin dimethyl ester - 2 pyrrole hydrogen atoms (602) + Zn (65) = 667].

Therefore daughter ions isobaric with N-methylprotoporphyrin or with its zinc complex were observed on fragmentation of the major griseofulvin pigment, depending on whether the pigment itself or its zinc complex were taken for tandem m.s. These results may therefore suggest that the attachment of griseofulvin to the pyrrole nitrogen is through a carbon atom and raise the possibility that the minor griseofulvin pigment, now conclusively identified as N-methylprotoporphyrin, may likewise arise in vivo by secondary degradation of the major griseofulvin pigment. It should be stressed, however, that such a sequential mechanism for the formation of the N-methylprotoporphyrin is still hypothetical, as it is only based on the m.s. fragmentation data, which are indirect and not conclusive. At this stage, alternative pathways for the formation of the N-methyl and N-griseofulvin derivatives should also be considered.

Previous work has shown that drugs can give rise to hepatic N-alkylated porphyrins by one of two different mechanisms, both involving reactive metabolites produced by cytochrome P-450 in a self-catalysed suicidal inactivation reaction [see Marks et al. (1988) for a recent review]. Either an alkylating derivative of the whole drug (plus an oxygen atom) is produced by monooxygenation, or a small alkylating moiety is generated from the drug by oxidative fragmentation. The present results appear to indicate the operation of yet another alkylating mechanism, involving as an alkylating species the whole of the drug (griseofulvin) without prior requirement for oxygen insertion. There is as yet no direct evidence that the N-alkylated porphyrins we have now isolated after griseofulvin treatment originate from cytochrome P-450; this is, however, suggested by the finding that after feeding griseofulvin to mice a loss of cytochrome P-450 is observed in the liver, which is accompanied by a loss of microsomal haem, but not of microsomal cytochrome b5 (Wada et al., 1968; Denk et al., 1977; Williams & Simonet, 1986). Also, in recent experiments in which liver haem was prelabelled with radioactive 5-aminolaevulinate before administrating griseofulvin, both pigments were found to be radioactive, suggesting that they originate from pre-existing haem (F. De Matteis, A. H.Gibbs & R. Milek, unpublished work).

Griseofulvin is extensively metabolized by liver cytochrome P-450 through O-demethylation at one of its methoxy groups, all three methoxy groups participating, though to different extents (Chang et al., 1973; Lin et al., 1973; Zia et al., 1979). This and other types of hydroxylation reactions catalysed by cytochrome P-450 are thought to operate through two discrete steps (Groves, 1986; Ortiz de Montellano, 1989): first, a carbon-centred radical intermediate is formed by hydrogen abstraction from the drug substrate to the activated ferryl oxygen of cytochrome P-450; in
a subsequent step the carbon radical undergoes recombination with the ferryl oxygen, leading to insertion of a hydroxy function in the substrate. With some drugs, particularly those where conjugated radicals are formed and the unpaired electron is therefore delocalized over more than one carbon atom, the half-life of the radical may be long enough to be measurable, for example by secondary rearrangement and loss of stereochemistry (Ortiz de Montellano, 1989). If the radical intermediate of griseofulvin were sufficiently long-lived to diffuse away, in part, before being trapped by the iron-bound oxygen, it could react, to some extent, with one of the pyrrole nitrogen atoms of the prosthetic group and give rise to the N-alkyloxyphyrin. This mechanism would be compatible with the whole drug (without an additional oxygen atom) being bound to the pyrrole nitrogen of the major griseofulvin pigment. In the very large number of O-demethylation reactions and other types of hydroxylations catalysed by cytochrome P-450 there is no known precedent for N-alkylation of the haem prosthetic group by a radical intermediate of the drug undergoing metabolism. However, this does not necessarily exclude a radical-mediated mechanism in the case of griseofulvin, as this drug may possess unusual structural features which make its interaction with mouse liver cytochrome P-450 potentially suicidal. Alternatively, it is possible that some degree of N-alkylation of the haem of cytochrome P-450 during mono-oxygenation of drugs may be of more common occurrence than suspected, and that the N-alkylated porphyrins produced have not yet been detected.

REFERENCES


Received 6 June 1990/24 September 1990; accepted 16 October 1990