Studies on the Mechanism of Griseofulvin-Induced Protoporphyria

A thesis submitted in completion of the requirements for the degree of Doctor of Philosophy at Leicester University.

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Abstract

Griseofulvin induces protoporphyria in the mouse. The porphyria is caused by inhibition of ferrochelatase, the last enzyme of the liver haem biosynthetic pathway. Two N-alkylprotoporphyrins can be isolated from liver of griseofulvin-fed mice. One, N-methylprotoporphyrin, is a known ferrochelatase inhibitor. In this thesis NMR spectroscopy was used to determine the structure of the second adduct, N-GfPP, which is an N-monosubstituted protoporphyrin in which griseofulvin (minus a hydrogen atom) is attached to the N\(_2\) pyrrole of protoporphyrin via an -N-CH\(_2\)-O- linking group derived from either the 4- or 6-OCH\(_3\) group of the drug. A preliminary investigation into possible break-down products of N-GfPP is also described and suggestions made for the mechanism(s) of adduct formation.

Individual regioisomers of N-alkylprotoporphyrins with varying N-alkyl groups were synthesised and separated. \(^1\)H and \(^13\)C NMR techniques were then used to identify structural differences between planar porphyrins and the synthetic N-alkylporphyrins. A discussion has been made of the effect of porphyrin ring currents on proton and carbon resonances.

The purification of rat liver ferrochelatase is also described; however only small amounts of highly labile protein were obtained. Experiments devised to optimise the purification procedure also met with little success. Therefore, inhibition studies used cholate-solubilised ferrochelatase and synthetic N-alkylporphyrins. These studies confirm that the ferrochelatase inhibitory activity of an N-alkylporphyrin is dependent on the size and position of the N-alkyl substituent.

The \(^1\)H NMR study of synthetic N-alkylporphyrins has also been linked to the inhibition analysis. This shows that the majority of N-alkylporphyrin enzyme inhibitors studied have equivalent 6 and 7 propionate groups, whereas the majority of N-alkylporphyrin non-inhibitors have distinctly non-equivalent 6 and 7 propionate groups. A proportion of the N-alkylporphyrins studied do not fit either description, but are still capable of inhibiting ferrochelatase. Mechanisms for the interaction of ferrochelatase with these compounds are therefore described.
Declaration

Except where indicated, all the experiments shown in this thesis were done by myself in the Biological NMR Centre at Leicester University or at the MRC Toxicology Unit, Carshalton Surrey. One paper has been accepted for publication by the Biochemical Journal: Bellingham, R.M.A., Gibbs, A.H., Lian, L.Y., de Matteis, F. & Roberts, G.C.K. - 'Determination of the Structure of an N-Monosubstituted Protoporphyrin Isolated from the Livers of Griseofulvin-Fed Mice.'
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I would also like to thank Francesco de Matteis and Gordon Roberts for their supervision and the Medical Research Council for supplying a studentship.

Most especially I would like to thank Andrew West for keeping me sane and my family for not losing patience.
### Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AIA</td>
<td>allylisopropylacetamide</td>
</tr>
<tr>
<td>ALA</td>
<td>5-aminolaevulinic acid</td>
</tr>
<tr>
<td>ALA-S</td>
<td>aminolaevulinic acid synthase</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>chloroform</td>
</tr>
<tr>
<td>COPRO'gen</td>
<td>coproporphyrinogen</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>DDC</td>
<td>3,5-diethoxycarbonyl-1,4-dihydrocollidine</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycholate (sodium salt)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DQF</td>
<td>Double Quantum Filter</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier Transform</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HMB</td>
<td>hydroxymethylbilane</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Coherence</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>N-BuPP</td>
<td>N-1-butylprotoporphyrin</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>N-ethylprotoporphyrin</td>
</tr>
<tr>
<td>N-GIPPP</td>
<td>griseofulvin-protoporphyrin adduct</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>N-1-propylprotoporphyrin</td>
</tr>
<tr>
<td>nOe</td>
<td>nuclear Overhauser enhancement</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect Spectroscopy</td>
</tr>
<tr>
<td>PBG</td>
<td>porphobilinogen</td>
</tr>
<tr>
<td>PP or PPLX</td>
<td>protoporphyrin IX</td>
</tr>
<tr>
<td>PP'gen</td>
<td>protoporphyrinogen</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>TTMS</td>
<td>3-[(2-(2,4,6-trimethylphenyl)thio)ethyl]-4-methylsydnone</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume ratio</td>
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<td>w/v</td>
<td>weight to volume ratio</td>
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1. INTRODUCTION

1.1. The Liver Haem Biosynthetic Pathway

1.1.1. Haem - Structure and Nomenclature

Haem is integral to life. This particular organic molecule with an iron atom at its centre constitutes a vital component of the haemoproteins, a large family of proteins which play essential roles in oxygen transport (using haemoglobin and myoglobin), electron transfer (the mitochondrial cytochromes) and oxygen activation e.g. cytochrome P450, cytochrome oxidase and tryptophan pyrrolase. They also play integral parts in the activation and decomposition of hydrogen peroxide using peroxidases and catalases respectively. The importance of haem is such that organisms lacking the capability to synthesise haem within the cell will eventually die if exogenously administered haem is in short supply.

Protoporphyrin IX is the name given to the organic part of haem and is a tetrapyrrolic structure in which the four pyrrole rings are linked by methene bridges. The iron atom is in the centre of this ring and is coordinated to all four pyrrolic nitrogen atoms (see Figure 1.1).

The Fischer nomenclature used with porphyrins is also indicated in the figure; the four pyrrole rings are lettered A-D as are the corresponding ring nitrogens, for example, the nitrogen of pyrrole ring A is termed N_A. The methene bridge carbons are termed the meso positions and are lettered α, β, γ and δ respectively. The IUPAC numbering convention is also displayed in the figure.

The tetrapyrrolic structure may also have substituents around its periphery, on the β-positions of the pyrrole rings. These β-positions are numbered 1-8. In haem the substituents
Fig. 1.1. The structure and nomenclature of haem.

A-D identify the four pyrroles

$N_{A:D}$ designates which pyrrole ring a particular nitrogen belongs to

Nos. 1-8 define the positioning of substituents around the porphyrin periphery.

Nos. (1) - (20) = IUPAC numbering convention

$\alpha, \beta, \gamma, \delta$ define the meso positions
are four methyl groups at the 1, 3, 5 and 8 positions, two vinyl groups at the 2 and 4 positions and two propionate groups at the 6 and 7 positions. These substituents identify the porphyrin as protoporphyrin. Fifteen possible arrangements of these substituents around the periphery are possible, but only one arrangement (isomer IX) is found in biological systems. This particular arrangement of substituents is asymmetric, a point of fundamental importance in the ensuing discussion.

1.1.2. Formation of Haem From its Precursors

As mentioned, haem is synthesised by most cells and plays an important role in many processes. The work described in this thesis involves trying to understand disturbances of liver haem biosynthesis - haem synthesised in the liver is used mainly for the prosthetic group of the family of detoxifying enzymes called the cytochromes P450.

The elucidation of the pathway\(^1\) (for a review see Dailey, 1990), was initiated in 1945 when Shemin injected himself with doses of \([15\text{N}]\)-glycine and found from mass spectrometric studies that haem from haemoglobin isolated from his blood was \([15\text{N}]\)-enriched (Shemin & Rittenberg, 1945). This observation showed that glycine (I) is a haem precursor. Degradation of haem by chronic acid then gave equally-enriched haem fragments (Wittenberg & Shemin, 1949) that suggested that glycine is not only a precursor of haem but actually provides the basic framework of the haem molecule (Shemin & Wittenberg, 1950), a conclusion further supported by the use of \([2-\text{14C}]\)-glycine. Using this labelled precursor haem was found to be radioactive (Shemin et al, 1948) but, upon controlled degradation to maleimides, only half the radioactivity could be recovered - the rest could be assumed to have existed at the meso positions of the final haem porphyrin framework (Wittenberg &

\(^1\)Intermediates of the liver haem biosynthetic pathway mentioned in the following written discussion are also numbered - bracketed numbers in italics refer to the structures drawn in Fig. 1.2.
Shemin, 1950), an assumption finally proven by using doubly labelled (both $^{15}$N and $^{14}$C) glycine which was found to provide the eight carbon atoms and four nitrogen atoms forming the basic porphyrin framework of haem (Muir & Neuberger, 1950).

It was then shown that synthesis of protoporphyrin from $^{14}$C-carboxyl labelled succinate gave rise to haem labelled at 26 carbon atoms. From this activity pattern it was proposed that acetate was utilised in the pathway by being converted to an unsymmetrical 4-carbon-atom compound via the tri-carboxylic acid cycle (Shemin & Kumin, 1952), and so succinyl Co-A was proposed to be the second haem precursor.

From looking at ways in which the two molecules glycine and succinyl Co-A could possibly interact, 5-aminolaevulinic acid (ALA(5)) was soon proposed as the next intermediate in the pathway, proved quickly by the demonstration of the same labelling pattern in haem using $[^5-^{14}]$ALA as using $[^2-^{14}]$glycine (Shemin et al, 1955). The enzyme responsible for the condensation reaction of glycine and succinyl Co-A is called 5-aminolaevulinic acid synthase. It was first identified in 1958 (Kichuchi et al, 1958; Gibson et al, 1958) and has since been found to be rate-limiting in the overall pathway.

The complete pathway was elucidated over the next three decades and is shown in Figure 1.2. It starts in the matrix part of the mitochondrion where the amino acid glycine and succinyl Co-A condense under the influence of the enzyme 5-aminolaevulinate synthetase (ALA-synthetase) to form 5-aminolaevulinate (ALA).

ALA moves out of the mitochondrion into the cytosol. Here two molecules of ALA condense to form the monopyrrole porphobilinogen (PBG, (4)). The enzyme found to be responsible for
Fig 1.2. Intermediates of the Liver Haem Biosynthetic Pathway - refer to text for detail. The sequence of formation starts with Glycine (1) and Succinyl Co-A (2) and proceeds anti-clockwise around the page. Intermediates of events taking place in the mitochondrion are enclosed in a box, other events and intermediates are present in the cytosol. Abbreviations: Me = Methyl (CH₃), Vi = Vinyl (CH₂CH₃), Pr = Propionate (CH₃CH₂COO⁻), Ac = Acetic acid (CH₃COOH).
This reaction in vivo is called porphobilinogen synthase and has been isolated from a variety of sources, such as human erythrocytes (Anderson & Desnick, 1979) and bovine liver (Bevan et al., 1980). Four of these monopyrroles are then joined together, helped in their reaction by the enzyme porphobilinogen deaminase (Bogorad, 1958), which tetrapolymerises porphobilinogen into a linear hydroxymethylbilane (HMB (5)) which is then taken up by a second enzyme (called uroporphyrinogen III synthase, Bogorad, 1958) which undertakes the cyclisation and rearrangement of hydroxymethylbilane to form uroporphyrinogen III (URO'III, (6)). Decarboxylation of carboxymethyl groups to methyl groups is mediated by a cytoplasmic decarboxylase called uroporphyrinogen decarboxylase, which has been isolated from human erythrocytes (Elder et al., 1983; de Verneuil et al., 1983), chicken erythrocytes (Kawanishi et al., 1983) and bovine liver (Straka & Kushner, 1983). The enzymic reaction yields coproporphyrinogen III (COPRO'gen III (7), which is taken up into the mitochondrion. The next step involves modification of the two propionate groups at positions 2 and 4 to vinyl groups. The enzyme for this reaction is coproporphyrinogen oxidase (Yoshinaga & Sano, 1980) and the product is called protoporphyrinogen (PP'gen (8)). Protoporphyrinogen is then oxidised to protoporphyrin IX (PPIX (9)) by the enzyme protoporphyrinogen oxidase (Poulson & Polglase, 1975; Dailey & Karr, 1987).

The final step of liver haem biosynthesis is central to this thesis. The reaction involved is the insertion of ferrous iron into protoporphyrin to form haem (10). That this could happen was seen in crude enzyme preparations as long ago as 1956 (Goldberg et al., 1956) but it was not until 1981 that the enzyme (ferrochelatase) was first purified to homogeneity from rat liver (Taketani & Tokunaga, 1981).
1.1.3. Regulation of Haem Biosynthesis

It is known that methods of haem regulation are different in mammals and eukaryotes such as yeast. Also, in mammals there are differences between erythroid and hepatic (or non-erythroid) cell types. The disorders of haem biosynthesis relevant to this thesis are those induced in the liver and so regulation of haem in hepatic cells is discussed here. The regulation of haem biosynthesis in these cells is understood well enough for a model to be proposed, unlike the situation with erythroid cell types.

As with other metabolic pathways the level of intermediates in the liver haem biosynthetic pathway is highly regulated. Porphyrins and their precursors are all normally present in only small amounts in the liver. The rate-limiting enzyme of the pathway is aminolaevulinic acid synthetase (ALA-S, the first enzyme of the pathway). ALA-S also plays an important role in the regulation of haem synthesis in the liver. Two models are proposed for the regulation of haem biosynthesis (see Figure 1.3.). One model (reviewed by May et al, 1986), proposes that ALA-S expression is controlled solely by haem, whereas Hamilton et al (1988) propose that, whereas haem does play a role in controlling ALA-S expression, other factors (such as certain drugs) also are important in controlling ALA-S expression.

Integral to both proposals is a hypothetical pool of regulatory 'free' haem within the cell. Although indirect evidence has been obtained for its existence (Druyan & Kelly, 1972, Grandchamp et al, 1981), definitive experimental proof is still required. It is thought that the haem of this pool is in dynamic equilibrium with various haemoproteins and newly synthesised haem. Schematics of both models are shown in Figure 1.3.

In model a), the feed-back regulation of haem biosynthesis is thought to operate on a number of levels. One method proposed is a direct inhibition of ALA-S activity by haem.
a) Prosthetic haem of various haemoproteins
E.g. cytochrome P450

Hypothetical pool of regulatory 'free' haem

- Glycine
- Succinyl Co-A

ALAS (5-Aminolaevulinic Acid)

Steps

1. Repression of mRNA for ALAS
2. Inhibition of translocation of ALAS from cytosol to mitochondrion
3. Direct inhibition of ALAS by haem

Further details of experimental proofs are provided in the text.

Key

- Proposed movement of haem within the cell
- Proposed methods of repression of ALAS

b) Proposed methods of repression of ALAS-Synthetase

Fig. 1.3. Two models for the role of the regulatory pool of 'free' haem in regulating haem synthesis.

a) A model in which haem is the sole regulator of ALAS synthesis
b) A model (taken from Hamilton et al., 1991) in which haem is not the sole regulator of ALAS synthesis. Phenobarbital-like (PB) drugs can stimulate transcription of the ALAS gene as well as induce cytochrome P450 genes. Haem regulates ALAS mRNA expression by decreasing its half-life, and this effect can be blocked by cycloheximide (CX). Haem also regulates ALAS synthase enzyme expression by blocking translocation of ALAS precursor protein into the mitochondrion.
Scholnick et al (1972) proposed such an inhibition effect when they found that the ALA-S from a partially purified rat liver preparation was inhibited by deuterohaemin, with a $K_i$ of $2 \times 10^{-5}$M. Whiting and Elliot (1972), using a much purer rat liver preparation, were also able to show that haemin is an inhibitor of mitochondrial ALA-S at levels of 10µM. They proposed that the close proximity of haem and ferrochelatase in the mitochondrion might make end-product inhibition of ALA-S a controlling factor of haem biosynthesis in mammalian liver. Wolfson et al (1979) were however unable to find evidence for such a control mechanism. In experiments which simultaneously monitored ALA-S and ferrochelatase activity they were unable to detect inhibition of ALA-S activity as the rate of haem formation was increased, even when the rates of haem generation were 75 times those assumed to be occurring in vivo. It is thought unlikely therefore that such a control mechanism is of physiological importance.

The haem pool is also proposed to repress the synthesis of m-RNA for ALA-S at both the transcriptional and translational levels. This would reduce ALA-S production which, in turn, leads to decreased production of ALA. Srivastava et al (1980) were able to demonstrate that nanomolar concentrations of haem can effectively regulate ALA-S synthesis and that haem acts at the level of transcription. Yamamoto et al (1982) were also able to provide evidence for a transcriptional inhibition of ALA-S synthesis by haem. They found that haemin caused a decrease in the ability of liver polysomes to direct cell-free synthesis of ALA-S. This decrease in activity had a rate comparable with the half-life of ALA-S mRNA which suggested that haem was capable of inhibiting transcription of mRNA for ALA-S.

Evidence has also been provided for the repression of ALA-S by haem at the translational level. Sassa and Granick (1970) used the chick-embryo liver cell culture to show that the apparent half-life of ALA-S is decreased when both actinomycin D (an inhibitor of RNA synthesis) and haemin are added to the system, compared to the half-life when actinomycin
alone is added. This suggested that haemin inhibits ALA-S synthesis at the translational level. Tyrell and Marks (1972) were also able to show that haemin inhibited a post-transcriptional phase of ALA-S induction, which was interpreted as inhibition of a translational process. More recently, Yamamoto et al (1983) carried out experiments which showed that translation of mRNA in a rabbit reticulocyte lysate system was specifically inhibited by addition of haemin. As previous work in their laboratory had found that haemin also acted at a transcriptional level (Yamamoto et al, 1982), they concluded that synthesis of ALA-S in the liver is regulated by haem at both transcriptional and translational levels.

On another level haem has also been proposed to inhibit translocation of the large, inactive precursor form of ALA-S (which is made in the cytosol) into the mitochondrion, the place where it is physiologically active. This effect was noted by Yamuchi and co-workers (1980) who used [3H]-leucine tracer experiments to show that haemin caused accumulation of a cytosolic form of ALA-S at the expense of the physiologically active mitochondrial ALA-S. It was concluded that the cytosolic form of ALA-S was a precursor in transit to the mitochondrion, and that haemin actually inhibits the intracellular translocation of ALA-S. The physiological validity of the proposal was tested using tryptophan pyrrolase activities as a marker for haem saturation in the rat liver (Yamamoto et al, 1981). It was found that the intracellular translocation of ALA-S was inhibited by concentrations of intracellular haem which were lower than those required to achieve a full saturation of tryptophan pyrrolase. Haem also inhibited the synthesis of ALA-S at these levels and so it was concluded that haem is capable of inhibiting ALA-S synthesis and translocation, and that these methods of inhibition are both capable of contributing to the feed-back regulation of haem biosynthesis under physiological conditions. It has also been proposed that haemin blocks the maturation of the pre-cursor form of ALA-S (Ades, 1983). Further work (Ades et al, 1983) showed that this particular inhibition by haemin was concentration dependent and also that the precursor form of ALA-S turned over with a half-life of thirty minutes. They proposed that,
in the presence of haemin, ALA-S is synthesised but is then degraded before becoming functionally active. In a 1987 paper Ades et al propose that post-transcriptional regulation of ALA-S may be entirely due to this process, and that no effects occur at the translational level.

In this model, drugs acting to alter the rates of haem biosynthesis (see next section) do so only by causing the haem pool to be depleted such that the repression and inhibition effects of haem described above are derepressed and ALA-S synthesis is induced. Haem is proposed to be the sole regulator of ALA-S activity in this particular model. A slightly different model has been proposed by others, in which the drugs themselves are able to initiate ALA-S synthesis. Haem than acts as a secondary regulator of the drug-induced ALA-S activity. Experimental proof for this model has been provided by Hamilton et al, 1988. They found that inhibition of protein synthesis by cycloheximide did not prevent induction of ALA-S mRNA by drugs and also that inhibitors of haem biosynthesis, when administered alone, caused only small increases in ALA-S mRNA compared to large increases caused following the administration of drugs alone. They also found that ALA-S mRNA levels could be increased without parallel in enzyme activity and vice versa. Drew and Ades (1989) went on to show that haem decreases the stability of ALA-S mRNA in hepatocytes and then (Ryan & Ades, 1991) that a short-lived protein may be involved in the degradation of ALA-S mRNA. Hamilton et al confirmed these findings in 1991 and proposed a model for the regulation of hepatic ALA-S expression shown in part b) of Figure 1.3. In this model haem regulates ALA-S primarily at the site of translocation whereas inducing drugs act at the transcriptional level to increase ALA-S mRNA.
1.1.4. Disturbances of Liver Haem Biosynthesis

Under normal conditions ALA-S is the only rate-limiting enzyme and intermediates (porphyrins and their precursors) accumulate or are excreted (mostly in the urine) in very small amounts. Conditions can occur, however, where this regulatory mechanism breaks down and intermediates accumulate to excess. Disorders of porphyrin metabolism in animals or man are called porphyrias and can occur either naturally (due to genetic defects (Whitcombe et al.; 1991, Straka et al., 1991)) or can be induced experimentally or exacerbated on administration of certain drugs (Marks et al., 1988). Each type of porphyria is the result of a specific decrease in the activity of one of the enzymes of liver haem biosynthesis. This can result in the affected enzyme becoming rate-limiting which will cause accumulation of the specific intermediate associated with the incorrectly functioning enzyme. Thus each porphyria is characterised by a particular pattern of tissue accumulation and excretion of porphyrins and their precursors. A correct identification of this pattern will lead to a clinical diagnosis.

It was proposed in 1966 (Granick, 1966) that drugs which caused disturbances of liver haem biosynthesis act by interfering with the 'specific' repressor function of haem, but it is only more recently that the actual mechanisms of the drug disturbances are being defined.
1.2. Inherited Human Porphyrias

1.2.1. Classification

Investigatory work into the porphyrias began in the late 19th century and evolved alongside the elucidation of the chemistry and biochemistry of the porphyrins. The first reported case of drug-induced porphyria (Harley, 1890) involved a woman treated with the new hypnotic Sulfonal who developed a blood-red-coloured urine and eventually died. The first formal classification of the porphyrias came in the early 20th century (Gunther, 1911) but was amended by Waldenstrom in 1957 to indicate the main site of accumulation of porphyrins or precursors (Waldenstrom, 1957). The main sites of production are either in the liver (hepatic) or in the bone-marrow (erythropoietic) as described more fully below.

1.2.2. Hepatic Porphyrias

Though the division of the porphyrias into hepatic and erythropoietic is a good starting point for classification, a further subdivision is needed for the hepatic porphyrias, into acute or non-acute forms. An acute attack results in abdominal pain and constipation and can also be associated with psychiatric problems such as anxiety, depression, disorientation, confusion and delirium as noticed by Waldenstrom (Waldenstrom, 1957). A non-acute porphyria will result in photosensitivity such that skin lesions appear in the areas exposed to light.

There are many types of hepatic porphyria. The acute hepatic porphyrias are Acute Intermittent Porphyria, Hereditary Coproporphyria, Variegate Porphyria and Doss Porphyria. The non-acute forms are Porphyria Cutanea Tarda and Hepatoerythropoietic Porphyria. Doss Porphyria has only more recently been identified.
and Hepatoerythropoietic Porphyria is very rare and so neither variety will be discussed further here.

Table 1.1 outlines the porphyrins and precursors present in the urine and faeces from patients with the acute hepatic porphyrias Acute Intermittent Porphyria, Hereditary Coproporphyria and Variegate Porphyria. It clearly demonstrates how specific patterns of porphyrins and precursors are diagnostic of a particular porphyria and also highlights the more severe disorder incurred in an acute attack. For example Hereditary Coproporphyria should be diagnosed from the large excess of coproporphyrin over protoporphyrin in a sufferer's stool sample.

Table 1.1. The biochemical features of the acute hepatic porphyrias (taken from Dailey, 1990). U = Uroporphyrin, C = Coproporphyrin, P = Protoporphyrin and PRE = Precursors.

<table>
<thead>
<tr>
<th>Porphyria</th>
<th>Attack or remission</th>
<th>URINE</th>
<th>FAECES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PRE</td>
<td>U</td>
</tr>
<tr>
<td>Acute Intermittent</td>
<td>Attack</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Porphyria</td>
<td>Remission</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Hereditary</td>
<td>Attack</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Coproporphyria</td>
<td>Remission</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Variegate</td>
<td>Attack</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Porphyria</td>
<td>Remission</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

This sort of early diagnosis can be important, as some subjects with latent or clinically expressed acute porphyrias may be precipitated into an acute attack by administration of certain drugs (see section on drug-induced porphyrias), a situation which can be avoided if the porphyria has been correctly diagnosed.
Porphyria Cutanea Tarda is the most common form of porphyria. It causes photosensitivity but acute attacks of this porphyria type are never observed. The disease may be Sporadic (usually observed in male patients with no family history of the disease) or Familial (where both sexes are affected from the onset of puberty). Uroporphyrinogen decarboxylase activity is affected in both the sporadic and familial types, but in sporadic porphyria cutanea tarda only the liver enzyme's activity is decreased (Moore et al, 1987).

The enzyme deficiencies associated with the hepatic porphyrias and also the major porphyrin or precursor which accumulates to excess are identified in Table 1.2.

Table 1.2. The hepatic porphyrias and their associated enzyme defects (after Cole & Marks, 1984).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Major Porphyrin</th>
<th>Enzyme Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Intermittent</td>
<td>porphobilinogen</td>
<td>porphobiligen</td>
</tr>
<tr>
<td>porphoria</td>
<td>δ-aminolaevulinic acid (ALA)</td>
<td>deaminase</td>
</tr>
<tr>
<td>Porphyria Cutanea</td>
<td>uroporphyrin</td>
<td>uroporphyrinogen</td>
</tr>
<tr>
<td>Tarda</td>
<td>(URO III)</td>
<td>decarboxylase</td>
</tr>
<tr>
<td>Coproporphyria</td>
<td>coproporphyrin</td>
<td>coproporphyrinogen</td>
</tr>
<tr>
<td>(COPRO III)</td>
<td></td>
<td>oxidase</td>
</tr>
<tr>
<td>Variegate Porphyrria</td>
<td>protoporphyrin</td>
<td>protoporphyrinogen</td>
</tr>
<tr>
<td>(PPIX)</td>
<td></td>
<td>oxidase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ferrochelatase</td>
</tr>
</tbody>
</table>

1.2.3. Erythropoietic Porphyrias

The second class of porphyrias is the erythropoietic porphyrias, which are clinically manifested by solar photosensitivity and the absence of acute attacks. Two forms have been documented, Congenital Erythropoietic Porphyria and Erythropoietic Protoporphyria.
The enzyme deficiencies associated with the erythropoietic porphyrias and also the major porphyrin or precursor which accumulates to excess are identified in Table 1.3.

Table 1.3. The erythropoietic porphyrias and their associated enzyme defects (compiled from Dailey, 1990).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Major Porphyrin Intermediate Excreted</th>
<th>Enzyme Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital</td>
<td>uroporphyrin</td>
<td>uroporphyrinogen</td>
</tr>
<tr>
<td>Erythropoietic Porphyria</td>
<td>(URO' I)</td>
<td>(III) cosynthetase</td>
</tr>
<tr>
<td>Erythropoietic Protoporphyria</td>
<td>protoporphyrin</td>
<td>ferrochelatase</td>
</tr>
</tbody>
</table>

Congenital Erythropoietic Porphyria is extremely rare, but was one of the first porphyrias to be reported in the literature, in 1874 (Schultz, 1874). This is no doubt due to the conspicuous appearance of afflicted individuals with severely mutilated ears, nose cartilage and fingers caused by severe skin photosensitisation complicated by inflammation and infection. Sufferers also develop mis-coloured teeth and hypertrichosis. Fortunately early diagnosis allows steps to be taken to prevent secondary infections and therefore severe scarring.

This form of porphyria is associated with an enzyme defect in uroporphyrinogen III synthase (Levin, 1968; Romeo & Levin, 1969), leading to a diversion of the haem biosynthetic pathway. The sequence of hydroxymethylbilane -> haem becomes hydroxymethylbilane-> uroporphyrinogen I. Uroporphyrinogen I converts spontaneously to coproporphyrinogen I which is the wrong isomer of coproporphyrinogen. This isomer cannot be subsequently utilised by succeeding enzymes of the pathway, consequently the disease state is incurred.
Erythropoietic Protoporphyria is the porphyria directly related to the work described in this thesis. It is a porphyria involving a deficiency of the mitochondrial enzyme ferrochelatase which is the enzyme responsible for inserting ferrous iron into protoporphyrin to form haem. In patients with erythropoietic protoporphyria ferrochelatase activity is 17% that of normal (Straka et al, 1991), although expected amounts of immunoreactive protein are present. This finding suggested a point mutation in the gene encoding for ferrochelatase.

In a study of afflicted families in the Netherlands (Went & Klasen, 1984) an autosomal-recessive mode of inheritance in conjunction with a 3-allele system to account for the variable penetrance of the disease had already been suggested. Since then the genetic defect present in erythropoietic protoporphyria has been mapped to chromosome 18q22 (Whitcombe et al, 1991), where a dinucleotide repeat polymorphism has been reported which may be instrumental in causing the enzyme defect (Whitcombe et al, 1993). In a different study of 6 affected families in Northern Ireland, a single base pair deletion (40 del G) was found in exon 1 of the ferrochelatase gene in three of the families studied (Todd et al, 1993). Many other molecular defects have, however, also been reported (Nakahashi et al, 1992, 1993(a), 1993(b)).

Erythropoietic protoporphyria is characterised biochemically by elevated levels of protoporphyrin in erythrocytes and blood plasma, as expected since ferrochelatase is the malfunctioning enzyme. Often (but not always) fecal protoporphyrin is increased whereas the urinary porphyrin excretion pattern is as normal (Poh-Fitzpatrick, 1986).

The clinical features of the disease in an affected individual are painful burning sensations on short exposure to sunlight, followed by edema and erythema (Magnus et al, 1961). As the patient ages, the skin may take a roughened weather-beaten look caused by the development of a hyperkeratotic, thick skin. The disease also has a hepatic aspect, and though overall, the
disease is generally benign, in very rare cases fatal liver disease with cirrhosis may develop (for example Donaldson et al, 1971). In such cases crystals of protoporphyrin IX have been found in the liver in sufficient concentrations to cause hepatic damage (Bloomer, 1982). Porphyrin-containing gallstones are also formed which causes the hepatic accumulation of protoporphyrin as biliary excretion is blocked.

An experimental model with biochemical characteristics similar to those of erythropoietic protoporphyria can be induced in the livers of rodents dosed with certain porphyrinogenic agents (For a review see Marks et al, 1988). In fact, a griseofulvin-induced mouse model for protoporphyria has been described which displays liver pathology and protoporphyrin crystals identical to those observed in the human disease (Gschnait et al, 1975). The in vivo models have contributed much to the understanding of the methods of regulation of haem biosynthesis in the liver and also to the elucidation of the molecular mechanisms involved in drug-induced porphyrias. This field of research is vital to the work presented in this thesis and so is described more fully in the section on drug-induced porphyrias (1.3.)
1.3. Drug Induced Porphyrias

1.3.1. Introduction

Hepatic porphyrias can be induced in experimental animals on administration of certain drugs. Of these drugs only a few such as allylisopropylacetamide (AIA) and other allyl-containing compounds, 3,5 diethoxycarbonyl-1,4-dihydrocollidine (DDC) and griseofulvin, are capable of inducing porphyria in the normal animal or human (Figure 1.4). Many others are capable of exacerbating porphyria in individuals having a genetic predisposition to this metabolic disorder.

The action of such drugs is to call for an increased demand for haem synthesis in the liver, which, in turn, causes an increase of ALA-S activity so that the enzyme deficiency in a particular porphyria becomes rate-limiting and porphyrin overproduction occurs capable of precipitating an acute attack. It may also be that the drugs themselves are capable of inducing ALA-S activity. One example of a group of drugs requiring increased haem synthesis is the barbiturates which induce a group of detoxifying enzymes with haem as their prosthetic group, the cytochromes P₄₅₀. On introduction of the barbiturates into medicine in 1903 an acute porphyric attack was soon documented, in this case precipitated by diethylbarbituric acid (Dobrschansky, 1906). Today lists of drugs which are known to be active in precipitating acute attacks in either the normal individual or patients with genetic enzyme defects are readily available for consultation prior to treatment.

Model porphyrias induced by drugs have been much used in studying and understanding liver haem biosynthesis and related disorders, as, depending on which active drug is administered, a different intermediate of the pathway accumulates and is excreted. With DDC and griseofulvin protoporphyrin accumulates and is excreted in excess in a condition known as Protoporphyria (for a review see Cole & Marks, 1984). With AIA and allyl
**Compound capable of Inducing Hepatic Porphyria in Rodents**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inactive analogue</th>
<th>'Green Pigment’ formed on drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethene</td>
<td>Unknown</td>
<td>N-2-Hydroxyethyl-Protoporphyrin IX (Ortiz de Montellano et al, 1981)</td>
</tr>
<tr>
<td>2-Allyl-2-isopropylacetamide (AIA)</td>
<td>2-Propyl-2-isopropyl acetamide (PIA)</td>
<td>N-Lactone Protoporphyrin IX (Ortiz de Montellano et al, 1979, 1983)</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>HET-Griseofulvin</td>
<td>Unidentified N-alkyl Porphyrin (de Matteis, 1966 Holley et al, 1991)</td>
</tr>
</tbody>
</table>

*Fig. 1.4. Diagram showing compounds capable of inducing a hepatic porphyria in rodents (left-hand column), inactive analogues of these same drugs (middle column) and the type of porphyrin that has been identified and isolated in each case (end column).*
containing compounds a mixture of different porphyrins accumulates (see de Matteis, 1978). The different porphyrin profiles caused by different drugs is dependent on the structure of the administered drug, but as of yet, no definitive structure/function relationships have been described.

1.3.2 'Green Pigments'

Apart from the accumulation of porphyrins inherent in drug-induced porphyrias, another feature of these conditions is the formation of 'green pigments' which can be isolated from the livers of rodents treated with ethene, AIA, griseofulvin or DDC. These pigments have been identified as N-alkylporphyrins which arise from the interaction between administered drug and the prosthetic haem of cytochrome P450 (see later).

1.3.3. Cytochromes P450 and Drug Metabolism

The cytochromes P450 are an important family of related haemoproteins which undertake the metabolism of numerous physiological substrates such as steroids, fatty acids, bile acids and cytokines. They are also capable of metabolizing a wide range of foreign chemicals such as drugs, environmental pollutants and natural products.

All members of the group have haem as the active prosthetic group, held in place by a linkage from a cysteine residue of the main protein. The haem iron lies in the centre of the porphyrin nucleus, four of its coordination valencies being occupied by the pyrrole nitrogen atoms in a plane which separates the fifth coordination site (the linkage to cysteine) and the sixth coordination site which is available for binding (de Matteis, 1987).

The cytochromes P450 can act as oxidases or reductases, but their most normal role is that of mono-oxygenase. This involves the incorporation of one oxygen atom into a substrate (AH) whilst the other oxygen atom is reduced to water with the intervention of a two-electron donor (DH2). This reaction is illustrated by the equation:
\[ \text{AH} + \text{DH}_2 + \text{O}_2 \rightarrow \text{AOH} + \text{D} + \text{H}_2\text{O} \]

In some drug-induced porphyrias it has been shown that the cytochromes P₄₅₀ interact with drugs such as ethene and DDC resulting in suicide inactivation of the cytochrome (for a review of suicide substrates of cytochrome P₄₅₀ see Ortiz de Montellano, ed., 1986 and detailed discussion in chapter 2).

Suicide inactivation of cytochrome P₄₅₀ occurs when a metabolite formed is so reactive that it seeks out and binds nucleophilic sites in the cytochrome itself. Less reactive metabolites diffuse away from the cytochrome and are either further metabolised e.g. by glutathione transferase or bind at some site far from the cytochrome. With the drugs under discussion here it is the haem prosthetic group that is modified by the reactive metabolites formed, and this modification has a marked effect on liver haem biosynthesis.

It has been proposed that the haem prosthetic group of cytochrome P₄₅₀ is in dynamic equilibrium with the pool (or pools) of regulatory free haem present in the liver (see 1.1.3.). Therefore any haem product derived from cytochrome P₄₅₀, once made, will leave the cytochrome which will be replenished with fresh haem. The haem pool is depleted and its negative feed-back effect diminished so that ALA-S activity is stimulated leading to increased production of porphyrins and their precursors. It may also be that cytochrome P₄₅₀-inducing drugs are capable of directly inducing ALA-S activity, which would also lead to increased porphyrin production. Either way, the biochemical picture is a hepatic porphyria.
1.3.4. DDC-Induced Protoporphyria

Administration of 3,5 diethoxycarbonyl-1,4-dihydrocollidine (DDC) to rodents causes protoporphyria (Oniswa and Labbe, 1963), suggesting that the last step of the liver haem biosynthetic pathway is blocked by inhibition or deficiency of ferrochelatase. Protoporphyrin accumulates in such large amounts that both blood plasma and erythrocytes become intensely fluorescent. Solid casts of protoporphyrin pigment can be found in the liver and pigmented stones in the bladder which can cause complications such as jaundice, photosensitivity and biliary cirrhosis after prolonged intoxication. These are symptoms of human erythropoietic protoporphyria, an inherited condition in which a defect in ferrochelatase is present as a genetic trait (see 1.2.3.). DDC-induced porphyria is therefore an experimental model for trying to understand how porphyrias come about.

It was soon found that the ferrochelatase inhibition observed in DDC-treated rodents could not be a direct inhibition of the enzyme as addition of DDC in vitro does not produce an inhibitory effect. The in vivo effect of DDC treatment was soon linked to a concomitant loss of cytochrome P₄₅₀ (Wada et al, 1968), substantiating the idea that the green pigment formed in the livers of treated rodents could result from the modification of the prosthetic haem of cytochrome P₄₅₀, as it displayed properties similar to N-alkyl porphyrins (Tephley et al, 1979). This porphyrin was capable of inhibiting ferrochelatase and was identified as N-methylprotoporphyrin (N-MePP, Tephley et al, 1981). The green pigment obtained with DDC treatment was also fully characterised and identified as N-methylprotoporphyrin using nuclear magnetic resonance (NMR) techniques and partially deuterated synthetic N-methylprotoporphyrin, (Ortiz de Montellano et al, 1981(b)). The work carried out to ascertain this is described in chapter 2, as the methods employed are relevant to work done characterising a green pigment derived from griseofulvin which is also described in this thesis (see chapter 4).
Work with analogues of DDC such as 4-desmethyl DDC (Figure 1.4.) gave additional evidence that the N-methyl group of the inhibitor originates from the 4-alkyl group of DDC, as the 4-desmethyl analogue of DDC does not induce porphyria. Other work, however, showed that 4-Ethyl DDC does induce porphyria and also causes formation of an N-ethylprotoporphyrin inhibitory green pigment (Cole et al, 1980, de Matteis et al, 1981).

In this type of porphyria cytochrome P<sub>450</sub> acts as an oxidase. One electron transfer occurs from the drug to haem during the oxidative aromatisation of the dihydropyridine molecule. If the methyl radical is formed, it then attacks the haem group of the cytochrome and a targeted inhibitor of ferrochelatase is formed (Loev & Snader, 1965, Augusto et al, 1982). Lack of haem causes ALA-S stimulation and ferrochelatase inhibition causes a block in haem synthesis. Both these factors contribute to the vast accumulation of protoporphyrin seen in DDC-induced porphyria.

1.3.5. AIA and Green Pigments

The phenomenon of accumulation of green pigments in the livers of rodents is also observed with the drug allylisopropylacetamide (AIA) where the induced porphyria has a biochemical profile of general porphyrin accumulation (de Matteis & Cantoni, 1979). Studies with ethylene, which is even more active that AIA but less likely to fragment in mass spectrometric studies, showed that the drug interacts with the haem prosthetic group of cytochrome P<sub>450</sub> (Ortiz de Montellano et al, 1980 (a) and (b), 1981 (c)). In this instance cytochrome P<sub>450</sub> is acting in its more usual role as a mono-oxygenase. The drug is still acting as a suicide substrate but in this case an oxygen atom is inserted to give a N-2-hydroxyethyl-protoporphyrin rather than a whole alkyl group being transferred without oxygen insertion, as is the case with DDC.
1.3.6. Griseofulvin and Green Pigments

The anti-fungal agent griseofulvin also causes protoporphyria (de Matteis & Gibbs, 1975). Two green pigments are found in the livers of griseofulvin-treated rodents (Holley et al, 1991). One (the minor product) has been identified as N-methylprotoporphyrin, the targeted inhibitor of ferrochelatase found on DDC treatment. The other (major product) has been less fully characterised but is proposed, on the basis of mass spectrometric studies, to be an N-alkylporphyrin consisting of protoporphyrin with the whole of griseofulvin (minus a hydrogen atom) attached to one of the pyrrole ring nitrogens. The determination of the structure of the major product (abbreviated as N-GPfPP) forms part of this thesis and is described in chapter 4.
2. THE STRUCTURE AND CHEMISTRY OF N-ALKYLPORPHYRINS AND RELATED COMPOUNDS.

2.1. General Features

2.1.1. Nomenclature and Stereochemistry of N-alkylporphyrins

N-alkylporphyrins are porphyrins with an alkyl group of some sort, for example a methyl group, attached to one of the pyrrole ring nitrogens of the porphyrin ring (see Figure 2.1.). Although di- and tri N-alkylporphyrins are known to exist, only the N-monoalkylated porphyrins will be considered here. There are four possible regioisomers of an N-alkyl porphyrin depending on which pyrrole nitrogen is substituted eg. $N_A$ denotes that the substituent is attached to the pyrrole nitrogen of ring A of the porphyrin.

It must also be considered that, each regioisomer i.e. $N_A$, $N_B$, $N_C$ or $N_D$ of an asymmetric N-alkylporphyrin exists as two corresponding enantiomers (see Figure 2.2.). This arises from the fact that the two faces of an asymmetric porphyrin such as protoporphyrin are not superimposable and so two enantiomers of certain N-alkylporphyrins can be formed, depending on whether the alkyl group attaches itself above, or below, the plane of the porphyrin ring.

Porphyrins with differing peripheral substituents have also been used for example in ferrochelatase binding studies to gain insight into the structure and mode of binding at the ferrochelatase active site. Most commonly used are mesoporphyrin and deuteroporphyrin - in mesoporphyrin the vinyl groups of protoporphyrin are replaced with ethyl groups and in deuteroporphyrin they are replaced with hydrogen atoms.
Fig. 2.1. The four possible regioisomers of an N-alkyl protoporphyrin (R=alkyl).

Fig. 2.2. The two enantiomers of an N\textsubscript{A}-alkyl protoporphyrin. The enantiomer formed depends on whether the alkyl group attaches above or below the porphyrin plane.
It is important to realise that the regioisomer and stereochemistry of a particular porphyrin must be taken into account when trying to understand the interaction of a drug with the haem of cytochrome P₄₅₀, or the inhibition of ferrochelatase. In the case of the latter effect, the type of porphyrin (e.g. whether protoporphyrin, mesoporphyrin or deuteroporphyrin is used), is also important.

2.1.2. Aromaticity

One of the outstanding features of the porphyrins is their highly conjugated aromatic macrocycle. In fact, when the structure was first proposed by Kuster in 1912 (Kuster, 1912), it was at first dismissed by Hans Fischer and other leaders in the porphyrin field, because it was thought that such a large molecule would be too unstable. There are 22\(\pi\) electrons within the tetapyrrolic structure, but only 18 of these are used in any one delocalization pathway, complying with Huckel's 4n+2 rule for aromaticity.

Further evidence for the aromaticity of the macrocycle stems from X-ray crystallographic data and Nuclear Magnetic Resonance (NMR) spectroscopy (see sections on these techniques). X-ray data have shown that non-alkylated metal-free and metalloporphyrins are planar (a pre-requisite for aromaticity) and NMR spectra of the porphyrins show all the characteristics of strong shielding and deshielding that a macrocycle with aromatic character (and therefore a strong secondary magnetic field) should induce.

Aromaticity is still evident upon N-alkylation of a porphyrin. X-ray crystallographic data indicates that distortion of the normally planar porphyrin macrocycle is incurred, but NMR spectra still display strong shielding and deshielding effects which are consistent with aromaticity of the macrocycle, despite distortion.
2.1.3. Acid-Base Equilibria

The acid-base equilibria of an N-alkyl and a non-alkylated porphyrin as designated by Phillips (Phillips, 1960) are displayed in Figure 2.3. and show the relationship between free-base, mono-protonated and di-protonated forms.

The acidic equilibria involve the dissociation of protons from pyrrole-type nitrogens and the basic equilibria are associated with the addition of protons to the pyrroleninic nitrogens. Formally pyrrole and pyrroleninic nitrogen cannot be distinguished due to the different tautomeric forms that the porphyrin exists in.

Compared to their parent compounds, N-alkyl porphyrins have been found to be much more strongly basic (Neuberger & Scott, 1952). Monocation formation is rarely observed with the parent compounds, but with N-alkyl compounds the addition of tri-fluoracetic acid (TFA) renders the monocation relatively easily, with subsequent formation of the di-cation being more difficult to observe.

Importantly, however, the monocatonic form of an N-alkyl porphyrin has a marked effect on residual free-base (Jackson & Drearden, 1973). In NMR studies, chemical shifts values of the mono-cationic form differ from chemical shift values of the free-base. Therefore a conscious effort has been made with the work described in this thesis to ensure that a particular form of a porphyrin is, in fact, under review, as it is vital to know which species one is working with to draw any conclusions from effects seen.
Fig. 2.3. Acid-base equilibria of non-N-alkylated (upper) and N-alkylated (lower, \( R = \text{alkyl} \)) porphyrins. Three forms are shown (a) free-base, (b) mono-cation and (c) di-cation.
2.1.4. Formation of Metal Complexes

In a metalloporphyrin, lone pairs on the central nitrogen atoms of the porphyrin are shared with a metal ion acting as a Lewis acid. The metal ion is also capable of displacing hydrogen atoms on pyrrole-type nitrogens so that it can make use of yet another two lone pairs. An elementary equation to describe metalation of a non-alkylated porphyrin is:

\[ \text{P(H}_2\text{)} + 2\text{H}^+ \rightarrow (\text{P})^{2-} + \text{M}^{2+} \rightarrow \text{M(P)} \]

This may be viewed simplistically as a porphyrin dianion \((\text{P})^{2-}\) (formed on removal of 2 hydrogen atoms) accepting a doubly positively-charged metal ion \((\text{M}^{2+})\) at its centre to form the metal complex \((\text{M(P)})\). This process is called metalation and may be reversed on addition of acid in a process called demetalation (in Smith, ed. 1975).

As with the acid-base equilibria, differences are seen between the behaviours of non-alkylated and N-alkylporphyrins. N-alkylporphyrins form metal complexes more easily than their parent compounds; this is thought to be due, in part, to their 'pre-deformed' structure which is already more similar to the structure of a metal complex than that of a planar non-alkylated porphyrin (Khrosropour & Hambright, 1972). Also, the N-alkyl porphyrins are more polar.

When a metal complex forms, the associated ligands of the metal ion (those of the solvent) are given up in preference for coordination with the lone pairs of the central nitrogen atoms of the porphyrin ring. Integral to this event taking place is an 'outer sphere' complexation where the metal ion and its associated ligands come into close vicinity of the porphyrin to facilitate the ligand exchange. A polar N-alkylporphyrin is more able to optimise favourable
interactions with the positive metal ion in this situation, and so eases metal complex formation.

A final difference between non-alkylated and N-alkylporphyrins is the number of protons dissociated during metalation. Non-alkylated porphyrins act as divalent anions as already mentioned. N-alkylporphyrins act as monovalent anions, hence only one proton is dissociated during metalation of an N-alkyl porphyrin and two protons are released during non-alkylated porphyrin metalation (in Lavallee ed., 1987).

2.1.5. Stability

The porphyrins used in this study are the free-base and metal-complex forms of N-alkylprotoporphyrins or protoporphyrin itself. Protoporphyrin has carboxylic acid groups at positions 6 and 7 of the porphyrin periphery which will ionise simultaneously with the nuclear nitrogen atoms, which has consequences for the chromatographic properties of the porphyrin. This complication is obviated, however, by using the methyl ester form of the porphyrin, protoporphyrin di-methyl ester (in Smith ed. 1975).

Protoporphyrin also has vinyl groups at position 2 and 4. Great care must be taken to ensure against undue exposure to light, which can cause rapid photoxidation to green photoproducts. Particular care must also be taken when using chloroform as a solvent, as a light-activated reaction can cause formation of phosgene (Cl₂CO) which may then attack the porphyrin's vinyl groups causing degradation.

Spontaneous N-dealkylation must also be watched-out for; the N-substituent of an N-alkylporphyrin can be dissociated in the presence of nucleophiles and this dissociation is more facile after a metal complex has been formed. The increased facility of N-dealkylation
of metal-complexed porphyrins actually forms the basis of a mass spectrometric technique for characterising the N-alkyl substituent of unknown N-monoalkylated porphyrins (Gibbs et al., 1990).

The porphyrin nucleus is, however, stable to concentrated sulphuric acid and neat tri-fluoracetic acid. As solvents, these acids are very useful for removing coordinated metal ions and reducing aggregation interactions (in Smith ed. 1975).

2.2 Electronic Absorption Spectra

The highly-conjugated nature of the porphyrins lend themselves to spectral analysis, a technique which is very useful in distinguishing between porphyrin types. For example, protoporphyrin IX has an aetio-type spectrum over the wavelength range 360-700nm, due to the fact that it has six peripheral positions of alkyl or carboxylic acid groups. The typical aetio-type spectrum for free-base protoporphyrin is displayed in Figure 2.4, and has four pronounced bands in the visible region (500-700nm) as well as the main Soret band at a lower wavelength. The Soret band absorbs intensely and so is used to define molar absorptivities which are then used in quantifying porphyrins.

Compared to a free-base non-alkylated porphyrin, the absorption bands of a free-base N-alkylporphyrin show shifts to the red. The spectra of mono-protonated and di-protonated forms of both alkylated and non-alkylated porphyrins show fewer absorption maxima when compared to their free-base spectra, but the molar absorptivities of the Soret in all forms are comparable.

The metal complexes of N-alkylated porphyrins also display spectra with marked differences to the spectra of the non-alkylated metalloporphyrins. A non-alkylated metalloporphyrin will
Fig. 2.4. The electronic absorption spectra of porphyrins. The upper half compares the Soret bands of the free-base and zinc-complexed forms of a non-alkylated porphyrin (protoporphyrin, PPIX), with the Soret band of a N-alkylated porphyrin (N-methylprotoporphyrin, N-MePP). The lower half compares the spectra (enlarged) displayed by these same porphyrins in the visible region of the spectrum. Differences between the zinc-complexed and free-base forms of the porphyrins and also the non-alkylated and alkylated porphyrins themselves are clearly apparent and are detailed in the text.
have two absorption maxima in the visible region, whereas an N-alkyl metalloporphyrin will typically show three absorption maxima (in Lavallee ed., 1987).

An electronic spectrum provides a quick and convenient way of quantifying porphyrins and also establishing what form a porphyrin is in. The shift in the wavelength of the Soret band upon N-alkylation or metalation provides a rapid check to see if conversion from parent compound to N-alkylporphyrin or from free-base porphyrin to metalloporphyrin has taken place.

2.3. X-Ray Crystallographic Data

X-Ray crystallographic studies are one way of characterising the effects of N-alkylation and metalation on the structure of a porphyrin but, to date, not very many N-alkylated structures have been defined and those that exist are of synthetic porphyrins rather than those of known biochemical interest. Enough evidence can, however, be picked out from existing data to be able to define structural differences.

Addition of an alkyl group to one of the central nitrogen atoms of a porphyrin can be expected to distort the normally planar porphyrin ring - three main differences are readily noted between the non-alkylated and N-alkylporphyrin forms:

1) changes in the bond lengths of the alkylated pyrrole ring
2) changes in hybridisation at the alkylated nitrogen atom
3) changes in the relative orientations of the pyrrole rings with respect to the central nitrogen atoms.
The aim of this section is to summarise the X-ray crystallographic work done which has highlighted these differences. The structures which will be referred to in doing this are set out below:

N-methyl-5,10,15,20-tetrakis(p-bromophenyl) porphyrin (N-CH₃TPPB₄) - which is an example of the free-base form of an N-alkylporphyrin (Lavallee, 1982)

21-ethylcarbonylmethyl-2,3,7,8,12,13,17,18-octaethyl-porphinium iodide (N-CH₂COC₂H₅HOEP⁺) - which is an example of the monoprotonated form of an N-alkylporphyrin (McLaughlin, 1974).

Chloro-N-methyl-5,10,15,20-tetraphenylporphinatozinc (II) (Zn(N-CH₃TPP)Cl) - which is an example of a Zn(II) complex of an N-methylporphyrin (Lavallee et al, 1978).

Chloro-N-methyl-5,10,15,20-tetraphenylporphinatoiron (II) (Fe(N-CH₃TPP)Cl) - which is an example of an Fe(II) complex of an N-methylporphyrin (Anderson et al, 1980).

The notation used to describe the different bonds of a porphyrin and the crystal structure of a free-base N-methylporphyrin are shown in Figure 2.5. Protonation of a planar non-alkylated porphyrin incurs a degree of 'ruffling' of the macrocycle. N-alkylation produces greater structural changes within a pyrroleninic ring than protonation. In an alkylated pyrrolenic ring of an N-alkylporphyrin, X-ray studies have shown

a) longer N-C₆ bonds (by about 0.05Å),
b) shorter C₆-C₆ bonds (by about 0.05Å)
c) a longer C₆-C₆ bond.

These changes in structural parameters are consistent with a greater degree of double bond character in the C₆-C₆-C₆-C₆ portion of the N-alkylated ring and indicate greater π-
Fig. 2.5. Bond lengths (Å) for the structure of N-methyl-5,10,15,20-tetrakis (p-bromophenyl) tetraphenylporphine (N-CH₃TPPB₄). The nomenclature used to define the carbon skeleton is shown in the lower half of the diagram (diagrams taken from Lavallee (ed.), 1987).
delocalization toward the porphyrin periphery. This is expected behaviour seeing as the porphyrin is having to withstand modification at one of its central nitrogen atoms.

As mentioned already, another matter of difference between non-alkylated and N-alkylporphyrins is the changes in hybridisation at the alkylated nitrogen atom. These changes are exemplified by the angle between the N-alkyl group carbon bond and the plane of the alkylated ring (Table 2.1.). As can be seen, this angle in the two N-alkylporphyrin examples quoted are intermediate between those for an sp² hybridised nitrogen and an sp³ hybridised protonated pyrrole. From this it can be deduced that the N-C₈ bond length changes already mentioned parallel a decrease in bond order that accompanies a change from sp² to sp³ hybridisation.

Table 2.1. Comparison of the Angle Between the N-alkyl Group Carbon Bond and the Plane of the Alkylated ring of Some N-alkylporphyrins and models of sp² and sp³ hybridised nitrogen (taken from data in Lavallee ed., 1987).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Angle between N-alkyl group carbon bond and plane of alkylated ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-CH₃TPPBr₄</td>
<td>120 degrees</td>
</tr>
<tr>
<td>N-CH₂CO₂H₂H₂OEP⁺</td>
<td>115 degrees</td>
</tr>
<tr>
<td>Protonated pyrrole (sp³)</td>
<td>109 degrees</td>
</tr>
<tr>
<td>Hybridised nitrogen (sp³)</td>
<td>180 degrees</td>
</tr>
</tbody>
</table>

A final difference of marked importance is the relative rotation of the pyrrole ring about the C₈-C₉ bonds. With non-alkylated porphyrins, distortions from planarity have been reported, but those in N-alkylporphyrins are more pronounced. Comparisons of various N-alkylporphyrin forms are tabled in Table 2.2. - The table includes two metalloporphyrins which, until now, have not been discussed.
Table 2.2. Showing deviations (in degrees) from the least-squares-plane of the unsubstituted nitrogen atoms. (Taken from Lavallee ed., 1987). N.A. = Not Applicable.

<table>
<thead>
<tr>
<th></th>
<th>N-substituted</th>
<th>Opposed</th>
<th>Adjacent</th>
<th>Out-of-plane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ring</td>
<td>Ring</td>
<td>Rings</td>
<td>Displacement</td>
</tr>
<tr>
<td>Free-Base</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-CH$_3$TPPBr$_2$</td>
<td>27.7</td>
<td>8.1</td>
<td>-11.9,-10.2</td>
<td>N.A</td>
</tr>
<tr>
<td>Mono-protonated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-CH$_3$COC$_2$H$_5$HOETPP$^+$</td>
<td>19.1</td>
<td>-11.7</td>
<td>-4.8,-2.2</td>
<td>N.A</td>
</tr>
<tr>
<td>Metalated</td>
<td>(Zn(N-CH$_3$TPP)Cl)</td>
<td>38.5</td>
<td>-5.3</td>
<td>-12.3,-11.6</td>
</tr>
<tr>
<td>Metalated</td>
<td>(Fe(N-CH$_3$TPP)Cl)</td>
<td>36.6</td>
<td>-6.4</td>
<td>-10.4,-8.3</td>
</tr>
</tbody>
</table>

The main difference between N-alkyl and non-alkylated metal complexes is a unique bond to the alkylated nitrogen atom and the out-of-plane orientation of the complexed metal ion that exists in a N-alkyl metalloporphyrin.

The coordination geometry of, for example, Fe(N-CH$_3$TPP)Cl can be considered as a distorted square-base pyramid in which the rehybridization of the alkylated nitrogen atom and the bulk of the N-alkyl substituent leads to displacement of the metal ion from the plane of the three non-alkylated nitrogen atoms towards the apical chloride ligand. The displacement is of the order of 0.62 Å as opposed to 0.42 Å seen in non-alkylated 5-coordinate high-spin Fe(II) complexes. The same situation is also seen when zinc (II) is the coordinated metal ion.

The general patterns emerging from this discussion are the increased distortion of an N-alkylporphyrin when compared to a non-alkylated porphyrin and also the even greater distortion incurred on complexing a metal ion to the porphyrin centre. These changes in structure become important when discussing the ability of different forms of a porphyrin to interact with an enzyme, as knowing the structures of particular forms and their inter-relationships establish their use as 'probes' of the enzymes' active site. Many porphyrins have
already been used to try and probe the ferrochelatase active site. Similar work has also been carried out as part of this thesis (see chapter 6).

2.4. Mass Spectrometric Studies.

Mass spectrometry is mentioned briefly here because it has been instrumental in structurally identifying the green pigments produced in the experimental porphyrias.

Electron Impact (EI) mass spectrometry, for example, played an important role in determining the N-alkyl group of the adduct formed in the DDC-induced porphyria (de Matteis et al, 1981). This was achieved by altering the size of the DDCs 4-alkyl group from a methyl to an ethyl group before administering both the drug and its altered analogue. The two adducts isolated after treatment with these compounds were characterised using EI mass spectrometry and gave mass ions of \textit{m/e} 604 and \textit{m/e} 618 equivalent to \((\text{protoporphyrin - hydrogen atom} + \text{methyl group})\) and \((\text{protoporphyrin - hydrogen atom} + \text{ethyl group})\) respectively. This indicated that the N-alkyl goup of the isolated adducts appeared to be derived from the 4-alkyl group of the administered drug. Conclusive evidence was provided using DDC in which the 4-alkyl group had been deuterated; the mass ion of the isolated adduct was \textit{m/e} 607. The increase in mass by three units gave firm evidence that the 4-methyl group of DDC was transferred intact, that is with its full complement of hydrogen atoms.

A drawback of the EI technique was that the N-alkylated products underwent facile thermal dealkylation in the mass spectrometer (Smith & Farmer, 1982). This observation was put to good use, however, in a technique where N-dealkylation was positively encouraged using copper to displace the N-alkyl group (Gibbs et al, 1990). The released N-alkyl group is then trapped by dodecylamine and the amine adduct identified by fast atom bombardment mass
spectrometry (f.a.b. m.s.), thus providing an efficient means of identifying the N-alkyl group of unknown porphyrins.

F.a.b. m.s. was in fact the preferred technique for later experiments, in conjunction with tandem mass spectrometry. For example, the two adducts isolated from griseofulvin-treated mouse-livers were characterised using the combination of these two techniques (Holley et al, 1991).
2.5. The Identification of N-Methylprotoporphyrin IX (N-MePP) as a Green Pigment Induced by Administration of 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC).

2.5.1. Introduction

3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) has already been mentioned in the section on drug-induced porphyrias. It is known that administration of this compound results in the disease protoporphyria, the biochemical lesion incurred being that of inhibition of ferrochelatase. DDC administration results in formation of a 'green pigment' which is isolable from the livers of treated animals and has been identified as N-methylprotoporphyrin (N-MePP), a targeted inhibitor of ferrochelatase.

It was already established (de Matteis et al, 1980(b), Tephley et al, 1981) that the green pigment isolated from the livers of DDC-treated rats was most likely N-MePP but it was the work of Ortiz de Montellano and co-workers that provided unequivocal proof that this was the case (Ortiz de Montellano et al, 1980(a) and (b), Kunze & Ortiz de Montellano, 1981). This was achieved by chemically synthesising all four regioisomers of N-MePP, characterising them fully using Nuclear Magnetic Resonance (NMR) and then comparing the spectra of the synthetic and biologically derived porphyrins to establish conclusively the porphyrin identity and, furthermore, establish which regioisomer of N-MePP was isolated predominantly from the liver.

This was no easy task, requiring specific assignment of every methyl group and meso proton signal in the NMR spectrum of each of the four regioisomers (see Figure 2.6.). Obtaining the necessary assignments involved
1) synthesis of the four N-MePP regioisomers from protoporphyrin with a known (but different) distribution of deuterium at the meso positions,

2) determination of the spin-lattice relaxation times ($T_1$ values) for all the protons in each of the regioisomers and

3) identification of the methyl group signals by measurement of the Nuclear Overhauser Effect (NOE) with respect to each of the meso protons.

2.5.2. Synthesis of Meso-Deuterated N-MePP

This part of the work made use of the known differences in the relative rates of acid-catalysed deuterium exchange into the four meso positions of protoporphyrin a property which Smith et al utilized in assigning all four meso protons in the NMR spectrum of dicyanoferrirprotohaem (Smith et al, 1979).

The result of such a deuterium exchange is an attenuation in the size of each meso proton signal in the NMR spectrum, allowing their individual identification. For example, the $\alpha$ meso proton is least deuterated and so will have the largest meso proton signal in the NMR spectrum of the deuterated N-MePP, whereas the $\gamma$ meso proton is most deuterated and so shall have the smallest meso proton signal in the same NMR spectrum. $\beta$ and $\delta$ meso protons in N-MePP were not however, unambiguously distinguished by this technique because they incorporate approximately equal amounts of the label.
Fig. 2.6. The $^1$H NMR spectra of the (a) $N_B$, (b) $N_A$, (c) $N_C$ and (d)$N_D$ zinc-complexed regioisomers of N-methylprotoporphyrin (taken from Ortiz de Montellano et al, 1981). The individual regioisomers were identified by a series of experiments described in the text. 

Meso proton ($H_\alpha$, $H_\beta$, $H_\gamma$, $H_\delta$) singlets lie in the range 10-10.5ppm, internal vinyl protons come at 7.9-8.3ppm and the methyl proton singlets are the truncated group of resonances in the region 3.3-3.8ppm of each spectrum. The structure shown is that of $N_A$-alkylated N-methylprotoporphyrin.
2.5.3. Spin-Lattice Relaxation Times ($T_1$ Values)

Looking at the protoporphyrin framework, some predictions can be made as to possible $T_1$ values of particular proton groups. Three of the meso protons ($\alpha$, $\beta$ and $\delta$) are surrounded by vinyl and/or methyl groups, whereas the $\gamma$ meso proton is flanked by two propionate groups. The $\gamma$ proton therefore has the means for more efficient relaxation, as first noted by Sanders et al (1978). Theoretically, the $\gamma$ meso proton should be readily distinguished from the other three meso protons by virtue of a shorter $T_1$ value.

Likewise, $T_1$ measurements should also distinguish between the methyl proton signal for a methyl group at the porphyrin periphery and the methyl ester proton signal for the methyl ester groups which are farther removed from the porphyrin edge and, as such, are unable to have their relaxation aided by protons in close proximity.

The $T_1$ measurements made by Ortiz de Montellano et al of all meso and methyl proton resonances for each regioisomer of N-MePP corroborated the predictions made above. One meso proton resonance of each regioisomer had a $T_1$ value significantly longer than those of the other three meso protons, so allowing assignment of the $H\gamma$ resonance in each regioisomer. These assignments made on the basis of $T_1$ values also backed up those made on the basis of differential deuteration.

Methyl proton and methyl ester proton resonances were also distinguished as, in each regioisomer, two resonances (which could have been of either type), were found to have longer $T_1$ values. This identified them as the methyl ester proton resonances and (mutually) identified the others as methyl proton resonances.

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2Relaxation theory is discussed in detail in Chapter 3; NMR: Experimental Methods and Theory.
2.5.4. Nuclear Overhauser (NOE) Enhancements

For porphyrins, the rigid framework of the macrocycle readily defines interproton distance. Sanders et al. (1978) reported that irradiation of the peripheral methyl groups of a porphyrin caused NOE enhancements of the meso protons and vice versa. This observation was picked up on by Ortiz de Montellano and coworkers who decided that it should be possible to assign all peripheral methyl groups using NOEs, as sequential irradiation of all methyl and methylene proton resonances should lead to enhancements of the (already assigned) meso proton resonances.

For example, in each regioisomers' spectrum, an NOE enhancement of one meso proton resonance was seen on irradiation of two different methyl group resonances. Thus, the meso proton showing this enhancement in each case was unambiguously identified as H₅ (the only meso proton in N-MePP that is flanked by two methyl groups), with the two corresponding methyl groups being identified as either ¹CH₃ or ⁵CH₃. Likewise only the H₇ meso proton in each regioisomer should show an NOE on irradiation of methylene protons and so could also be identified. H₆ in each regioisomer should only show an NOE on irradiation of ³CH₃ and H₅ should only show an NOE on irradiation of ⁵CH₃. This sort of analysis of the NOE data for each regioisomer led to complete meso proton and methyl group assignments except for the differentiation of the methyl groups at positions 1 and 8 (¹CH₃ and ⁸CH₃), an assignment which was made on identification of which regioisomer had been alkylated in each of the four regioisomers.

The combined results of this series of experiments led to complete assignments for all four regioisomers of N-MePP. These spectra were then compared to that of the biologically-
derived porphyrin (Ortiz de Montellano et al, 1981(b)) which had been isolated mainly as one form. This form was identified as the NA regioisomer of N-MePP: other regioisomers were also isolated but were found only in much smaller amounts.

2.5.5. Mechanism of N-MePP Formation

The formation of N-MePP on DDC administration is coupled to a loss of hepatic haem and cytochrome P450 suggesting that the cytochrome is involved in N-alkylporphyrin formation (Wada et al, 1968). A precedent for this lay in work with other compounds (such as ethene) which had demonstrated both the role of the cytochrome in N-alkylporphyrin formation and the structures of the resulting porphyrins (Ortiz de Montellano et al, 1980(b)).

With DDC, a lot of work had been done which showed that the mechanism by which the N-MePP was formed involved transfer of the 4-alkyl group of the dihydropyridine to the prosthetic haem group of the cytochrome. This can be achieved in chemical systems by oxidative aromatisation of the dihydropyridine with competitive loss of either the 4-alkyl group or 4-H (Loev and Snader, 1965). The preference for loss of the 4-alkyl group increases in proportion to its cationic (or radical) stability. The question of whether this was indeed the mechanism of N-alkylporphyrin formation induced by DDC was verified by spintrapping experiments (Augusto et al, 1982), which also determined whether the process was one involving extrusion of the radical or cationic form of the 4-alkyl group.

The possible pathways which could be envisioned, supposing cytochrome P450 involvement and transfer of the 4-alkyl group during oxidative aromatisation of the dihydropyridine, are outlined in Figure 2.7. This schematic is derived from the work done by Ortiz de Montellano's group, who utilised an analogue of DDC with an ethyl group rather than a methyl group at the 4 position (DDEP). This analogue was shown to cause greater
Fig 2.7. Three possible mechanisms proposed by Augusto et al. (1982) for the aromatisation of DDEP. The pathway taken was shown to be pathway a), as determined by spin-trapping experiments (See text for details).
accumulation of N-ethylprotoporphyrin (N-EtPP) than N-MePP accumulation on DDC administration (Augusto et al., 1982). This suggested that the N-alkylporphyrin pathway was more favoured with this compound and, as this was the pathway to be studied, the DDEP analogue was used.

Three possible pathways were considered

a) one electron oxidation of DDEP to yield a radical cation which would then undergo an aromatisation reaction with ejection of a 4-Et radical. This process would be competitive with pathways b) and c).

b) two electron oxidation of DDEP to give an unstable hydroxylamine intermediate. This could then either lose 4-Et as a cation during the succeeding aromatisation or

c) undergo further oxidation to yield a nitroxide radical which would then rearrange to the aromatic molecule with loss of 4-Et as a radical.

Paths b) and c) were the more expected because the cytochromes P₄₅₀ often act as monooxygenases, but the generation of ethyl radicals during the cytochrome P₄₅₀ catalysed reaction of DDEP led to the adoption of path a) as the reaction pathway. This was because paths b) and c) (which involve hydroxy formation at the N-position) were discounted when an N-ethyl derivative of DDEP was found to destroy cytochrome P₄₅₀ and give rise to ethyl radicals as effectively as DDEP itself. It was assumed that an N-substituent should have effectively blocked the b) and c) pathways if they were involved in ethyl radical generation (and therefore N-alkylporphyrin formation).
More recently (Kennedy & Mason, 1990, Rumyantseva et al 1991) some doubt has been cast on this spin-trapping evidence though, as it has been found that the free radicals produced in the system used may well arise from trace transition metal-catalysed reactions. However, even if the evidence is not fully conclusive, it must be upheld that a new mode of function of the cytochromes P450 had been brought to light, which involves alteration of the cytochrome P450 prosthetic haem without oxygen insertion, a reaction of relevance to work described in this thesis.
2.6. Identification of Other N-alkylporphyrins Derived from the Prosthetic Haem of Cytochrome P450.

2.6.1. Introduction

It is of interest here to describe some other mechanisms which have been elucidated for the formation of other N-alkylporphyrins also derived from the prosthetic haem group of cytochrome P450. These arise from the administration of different drugs such as ethylene, allylisopropylacetamide (AIA) and novonal, propyne, and the sydnones.

2.6.2. Ethylene and AIA

Work began on ethylene when it was found (Ortiz de Montellano & Mico, 1980(c)) that the unsaturated bond of allylisopropylacetamide (AIA) was the only functional component required for cytochrome P450 destruction in a system that contained catalytically competent enzyme, NADPH and molecular oxygen. Mass spectrometric studies on the adduct formed on ethylene administration (Ortiz de Montellano et al, 1980(b)) gave a mass ion corresponding to the molecular sum of protoporphyrin dimethylester + ethylene + an oxygen atom. Such a metabolite suggested covalent attachment of the olefinic substrate to the protoporphyrin framework of the prosthetic haem of the cytochrome by a mechanism which also results in incorporation of an oxygen atom. This proposal was verified on identification of the N-alkylporphyrin as N-(2-hydroxyethyl) protoporphyrin (Ortiz de Montellano et al, 1981(c)).

The mechanism put forward for adduct formation is shown in Figure 2.8. and proposes that haem is N-alkylated by a transient species generated during catalytic transfer of oxygen to the π-bond of ethylene.
Possible intermediates proposed for the destruction mechanism involving these compounds were the epoxides (de Matteis et al, 1980(b)), because the catalytic action of cytochrome P₄₅₀ enzymes on olefinic \( \pi \)-bonds results in epoxide formation. This proposal, however, was discounted by the finding that the epoxide metabolite of AIA was able to 'bind' cytochrome P₄₅₀ without destroying it (Ortiz de Montellano et al, 1979). Ortiz de Montellano et al proposed instead, based on their ethylene findings, that oxygen insertion should occur via a hydroxy intermediate which could then undergo secondary rearrangement involving loss of a small molecule such as ammonia during chemical lactonization, a process which would yield an N-lactoneprotoporphyrin as the resulting adduct.

This proposal was verified when the AIA and novonal prosthetic haem adducts were identified (Ortiz de Montellano et al, 1983). Novonal is a prescription drug similar to AIA but lacking a chiral centre which made subsequent identification of the isolated adduct easier. With these compounds the N-alkyl groups of the isolated N-alkylporphyrins were indeed lactones proposed to be formed by addition of a hydroxyl group to internal carbon and of a porphyrin nitrogen to the terminal carbon of the \( \pi \)-bond in each drug. The proposed mechanisms of formation of the ethylene, AIA and novonal prosthetic haem adducts are shown in figure 2.9. It is interesting to note that this class of compounds, containing a double bond, give rise predominantly to the ND regioisomer of the resulting haem adducts.

2.6.3. Propyne

As well as the double bond in an olefin, it has been found that the triple bond in acetylenes can also cause the formation of haem-derived N-alkylporphyrins (Ortiz de Montellano & Kunze, 1980). The cytochrome P₄₅₀ monooxygenase system is efficiently destroyed by monosubstituted acetylenes in which the attached carbon may be mono-, di- or even tri-substituted, a finding which ruled out the possibility of a mechanism in which a delocalised
Fig. 2.8. The proposed mechanism of formation of \(N\)-(2-hydroxyethyl) protoporphyrin from ethylene at the cytochrome P\(_{450}\) active site. The mechanism shown would involve 2e\(^{-}\) transfer from oxygen to the \(\pi\)-bond of the substrate. The oxenoid formed during this process is highly reactive and so quickly forms a covalent bond with predominantly the nitrogen of ring D of the porphyrin ring (after Ortiz de Montellano et al., 1981).

Fig. 2.9. The proposed reaction mechanism for the formation of the AIA and Novonal prosthetic haem adducts (pathway (a)) and also the reaction pathway (b) for formation of the principle metabolite of AIA. The pathway also involves electron transfer from the oxygen to the \(\pi\)-bond of the substrate. With these substrates, however, the initial alcohol product undergoes a secondary rearrangement (with loss of ammonia) to yield the isolated adduct (taken from Ortiz de Montellano et al., 1983).
radical species is formed by abstraction of an allylic hydrogen atom. Characteristic abnormal green pigments were found in rats treated with terminal monosubstituted acetylenes, not the disubstituted derivatives.

Oxygen insertion was again proposed as part of the cytochrome P₄₅₀ destruction mechanism because compounds containing triple bonds such as N-(1,1-dimethylpropynal)-3,5-dichlorobenzamide, yield metabolites with, for example, α-hydroxy acid functions during normal metabolism, which suggests that an oxidative mechanism may be involved. This could either involve oxygen transfer to the acetylenic triple bond or oxygen insertion into the acetylenic carbon-hydrogen bond. The structure of the prosthetic haem adduct with propyne was finally identified as the Nₐ regioisomer of N-(2-oxopropyl)protoporphyrin.

As with DDC, a one electron or a two electron pathway can be envisioned (see Figure 2.10.). Ortiz de Montellano et al (1981(d)) proposed an autocatalytic mechanism in which oxygen transfer to the acetylenic triple bond gives rise to a highly unstable species able to react with the prosthetic haem of cytochrome P₄₅₀. This species could be either an oxirene or a transient acyclic oxirene precursor. Both these species are highly unstable which, the authors would suggest, is in concordance with the strict regiospecificity of the haem-alkylation reaction undergone by propyne (to yield mainly the Nₐ regioisomer).

Such regiospecificity would call for a short-lived, oxygen anchored species as shown in Figure 2.10. Both these pre-alkylation complexes could, in theory, give rise to two structurally distinct adducts, a ketone or an aldehyde, dependent on whether the haem nitrogen reacts with the substituted or unsubstituted carbon atoms of the acetylene function.

In practice, only the ketone adduct is isolated, which would suggest that N-alkylation is limited to reaction at the unsubstituted end of the acetylene function. Alternatively though, it must be considered that
Fig. 2.10. Mechanism for the formation of the prosthetic haem adduct with propyne. Two possible pathways are outlined, either (a) involving 2e' transfer from oxygen with an intermediate oxenoid structure which rearranges to give the resulting porphyrin adduct or (b) which is a 1e' process involving a radical cation precursor, then a free radical that interacts with the porphyrin to form the final adduct (after Ortiz de Montellano and Kunze, 1981).

Fig 2.11. Mechanism proposed for the N-vinyl protoporphyrin adduct. Pathway (1) outlines the oxidative fragmentation of the sydnone initiated by electron transfer from cytochrome P₄₅₀. This pathway produces pyruvic acid and a reactive diazooalkane capable of further reaction. Pathway (2) outlines the sequence of events proposed to lead to formation of the isolated adduct, N-vinyl protoporphyrin (taken from Ortiz de Montellano and Grab, 1986)
any aldehyde products may not be detected by the isolation procedure.

2.6.4. Sydnones

The sydnones are mentioned here as a more recent discovery of a group of drugs able to induce protoporphyria. Sydnones have many useful medicinal properties but, unfortunately, also cause accumulation of protoporphyrin in the livers of rats and dogs (Stejskal et al, 1975). Sutherland et al observed the same protoporphyrin accumulation in cultured chick-embryo liver cells (1986) and were able to pinpoint the accumulation to inhibition of ferrochelatase. It has already been mentioned (see section 1.3. on drug-induced porphyrias) that N-methylprotoporphyrin (N-MePP) is a targeted inhibitor of ferrochelatase. In the case of one particular sydnone 3-[(2,4,6-trimethylphenyl)thio]ethyl]-4-methylsydnone (TTMS), an N-alkylporphyrin has been isolated which has been identified as mainly the Nα and Nβ regioisomers of N-vinylprotoporphyrin which have also been found to be targeted inhibitors of ferrochelatase (Ortiz de Montellano & Grab, 1986). N-vinylprotoporphyrin formation is outlined in Figure 2.11. It involves oxidative fragmentation to yield a reactive diazoalkane and pyruvic acid which is released in measurable quantities. In N-alkylporphyrin formation, the diazoalkane goes on to form an iron-nitrogen bridged porphyrin intermediate which collapses to give the N-vinylhaem. Chemical precedents are known for the proposed model, which outlines yet another method of prosthetic haem-derived N-alkylporphyrin formation (Lange & Mansuy, 1981, Callot & Schaeffer, 1980).
3. Nuclear Magnetic Resonance (NMR) : Experimental Methods and Theory

3.1. The One-Dimensional Experiment

A sample which contains nuclei with a spin number (I) greater than, or equal to, 1/2 will generate a bulk macroscopic magnetization if placed in a spectrometer magnet. This magnetization can be perturbed by a second field which is oscillating at an appropriate radio-frequency such that resonance occurs. The main use of nuclear magnetic resonance (NMR) lies in this observation; that the characteristic absorption frequency of a nucleus placed in a magnetic field is dependent to a small but measurable extent on the molecular environment of the nucleus.

In Fourier transform (FT) spectroscopy the system is perturbed by a pulse of radiofrequency (rf) energy, and the response of the system is measured as a function of time in the form of a free induction decay (FID). A frequency spectrum is then generated mathematically by Fourier transformation, which converts the time domain data to the frequency domain spectrum. In the frequency spectrum resonances are seen as lines at different resonance frequencies. This is because the electrons magnetically screening a particular nucleus ensure that the magnetic field experienced by that nucleus is not the same as the applied magnetic field. In an NMR spectrum resonance frequencies of such nuclei are given 'chemical shift' values (in parts per million (ppm)) referenced to the resonance of a standard compound which is given a chemical shift value of zero.

\[ \delta \text{ (chemical shift value)} = \frac{\nu_{\text{sample}} - \nu_{\text{reference}}}{\nu_{\text{reference}}} \times 10^6 \]

which can be simplified to

\[ \delta = \frac{\Delta \nu}{\text{observing frequency}} \times 10^6 \]
Tetramethylsilane (TMS) is commonly used as the internal standard for proton NMR and the δ value for this reference compound is, by definition, zero.


Some discussion has to be made of the magnetic shielding of atomic nuclei because shielding plays a very important role in the NMR spectra of porphyrins.

If a proton (which has a simple symmetrical electron distribution) is placed in a uniform magnetic field a secondary magnetic field is induced due to the flow of electrons. The induced magnetic field will, at the nucleus, be opposed to the applied field. This means that the nucleus experiences a resultant magnetic field which is less than the applied field. The nucleus is said to be 'shielded'.

The spherical symmetry of an isolated nucleus is lost, however, on moving to a more real-life scenario such as a molecule. Here the electron circulation cannot be so easily defined. One treatment (by Pople, 1958 (a) & (b)) splits the electron circulation into three components:

1) Local Diamagnetic Currents - This is similar to the situation described for an isolated nucleus and it depends only on the electron density around the nucleus concerned. The effect of this type of contribution to overall shielding of the molecule is positive shielding (see Figure 3.1., a.)

2) Paramagnetic Currents - This contribution to overall shielding allows for the ease of movement of electrons as this can depend on the orientation of the molecule with respect to the applied magnetic field (magnetic anisotropy). This contribution produces a secondary magnetic field at the nucleus which is parallel to the applied field and so decreases shielding. This effect is not important for protons but other nuclei such as
Fig. 3.1. Schematic showing a) the diamagnetic shielding of the nucleus of an isolated atom, b) the shielding of acetylenic protons (1) in terms of induced paramagnetism and (2) in terms of diamagnetic anisotropy, and c) the shielding of aromatic protons (after Jackman, 1959).
carbon or oxygen will have shielding effects on neighbouring protons (see Figure 3.1., b)).

3) Interatomic Diamagnetic Currents - Some molecules such as benzene, have overlapping molecular orbitals which result in a delocalisation pathway through which electrons are free to move eg. the π-system of benzene. The secondary magnetic field induced by such a motion is very strong and is outlined in Figure 3.1., c). It results in deshielding of the hydrogen atoms around the periphery of the benzene ring.

These components are not the only contributors to the magnetic shielding of a nucleus. In particular cases the electrical field effect of polar groups and the Van der Waals effect can be important and so shall be mentioned briefly here.

Highly polar groups (such as the nitro group in nitrobenzene) possess electrical dipole moments which can cause changes in the charge density at particular protons. The effect of the dipole on protons involved in bonding will be to move the bonding electrons either towards, or away from, the protons. This results in shielding, or deshielding, of the protons involved respectively. Van der Waals effects arise when a particular proton is sterically constrained by a neighbouring group such that its electron distribution is no longer spherical. Loss of symmetry will cause a paramagnetic shielding effect as discussed earlier.
3.1.2. NMR of Porphyrins - The Ring-Current Induced Shift

In the proton NMR spectra of diamagnetic porphyrins a major contributor to chemical shift is the interatomic diamagnetic current (in Smith ed. 1975). The \( \pi \)-system of a porphyrin is much bigger than that of benzene as it contains 18\( \pi \) electrons. When this system is subjected to an external magnetic field the closed loop of electrons starts to precess and the electron circulation gives rise to an exceedingly strong secondary magnetic field, an example of which, for phthalocyanine, is given in Figure 3.2.

The main effects of this strongly anisotropic magnetic field are positive shielding over the centre of the porphyrin macrocycle and negative shielding (deshielding) at the porphyrin periphery. These effects are strong enough to be collectively known as ring-current induced shifts which are highly characteristic of porphyrin NMR, indeed ring-current induced shifts can be used as a probe for structural changes in a porphyrin.

For example, in the NMR spectra of the N-alkyl porphyrins resonances appear at extreme high field which is indicative of positive shielding effects. Such severe shielding tells us that the nuclei responsible for these resonances must lie in the positive-shielding area of the porphyrin, directly over the macrocycle and so the site of alkylation (and therefore structural change) is identified as one of the central pyrrole nitrogens. Alternatively, if such resonances were deshielded alkylation could be identified as being at the porphyrin periphery, because only nuclei in this area should show such deshielding effects.
Fig. 3.2. The magnetic anisotropy of the phthalocyanine ring system (the inner tetraazoporphyrin system), showing the isoshielding lines for this system. Values shown indicate the shifts (in ppm) either to high field (positive numbers) or to low field (negative numbers) that a proton situated in the vicinity of that line can be expected to experience. The abscissa gives the radial distance from the centre of the macrocycle, and the ordinate the z (out-of-plane) coordinate. The three-dimensional picture is obtained by rotating this cross-section around the z axis (taken from Scheer and Katz, 1975).
3.1.3. Relaxation processes

Spin-lattice (or longitudinal) relaxation.

In modern NMR spectroscopy, radiofrequency energy is supplied to the system in the form of a 'pulse', the duration of which can be carefully regulated. A 90° pulse supplies enough energy to bring the initial magnetization ($M_0$, aligned along the Z axis) into the XY plane. After this perturbation the system tries to regain the equilibrium condition ($M_Z = M_0$). The rate at which this occurs is determined by the spin-lattice relaxation time, $T_1$:

$$\frac{dM_Z}{dt} = -\frac{M_Z - M_0}{T_1}$$

The main contributor to $T_1$ is dipolar relaxation. This occurs when a nucleus is surrounded by other moving magnetic nuclei which form the 'lattice'. Movement of these nuclei cause fluctuations in the magnetic field experienced by the nucleus under study and, if the fluctuating field can supply a component of appropriate frequency, a nuclear spin transition can be induced. Energy is therefore transferred to the lattice so that the system can try and move to meet the equilibrium condition once more.

Spin-spin (or transverse) relaxation

A Y component of magnetization brought into the XY plane by a 90° pulse will evolve according to the equation

$$\frac{dM_Y}{dt} = -\frac{M_Y}{T_2}$$

The time-constant $T_2$ is the spin-spin or tranverse relaxation time and arises mainly from a lack of magnetic field inhomogeneity. As the applied magnetic field is unlikely to be
uniform, nuclei in different areas will experience slightly different field strengths. Therefore, with time, the tranverse magnetization components will no longer precess at the same rate. The amount of $M_Y$ magnetization detected in the XY plane will therefore decay as the $M_Y$ magnetization is the resultant of all its individual components which are now precessing at different rates. This form of relaxation has nothing to do with the energy of the spin-system, as population levels are not affected. It has more to do with the entropy of the system, as disorder is increased with time as the individual components begin to precess at different rates.

3.1.4. Two-dimensional NMR spectroscopy

In two-dimensional NMR spectroscopy a sequence of rf pulses is applied to the sample under study. A typical sequence is made up of four distinct sections: a preparation period, a variable evolution period $t_1$, an optional mixing period and a detection period $t_2$ during which the free-induction decay is recorded.

The 2D experiment is actually comprised of a series of experiments. In each successive experiment (made up of one complete pulse sequence) the evolution period $t_1$ is incremented, as displayed schematically in Figure 3.3. In this way a complete matrix of signals $S(t_1,t_2)$ is built up. The complete matrix is subjected to a double Fourier transform, once with respect to $t_2$ and once with respect to $t_1$. An example of the resultant 2D spectrum for a one-line spectrum is given in the figure. The signal comes in F2 at the precession frequency of that nucleus in $t_2$ and in F1 at its precession frequency during $t_1$. The usefulness of 2D NMR experiments is that they allow correlations to be made between nuclei. For example, through-space connectivities can be made using the NOESY experiment and through-bond connectivities can be made using the COSY experiment. The type of correlation made depends on the pulse sequence used. Various pulse sequences used in this thesis are described in the next section.
Fig. 3.3. Schematic representation of a 2-dimensional (NOESY) experiment for a one-line spectrum (taken from Neuhaus and Williamson, 1989). The first 90° pulse is followed by a time $t_1$, which is incremented in regular steps from zero to $t_{1\text{max}}$. This is followed by a second 90° pulse, a mixing time $t_m$ and then a third 90° pulse, after which the FID is collected during the time $t_2$ (see top half of diagram). The experiment yields a matrix of points $S(t_1,t_2)$. The first Fourier transform (with respect to $t_2$) turns this into an array of 1D spectra in which the line intensity varies as a function of $t_1$ (see RHS of bottom half of diagram). The second Fourier transform (with respect to $t_1$) creates the final 2D spectrum (see LHS of bottom half of diagram).
3.2. NMR Techniques

3.2.1. $T_1$ Measurements

$T_1$ measurements were made using the inversion recovery method. A general pulse sequence is as below:

\[ t_D \quad 180^\circ \quad VD \quad 90^\circ \quad t_2 \]

The initial delay ($t_D$) is the time required for the system to relax fully between excitations. This is usually $5T_1$, but since the $T_1$ value is not necessarily known a suitably long value of (10 seconds or more) should be used for this experiment. The first $180^\circ$ pulse causes all the energy populations to be inverted. After an appropriate time interval (VD) a $90^\circ$ pulse is applied to bring any remaining magnetization (or a component thereof) into the XY plane, where its intensity may be measured by acquiring a free induction decay (FID) during the time $t_2$. The detected signal intensity reflects the extent to which the populations have returned towards the Boltzmann equilibrium value in the time VD. By varying the value of VD the $T_1$ values of different protons can be determined, as for each line in the proton spectrum a curve of residual signal intensity (at the end of the pulse sequence) against time is obtained. The number of points in this curve depends on the number of VD values used. More than one experiment is carried out for each point in the curve so that an average value can be obtained. The $T_1$ value for each line in the spectrum is then calculated by least-squares fitting of each experimental curve.
3.2.2. Correlation Spectroscopy (COSY)

A general pulse sequence for the double-quantum filtered phase sensitive COSY experiment is as below:

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90 90 90 ...
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The basis of the double-quantum filtered COSY experiment lies in specifically selecting (through careful use of phase cycling of the receiver and transmitter) magnetisation that existed as double quantum coherence in the second delay, $t_{DQ}$. The double quantum transition is not directly observable, but can be made observable, by the use of a 90° 'read' pulse. Two coupled spins are required to give rise to double-quantum coherence. The pulse sequence outlined above manipulates the nuclear magnetization such that a signal processing at chemical shift $\delta_A$ during $t_1$ is partially transferred to its coupling partner by the second pulse and then precesses at the chemical shift $\delta_B$ of its partner during $t_{DQ}$. After detection by the third pulse (the double-quantum filter) and 2D Fourier transformation, these transferred signals appear as cross-peaks in the 2D spectrum at $(\delta_A,\delta_B)$. Any non-transferred magnetization gives rise to a diagonal peak (as a signal precessing at $\delta_C$ during delay $t_1$, precesses at the same frequency during delay $t_{DQ}$ and so gives rise to a cross-peak in the final spectrum at $(\delta_C,\delta_C)$). The COSY experiment is therefore used to identify through-bond connectivities of protons - the cross-peaks present in such a spectrum are related to vicinal and geminal couplings of the protons.
3.2.3 Nuclear Overhauser Effect Spectroscopy (NOESY)

A proton (I), which relies on another proton (S) for its dipolar relaxation, will undergo a change in intensity if the S resonance is saturated or perturbed. This change in intensity is called the nuclear Overhauser effect (NOE) and has been shown to have an inverse dependence on the distance between the two protons in question:

\[ \text{NOE} \propto \frac{1}{r^6} \]

The NOE can therefore be useful in defining chemical structure as it determines which protons are close in space. The NOE forms the basis of the two-dimensional NMR experiment (NOESY).

The four energy levels of a two-spin system are shown in Figure 3.4., where, at equilibrium, \( \alpha \alpha \) is the lowest energy state and so has a slight population excess. \( \beta \alpha \) and \( \alpha \beta \) have approximately equal populations and \( \beta \beta \) will have a slight population deficit. The energy levels are linked by transition probabilities (given the symbol W). The single quantum transition probabilities (W\( \alpha \) and W\( \beta \)), involve the flipping of one nuclear spin, for example the \( \alpha \alpha \) energy state becomes \( \beta \alpha \). The transition probabilities W\( \alpha \) (zero-quantum) and W2\( \alpha \) (double-quantum) involve simultaneous flipping of both nuclear states and are central to the NOE phenomenon, for example \( \alpha \alpha \) becomes \( \beta \beta \).

At equilibrium, there is the same population difference across all single-quantum transitions but, when the S resonance is saturated, the populations of the \( \alpha \alpha \) and \( \alpha \beta \) energy levels are equalized, as are the \( \beta \alpha \) and \( \beta \beta \) energy levels. Overall this increases the population levels of \( \beta \beta \) and \( \alpha \beta \) whilst decreasing the \( \alpha \alpha \) and \( \beta \alpha \) population levels. The population differences across the I transitions remain unchanged, however. The W2\( \alpha \) relaxation pathway now comes into play to countermand the effect of the saturation of S by decreasing the \( \beta \beta \) population and increasing the \( \alpha \alpha \). Saturation of S still requires that the population difference between energy levels be the same and so this
Analogously, relaxation via the W0IS pathway would result in a negative NOE as it acts to decrease the $\alpha\alpha$ population level.

![Diagram](image)

Fig. 3.4. Diagram adapted from Neuhaus and Williamson, 1989 which shows the energy levels and transition probabilities of a two-spin system. Single quantum transitions are shown with hashed lines, whereas the double and zero-quantum coherences important in the NOE are shown by arrows.

The relaxation pathway (W2IS or W0IS) which is favoured in a particular molecule depends on the molecular correlation time $\tau_C$. This parameter approximates the random motion of a molecule, and as such is a function of molecular weight, solution viscosity, temperature and other factors such as pH and hydrogen bonding. Small molecules in non-viscous solvents are known to give positive NOEs as the W2 transition is favoured over the W0 and this is the situation found in the described experiments with porphyrins (m.w. 590-1003) in chloroform.
A schematic of the pulse sequence used to obtain the NOESY spectra is outlined below:

The mixing time \( t_m \) is the time required for the system to regain thermal equilibrium after a pulse sequence has been completed. The first 90° pulse causes the initial equilibrium magnetization to be bought into the XY plane. Here, during time \( t_1 \), precession occurs at frequencies dependent on the distance between the carrier frequency and the resonances of interest such as I and S (\( \Delta \nu \)). If I has a greater \( \Delta \nu \) than S it will travel through a greater angle during the time \( t_1 \) than S as I has a higher precession frequency.

The second 90° pulse then brings magnetization of I and S into the YZ plane for the time \( t_m \) (the mixing time). Of this magnetization the Z components are the most important, as it is the difference in the magnitudes of the Z components of I and S which give rise to the transient NOE. The magnitude of these components is dependent on the time \( t_1 \) and their precession frequency. If these components only are immediately bought back into the XY plane by a third 90° observe pulse this constitutes a \( t_m \) value of zero. A 2-dimensional experiment performed with such a setting would give a 2D spectrum with two diagonal peaks. Peaks will be seen at \((\delta_5, \delta_5)\) and \((\delta_5, \delta_1)\) because the spins travel at the same frequency during \( t_1 \) and after the third 90° pulse; and this is because nothing has happened to alter the states of the spins between these two times.

In the 2D NOESY experiment, however, \( t_m \) does not equal zero. \( t_m \) is commonly known as the mixing time and is a delay introduced into the pulse sequence to allow the two spins involved to exchange magnetization, either by cross relaxation (the W2IS and W0IS mechanisms mentioned earlier which give rise to an NOE enhancement), or by chemical exchange. These two processes give rise to the cross-peaks in the NOESY experiment.
For example, with the spins I and S already mentioned, we know that the magnitude of the longitudinal components after the second 90° pulse has an angular dependence on the time $t_1$. Thus, at certain values of $t_1$ one or other of the two spins which are at equilibrium at the start of the pulse sequence, will be the 'more inverted' by the succeeding two 90° pulses. During the mixing time ($t_m$) that follows, the more inverted spin will cause a transient NOE at the less inverted spin as cross-relaxation processes occur.

The 2D NOESY experiment therefore consists of a series of 1D experiments of the pulse sequence shown, where $t_1$ is incremented in each experiment so that all transient NOEs are detected in both directions, both at I from S and at S from I. The mixing time ($t_m$) remains unaltered so that cross-relaxation can occur. The FID recorded for each experiment contains information about the precession frequencies of each spin during $t_1$ (extracted by the first fourier transform with respect to $t_2$) and also the size and sign of the transient NOEs between them (extracted by the second fourier transform which is with respect to $t_1$). The resulting 2D spectrum therefore has a diagonal corresponding to non-transferred magnetization and cross-peaks corresponding to magnetization transfer between spins (for a schematic example of the 2D experiment for a one-line spectrum refer to Figure 3.3).
3.3. Carbon ($^{13}$C) NMR

3.3.1 Introduction

The most abundant isotope of carbon ($^{12}$C) has no nuclear spin and consequently is not observable in NMR experiments. $^{13}$C, however, with a natural abundance of 1.1%, is a spin-half nucleus and therefore observable in the NMR experiment. The main draw-back to $^{13}$C NMR experiments is the lower gyromagnetic ratio of $^{13}$C when compared to $^1$H (approximately 1/4), which lowers experimental sensitivity. However, if larger amounts of material or $^{13}$C-labelled samples are available, $^{13}$C NMR spectroscopy is feasible and has the advantage that the chemical shift dispersion spans more than 200ppm.

In terms of porphyrins, $^{13}$C NMR can be used to probe the magnetic anisotropy at sites inaccessible to study via proton NMR (such as the carbon atoms making up the conjugated system of the porphyrin ring). The contributions to $^{13}$C chemical shift are much the same as those for protons, but the $^{13}$C nucleus is more shielded from the applied magnetic field and can have many hybridization states, reflected in the range of $^{13}$C chemical shift spanning a couple of hundred ppm. This means that ring-induced shifts of the magnitude of 5-10ppm for protons, will have considerably less effect on the total chemical shift of a $^{13}$C nucleus, but, nevertheless, can be very useful in monitoring deformation of the porphyrin macrocycle.

Part of the work described in this thesis has been to compare the $^{13}$C spectra of Protoporphyrin and its zinc complex form with the $^{13}$C spectra of the free-base and zinc forms of the $^{N}_C$ regioisomer of N-Methylprotoporphyrin, to see if the $^{13}$C chemical shifts do indeed undergo changes on N-alkylation and metal complexation. This has involved the use of homonuclear broadband decoupling experiments and the reverse proton-carbon shift correlation experiment as detailed below.
3.3.2. Broadband Proton Decoupling and Composite Pulse Decoupling.

$^{13}$C NMR spectra often have many lines due to coupling with hydrogen nuclei. This complexity can be avoided by decoupling the protons during the recording of the spectrum. The method used to achieve this in this thesis has been broad-band proton decoupling or composite pulse decoupling.

Broad-band proton decoupling uses a radio-frequency generator which provides a frequency in the range of proton resonances. This frequency is then modulated to produce a broad band of frequencies that covers the entire proton spectral range. The generator is of sufficient power to ensure that all proton resonance transitions are saturated. The result of this is that all signal splittings caused by proton-carbon couplings are eliminated, leaving a $^{13}$C NMR spectrum made up entirely of single lines.

As the high-power needed for broad-band proton decoupling may lead to sample heating, a viable alternative is to use composite pulse decoupling. The idea behind this method is to use a series of 180° pulses on the nucleus to be decoupled (in this case protons). The effect of this on the carbon magnetization is that, at certain times, the $^{13}$C magnetisation is decoupled from the protons. If the $^{13}$C magnetization were sampled at this time the resulting carbon spectrum would be proton decoupled. Perfect 180° pulses are, however, very difficult to achieve, and imperfections will lead to incomplete decoupling. Composite pulses (for example GARP 16) have therefore been designed to overcome the problem of imperfect pulses and field inhomogeneities. These are sequences of pulses, very much smaller than a 180° pulse which have a nett equivalent of a 90° or 180° pulse. These sequences are able to supply 180° pulses which are much more accurate and therefore capable of decoupling a carbon spectrum completely, without sample heating.
3.3.3. Reverse Proton-Carbon Correlation Spectroscopy

This experiment allows correlations to be made between the carbon resonances of a compound's $^{13}$C spectrum and proton resonances in the $^1$H spectrum of the same compound. If a correlation between a carbon and a proton resonance is seen, the carbon and proton nuclei responsible for these resonances must be coupled. The experiment is therefore useful in aiding assignment of the (unknown) $^{13}$C spectrum if the proton spectrum has already been assigned.

A 'normal' proton-carbon shift correlation experiment will yield a 2-dimensional spectrum with (the more complex) proton spectrum along the F1 axis (where resolution is less good) and the carbon spectrum along the F2 axis (where good resolution is more easily achieved).

The reverse experiment results in a change of axes, such that the proton spectrum appears in the better-resolved F2 dimension whilst the carbon spectrum appears in F1. In terms of porphyrin NMR spectra, this allows for a better chance of being able to assign the $^{13}$C spectrum from the $^1$H spectrum. Regions of the porphyrin spectrum (for example the methyl/methoxy region between 3.5 and 3.8ppm) have almost overlapping resonances which would not be distinguished were the proton spectrum to be placed in the less well-resolved F1 dimension. The carbon spectrum is, however, comparatively well-dispersed and so its being placed in F1 does not detract significantly from its resolution.

The experiment also benefits from enhanced sensitivity over its 'normal' relation, as it uses proton equilibrium magnetization which is proportional to the difference in energy of the high and low energy levels of a nucleus. This difference is larger for proton than for carbon nuclei (protons have the higher resonance frequency) and so use of this magnetization therefore results in larger NMR signals. Improved sensitivity is of
considerable importance, as the amounts of porphyrins isolated by the methods reported here do not exceed a few milligrams.

A schematic for the pulse sequence used is shown below (adapted from Cavanagh et al, 1988)

\[ \text{\textsuperscript{1}H} \]

\[ 90 \rightarrow 180 \]

\[ \text{\textsuperscript{13}C} \]

\[ -1/2J \rightarrow 90 \rightarrow t_1 \rightarrow 90 \rightarrow 1/2J \rightarrow t_2 \]

This particular form of the reverse experiment makes use of multiple quantum filtering methods and difference spectroscopy. Proton magnetization generated by the first 90° \textsuperscript{1}H pulse evolves, and then any antiphase \textsuperscript{13}C-\textsuperscript{1}H couplings are selectively transferred into heteronuclear multiple quantum coherence (double and zero quantum). These coherences are allowed to evolve for a variable time \( t_1 \). During this time the 180° proton pulse refocuses proton chemical shifts and heteronuclear couplings such that a clean carbon spectrum, devoid of couplings is seen in F1. The last \textsuperscript{13}C 90° pulse regenerates the observable proton magnetisation that was taken into multiple quantum coherence by the first 90° carbon pulse. This magnetization will still be anti-phase with respect to the proton-carbon couplings and so a delay is inserted to allow the couplings to come in phase. This delay is 1/2J, where J is the mean one bond proton-carbon coupling. This delay can be changed as necessary to accommodate observation of two or three bond couplings. The magnetization not involved in multiple quantum coherence and which is therefore not transferred (that of protons which are not coupled to carbon in any way) is removed through phase cycling. All unwanted proton signals are removed by subtracting two successive free-induction decays.

4.1. Introduction

Griseofulvin (see Figure 4.1.) is a porphyrinogenic agent capable of inducing protoporphyria in rodents. It was first isolated from Penicillium griseofulvin and found to have anti-fungal properties if administered orally. The drug is now in widespread use in the treatment of finger- and toe-nail ringworm infections (Kerridge, 1986). Of the four possible stereoisomers only one form (the 2S,6'R or (dd)) has anti-fungal activity which is quickly lost with most structural modifications (Delgado et al, 1992).

The metabolism of griseofulvin has been studied both in rodents and man. Work with liver microsomes of rats and mice indicate that griseofulvin is oxidatively demethylated to 4- and 6-desmethyl griseofulvin (Chang et al 1973 ; Lin et al, 1972). This system requires NADPH for oxidation to occur, suggesting that a NADPH-dependent system such as that of cytochrome P₄₅₀ may be involved in the metabolic process. Metabolism of griseofulvin in humans causes formation of 6-desmethyl griseofulvin which can be isolated from the urine, but the 4-desmethyl metabolite is not seen. Instead griseofulvic acid (see Figure 4.1.) has been identified as a second urinary metabolite in a ratio to 6-desmethyl griseofulvin of 1:15 (Zia et al, 1979 ;Lin et al, 1973, Hathaway, 1975 & 1980). Griseofulvic acid formation is proposed to occur via microsomal demethylation at the 2' position followed by tautomerisation of the 2' enol to the 2'4' dione.

Griseofulvin-induced protoporphyria was first reported in 1963 (de Matteis & Rimington, 1963) when pigments with red fluorescence (identified as protoporphyrin) were found in liver cells from mice given a 2.5% oral dosage of the drug. Elevated levels of faecal
Fig. 4.1. The structures of a) griseofulvin, b) isogriseofulvin and c) griseofulvic acid. The given nomenclature for griseofulvin is used throughout.
protoporphyrin and urinary coproporphyrinogen were also noticed. This soon led to the discovery that griseofulvin, like 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), causes a fall in the enzyme activity of ferrochelatase, the last enzyme of the liver haem biosynthetic pathway (de Matteis et al, 1975). This immediately suggested that a deficiency or inhibition of ferrochelatase was responsible for the drug-induced porphyria.

In 1980, a porphyrin with inhibitory activity towards ferrochelatase was isolated from the livers of mice given isogriseofulvin, an analogue of griseofulvin in which the 2'-carboxymethyl takes the place of the 4'-ketone and vice-versa (de Matteis & Gibb (1980(a), see Figure 4.1.). Later, it was established that this pigment was comprised of two different N-monosubstituted protoporphyrins (Holley et al, 1991). One of these (termed the minor adduct) was identified as predominantly the N-methylprotoporphyrin (N-MePP) and as the adduct responsible for ferrochelatase inhibition.

The other porphyrin (termed the major adduct and abbreviated from here on as N-GfPP) was present in far greater amounts. A positive ion f.a.b. mass spectrum obtained for this major griseofulvin pigment gave a molecular ion at (MH^+) m/z 941, a mass consistent with an N-alkylated structure where the whole of griseofulvin is bound to one pyrrole nitrogen of protoporphyrin. During tandem m.s. of this molecular ion three daughter ions resulted, one at m/z 591 (protoporphyrin dimethyl ester), another at m/z 353 (griseofulvin) and a further one at m/z 605. The m/z 605 molecular ion was attributed to protonated N-MePP and this formed the basis of a hypothesis that the minor adduct (N-MePP) might be formed by from secondary degradation of the major griseofulvin pigment (N-GfPP).

Further work (de Matteis et al, 1991) on the two adducts established that they are chiral (suggesting formation on an enzymic template) and are labelled from 5-amino-[4-14C]-laevulinate (suggesting that it is endogenous haem in the liver cells that is used in N-
alkylporphyrin production). Furthermore, it was found that pretreatment with a suicide substrate of cytochrome P_450 prevented both liver accumulation of the adducts and the porphyria. These findings suggested a role for cytochrome P_450 in the porphyria and in the N-alkylporphyrin production. Precedence for such a role does exist, as already discussed in detail in the previous chapter, cytochrome P_450 has been implicated in the production of N-alkylporphyrins during suicide inactivation of the enzyme by a variety of compounds (see previous chapter and Guengerich et al, 1991).

4.2. Statement of Aims

The aim of the work described in this chapter was to fully establish the structure of the major adduct (N-GfPP) that is found in the livers of mice made protoporphyric by oral administration of griseofulvin. This work required that;

a) the presence of the whole griseofulvin drug in the major adduct (as indicated by mass spectrometric studies) be confirmed,

b) the site of linkage between the drug and porphyrin framework be defined and

c) the predominant regioisomer of the adduct isolated from the liver be identified.

It was also interesting to study a molecular model of the adduct once the structure had been determined, and also to make preliminary investigations into possible adduct break-down products.
4.3. Methods

4.3.1. Isolation of the Major Griseofulvin-Protoporphyrin Adduct (N-GfPP)

Treatment of Animals

Typically 21 male mice of the MF1 strain were maintained on a powdered expanded diet containing 2% arachis oil for a minimum of 3 days before being swapped to the same diet containing 1% griseofulvin for 2 days. Mice were usually 7 weeks old at sacrifice.

Extraction and Homogenisation of Livers

Livers were extracted quickly after death and pooled (in batches of ~15g) in a beaker on ice. Homogenisation buffer (20mM Tris/HCl, pH 8.0 containing 0.25M sucrose) was added to give a 50% w/v final homogenate. Homogenisation was carried out using an Ultraturax in 30-second bursts, the beaker being kept on ice at all times to avoid heating.

Organic Extraction of liver porphyrins

Liver homogenate (~30ml) was added dropwise with vigorous stirring to 5% H₂SO₄ in methanol (350ml). The mixture then stood for 1 hour before being filtered on a Buchner funnel and washed through with another 25ml H₂SO₄/MeOH. The filtrate was left to stand overnight in the dark at 4°C. Next day, the filtrate was transferred to a 1 litre separating funnel and CHCl₃ (50ml) added and mixed to extract the esterified porphyrins. H₂O (600ml) was then added, the funnel inverted several times, and then the phases allowed to separate. The lower (organic) phase was run off and 2x25ml of distilled water added consecutively to the funnel with funnel inversion and running-off of the separated organic
phase as before. All organic extracts were combined, returned to the separating funnel and
rewashed (2x200ml). The extracts were then dried (a 5x1.5cm anhydrous Na$_2$SO$_4$ column
washed through with CHCl$_3$), and rotary-evaporated under vacuum almost to dryness.

Separation of Porphyrin Products by Column Chromatography

The oily residue from the previous procedure was placed on a silica column (7x1.5cm,
Merck silica 60, 70-230 mesh) prepared from a hexane slurry. The column was washed with
hexane (100ml) to bring off a brown eluate which is mainly lipid. CHCl$_3$ (65ml) was then
used to elute protoporphyrin. All other chromophores were then eluted with MeOH/CHCl$_3$
(50:50 V/V, 30ml) into a round-bottomed flask containing freshly prepared saturated zinc
acetate in MeOH (120µl). This eluate was washed with water (1x30ml) to remove excess
methanol and the remaining CHCl$_3$ containing the zinc-complexed porphyrins dried
(through Na$_2$SO$_4$ and then rotary evaporated under vacuum as previously).

The residue (still warm from the rotary evaporation at ~40°C) was taken up in MeOH (4ml)
and applied to a fresh silica column (7x1.5cm) prepared from an (at least) day-old MeOH
slurry. The column was washed with MeOH (35ml) to remove any remaining haem and then
the major and minor griseofulvin-protoporphyrin adducts (N-GfPP and N-MePP
respectively) were eluted with MeOH (40ml) containing 50mM HCl (222µl conc.
HCl/100ml MeOH). The adducts were then immediately extracted into CHCl$_3$ (6ml) and
washed with H$_2$O (3x20ml) to remove the acidic methanol. The adducts were then dried
(Na$_2$SO$_4$ as previously, and rotary evaporation).
Thin-layer Chromatography of Griseofulvin-Protoporphyrin Adduct

The products from the column chromatography on silica were taken up in CHCl₃ and streaked onto a silica gel 60 TLC plate. The plate was developed in a CHCl₃:MeOH (20:3 v/v) system. After drying the plate was scraped, collecting the relevant bands separately (Rf = 0.77 for N-GiPP and Rf = 0.72 for N-MePP). The adducts were eluted with MeOH, dried (N₂) and stored at -20°C.

Crystallisation of N-GiPP

Preliminary 1D proton NMR studies showed a number of peaks in the region 0-2ppm due to impurities and so the N-GiPP was purified further by crystallising the zinc complex from methyl acetate by drop-wise addition of heptane. The free-base was then regenerated by mixing with 5% H₂SO₄ in MeOH, extracting into CHCl₃ and drying under N₂. The crystals were then exposed to ammonia vapour to ensure that the free-base form pre-dominated.

4.3.2. Preparation of a Deuterated Sample

A sample of N-GiPP was made and recrystallised as described in the previous sections. This sample was placed in a petri dish over KOH and covered with 6N HCl to hydrolyse its methyl ester groups. These groups were then re-esterified using 5% D₂SO₄ in CD₃OD. This procedure resulted in approximately 100-150µg deuterated sample.
4.3.3. Chemical synthesis and separation of regioisomers of N-MePP

The methods used to achieve the synthesis and separation of the four regioisomers of N-MePP, which have been used as models to determine the regiosomeric type of N-GfPP are described in the methods section of chapter 5.

4.3.4. NMR Methods

A full discussion of the NMR experiments undertaken and the theory behind them is included in Chapter 3 ; NMR: Experimental Methods and Theory. General precautions taken in handling the pigment are, however, as follows:

All NMR tubes were flushed with N₂ prior to use, as presence of oxygen could lead to porphyrin degradation. Care was taken to protect the N-GfPP adduct from light, which could cause photolysis. Spectra were run using the free-base form of N-GfPP as its methyl ester unless otherwise stated. This was because the zinc form of the adduct appeared to undergo quite facile degradation. The solvent was either 99.8% deuterated chloroform with 0.003% TMS as internal standard or Chloroform-d 99.8% to which TMS was added. All spectra were referenced to the CDCl₃ peak at 7.27ppm. Further experimental detail is also provided with the relevant spectra shown in the results section.
4.4. Results from NMR Experiments

4.4.1. Comparison of the $^1$H NMR spectra of Griseofulvin and N-GfPP

The proton NMR spectrum of griseofulvin has been reported at 60MHz (Arison et al. 1963). It was necessary therefore to obtain a spectrum at higher resolution to be able to compare the spectrum of the drug with the spectrum of the isolated adduct.

The proton spectrum and structure of griseofulvin is shown in Figure 4.2. All peaks were integrated and assignments made as labeled in the figure. A DQF-COSY spectrum was also obtained to verify assignment of the spin-system of ring C (which includes $H_C$, $H_D$, $H_E$ and $6'CH_3$, results not shown).

The proton spectrum and proposed structure for N-GfPP is shown in Figure 4.3. This spectrum has also been integrated, but it must be stressed that the spectrum is not completely clean (due to its biological origin) and that the relaxation delay used to acquire this spectrum (1.5sec) is not sufficient to ensure complete relaxation, so care has been taken in interpretation of the integration data.

The single most striking feature of the spectrum is the appearance of a resonance at extreme high-field (~3.03)ppm. On close inspection (see inset Figure 4.3.) this resonance appears to be approaching an AB quartet form and integrates (approximately) for two protons.

This observation has important consequences for identifying the adduct's structure, as a resonance at such high-field is characteristic of an N-alkyl porphyrin. The extreme high-field position of the resonance is due to the porphyrin ring current which is capable of strong shielding effects. Protons directly over the porphyrin plane are very strongly shielded and so
Fig. 4.2. 500MHz $^1$H NMR spectrum of griseofulvin. Sample 1M in deuterated chloroform containing tetramethylsilane (TMS) as internal standard. Resonance assignments are indicated.
appear at extreme high-field. From this observation, it can also be deduced that the protons giving rise to this signal must be those that make up the linkage of the drug to the porphyrin ring and that the linkage is almost certain to be to a porphyrin pyrrole ring nitrogen, as any modification around the porphyrin periphery should give arise to deshielding (not shielding) events. An integral value of approximately two protons for this resonance and the AB quartet structure also indicates that the linkage group may well be a methylene group (-CH\_2-), which would indicate that the OCH\_3 groups at positions 4, 6, and 2'OCH\_3 of griseofulvin are likely candidates for the bonding interaction.

The not-fully-resolved form of this multiplet suggests that the two protons making the methylene linkage have distinct, but similar, chemical shifts and also that the orientation of the drug over the porphyrin may well be fixed. This is proposed because the methylene protons in a freely rotating bond are not expected to display the geminal coupling which is seen in the resonance at ~3.03ppm. The almost quartet-like form can be explained in terms of two chemically distinct protons showing geminal couplings to each other but the chemical shift difference between the two protons must be of the same order as the geminal coupling constant, for that which is expected to be in the form of two almost overlapping double-doublets takes on the appearance of a quartet.

The proton spectrum of the adduct also has other similarities when compared to the spectra of other N-alkylprotoporphyrins. There are four meso proton resonances in the region 10.05-10.25ppm, internal vinyl resonances (two double doublets at 8.13 and 8.32ppm respectively) and terminal vinyl resonances (in the region 6.09-6.44ppm). The peripheral methyl groups and propionate methyl ester groups appear as singlets in the region 3.43-3.77ppm. As the whole of the griseofulvin drug was proposed to involved in adduct formation (Holley et al, 1991) it was hoped that drug resonances would also be easily
Fig. 4.3. 500MHz $^1$H NMR spectrum of N-GfPP (0.74mM sample in deuterated chloroform). The assignments of some resonances are indicated. Acquired with 4K data points and zero-filled to 8K. Processing also included an exponential multiplication window function with line-broadening of 0.5Hz. The proposed structure for the adduct is also shown.
distinguished in the spectrum, but overcrowding in the region 1.5-3.77ppm makes analysis very difficult.

4.4.2. Assignment of the γ-meso Proton and the 6 and 7 Methyl Ester Groups of N-GfPP using $T_1$ Measurements and a Deuterated Sample.

$T_1$ measurements were obtained as described in chapter 3; NMR: Experimental methods and theory. The values obtained are included in Table 4.3. at the end of this chapter.

The assignment of the γ-meso proton will be considered first. It was proposed, based on the findings of other workers (Sanders et al, 1978; Kunze & Ortiz de Montellano, 1981), that the γ-meso proton should relax faster than the other meso protons because of dipolar interactions with the flanking propionate groups. It should therefore have a shorter $T_1$ value than the other meso protons. On measuring the $T_1$ values of the four meso protons at lowest field in the N-GfPP spectrum one resonance did indeed have a significantly lower $T_1$ value (0.657s as opposed to 0.947s or greater) and so could be assigned to $H_γ$.

It was also expected that the methyl groups at position 1, 3, 5, and 8 of the porphyrin periphery (CH$_3$, 2CH$_3$, 5CH$_3$ and 8CH$_3$) would have shorter $T_1$ values than the methyl ester groups at positions 6 and 7 of the porphyrin periphery.

The methyl ester and the methyl group resonances were known to lie in the group of singlets in the region 3.44-3.75ppm. Measurement of the $T_1$ values of these resonances gave a means of distinguishing methyl resonances from those of the methyl esters. Four of the resonances in this region have significantly shorter $T_1$ values (of between 0.542 and 0.675 seconds), when compared to the $T_1$ values of the other resonances (which lie between 1.016 and 1.774 seconds). Two of these resonances must therefore be the 6-OMe and 7-OMe
resonances, leaving the other four resonances to be (mutually) the four methyl resonances. The 6-OME and 7-OME resonances were finally definitively distinguished using a deuterated sample.

A portion of the proton spectrum of the adduct is compared to the same portion of the proton spectrum of a deuterated sample in Figure 4.4. The parts of the adduct which have been deuterated are the 6 and 7-OME groups (see methods). Deuterium nuclei are not observed in a proton spectrum, and so this spectrum conclusively identifies the 6 and 7-OME resonances as being those at 3.71 and 3.68 ppm.
Fig. 4.4. Comparison of a region of a) 500MHz $^1$H NMR spectrum of N-GfPP (0.74mM sample in deuterated chloroform: acquired with 4K data points and zero-filled to 8K. Processing included an exponential multiplication window function with line-broadening of 0.5Hz) and b) 600MHz $^1$H NMR spectrum of N-GfPP deuterated at the 6- and 7-OMe positions (0.32mM in deuterated chloroform: acquired with 4K data points and zero-filled to 8K. Processing included a gaussian multiplication (lb = -10Hz, GB = 0.1). The lack of the 6- and 7-OMe resonances in the deuterated sample is clearly demonstrated.
4.4.3. Use of Correlation Spectroscopy (COSY) to Aid Assignment of Drug-Derived Resonances of N-GfPP

Figure 4.5. shows the region of the DQF-COSY spectrum that clearly picks out the spin-system of ring C of the drug. From this spectrum the resonances for $H_C$, $H_D$, $H_E$ and $6'CH_3$ in the N-GfPP spectrum can be assigned (see inset Figure 4.5. and Table 4.2.). It is interesting to note that the assigned chemical shift values differ by only 0.16ppm, at the very most, from the chemical shift values for the same resonances in the proton spectrum of griseofulvin alone. Such small differences in chemical shift value can be interpreted as defining the position of ring C of the drug in relation to the porphyrin ring; as no significant shielding of these resonances occurs, it can be stated that this ring of the drug must lie beyond the shielding zones of the porphyrin ring. For this to be so it is highly unlikely that the drug-to-porphyrin linkage can be through the methoxy group at position 2' of ring C of griseofulvin. Table 4.1. clearly shows that, in N-GfPP, only ring A of griseofulvin is experiencing significant changes in chemical shift, probably due to ring-current induced shielding effects.
Fig. 4.5. Portion of the $^1$H-$^1$H DQF-COSY spectrum of N-GiPP which picks out the ring system of ring C of griseofulvin (structure shown in inset). This spectrum was acquired with a 2K x 512 data matrix and was processed with zero-filling in the F1 dimension prior to applying a sinebell window function.
Table 4.1. Table highlighting the changes in chemical shift experienced by griseofulvin-associated group resonances when (a) part of the drug alone and (b) part of the N-GfPP adduct.

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical shift of group in griseofulvin /ppm (a)</th>
<th>Number of protons (obtained by integration of the griseofulvin spectrum)</th>
<th>Chemical shift of group in N-GfPP adduct /ppm (b)</th>
<th>Ring of griseofulvin to which group is attached</th>
<th>Has group experienced a significant change in chemical shift?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'OCH₃</td>
<td>3.61</td>
<td>3</td>
<td>3.77</td>
<td>C</td>
<td>NO</td>
</tr>
<tr>
<td>H₉</td>
<td>5.53</td>
<td>1</td>
<td>5.45</td>
<td>C</td>
<td>NO</td>
</tr>
<tr>
<td>H₁C</td>
<td>3.02</td>
<td>1</td>
<td>3.09</td>
<td>C</td>
<td>NO</td>
</tr>
<tr>
<td>H₁D</td>
<td>2.42</td>
<td>1</td>
<td>2.38</td>
<td>C</td>
<td>NO</td>
</tr>
<tr>
<td>6'CH₃</td>
<td>0.95</td>
<td>3</td>
<td>0.81</td>
<td>C</td>
<td>NO</td>
</tr>
<tr>
<td>H₂E</td>
<td>2.83</td>
<td>1</td>
<td>2.73</td>
<td>C</td>
<td>NO</td>
</tr>
<tr>
<td>6OCH₃</td>
<td>4.03</td>
<td>3</td>
<td>-3.03</td>
<td>A</td>
<td>YES</td>
</tr>
<tr>
<td>H₁A</td>
<td>6.13</td>
<td>1</td>
<td>2.04</td>
<td>A</td>
<td>YES</td>
</tr>
<tr>
<td>4OCH₃</td>
<td>3.97</td>
<td>3</td>
<td>2.64</td>
<td>A</td>
<td>YES</td>
</tr>
</tbody>
</table>

This was a surprising finding, as it was previously supposed that the 2'-methoxy group may well be the point of covalent attachment, as a thioether substitution at the 2' position completely inactivates the porphyrinogenic properties of the compound. The importance of the structure of ring C of griseofulvin in inducing porphyria was also illustrated by the finding that isogriseofulvin (see Figure 4.1.) is even more potent as a protoporphyrinogenic agent than griseofulvin itself (de Matteis et al., 1975). Supplementary evidence for the fact that the 2'methoxy is not the site of the drug-porphyrin linkage is given later, provided by data from a NOESY experiment.

The COSY spectrum also provides information on the assignments of the methylene resonances of the propionate groups at positions 6 and 7 of the porphyrin component of the

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1 These assignments have been made from experimental data which will be discussed later. They are provided here so that a comparison can be made.
adduct. From Figure 4.6, it can be seen that two of the four proximal methylene protons are magnetically equivalent and come at 4.25ppm. These are coupled to two magnetically equivalent distal protons at 3.25ppm. The remaining two proximal methylene protons are not equivalent but do couple to two almost equivalent distal methylene protons.

This is unlike the situation in protoporphyrin where the planarity and (near) symmetry means that all proximal and all distal methylene protons are practically equivalent (Scheer & Katz, 1975). Lack of equivalence in the adduct can be best explained by considering the effect the drug may have in hindering rotation of the normally 'free' propionate groups. It would appear that the effect of the drug is in fact to hinder rotation of just one of the propionate groups. The cross-peak labelled 1 in Figure 4.6 shows the through-bond connectivities of two equivalent proximal protons (at 4.25ppm) to two almost equivalent distal protons (at 2.94ppm) in the same propionate residue, one which is free to rotate. The other cross-peak (labelled 2 in Figure 4.6.) shows the coupling of two non-equivalent proximal methylene protons (at 4.20 and 4.30ppm respectively) in the propionate residue which is not free to rotate, to two equivalent distal protons in the same residue (at 3.25ppm).
Fig 4.6. Portion of the $^1$H-$^1$H DQF-COSY spectrum of N-GfPP (see figure 4.5. for experimental details). The region shown highlights the coupling of the proximal methylene protons (signals between 4.2 and 4.4ppm) of the methyl ester groups at positions 6 and 7 of the porphyrin periphery with the distal methylene protons (at 3.25 and 2.94ppm).
The DQF-COSY spectrum also provides a nice means of differentiating between the vinyl groups of the porphyrin framework of N-GfPP and showing how, in ideal first order spectra, passive and active couplings may be differentiated.

Figure 4.7. is part of the $^1H-^1H$ DQF-COSY spectrum of N-GfPP. As the adduct is an N-alkyl porphyrin the porphyrin macrocycle is no longer planar and so now the groups at positions 2 and 4 of the porphyrin periphery can be differentiated because the lack of planarity causes one set of resonances to be shifted to higher field.

The cross-peaks shown in the figure are from the internal vinyl resonances ($H^1$ and $H^{1'}$ at 8.32 and 8.13ppm respectively to the terminal vinyl resonances ($H^2$ and $H^{2'}$, $H^3$ and $H^{3'}$). The terminal vinyl resonances would be very difficult to assign without access to this spectrum as they all give rise to overlapping double doublets. Active couplings can be seen in the spectrum and are between peaks of opposing phase (those labelled in red), whereas passive couplings are between peaks of the same phase and are labelled in black.

The cross-peak labelled 1 can be identified as representing the cis coupling of $H^1$ to $H^{2'}$, as the active coupling is small and the passive coupling is large. Vinyl groups have defined stereochemistry and it is well documented that the cis coupling is smaller than the trans (Friebolin, 1993). Likewise, the cross-peak labelled 2 can be identified as representing the trans interaction of $H^1$ with $H^{3'}$, as the active coupling is large and the passive coupling small. Geminal couplings between $H^2$ and $H^{3'}$ are very small and so are not seen to complicate the pattern. The same reasoning can be applied to the set of resonances caused by the $H^1$, $H^2$, $H^3$ vinyl group. Coupling constants derived from the spectrum are given in Table 4.2.
Fig. 4.7. Portion of the $^1$H-$^1$H DQF-COSY spectrum of N-GfPP (see figure 4.5. for experimental details). The region plotted shows the coupling between internal and terminal protons of the vinyl groups at positions 2 and 4 of the porphyrin ring. Active couplings are highlighted in red, whereas passive couplings are shown in black.
Table 4.2. Assignments and couplings of the vinyl groups at positions 2 and 4 of the N-GfPP adduct. (u.l. = upper limit of resolution).

<table>
<thead>
<tr>
<th>PROTONS INVOLVED (ppm)</th>
<th>INTERACTION</th>
<th>COUPLING (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsubscript{a} (8.32) and H\textsubscript{g} (6.29)</td>
<td>cis</td>
<td>10.6</td>
</tr>
<tr>
<td>H\textsubscript{a} (8.32) and H\textsubscript{c} (6.44)</td>
<td>trans</td>
<td>18.2</td>
</tr>
<tr>
<td>H\textsubscript{g} (6.29) and H\textsubscript{c} (6.44)</td>
<td>gem</td>
<td>u.l.</td>
</tr>
<tr>
<td>H\textsubscript{A\textsuperscript{'}}} (8.13) and H\textsubscript{g} (6.09)</td>
<td>cis</td>
<td>10.6</td>
</tr>
<tr>
<td>H\textsubscript{A\textsuperscript{'}}} (8.13) and H\textsubscript{c} (6.31)</td>
<td>trans</td>
<td>18.2</td>
</tr>
<tr>
<td>H\textsubscript{g} (6.09) and H\textsubscript{c} (6.30)</td>
<td>gem</td>
<td>u.l.</td>
</tr>
</tbody>
</table>

4.4.4. Use of Nuclear Overhauser Effect Spectroscopy (NOESY) to Determine How Griseofulvin Attaches to the Porphyrin Ring.

The work described so far has allowed the assignment of various resonances from the drug component of the adduct such as those of ring C. These resonances appear at chemical shifts similar to those in griseofulvin itself, unlike those of griseofulvin's ring A. Porphyrin resonances have also been identified, though not all have been individually distinguished as yet. For example, the meso protons have been identified but, so far, only the \textit{p}-meso proton has been individually assigned. The same is true for the the methyl ester resonances (6- and 7-OMe). Such distinctions rely heavily on how the drug attaches itself to the porphyrin ring and also which regioisomer of N-GfPP is formed.

The first part of this two-fold question was answered by a NOESY experiment, a portion of which is reproduced in Figure 4.8. Such an experiment links together the resonances of protons which are close in space in the molecule under study.

It is interesting to note the presence of an NOE (cross-peak 1 in the spectrum) from the resonance at very high-field (\textasciitilde3.03ppm) to a resonance at 2.04ppm. Another NOE (cross-
peak 2 in the spectrum) is also apparent from this 2.04ppm singlet to another at 2.64ppm. The presence of these two 'sequential' NOEs can be explained in the following way:

As mentioned already, the proposed site of structural modification of the porphyrin is a pyrrole ring nitrogen. The site of modification of the drug was proposed to be the 2'0CH$_3$ group of ring C, but the lack of ring-current shifts for all proton members of this ring provided quite strong evidence against this proposal. As the 2'0CH$_3$ group appears not to be involved in the drug-to-porphyrin-ring bonding, the other likely candidates are the 4OCH$_3$ and the 6OCH$_3$ groups of ring A. Such a suggestion fits in with the NOESY data.

The high-field resonance at ~3.03ppm has already been assigned as a methylene linkage (-CH$_2$-) of drug-to-porphyrin-ring, such that the complete linkage will be (-O-CH$_2$-N(pyrrole)-). This could be derived from either the 4OCH$_3$ or the 6OCH$_3$ groups. The NOE (1, Figure 4.8.) can then be interpreted as being from the methylene linkage to H$_A$ of the aromatic ring A of the drug, followed by another NOE (2, Figure 4.8.), from H$_A$ to the unaltered (other) OCH$_3$ group. Unfortunately there is no easy means whereby the 4OCH$_3$ and the 6OCH$_3$ groups can be distinguished and so it can only be stated with certainty that the linking group between the drug and the porphyrin is a methylene group derived from one, or other, of the OCH$_3$ groups of ring A of griseofulvin.

Such an assignment also makes sense in the context of the orientation of the drug over the porphyrin. As the point of attachment is deemed to be through ring A of the drug, the protons on this ring should certainly experience porphyrin ring-induced shifts. This is definitely the case. The OCH$_3$ group responsible for the drug-to-porphyrin-ring linkage experiences a chemical shift to high-field of approximately 7ppm, whereas the H$_A$ singlet and the other OCH$_3$ are shifted around 4 and 1.4ppm respectively, also to high-field. These ring current-induced
Fig. 4.8. Portion of the $^1$H-$^1$H NOESY spectrum of N-GfPP showing the identification of the -O-CH$_2$-N- linkage between the porphyrin and griseofulvin moieties and of the methoxy group in ring C of griseofulvin (details of assignment described in the text). Data was processed with zero-filling in both dimensions and a gaussian multiplication ($lb = -10$Hz, $GB = 0.1$).
shifts explain the failure to assign these resonances hitherto, as they come at unexpected chemical shifts when compared to their shifts in the griseofulvin spectrum.

The NOESY spectrum also establishes that the 2'OCH₃ resonance comes at 3.77ppm, coincident with a methyl resonance. The cross-peak labelled 3 in Figure 4.8. represents an NOE between 2'OCH₃ (at 3.77ppm) and 3'H₃ (at 5.45ppm). Both these substituents are on ring C of the drug and, as such, experience only very slight ring-induced shifts of 0.16ppm or less.

4.4.5. Comparison of the ¹H NMR Spectra of N-GfPP and Model N-Methylporphyrins to Determine which Regioisomer of N-GfPP is Predominantly Isolated from Mouse Liver.

The principal question that now remains unanswered is the one of which regioisomer of N-GfPP is isolated from the liver, as this will have implications for the mechanism of adduct formation and for its role in the porphyria. Definitive identification of the structural isomer predominating in the DDC-induced porphyria came from comparing NMR spectra of the biological pigment with those of individual regioisomers of synthesised N-methylprotoporphyrin (N-MePP, Kunze & Ortiz de Montellano, 1981). Unfortunately, chemically synthesised standards are not available for the much more complex N-GfPP adduct and so it was decided to use the four regioisomers of N-MePP whose synthesis is described in the following chapter as model compounds for the adduct instead. Also, the work already cited was undertaken using the zinc-complex forms of the DDC-adduct and the N-MePP regioisomers. The work described so far in this thesis has made use of the free-base form of the pigment a) because it is less labile and b) the ¹H NMR spectrum of the free-base form has fewer impurities and therefore greater clarity, than the zinc-complex form. It was decided that the zinc and free-base forms of N-GfPP should be compared to the
zinc and free-base forms of all four regioisomers of chemically synthesised N-MePP. Figure 4.9 shows a comparison of regions of the proton spectra of the free-base and zinc-complex forms of N-GfPP and the free-base and zinc-complex forms of the four regioisomers of N-MePP.

Differences between the two forms (zinc complex and free-base) of the four regioisomers of N-MePP are immediately apparent, but are not be discussed in detail here as they form the basis for discussion in the following chapter. What is vital to the discussion here is any similarities between these spectra and the zinc and free-base forms of N-GfPP. Marked similarities are found and can provide evidence for the predominant regioisomer of N-GfPP isolated from the liver. These similarities are detailed below.

1) there is a clear resemblance between the pattern seen for the meso proton resonances in the zinc form of N-GfPP and the zinc form of the $N_C$ regioisomer of N-MePP. Likewise, there is also a clear resemblance between the meso proton resonances in the free-base form of N-GfPP and the free-base form of the $N_C$ regioisomer of N-MePP (see Figure 4.9.).

2) the behaviour of the resonances for the internal vinyl protons is also very similar between the zinc complex and free-base forms of N-GfPP and the corresponding forms of the $N_C$ N-MePP regioisomer. Two sets of internal vinyl proton resonances are seen in the free-base form of N-GfPP and only one multiplet in the zinc complex form (see Figure 4.9.). This is behaviour indicative of a $N_C$ or $N_D$ substituted N-allylporphyrin, as coordination of zinc to the porphyrin centre confers rigidity on the molecule. If the N-substituent is on either ring C or ring D of the porphyrin, zinc complexation causes the the unsubstituted A and B rings to come into the same plane, such that only one multiplet is seen for the internal vinyl resonances. In the more flexible free-base form such restrictions are not imposed and so all rings of the porphyrin are free to move relative to each other. In this form then, the internal
Fig. 4.9. Comparison of the proton spectra of N-GfPP and the four regioisomers of N-MePP. A. in the free-base forms and B. as the zinc complexes. In both A and B, spectra are shown for a) N-GfPP, b) N$_A$-methylprotoporphyrin, c) N$_B$-methylprotoporphyrin, d) N$_C$-methylprotoporphyrin and e) N$_D$-methylprotoporphyrin. Sections of the spectra are shown to illustrate the resonances of, from low to high field, the meso, internal and terminal vinyl and proximal and distal methylene protons: Refer to the full spectrum of N-GfPP in figure 4.2.
vinyl groups will give rise to two multiplets. Conversely, an $N_A$ or $N_B$-alkylated porphyrin would be expected to show two internal vinyl multiplets in both the free-base and zinc complex forms, as the steric constraints imposed by the presence of an N-alkyl substituent on either of these rings do not allow the vinyl groups to come into the same plane, even on zinc complexation.

3) the resonances attributable to proximal and distal methylene protons are also reasonably similar to the situation in a $N_C$ or $N_D$ regioisomer, although a discrepancy is also apparent. A dispersed multiplet for the proximal methylene protons is observed in the N-GfPP free-base spectrum as opposed to two or three distinct multiplets clearly visible in a free-base $N_C$ or $N_D$ N-MePP regioisomer. Two multiplets are, however, clearly observable for the distal methylene protons in the N-GfPP free-base spectrum (see Figure 4.9.) which corresponds closely to the situation in a $N_C$ or $N_D$ free-base regioisomer. The discrepancy can be accounted for however. The role of the drug in affecting the proximal and distal methylene resonances has already been discussed during interpretation of the COSY data (see 3.4.3.), where a proposal was put forward that the drug was able to hinder rotation of at least one of the two methyl ester groups, causing the proximal methylene protons to come at three chemical shifts close enough together to give rise to the overlapping dispersed multiplet structure seen in the N-GfPP spectrum. The much smaller size of the N-substituent in the $N_C$ and $N_D$ N-methylporphyrins means that the N-alkyl substituent can not be expected to interact directly with any of the methylene protons. The distinctly separate proximal methylene proton multiplets in these regioisomers can be accounted for by N-alkylation causing the C and D rings to be in different planes relative to each other. Hence each methyl ester is in a different environment and so the proximal and distal methylene protons for each of these residues come at distinct chemical shifts. Similarities of these resonances between the N-GfPP zinc complex spectrum and the N-MePP zinc complex regioisomers are difficult to define precisely due to contaminants in the zinc complex spectrum but two multiplets
which may be attributable to distal methylene protons are distinguished in Figure 4.9., as is a highly complex multiplet pattern for the proximal methylene protons, making the situation very similar to that in a \( \text{N}_C \) or \( \text{N}_D \) alkylated N-MePP. With zinc-complexed \( \text{N}_C \) or \( \text{N}_D \) N-MePP regioisomers, a dispersed multiplet is seen for the proximal methylene protons (though this is far from being as complex as that seen in the N-GfPP zinc spectrum) and two distinct multiplets are seen for the distal methylene protons.

It can be concluded therefore, that the behaviour of the free-base N-GfPP upon zinc complexation is most consistent with the behaviour of a \( \text{N}_C \) or \( \text{N}_D \) N-alkylporphyrin, as monitored by the changes in the appearance of particular resonances in the proton NMR spectra of the four regioisomers of N-MePP and N-GfPP. The NMR data also suggests that the regioisomer of N-GfPP predominantly isolated from the liver of mice is the \( \text{N}_C \) regioisomer, the distinction of \( \text{N}_C \) as opposed to \( \text{N}_D \) being made on the marked resemblance of the meso proton resonances of N-GfPP (zinc and free-base forms) when compared to N-MePP (all regioisomers, zinc and free-base forms).

### 4.4.6. Complete Assignment of the \( ^1\text{H} \) NMR Spectrum of N-GfPP (free-base).

Figure 4.10. shows a region of the NOESY spectrum linking the meso protons of the N-GfPP adduct (four resonances between 10.07 and 10.20ppm) to the internal vinyl multiplets (at 8.13 and 8.32ppm), the methyl/methoxy (in the region 3.43-3.77ppm) and the multiplet for the proximal methylene protons (centred at 4.25ppm). These NOEs are useful aids to assigning the porphyrin-related resonances that remain unassigned thus far.

The meso proton at lowest field (10.20ppm) has already been assigned to \( \text{H}_7 \) on the basis of \( T_1 \) measurements (see 3.4.2.). This assignment is confirmed by the NOE data as the \( \text{H}_7 \) resonance shows two NOEs (labelled peak 1 in Figure 4.10) to the proximal methylene...
proton resonance at 4.25ppm. Considering the structure of N-alkylprotoporphyrins only 
H_y, flanked by two propionate methyl ester groups, could be expected to exhibit these 
NOEs.

The *meso* proton resonance at 10.13ppm shows an NOE to a high-field internal vinyl 
resonance (at 8.13ppm, peak 2 in Figure 4.10.) and also to a methyl group singlet at 
3.77ppm (peak 3 in Figure 4.10). Considering that N-GfPP is the N_C regioisomer, X-ray 
data indicates that the alkylated ring of a free-base N-alkylporphyrin and the ring opposite 
the alkylated ring are out-of-plane in a positive sense by 27.7 and 8.10° respectively (Lavallee & Anderson, 1982), whereas the non-alkylated rings are out of plane by ~11.9 and ~10.2°. In 
the NMR spectra of free-base N-alkylporphyrins it has been noted (Jackson & Drearden, 
1973) that this distortion of the porphyrin macrocycle causes groups on the periphery of the 
alkylated pyrrole ring to experience a high-field shift. A slight extension of this reasoning 
would lead to the assumption that the pyrrole ring opposite the N-alkylated ring will also 
experience a smaller high-field shift, as this ring is also out-of-plane relative to the plane of 
reference of the other two unalkylated rings.

In the case of N_C alkylation of N-GfPP considered here, the ^5CH_3 and 6-OMe associated 
resonances can be expected to experience high-field shifts (as they are on the alkylated ring) 
as can also the the ^2Vi and ^1CH_3 resonances (as they are on the ring opposite the alkylated 
ring).

In terms of the NOE data, this allows assignment of the 10.13ppm *meso* proton resonance to 
H_{ZL}, as it shows NOEs to the higher-field of the two internal vinyl multiplets (at 8.13ppm, 
which on the basis of the argument above can be assigned to H_A' of ^2Vi) and also to a 
methyl resonance at relative low-field (3.77ppm) which can be assigned to ^3CH_3.
Fig. 4.10. Portion of the $^1$H-$^1$H NOESY spectrum of the N-GfPP adduct. The region plotted shows NOEs between the meso protons ($H_\alpha$, $H_\beta$, $H_\gamma$, $H_\delta$) and internal vinyl (8.13 and 8.32ppm), proximal methylene (centred at 4.22ppm) and methyl/methoxy singlets (in the range 3.43-3.77ppm). These NOEs are used to complete assignment of the free-base form of N-GfPP (for details see text). The spectrum was acquired with a 2K x 512 data matrix, with a relaxation delay of 3s and a mixing time of 1s. Data was processed with a gaussian multiplication (GB = 0.1, lb = 10Hz).
The meso proton at 10.07ppm can likewise be assigned to Hβ, as it shows an NOE to the lower field of the two internal multiplet resonances (assignable to Hα of 4Vi at 8.32ppm, peak 4 in Figure 4.10) and also to a group of three high-field methyl resonances, one of which must be assignable to 5CH3 (peak 5 in Figure 4.10). The last meso proton (at 10.06ppm) must, therefore, be Hg. This assignment is verified by the NOE data, as NOEs are seen from this resonance to the group of three methyl resonances (peak 5 in Figure 4.10) two of which must be attributable to 1CH3 and 8CH3 as only Hg is flanked by these two groups. There is only a small chemical shift difference between the methyl group resonances (3.42, 3.44 and 3.48ppm) and the indeterminate shape of cross-peak 5 makes distinction difficult. It must now be considered that the drug, being much larger than an N-methyl substituent, will have an effect on the distortion of the porphyrin ring in the adduct and may also directly affect the positions of some resonances by virtue of its own structure and presence over the porphyrin ring. The 1CH3 resonance can be expected to exhibit a high-field shift and indeed, has to, if sense is to be made of the clearly-seen differences in chemical shift of the internal vinyl multiplet protons. N-alkylation by griseofulvin, however, may well disguise the expected high-field shift of the 5CH3 resonance as the effect of the drug, if lying over 5CH3, may be to affect its chemical shift value such that the high-field shift may be masked, or may not be observed at all. This makes sense of the fact that the 5CH3 and 8CH3 resonances seem to come at similar chemical shifts. On this basis, as the 3CH3 resonance lies at 3.77ppm, the resonance at 3.48ppm shall be assigned to 1CH3. Such an assignment makes the chemical difference between these groups (0.32ppm) the most similar in size to the difference in chemical shifts between the internal 2Vi and 4Vi multiplets (0.18ppm). The resonance at 3.45ppm is thus assigned to 8CH3, leaving the resonance at highest field in the group (3.42ppm) assignable to 5CH3, in keeping with its position on the N-alkylated ring.
The same argument can also be extended to differentiating the 6- and 7-OMe resonances, where it has already been commented upon that the proximal methylene protons exhibit similar chemical shifts such that a dispersed multiplet structure is seen rather than the distinctly separate multiplets seen for the same resonances in a N_C or N_D N-MePP regioisomer. The reasoning applied to the case of the masked, or not observed, high-field shift of the 5-CH3 resonance can also be applied to the 6-OMe resonance, making the observed dispersed multiplet structure understandable. The assignment of the 6- and 7-OMe resonances is based on the assumption that any shifts seen for the distal methylene groups are more indicative of which pyrrole ring these resonances are associated with. This is because these protons are farther from, and therefore less likely to be affected by, any effects that the drug may have on the chemical shifts of protons within its vicinity. Thus the distal methylene proton multiplet at highest field (2.94ppm) is assigned to the 6-OMe distal protons, as protons associated with the 6-OMe group, by virtue of being on the N-alkylated ring, should experience a high-field shift. This multiplet is linked, by the COSY experiment (see figure 4.6.), to two equivalent proximal methylene protons (now assigned to the proximal methylene protons of the 6-OMe group) whose resonance (at 4.25ppm) makes up part of the dispersed proximal methylene proton multiplet which is also centred at this chemical shift value. By exclusion, this leaves the other distal methylene proton multiplet at 3.25ppm assignable to the 7-OMe group, coming at lower field because it is not on an alkylated ring. The COSY experiments then links this resonance with two non-equivalent proximal methylene protons at 4.20 and 4.30ppm, now assigned to the proximal methylene protons of the 7-OMe group.

The final outstanding assignments are those of the 2- and 4- terminal vinyl protons. As the 2- and 4- internal vinyl protons have now been assigned, the COSY experiment (see Figure 4.7.) quickly allows assignment of these other resonances, as through-bond connectivities of internal to terminal vinyl protons are clearly visible. Thus H_1 of 2Vi is assigned to a double
doublet centred at 6.30ppm whereas \( H_C \) of \(^2^V\)i is assigned to a double doublet at 6.09ppm. Likewise \( H_D \) of \(^4^V\)i is assigned to a double doublet at 6.29ppm, whereas \( H_C \) of \(^4^V\)i is assigned to a double doublet at 6.44ppm.
Table 4.3. Complete assignments of the griseofulvin-protoporphyrin adduct (N-GfPP).

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical Shift (ppm) from tetramethylsilane in N-GfPP</th>
<th>Multiplicity(^2)</th>
<th>Chemical Shift (ppm) from tetramethylsilane in griseofulvin</th>
<th>Chemical Shift (ppm) from tetramethylsilane in N(\text{-})MePP</th>
<th>(T_1) Value (s)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^{\text{CH}})</td>
<td>3.48 s</td>
<td>-</td>
<td>3.75</td>
<td>0.542</td>
<td></td>
</tr>
<tr>
<td>2(^{\text{CH}})</td>
<td>3.77 s</td>
<td>-</td>
<td>3.38</td>
<td>0.675</td>
<td></td>
</tr>
<tr>
<td>5(^{\text{CH}})</td>
<td>3.43 s</td>
<td>-</td>
<td>3.47</td>
<td>0.641</td>
<td></td>
</tr>
<tr>
<td>6(^{\text{CH}})</td>
<td>3.45 s</td>
<td>-</td>
<td>3.49</td>
<td>0.667</td>
<td></td>
</tr>
<tr>
<td>6(^{\text{CH2}}) (proximal)</td>
<td>4.23 m</td>
<td>-</td>
<td>4.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6(^{\text{CH2}}) (distal)</td>
<td>2.94 m</td>
<td>-</td>
<td>2.76</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6(^{\text{OMe}})</td>
<td>3.68 s</td>
<td>-</td>
<td>3.86</td>
<td>1.774</td>
<td></td>
</tr>
<tr>
<td>7(^{\text{CH2}}) (proximal)</td>
<td>4.20, 4.30 m, m</td>
<td>-</td>
<td>4.25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7(^{\text{CH2}}) (distal)</td>
<td>3.25 m</td>
<td>-</td>
<td>3.24</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7(^{\text{OMe}})</td>
<td>3.71 s</td>
<td>-</td>
<td>3.70</td>
<td>1.165</td>
<td></td>
</tr>
<tr>
<td>2(^{\text{Vi}}) (internal)</td>
<td>8.13 dd</td>
<td>-</td>
<td>8.34</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2(^{\text{Vi}}) (terminal)</td>
<td>6.30, 6.09 dd</td>
<td>-</td>
<td>6.23, 6.39</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4(^{\text{Vi}}) (internal)</td>
<td>8.32 dd</td>
<td>-</td>
<td>8.18</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4(^{\text{Vi}}) (terminal)</td>
<td>6.29, 6.44 dd, dd</td>
<td>-</td>
<td>6.10, 6.25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(\alpha)-meso</td>
<td>10.13 s</td>
<td>-</td>
<td>10.10</td>
<td>0.947</td>
<td></td>
</tr>
<tr>
<td>(\beta)-meso</td>
<td>10.08 s</td>
<td>-</td>
<td>10.02</td>
<td>1.102(\text{av})</td>
<td></td>
</tr>
<tr>
<td>(\gamma)-meso</td>
<td>10.20 s</td>
<td>-</td>
<td>9.94</td>
<td>0.657</td>
<td></td>
</tr>
<tr>
<td>(\delta)-meso</td>
<td>10.07 s</td>
<td>-</td>
<td>9.94</td>
<td>1.102(\text{av})</td>
<td></td>
</tr>
<tr>
<td>N(^{-})CH(_2)-O</td>
<td>-3.03 c</td>
<td>(4.03 or 3.97)(^4)</td>
<td>(4.48)(^4)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>H(_A)</td>
<td>2.04 s</td>
<td>6.13</td>
<td>-</td>
<td>0.618</td>
<td></td>
</tr>
<tr>
<td>H(_B)</td>
<td>5.45 s</td>
<td>5.53</td>
<td>-</td>
<td>0.667</td>
<td></td>
</tr>
<tr>
<td>6(^{\text{CH3}})</td>
<td>0.81 dd</td>
<td>0.95</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2(^{\text{OCH3}})</td>
<td>3.77 s</td>
<td>3.61</td>
<td>-</td>
<td>1.012</td>
<td></td>
</tr>
<tr>
<td>4(-6)^{\text{OCH3}}\</td>
<td>2.64 s</td>
<td>4.03 or 3.97</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^2\) s = singlet, d = doublet, m = multiplet, dd = doublet of doublets, c = AB quartet

\(^3\) A slight difference was observed in the \(1^H\) NMR spectrum of this particular batch of adduct used for making \(T_1\) measurements, in that two superimposed singlets that came at 3.77ppm in previous spectra, were actually resolved into two separate singlets at very similar chemical shifts in this spectrum (3.75 and 3.72ppm respectively). \(T_1\) values were calculated for each of these singlets. The 'extra' singlet seen at 3.72ppm in this experiment is not seen in other spectra of the adduct. It has a \(T_1\) value of 1.016 seconds, suggesting that it may well be a carboxy-methyl group (OCH\(_3\)). Comparison of the proton spectra of this particular batch of adduct and griseofulvin suggests that the group responsible for this resonance is the 2\(^{\text{OCH3}}\) group on ring C of the drug, as the chemical shifts of this group in both spectra are very similar, coming at 3.62ppm in griseofulvin and 3.77ppm in the adduct. In all other spectra obtained for the adduct it appears as though this resonance is coincident with a methyl group resonance and so gives rise to two superimposed singlets at 3.77ppm.

\(^4\) 4 or 6\(-\text{OCH3}\) in griseofulvin, N=CH\(_2\) in N\(\text{-}\)MePP

\(^5\) Specific assignment not made in N-GfPP
4.4.7. Summary

The NMR work described in this chapter started with a comparison of the proton NMR spectra of griseofulvin and N-GfPP. This comparison showed that

- a) N-GfPP is an N-alkylporphyrin,
- b) the drug-to-porphyrin linkage is a methylene group,
- c) resonances attributable to protoporphyrin are found in the adduct and
- d) overcrowding of the N-GfPP spectrum makes distinction of griseofulvin-derived resonances difficult.

\[\text{T}_1\] experiments were then carried out which allowed

- a) assignment of the H\(_r\) meso proton, and
- b) distinction of the four peripheral porphyrin methyl groups (\(1\text{CH}_3, 3\text{CH}_3, 5\text{CH}_3\) and \(8\text{CH}_3\)) from the 6-OMe, 7-OMe and 2'OCH\(_3\) resonances.

Use of a sample with deuterated 6- and 7-OMe groups then allowed

- a) assignment of the 6- and 7-OMe resonances

Use of correlation spectroscopy (COSY) then showed that

- a) the chemical shift values of protons in the spin-system of ring C of griseofulvin are not altered from their chemical shift values in the proton spectrum of griseofulvin alone, and therefore that
- b) the orientation of the drug over the porphyrin ring is such that ring C of the drug is beyond the shifting capabilities of the porphyrin ring,
- c) the linkage of drug-to-porphyrin is unlikely to be through 2'OCH\(_3\),
- d) the propionate methyl ester groups at positions 6 and 7 of the porphyrin periphery appear to experience hindered rotation, and
- e) the vinyl groups at positions 2 and 4 of the porphyrin periphery can be differentiated.
Use of nuclear Overhauser effect spectroscopy (NOESY) then established that
a) the attachment of griseofulvin to the porphyrin is through a methylene linkage derived from either the 4- or 6-methoxy groups of ring A of griseofulvin,
b) all proton resonances associated with ring A of griseofulvin experience high-field shifts whereas,
c) 2′OCH₃ and 3′Hₓ of ring C of griseofulvin come at expected chemical shift values.

Comparison of the 1D spectra of the zinc and free-base forms of N-GfPP with spectra of the zinc and free-base forms of all four regioisomers of N-MePP was then able to establish
a) that N-GfPP appears to be predominantly the N₇ regioisomer

Following on from identification of the predominant regioisomer of N-GfPP formed, complete assignments for the free-base form of N-GfPP could now be obtained and included
a) assignment of all meso protons on the basis of NOEs from meso protons to methyl, internal vinyl and proximal methylene groups,
b) likewise assignment of all methyl group resonances and internal vinyl resonances, on the basis of NOEs and assumptions made on the effect of the drug in affecting chemical shift values,
c) differential assignment using COSY data of the proximal and distal methylene groups of the propionate methyl ester groups at positions 6 and 7 of the porphyrin periphery, again on the basis of assumptions made on the effect of the drug in affecting chemical shift values, and
d) assignment of the 2- and 4- terminal vinyl protons using data from the COSY experiment.
4.5. Results from Molecular Modelling of N-GfPP

The NMR studies of N-GfPP had raised a number of interesting questions, such as
1) how much distortion of the porphyrin plane is incurred on N₇ alkylation?
2) can the proposal that the positioning of the drug over the porphyrin ring will affect the
   5CH₂ and 6-proximal methylene resonances be merited?
3) in what way is rotation of the 6- and 7-propionate methyl ester groups hindered?

Although molecular modelling of N-GfPP is not in any way proposed to provide conclusive
answers, it was hoped that studying a molecular model would help clarify the stereochemical
situation that prevails in the adduct.

The first drawback to making the model was the lack of any 'intermolecular' NOEs between
the griseofulvin drug and the porphyrin ring, which would have given an idea of distance
restraints. All that could be done was to attach the griseofulvin drug to the proposed ring C
pyrrole nitrogen of the porphyrin and then energy minimise the resulting structure. The point
of attachment was chosen as the 6-methoxy group (as opposed to the 4-methoxy group)
because, during normal metabolism of griseofulvin, the 6-desmethyl metabolite predominates
over the 4-desmethyl metabolite (Chang et al, 1973). The results of this procedure are
shown in Figure 4.11.

In this model the proton H₄ is 5.27 and 5.46Å away from the pyrrole ring B and the pyrrole
ring D nitrogens respectively and so lies almost over the centre of the porphyrin. The view
tries to show which protons of griseofulvin are in the vicinity of the porphyrin centre and can
therefore be expected to experience ring-induced shifts. These protons are, in decreasing
order of the size of the expected high-field shift 1) the protons of the N-CH₂-O linkage, 2)
the H₄ proton and 3) the protons of the 4-OCH₃ group. The structural form of the model

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Fig 4.11. Proposed structure of the N-GfPP adduct. The linkage point shown is through the 6-OCH₃ group of griseofulvin (see text). The conformation shown, generated using Insight II (Biosym), is essentially arbitrary, but is consistent with the observation that the resonances of protons of ring C of the griseofulvin moiety are not affected by the ring current of the porphyrin.
coincides closely with the observed NMR data, where the resonances assigned to these protons experienced high-field shifts of 7, 4 and 1.4 ppm respectively, when compared to their chemical shift values in a proton spectrum of griseofulvin alone.

The NMR data also suggested that the drug was not completely free to rotate about the N-CH$_2$-O linkage bond, as the two protons in this group gave rise to distinct resonances and displayed geminal couplings. As methylene protons which are free to rotate are equivalent, the observed results implied a lack of rotation. The hypothesis was tested by fixing the CH$_2$-O bond of the linking group. This bond was chosen as the axis of rotation because it was assumed that the N-CH$_2$ and O-Ring A (griseo) bonds would be 'fixed' stereochemically as they form substituents of aromatic components of the model in each case. Upon trying to rotate the drug around this axis it became clear that, in this model, such a rotation is a physical impossibility, as it results in the drug travelling through the porphyrin plane. This therefore implies that the drug will take up at least one preferred orientation in keeping with the interpretation of the NMR data. The model also clearly defines the position of ring C of the drug as being beyond the shifting capabilities of the porphyrin ring, as already proposed during interpretation of the NMR data. It is also clear from this viewpoint that griseofulvin may well have some effect on the positioning of the 6 and 7 propionate methyl ester residues as the griseofulvin drug does lie more over ring C of the porphyrin and its associated groups ($^5$CH$_3$ and 6-OMe). In interpreting the NMR data it was proposed that the drug may have an effect on the $^5$CH$_3$ and 6-proximal methylene protons such that any induced chemical shifts may well be masked, or not observed and this model, if taken to be a true representation of the N-GfPP adduct, helps to verify such a proposal.

In conclusion, the molecular model for N-GfPP coincides remarkably well with the NMR data and has been used to enhance understanding of the three-dimensional structure of the adduct. It has been used to explore the possibilities of porphyrin ring distortion and various
rotational effects that are difficult to envision otherwise and has also been highly instrumental in defining the probable position of the griseofulvin drug over the porphyrin ring.
4.6 Results of Investigation into Possible Break-down Products of N-GfPP

4.6.1. Discovery of Breakdown Products

Batches of N-GfPP which no longer yielded good NMR spectra were rerun on tlc as described in the methods section of this chapter. Of the three batches investigated, only one appeared to have undergone considerable degradation, as shown by a total of seven distinct tlc bands. The other two batches appeared only to resolve into two components, one the expected band of N-GfPP near the leading edge of the solvent system and the other a green fluorescent smudge at an Rf of approximately 0.39.

It was this second band which was of interest as a possible break-down product of N-GfPP. As mentioned already, two N-alkylporphyrins are produced in the griseofulvin-induced protoporphryia (Holley et al., 1991). One is N-GfPP and the other the Nα regioisomer of N-MePP. Secondary fragmentation of N-GfPP occurs in the mass spectrometer to yield an N-MePP daughter ion, which led to the proposal that a secondary fragmentation of N-GfPP may occur in vivo. It was therefore conceived possible that this second unknown band appearing in the tlc of NMR'd N-GfPP might be an N-methylporphyrin. To try and verify this, the band was scraped, eluted, treated with acid to remove zinc contaminants and an electronic UV/Vis spectrum obtained (see (a), Figure 4.12.).

The spectrum is clearly that of a porphyrin, with a Soret at 411nm and other, barely distinct peaks in the region 540-660nm. The overall form appears to be a free-base, but the shape of the Soret is suspiciously broad, indicating that more than one form of porphyrin may be present. A free-base protoporphyrin is meant to absorb at 407nm, whereas free-base N-MePP is meant to absorb at 419nm (Falk, 1964). A value of 411nm is an intermediate between the two extremes. It was decided to use high pressure liquid chromatography (hplc) to try and resolve the sample into all its constituent components.
4.6.2. HPLC Analysis of Break-Down Products.

The hplc system used is described fully in the methods section of chapter 5. It was devised to allow the separation of \( N_{A+B} \) N-MePP from \( N_{C+D} \) N-MePP. The sample under investigation was compared, using this system, to a standard mixture of chemically synthesised \( N_{A+B} \) and \( N_{C+D} \) regioisomers of N-MePP. The results are shown Figure 4.12., (b).

Trace A) is of a sample of the standard mixture of \( N_{A+B} \) and \( N_{C+D} \) N-MePP regioisomers. The 1.03 and 1.63 min retention peaks are unknown compounds and protoporphyrin respectively. The 3.58 min retention peak corresponds to \( N_{A+B} \) N-MePP regioisomers and the 5.67 min retention peak to \( N_{C+D} \) N-MePP regioisomers.

Traces B) and C) are of the unidentified band collected from the tic of N-GiPP. These also appear to have a retention peak at \( \sim 0.93 \) and \( \sim 1.62 \) min corresponding to unknown compounds and protoporphyrin but the peak of real interest is at \( \sim 5.75 \) min. This peak is compatible with the retention peak for \( N_{C+D} \) regioisomers of N-MePP in the standard. No peak appears at \( \sim 3.58 \) min compatible with \( N_{A+B} \) regioisomers of N-MePP.

It was therefore concluded from this investigation that N-GiPP appears to breakdown into an N-alkylporphyrin of a \( N_C \) or \( N_D \) regioisomeric type.
Fig. 4.12. Investigation into the break-down products of N-GfPP. 1. shows the electronic absorption spectrum of the proposed break-down product of N-GfPP. 2. shows hplc traces of A) standard mixture of all four regioisomers of N-methylprotoporphyrin, B) and C) proposed N-GfPP break-down product. A comparison of these traces indicates that the N-GfPP break-down product may be a $N_C$ or $N_D$ regioisomer of N-methylprotoporphyrin (for details see text).
4.7. Discussion

The main aim of the work described in this chapter was to establish the structure of the N-GfPP that forms, and can be isolated from, the livers of griseofulvin-fed mice. This structural characterization produced two unexpected results:

1) The point of attachment of griseofulvin to the porphyrin appears to be through the 4- or 6-OCH$_3$ group of ring A of griseofulvin, and does not appear to involve ring C.

2) The N$_2$ regioisomer of N-GfPP appears to be the predominant form isolated from the liver.

The basis for presuming that the drug would attach via the 2'OCH$_3$ came from data (de Matteis et al, 1975) showing that a thioether substitution at the 2' position causes complete inactivation of the porphyrinogenic properties of the compound, whereas isogriseofulvin (with a ketone at the 2' position and a methoxy at the 4' position) has enhanced activity. These findings show that the 2' position, or ring C as a whole, are clearly involved in causing porphyria. Normal metabolism of griseofulvin causes the production of the 4- and 6-desmethyl metabolites in the urine (Chang et al, 1973; Lin et al, 1972), suggesting, on the other hand, that ring A is the normally-favoured site of griseofulvin modification.

The NMR structural characterisation of N-GfPP has determined that the site of modification of griseofulvin is at the 4- or 6-OCH$_3$ group. The assignment is based on NOE data and distinct ring-induced shifts of Ring A (griseo) and not ring C (griseo) resonances, behaviour which is unaccountable if 2'OCH$_3$ were to be the site of modification.

The NMR studies have also shown that the N$_2$-GfPP regioisomer appears to be the main form isolated from the livers of griseofulvin-fed mice. Again, this was not an expected result as the minor adduct isolated from the same livers is the N$_A$ regioisomer of N-MePP.
tandem mass spectrometric studies the N-GfPP adduct gave rise to a N-MePP daughter ion which suggested that N-GfPP might give rise to N-MePP as a secondary product (Holley et al., 1991). N-MePP plays a role in the porphyric state by inhibiting ferrochelatase, the last enzyme of the liver haem biosynthetic pathway.

It is highly unlikely that N^-GfPP would give rise to N/^MePP, as it seems improbable that, once a covalent bond of drug-to-porphyrin has been formed, this same bond will then break and the N-alkyl group relocate to another pyrrole ring nitrogen. Further evidence against such a sequence of events is provided by the investigation into the break-down products of N-GfPP. This study is not exhaustive, but would suggest that an N-alkyl porphyrin (most likely N-MePP) of N_C or N_P regiosomeric type is formed as a break-down product of N-GfPP. The data described in this chapter would therefore suggest that the N_C-GfPP which is isolated predominantly from the liver can, with time, break down into (most likely) a N_C or N_P N-MePP.

Such behaviour is compatible with more recent findings (de Matteis et al, abstract for meeting) concerning the ferrochelatase inhibitory activity of N-GfPP. Previously, it was proposed that N-GfPP had no ferrochelatase-inhibitory activity up to concentrations of 170nm (Holley et al., 1991), but the more recent studies have shown that preincubation of N-GfPP with liver homogenate is required for inhibitory activity to be observed. At maximal inhibition, obtained with approximately 20 mins preincubation, the inhibitory activity of N-GfPP is very similar to that of N-MePP, a finding consistent with time-dependent conversion of N-GfPP to N-MePP.

The scenario then, is that N-alkylporphyrins of more than one regiosomeric type are present in the livers of griseofulvin-fed mice. One study (Holley et al., 1990), demonstrated that all four structural regiosomers of N-MePP are found (with the N_A regioisomer predominating) in treated mice and the work described here has identified the N-GfPP adduct of being
primarily $N_C$ regioisomeric type. The presence of only small amounts of $N_C$-MePP does not invalidate the proposal of a break-down mechanism of $N$-GfPP, as the data only suggests that $N$-GfPP only breaks down in (amongst other things) a time-dependent manner. This time-course is of a few days in an NMR tube (which even then only gives rise to a very small amount of product) or 20mins preincubation with liver homogenate. This suggests that the $N$-GfPP adduct is actually quite stable and is unlikely to break down spontaneously. Indeed, if spontaneous break-down were to occur, an $N$-MePP should logically be the predominant isolated adduct. As it stands, $N$-GfPP and $N$-MePP are isolated in a ratio of approximately 10:1, so it can be assumed that breakdown of $N$-GfPP is not likely to be a very prominent process in operation in the liver which would account for the small amounts of isolated $N$-MePP regioisomers. It should also be remembered that $N$-MePP has also been found in very small amounts in the livers of untreated mice (Holley et al, 1990), where the $N_A$ and $N_C$ regioisomers appear to be present in approximately equal amounts (though numbers are not given).

So how do the $N_A$-MePP and $N_C$-GfPP adducts arise in the first place? The existence of two regioisomeric forms of $N$-alkylporphyrins, with differing alkyl groups, leads to the suggestion of two different mechanisms of $N$-alkylporphyrin formation in operation in the liver, as the formation of $N_A$-MePP from $N_C$-GfPP has been discussed and appears unlikely. Of course, it may be that an $N_A$ regioisomeric component of $N$-GfPP is made in vivo which, for some reason, is more labile than $N_C$-GfPP and so quickly breaks down into the isolated $N_A$-MePP. Such an eventuality is very difficult to investigate.

Both the $N$-alkylporphyrins isolated from the livers of griseofulvin-fed mice are chiral and are labelled from 5-amino[4-14C]laevulinate (de Matteis et al, 1991), which has led to the suggestion that liver cytochrome $P_{450}$ may be involved in adduct formation. There are many instances cited in the literature of mechanisms whereby drug metabolism leads to $N$-alkylporphyrin formation and results in suicide inactivation of the enzyme.
For example, formation of N-MePP at the cytochrome P₄₅₀ active site has been well-documented in accounts of the DDC-induced porphyria (see section 2.6.1.), where a methyl radical, lost during oxidative aromatisation of DDC, reacts with haem at the cytochrome P₄₅₀ active site to form mainly Nₐ-MePP (Kunze & Ortiz de Montellano, 1981). Such a mechanism cannot account for the N-MePP formed in the griseofulvin case. The structures of DDC and griseofulvin are markedly different, most importantly in that ring A in griseofulvin is already aromatic and so oxidative aromatisation can not occur. A methyl radical could be generated from heterolytic cleavage of the 4- or 6- OCH₃ bond. If this step were preceded by a 1e⁻ reduction by cytochrome P₄₅₀ at oxygen (which would confer instability on the bond and make cleavage more likely), this would also give rise to a griseofulvin radical which could either take up a proton from its surroundings (to form the 4- or 6-desmethyl derivatives), or recombine with the methyl radical. The methyl radical could either recombine or react with a pyrrole ring nitrogen of the prosthetic haem. Presumably there would be some limits on which pyrrole ring nitrogen is most available for reaction, which would explain the dominance of the isolated Nₐ-MePP. Such a mechanism is completely hypothetical, however, and has no precedent. It should be remembered that formation of N-MePP was documented in low but measurable quantities in the livers of control mice in the DDC-induced porphyria (Tephley et al, 1979; Holley et al, 1990), which would argue that there is an in vivo mechanism for N-MePP formation not necessarily influenced by drug administration in any way.

The griseofulvin adduct also poses a problem mechanistically, as it appears to defy the known precedents of N-alkylporphyrin formation and cytochrome P₄₅₀ catalysed reactions. It is proposed, to make sense of the resulting N-GiPP structure, that hydrogen abstraction must occur at the 4- or 6-OCH₃ group, followed by combination of this group with the nitrogen of pyrrole ring C of the prosthetic haem of cytochrome P₄₅₀.
Cytochrome P₄₅₀ most often works as a monooxygenase. In suicidal inactivation of the cytochrome by N-alkylporphyrins, the alkylating species is usually

a) the drug with an oxygen atom inserted or

b) a small portion of the drug produced by fragmentation of the drug during metabolism.

Here, griseofulvin appears to attach itself to a N₉ pyrrole ring nitrogen without oxygen insertion or fragmentation. Another type of alkylating species appears to have been uncovered.

One possible mechanism, completely hypothetical, could be that the cytochrome accepts the drug and tries to carry out carbon hydroxylation of the 4-OCH₃ or the 6-OCH₃ group. The reaction is as follows and has been chosen as the starting point of the mechanism because it is the only documented type of cytochrome P₄₅₀ reaction that begins with hydrogen abstraction:

\[
[\text{FeO}]^{3+} + \text{H-CH}_2 \rightarrow [\text{FeOH}]^{3+} \cdot \text{CH}_2 \rightarrow \text{Fe}^{3+} + \text{HOCH}_2
\]

After hydrogen abstraction from the drug substrate (in this case griseofulvin) to the activated ferryl oxygen of cytochrome P₄₅₀, the newly-formed carbon centred radical may be able to combine with a pyrrole ring nitrogen rather than the recombination event with the ferryl oxygen to give the hydroxy product. Such a process might be preferred if the griseofulvin radical was reasonably stable and therefore able to diffuse slightly from the immediate vicinity of the haem iron. Again, as with the N-MePP adduct, there must be some limits on the availability of particular pyrrole ring nitrogen for bonding interactions, as the N₉ regioisomer of N-GiPP appears to predominate.
Summary - The structure of the N-GfPP adduct has been identified as protoporphyrin with the whole of griseofulvin (minus a hydrogen atom) attached via the 4- or 6-OCH₃ group. The N₇ regioisomer appears to predominate. A brief investigation has also been made into possible break-down products of N-GfPP, with the result that a N₇ or N₉ N-alkylporphyrin appears to arise in a time-dependent manner from the parent N₇-GfPP. The presence of two N-alkylporphyrins of differing N-alkyl group and regioisomeric type (N₇-MePP and N₇-GfPP) makes it unclear whether there are one of two methods of N-alkylporphyrin formation in operation in the liver. Hypothetical mechanisms have been proposed for the formation of both adducts.
5. \textit{\textsuperscript{1}H and \textsuperscript{13}C NMR Studies of Synthetic N-alkylporphyrins}

5.1. Statement of Aims

The synthesis of N-alkylporphyrins was undertaken for three reasons:

1) To provide model compounds for the biological N-GfPP
2) To study the NMR spectral characteristics of N-alkylporphyrins with differing N-alkyl groups and,
3) To provide a range of N-alkylporphyrins for use in studies of their inhibition of ferrochelatase.

The first point has already been covered in the preceding chapter, where comparison of the proton spectra of free-base and zinc forms of the four regioisomers of N-methylprotoporphyrin aided identification of the regioisomer of N-GfPP isolated from the livers of griseofulvin-fed mice.

The third point is of considerable importance, but its discussion is delayed until later (chapter 6), where the results of inhibition studies with solubilised ferrochelatase, using N-alkylporphyrins, will be discussed within the context of the form and function of the ferrochelatase active site.

The second point forms the basis for discussion of this chapter, where various N-alkylporphyrins have been synthesised and their NMR spectra recorded. The proton spectra of the individual regioisomers of N-methyl and N-ethylprotoporphyrin have been compared and contrasted to the spectra of the planar porphyrin, protoporphyrin, to see the effects of N-alkylation and metalation on the various porphyrin resonances. These effects are marked because of the highly anisotropic nature of the porphyrin ring current and so the validity of
using shifts of resonances as a probe for porphyrin structure is discussed. The spectra of the free-base and zinc-complexed regioisomers of both the N-methyl and N-ethylprotoporphyrins have been assigned from NOESY data. The $^1$H NMR spectra of both forms of the regioisomeric pairs of N-1-propyl and N-1-butylprotoporphyrin are also discussed to see if the NMR behaviour of N-alkylporphyrins with larger N-alkyl groups differs in any way from that of the N-methyl and N-ethylprotoporphyrins. $^{13}$C NMR spectroscopy has also been investigated as a means for probing porphyrin structure by comparing the $^{13}$C spectra of the zinc and free-base forms of protoporphyrin with those of the $N_C$ regioisomer of N-methylprotoporphyrin. General assignments have been made for the $^{13}$C spectra of both forms of $N_C$-methylprotoporphyrin through the use of carbon-proton correlation spectroscopy. Throughout this discussion the term 'both forms' refers to the zinc-complex and free-base derivatives of the N-alkylprotoporphyrin dimethyl esters.
5.2. Methods

5.2.1. Quantitating Porphyrins by Spectral Analysis

Porphyrins can form either free base or metal complexes with some metals e.g. Zn$^{2+}$ or Co$^{2+}$. Both metal complexes and the free-base have distinctive electronic absorption spectra over the range 360-700nm. Porphyrins can be estimated from absorbance of the main Soret band, using known molar absorption coefficients (see Appendix). Porphyrins are dissolved in chloroform as the methyl esters for spectral analysis.

5.2.2. Interconversion of Free-base and Zinc Complex forms of N-alkylporphyrins.

Conversion between metal complex and free-base is initiated by addition of 50mM HCl in methanol. The free-base form is extracted into CHCl$_3$ and dried by rotary evaporation. The zinc complex is generated from the free-base by adding saturated zinc acetate (in MeOH), to a solution of porphyrin in CHCl$_3$. This is washed to remove acetate (by shaking with distilled water and then centrifuging, the process is repeated three times) and then saturated NaCl (in MeOH) added to form the chloride zinc counterion. This is washed to remove excess chloride (three water washes are used) and then dried (by passing through a Na$_2$SO$_4$ column, and then by rotary evaporating).

5.2.3. Conversion of Protoporphyrin Dimethyl Ester to the Free-acid Form.

The dimethyl ester form of protoporphyrin IX (PPIX) is used for all syntheses and NMR work, but the free-acid form of the compound is less expensive and available from Sigma. The conversion of free-acid to ester is achieved by dissolving the porphyrin in 5% sulphuric
acid in MeOH and leaving the reaction to proceed overnight in the dark at 4°C. Next day
the dimethyl ester is extracted into CHCl₃, and dried (rotary evaporation).

5.2.4. Synthesis of N-alkylporphyrins

Protoporphyrin IX dimethyl ester (~8mg, produced as above) is dissolved in the appropriate
alkyl iodide (~4ml, from Aldrich) and heated (100°C, 3hrs (methyl iodide), 5hrs (ethyl
iodide) or 24hrs (propyl and butyl iodide) in a test-tube with a teflon-lined screw-cap.
Throughout the heating procedure, the tubes are protected from light by covering with tin­
foil. The heat source is a dri-block. After this time the tubes are cooled, the products dried
under N₂ or rotary evaporated and stored at ~20°C. The syntheses of N-
methylprotoporphyrin (N-MePP, 44% yield), N-ethylprotoporphyrin (N-EtPP, 14.1% yield),
N-1-propylprotoporphyrin (N-PrPP, 17.8% yield), and N-butylprotoporphyrin (N-BuPP,
11.8% yield) were undertaken.

5.2.5. Separation of N-alkylporphyrin Regioisomers by Thin Layer Chromatography
(tlc)

The products from the N-alkylation reaction are dissolved in CHCl₃ (~1ml) and streaked
across two Merck silica 60 pre-coated tlc plates (20cm x 20cm, 0.25cm thick without
fluorescent indicator). These are developed in a closed glass tlc tank lined with blotting
paper and preequilibrated with a CHCl₃:MeOH (20:3, v/v) developing system. Light was
excluded during development of the plates by covering the tank with tin-foil. The first run in
this system separates the N-alkylated products from unreacted protoporphyrin and tri- and
di-alkylated products. Protoporphyrin runs as a dark brown leading band with a tailing green
fringe (which fluoresces orange under long-wave UV but loses fluorescence with time). The
next band is a clearly-defined dark-red band (with permanent deep orange/red fluorescence
under long-wave UV) which is collected as it constitutes the mono-alkylated products. A final dark-brown band just above the base-line constitutes the di- and tri-alkylated products. The required products are scraped from the plate, eluted from the tlc silica with MeOH and extracted into CHCl₃. The sample is then washed (as before with 3 × H₂O) and dried (through Na₂SO₄ and then rotary evaporation) and stored at −20°C.

This procedure is then repeated to separate the Nₐ+B regioisomers of N-alkylporphyrins from the Nₐ+C regioisomers. After this run the leading band (which is not at the solvent edge) is a green band which may be followed by a distinct brown line. The two bands of interest come next, the dark-red band nearest the solvent edge constitutes the Nₐ+B regioisomers and the next dark-red band the Nₐ+C regioisomers. The regioisomeric pairs are collected as separate bands (Rₐ(Nₐ+B) = 0.36 ; Rₐ(Nₐ+C) = 0.26) which are eluted from the tlc silica with MeOH and extracted into CHCl₃. The samples are then washed (3 × H₂O as before to remove as much silica as possible), dried (through Na₂SO₄ then N₂ or rotary evaporation) and stored at −20°C.

Individual regioisomers are separated by multiple development in a heptane:methyl acetate (3:2, v/v) solvent system using solvents of normal spectrophotometric grade. The Nₐ+B regioisomers are plated out separately from the Nₐ+C regioisomers. The Nₐ regioisomer moves fastest in this system, followed by Nₐ, N₉ and then N₉. Usually at least three developments are needed, often four, especially with the N-ethylprotoporphyrin, to see individually distinct bands accounting for the four regioisomers. With N-alkyl groups larger than a methyl group the Nₐ+B sample will yield Nₐ and Nₐ bands only, the Nₐ+C sample often yields Nₐ, Nₐ, N₉ and N₉ bands. Each band is collected separately, freed from the silica using CHCl₃:MeOH (20:3 v/v) or 50mM HCl in MeOH if necessary and extracted immediately into CHCl₃ before degradation of the vinyl groups can occur (if acid is used).
The samples are washed (as before, 3 x H₂O), dried (through Na₂SO₄ and then N₂ or rotary evaporation) and stored at -20°C.

Care is taken at all times to protect the samples from light, which could induce photodegradation. The separated regioisomers are quantified by spectral analysis.

5.2.6. Separation of N-alkylporphyrin Regioisomers by High Pressure Liquid Chromatography (hplc)

Some N-alkylporphyrins isolated by the tlc procedure had a broad band of impurity in their NMR spectra between 0.4 and 3ppm. These samples were therefore subjected to a hplc clean-up procedure.

Regioisomeric pairs (N_{A+B} and N_{C+D}) of N-alkylporphyrins can be separated using a Nucleosil 5 silica column (4.6 x 250mm, de Matteis et al, 1982). The mobile phase is dichloromethane: methanol: ammonia (50:50:0.1, v/v/v), run isocratically, monitoring at 417nm. This system was used to clean-up samples of separate regioisomers of impure N-alkyl porphyrins.

The system was also used in the separation of regioisomeric pairs of N-PrPP and N-BuPP, where the increased size of the N-substituent made separation on tlc difficult to achieve.

5.2.7. NMR Methods

Proton and carbon NMR studies were carried out on samples of concentration ranging between 1-6mM in CDCl₃, 1.25% w/v deuterated pyridine was added to ensure formation of the free-base form and was also added to studies with the zinc complex forms of the
porphyrins for continuity. Proton studies have been made of the separate regioisomers of the zinc and free-base forms of N-MePP and N-EtPP, and the regioisomeric pairs of N-1-PrPP and N-1-BuPP. Carbon studies have used the zinc and free-base forms of protoporphyrin and the zinc and free-base forms of the N⁷ regioisomer of N-methylprotoporphyrin.

The reader is referred to Chapter 3 (NMR : Experimental Methods and Theory) for a more detailed description of the applicability of carbon NMR to porphyrins and also of the NMR experiments used. Relevant experimental details are, however, included with the appropriate figures.
5.3. Results From $^1$H NMR Studies of N-alkyl Porphyrins and Related Compounds

5.3.1. A Study of the Planar Porphyrin Protoporphyrin IX Dimethyl Ester.

The free acid form of protoporphyrin is utilised by ferrochelatase. Furthermore, X-ray crystallographic studies have shown that non-alkylated porphyrins such as protoporphyrin are planar compounds (Little et al, 1975; Little & Ibers, 1975), which makes this enzyme substrate an ideal reference compound for NMR studies aimed at identifying structural differences between the non-alkylated and N-alkyl porphyrins.

The proton spectra of protoporphyrin IX dimethyl ester has been studied in detail and assigned at 220MHz (Janson & Katz, 1972). The spectra shown in Figure 5.1. were recorded at 500MHz. The assignments made for both the free-base and zinc form of protoporphyrin are given in Table 5.1.

The molecular structure of protoporphyrin is shown as an inset in the figure, and the most important resonances are clearly marked. The two spectra show clearly the differences between the free base and zinc complex forms of a porphyrin.

Firstly, an extremely high-field resonance is seen at 4.05ppm in the free-base spectrum but not in the zinc complex spectrum. This resonance is attributable to the NH protons of the free-base. They come at such high-field because of their position directly in the centre of the porphyrin macrocycle. Upon metalation the porphyrin loses its two NH protons (section 2.1.4.) and so no signal is seen at such high field in the spectrum of the zinc complex.
Fig. 5.1. Proton NMR spectra of a) free-base and b) zinc-complexed protoporphyrin. Samples were 5mM in CDCl₃.
Coordination of a metal atom to the four central nitrogen atoms of protoporphyrin appears to have little effect on the spectrum of the molecule. Proximal and distal methylene groups are in similar environments in both forms of the porphyrin, as exemplified by the triplets to low and high-field of the methyl/methoxy singlets lying between 3.61-3.73 ppm. Likewise, the 2 and 4 vinyl groups are in similar environments in both forms as indicated by the multiplets and distinct doublets in the range 8.3-6.0 ppm.

Even the methyl/methoxy group resonances span similar ranges in each form. In the free-base form $^{1}CH_{3}$ and $^{3}CH_{3}$ (which are on vinyl-substituted pyrrole rings A and B) come at similar chemical shift values of 3.71 and 3.70 ppm. Likewise, $^{5}CH_{3}$ and $^{8}CH_{3}$ (which are on propionate-substituted pyrrole rings C and D) are also closely grouped together at 3.61 and 3.62 ppm. In the zinc-complex the resonances for $^{1}CH_{3}$ and $^{3}CH_{3}$ (and $^{5}CH_{3}$ and $^{8}CH_{3}$) are not distinguished. In both forms, however, the groups on vinyl substituted rings ($^{1}CH_{3}$ and $^{3}CH_{3}$) come to low field compared with those on propionate substituted rings ($^{5}CH_{3}$ and $^{8}CH_{3}$), probably as a consequence of decreased shielding caused by the 2 and 4 vinyl groups.
Table 5.1: Assignments of the Zinc-Complex and Free-Base Forms of Protoporphyrin IX.

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical Shift Values (in ppm) for Protoporphyrin IX Dimethyl Ester</th>
<th>Chemical Shift Values (in ppm) for Protoporphyrin IX Dimethyl Ester</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FREE BASE</td>
<td>ZINC COMPLEX</td>
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<tr>
<td>Hα</td>
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<tr>
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<td>9.98</td>
</tr>
<tr>
<td>2Vi (int)</td>
<td>8.30</td>
<td>8.39</td>
</tr>
<tr>
<td>4Vi (int)</td>
<td>8.30</td>
<td>8.39</td>
</tr>
<tr>
<td>2Vi (term)</td>
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</tr>
<tr>
<td>4Vi (term)</td>
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<td>4.44 (6&amp;7)</td>
</tr>
<tr>
<td>1CH₃</td>
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</tr>
<tr>
<td>7-OMe</td>
<td>3.66</td>
<td>3.67</td>
</tr>
<tr>
<td>Distal methylene</td>
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<td>3.31 (6&amp;7)</td>
</tr>
<tr>
<td>NH</td>
<td>4.05</td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>
5.3.2. Comparison of the $^1$H NMR Spectra of Free-Base Protoporphyrin and Free-Base N-Methylprotoporphyrin, $N_A$ and $N_C$ Regioisomers.

The $N_A$ and $N_C$ regioisomers of N-methylprotoporphyrin (N-MePP) have been chosen for comparison with protoporphyrin because they are examples of porphyrins alkylated on vinyl-substituted ($N_A$) and propionate-substituted ($N_C$) rings. Subtle differences are seen in the spectra of these compounds (see Figure 5.2) when compared to that of free-base protoporphyrin which are dependent both on N-alkylation itself and also the type of ring that is alkylated.

Firstly, the 2 and 4 vinyl groups are no longer in similar environments as is the case with free-base protoporphyrin. N-alkylation of a porphyrin, as mentioned already, causes distortion of the porphyrin. Moieties on out-of-plane pyrrole rings resonate at higher field, as their changed position relative to the interrupted porphyrin ring current results in their lessened deshielding. This is clearly demonstrated by the 2 and 4 internal vinyl resonances in the spectra of both N-alkylporphyrins, as whereas one multiplet for these groups is clearly seen at 8.30ppm in the free-base protoporphyrin spectrum, two are seen in the $N_A$ and $N_C$ N-MePP spectra. Significantly, the shift to high-field of one of these multiplets is larger in the $N_A$-MePP spectrum than the $N_C$-MePP spectrum (0.31ppm as opposed to 0.16ppm). This correlates well with the idea that the alkylated ring will have greater out-of-plane character than any other ring of the porphyrin, as $^2$Vi is on an alkylated ring in $N_A$-MePP, but only opposite an alkylated ring in $N_C$-MePP the ring-induced shift for this resonance can reasonably be expected to be larger in $N_A$-MePP, and this is actually found. The 2 and 4 terminal vinyl resonances are also affected, as they no longer give distinct doublets of-doublets as in the free-base protoporphyrin. Instead, the doublets of-doublets cover a range of 6.04-6.39ppm as alkylation is instrumental in causing shifts of some of these resonances to higher field in each N-alkylated regioisomer.
Secondly, in free-base protoporphyrin, the 6 and 7 proximal and the 6 and 7 distal methylene resonances have identical chemical shifts, implying that they are in closely similar magnetic environments. In \( N_A \) and \( N_C \) N-MePP, there are two proximal methylene resonances, again indicating that alkylation is causing a shift of resonances to higher field. As it is propionate resonances that are affected, the difference in chemical shift to higher field is larger in the \( N_C \)-alkylated porphyrin (which is propionate-substituted) rather than the \( N_A \)-alkylated porphyrin, though only for the distal methylene resonances. In \( N_A \)-MePP these resonances are superimposed (at 3.32ppm), whereas in \( N_C \)-MePP a shift to high-field of 0.48ppm occurs for the 6 distal methylene resonance. This implies that the distortion of the \( N_C \) ring caused by \( N_C \) alkylation is such that the 6 and 7 distal methylene protons in this porphyrin are in distinctly different magnetic environments, whereas the 6 and 7 distal methylene protons in \( N_A \)-MePP must be in similar magnetic environments as they have the same chemical shift value.

Some sort of dynamic picture of these porphyrins in solution can also be built up from this comparison since, in the free-base protoporphyrin, both proximal and distal methylene resonances are triplets, indicating free rotation around the carbon-carbon bonds of the 6 and 7 propionate groups. The triplet form of the multiplet arises from two magnetically equivalent protons coupling to the two non-equivalent protons of the neighbouring methylene group. In both \( N_A \) and \( N_C \) N-MePP, only one of the proximal methylene resonances is a triplet, the other appears as a complex multiplet. The complex multiplet implies that the two protons of one of the adjacent methylene groups are no longer magnetically equivalent and so are coupling to each other as well as their neighbouring distal methylene protons. Such a situation could arise if N-alkylation causes hindered rotation of one or other of a porphyrin's propionate groups.
Fig. 5.2. Spectra of the free-base forms of a) protoporphyrin (5mM in CDCl₃), b) Nₐ-methylprotoporphyrin (4.16mM in CDCl₃) and c) Nᵦₐ-methylprotoporphyrin (5.59mM in CDCl₃). Regions from low to high field are: meso protons, internal and terminal vinyl, proximal methylene, methyl/methoxy singlets and distal methylene and the N-CH₃ proton singlet.

Fig. 5.3. Spectra of the zinc-complexed forms of a) protoporphyrin (5mM in CDCl₃), b) Nₐ-methylprotoporphyrin (2.76mM in CDCl₃) and c) Nᵦₐ-methylprotoporphyrin (3.69mM in CDCl₃). Regions from low to high field are: meso protons, internal and terminal vinyl, proximal methylene, methyl/methoxy singlets and distal methylene and the N-CH₃ proton singlet.
5.3.3. Comparison of the $^1$H NMR Spectra of Zinc-Complexed Protoporphyrin and Zinc-Complexed N-Methylprotoporphyrin $N_A$ and $N_C$ Regioisomers.

Figure 5.3. shows a comparison of the $^1$H NMR spectra of the zinc complexes of protoporphyrin, $N_A$-MePP and $N_C$-MePP.

The spectral range is larger for the N-alkylated zinc complexes than the protoporphyrin zinc complex as $N_A$ and $N_C$ N-MePP have extremely high-field resonances not apparent in the zinc-protoporphyrin spectrum. Also, the meso protons in the N-alkylated zinc complexes appear to be shifted slightly to low field when compared to their equivalents in the zinc-protoporphyrin spectrum. The N-CH$_3$ proton resonances appear at such high-field because of their position directly over the porphyrin macrocycle. The increased deshielding of the meso resonances, however, is more complex to explain as it arises from a combination of factors:

1) N-alkylation. This causes distortion of the porphyrin macrocycle such that the conjugation pathway for the π-electrons that give rise to the porphyrin ring current is disrupted. As a result, the ring current is decreased and so peripheral proton resonances should come at higher field (as seen in the free-base spectra).

2) Metalation. Coordination of zinc to three of the central pyrrole nitrogens causes increased resonance stabilisation of the porphyrin (Abraham, 1961). This causes the ring current to become stronger and leads to deshielding of peripheral proton resonances.

In the case of $N_A$ and $N_C$ N-MePP, it appears that the ring deshielding effects caused by metalation play a prominent role, resulting in deshielding of these meso protons compared to those in the protoporphyrin zinc complex.
A lack of equivalence of various group resonances is clearly seen in figure 5.3. For example, the methyl/methoxy resonances are distinct and come over a larger range in both the N-alkylated zinc complexes when compared to the zinc protoporphyrin. Internal vinyl resonances are equivalent in NC-MePP, but not in NA-MePP. Conversely distal methylene resonances are (almost) equivalent in NA-MePP, but not in NC-MePP. These observations are explained in terms of the zinc atom coordinating to the three non-alkylated pyrrole nitrogen atoms at the porphyrin centre. As a result of coordination, these three rings will be held more nearly in the same plane relative to the alkylated ring which will be distinctly out-of-plane. Therefore porphyrins alkylated on propionate-substituted rings (NC and ND) will have distinctly non-equivalent proximal and distal methylene proton resonances and almost equivalent 2Vi and 4Vi group resonances. Porphyrins alkylated on vinyl-substituted rings (NA and NB) will, however, have distinct non-equivalent 2Vi and 4Vi group resonances and almost equivalent proximal and distal methylene resonances, a point referred to in more detail in the next section.

5.3.4 Comparison of the $^1$H NMR Spectra of Free-Base and Zinc Complex Regioisomers of N-Methylprotoporphyrin.

Regions of the $^1$H NMR spectra of the free-base and zinc-complex forms of the four regioisomers of N-methylprotoporphyrin (N-MePP), are shown in Figures 5.4 and 5.5 respectively. The following is a qualitative discussion of differences in the spectra between the two forms. The spectra shall be discussed in an orderly manner, moving from low-field to high-field resonances.

Firstly, it can be seen that the meso proton resonances at about 10ppm have a distinctive pattern in the free-base spectra (see Figure 5.4.). The pattern is dependent on the regioisomer under study. Upon incorporation of zinc (which coordinates to the three
unalkylated pyrrole ring nitrogen) a change is seen in this pattern (see Figure 5.5.) which arises from the effects of zinc binding on the conformation and electron distribution of the macrocycle. The pattern is dependent, again, on the regioisomer under study but the pattern is not the same for a particular regioisomer in both the free-base and zinc-complex forms.

Secondly, differences are seen in the resonances of vinyl groups of the porphyrin ring. Internal vinyl protons come between 7.6 and 8.4 ppm, and the terminal vinyl protons come between 5.8 and 6.4 ppm. In the free-base form (see Figure 5.4.) there are two internal vinyl proton resonances in the $^1$H NMR spectrum of each regioisomer, whereas in the $^1$H NMR spectra of the zinc-complex (see Figure 5.5.), two sets of internal vinyl proton resonances are seen only in the $N_A$ and $N_B$ regioisomers.

This behaviour is caused by the greater flexibility of the free-base formed as discussed previously (section 5.3.2.). The N-alkylated ring and the one opposite are distorted from planar in a free-base porphyrin. Hence in no instance will both vinyl-substituted pyrrole rings (A+B) lie in the same plane relative to each other. The separation of the two internal vinyl multiplets (doublet of doublets) is greater in the $N_A$ and $N_B$ regioisomers of free-base N-methylprotoporphyrin because a vinyl-substituted ring is N-alkylated (and the move to high-field of a resonance is always greatest for protons of those groups on the N-alkylated ring). The same behaviour is not seen in the spectra of the zinc complex because zinc coordination holds three of the pyrrole rings within the same plane. Two distinct internal vinyl multiplets will therefore only be seen when a vinyl-substituted ring is N-alkylated. It should also be noted that a characteristic pattern, dependent on both regioisomer and form, is seen for the terminal vinyl protons (5.8-6.5 ppm).

Thirdly, the proximal methylene protons appear between 4.4-5.5 ppm. In the spectra of the free-base form (see Figure 5.4.), two complex multiplets (at least) are apparent and these
Fig. 5.4. Comparison of regions of the proton spectra of the free-base forms of a) $N_A$-methylprotoporphyrin (4.16mM in CDC$\textsubscript{3}$), b) $N_B$-methyl protoporphyrin (3.22mM in CDC$\textsubscript{3}$), c) $N_C$-methylprotoporphyrin (5.59mM in CDC$\textsubscript{3}$) and d) $N_D$-methylprotoporphyrin (6.20mM in CDC$\textsubscript{3}$). Regions from low to high field are: meso protons, internal and terminal vinyl, proximal methylene, methyl/methoxy singlets and distal methylene and the N-CH$_3$ proton singlet.

Fig. 5.5. Comparison of regions of the proton spectra of the zinc-complexed forms of a) $N_A$-methylprotoporphyrin (2.76mM in CDC$\textsubscript{3}$), b) $N_B$-methylprotoporphyrin (2.43mM in CDC$\textsubscript{3}$), c) $N_C$-methylprotoporphyrin (3.69mM in CDC$\textsubscript{3}$) and d) $N_D$-methylprotoporphyrin (6.68mM in CDC$\textsubscript{3}$). Regions from low to high field are: meso protons, internal and terminal vinyl, proximal methylene, methyl/methoxy singlets and distal methylene and the N-CH$_3$ proton singlet.
appear at higher field in the \( N_C \) and \( N_D \) regioisomers. In the spectra of the zinc-complex form (see Figure 5.5.), at least one complex multiplet appears at a similar chemical shift in all regioisomers. Differences are also seen for the distal methylene resonances (2.6-3.4 ppm). In the free-base form (Figure 5.4.) one multiplet appears in the \( N_A \) and \( N_B \) regioisomers at ~3.2 ppm, and two multiplets (at 3.24 and 2.76 ppm) in the spectra of the \( N_C \) and \( N_D \) regioisomers. A similar pattern is seen in the spectra of the zinc-complexed regioisomers (Figure 5.5.). This behaviour can be explained using the arguments already described for the behaviour of the internal vinyl protons i.e. that N-alkylation causes the resonances of protons of groups situated on the alkylated ring to appear at higher field.

The methyl and methoxy resonances in the range 3.2-3.6 ppm (free-base form) and 3.5-3.75 ppm (zinc-complex form) should also be mentioned here. The larger range of ppm values found for these resonances in the free-base form indicates that the methyl and methoxy groups of a free-base N-alkylporphyrin are more varied in their positions relative to the ring-current of the porphyrin macrocycle than the same groups in a zinc-complexed N-alkylporphyrin. The greater flexibility of the free-base form as opposed to the zinc-complex is again illustrated.
5.3.5. Specific Assignment of the $^1$H NMR Spectra of the Zinc-complexed Regioisomers of N-Methylprotoporphyrin and N-Ethylprotoporphyrin.

Purified N-alkylprotoporphyrins made by established protocols were used in the present study. Portions of the NOESY spectrum of $N_A$-alkylated N-methylprotoporphyrin are shown in Figure 5.6. Part (a) shows the NOEs between meso protons and methyl/methylene resonances and is the starting point for assignment.

$N_A$ alkylation will mean that the resonances of the $^1$CH$_3$ and internal 2Vi groups will be moved to higher field by virtue of their being on the alkylated ring. Therefore $H_{12}$ in an $N_A$ alkylated porphyrin will show NOEs to a high-field internal 2Vi resonance and a 'normal' 3CH$_3$ resonance. $H_D$ will show NOEs to a low-field internal 4Vi resonance and a 'normal' 5CH$_3$ resonance. $H_Y$ will show NOEs to both 6 and 7 proximal and distal methylene groups, and $H_5$ will show NOEs to a 'normal' 8CH$_3$ resonance and a 1CH$_3$ resonance that has been shifted to high-field.

In terms of interpreting the region of spectrum shown in Figure 5.6 (a), this means that the meso proton to lowest field (at 10.43ppm) must be the $H_{12}$ meso proton as this resonance shows an NOE to a high-field internal vinyl resonance which can be assigned to 2Vi (not shown in the diagram), and another NOE to a methyl resonance which can be assigned to 3CH$_3$ (peak 1 in the diagram). Therefore the other meso proton at 10.29ppm which shows NOEs to both an internal vinyl (not shown) and a methyl resonance (peak 3 in the diagram) must be the $H_3$ resonance, meaning that the methyl resonance at 3.58ppm is assignable to 5CH$_3$ and the low-field internal vinyl resonance to 4Vi. The meso proton resonance at highest field (10.21ppm) shows two NOEs, to the proximal methylene resonances (peak 5 in the diagram) and the distal methylene resonances (peak 4 in the diagram). This pattern of NOEs can only be displayed by a $H_Y$ meso proton as it is flanked by the 6 and 7 propionate...
Fig. 5.6. Portions of the NOESY spectrum of zinc-complexed $N_{meso}$-methylprotoporphyrin (2.32mM in CDCl$_3$). Details and explanation of assignments are given in the text. Acquired with a data matrix of $1K \times 512$ over 512 increments of 32 scans. Relaxation delay ($D_1$) = 3s and mixing time ($D_9$) = 1s. Both dimensions had zero-filling and a gaussian window function ($GB = 0.1$ and $lb = -10$Hz) applied prior to fourier transform.

a) Region showing NOE connectivities between the $meso$ protons (10.2-10.5ppm) and methyl (3.3-4.4ppm) and proximal methylene (4.37ppm) group resonances

b) Region showing NOE connectivities from the $meso$ protons (10.2-10.5ppm), internal vinyl (8.11 and 8.27ppm) and proximal methylene groups (4.37ppm) to the methyl group resonances (in the range 3.5-3.7ppm).
groups. The last meso resonance at 10.34 ppm has to be, by exclusion, the H₅ meso proton resonance, which should show NOEs to the ¹CH₃ and ⁸CH₃ resonances. Peak 2 in the diagram indicates that these methyl groups must come very close together in the ¹H NMR spectrum as N-alkylation will cause the ¹CH₃ resonance to move to higher field from its position which is normally to low-field of the methyl groups on propionate-substituted rings.

This assignment strategy results in the assignment of the meso protons, internal vinyl, methyl and proximal and distal methylene resonances. Terminal vinyl resonances are assigned by virtue of NOEs between internal and terminal vinyl resonances. 6 and 7-OMe resonances are, by exclusion, the two singlets in the methyl region of the spectrum which are not assigned by the NOE experiments. These are assigned on the assumption that the resonance of the OMe group on, or opposite, the N-alkylated ring will be at the higher field of the two resonances.

Figure 5.6. (b) then shows other NOEs which are useful in confirming the validity of the assignment scheme chosen. As all methyl resonances have already been assigned on the basis of meso to methyl NOEs, it is pleasing to see that not a single assignment is made invalid on inspection of this region of the same spectrum. (The meso proton NOEs shown plot (a) are seen again in the left-hand part of (b)).

Two NOEs (6 and 7) are from the assigned internal and terminal ⁴Vi resonances respectively, to the assigned ³CH₃. NOEs (8 and 9) are from the assigned internal and terminal ²Vi resonances to the assigned ¹CH₃. NOE (10) must be from the 7 proximal methylene resonance to the assigned ⁸CH₃ and NOE (11) must be from the 6 proximal methylene resonance to ⁵CH₃. Backtracking from these assignments (hatched lines) verifies assignments already made and also brings to light the position of the two methyl resonances.
(¹CH₃ and ⁸CH₃) that H₅ is meant to show NOEs to, but which had not previously been individually defined.

Figure 5.7. exemplifies the differences in assignment of a propionate-substituted porphyrin. The portion of the NOESY spectrum shown belongs to N₆-MePP. The assignment strategy follows the same sort of reasoning already applied to the N₅ regioisomer. In the N₆ alkylated porphyrin, however, high-field shifts are expected for the ⁵CH₃ and ⁶ propionate-associated resonances, as these groups are on the alkylated ring. H₆ should therefore show NOEs to a 'normal' ³CH₃ and a 'normal' ²Vi resonance. H₅ should show NOEs to a normal ⁴Vi resonance and a high-field ⁵CH₃ resonance. H₇ should show NOEs to proximal methylene resonances at different chemical shifts and H₄ should show NOEs to two methyl group resonances at the same chemical shift. Such assignments are easily made as demonstrated in the figure.

Similar assignment arguments apply to the N₃ and N₄ alkylated N-methylprotoporphyrins and have also been extended to include all regioisomers of zinc-complexed N-ethylprotoporphyrin. Assignments of all four regioisomers of zinc-complexed N-methylprotoporphyrin and N-ethylprotoporphyrin are included in Tables 5.2. and 5.3.
Fig. 5.7. Portion of the NOESY spectrum of zinc-complexed N_C^-methylprotoporphyrin (~1.62mM in CDCl_3) showing NOE connectivities between the meso protons (10.21-10.40ppm) and internal vinyl (8.17 and 8.18ppm), terminal vinyl (6.22ppm), methyl (3.41-3.62ppm) and proximal methylene (4.22 and 4.29ppm) group resonances. Details and explanation of assignments are given in the text. Acquired with a data matrix of 1K x 512 over 512 increments of 32 scans. Relaxation delay (D1) = 3s and mixing time (D9) = 1s. Both dimensions had zero-filling and a gaussian window function (GB = 0.1 and lb = ^10Hz) applied prior to fourier transform.
Table 5.2: Assignments of the four zinc-complexed regioisomers of N-methylprotoporphyrin

<table>
<thead>
<tr>
<th>Group</th>
<th>( \delta/\text{ppm} ) N(_A)-MePP</th>
<th>( \delta/\text{ppm} ) N(_B)-MePP</th>
<th>( \delta/\text{ppm} ) N(_C)-MePP</th>
<th>( \delta/\text{ppm} ) N(_D)-MePP</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H}_{10} )</td>
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<td>10.38</td>
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<td>10.37</td>
</tr>
<tr>
<td>( \text{H}_9 )</td>
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<td>10.34</td>
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<tr>
<td>( \text{H}_8 )</td>
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<tr>
<td>( \text{H}_7 )</td>
<td>10.34</td>
<td>10.24</td>
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</tr>
<tr>
<td>2( \text{Vi} ) (int)</td>
<td>8.11</td>
<td>8.24</td>
<td>8.17</td>
<td>8.27</td>
</tr>
<tr>
<td>4( \text{Vi} ) (int)</td>
<td>8.27</td>
<td>8.06</td>
<td>8.18</td>
<td>8.26</td>
</tr>
<tr>
<td>2( \text{Vi} ) (term)</td>
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<td>6.34 (weak)</td>
<td>6.45</td>
</tr>
<tr>
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<td>6.42x</td>
<td>6.24</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>4.37 (6 &amp; 7)</td>
<td>4.34 (6 &amp; 7)</td>
<td>4.29 (7)</td>
<td>4.39 (6)</td>
</tr>
<tr>
<td>( \text{CH}_1 )</td>
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<td>3.58</td>
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<tr>
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<td>3.66</td>
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<td>3.59</td>
<td>3.41</td>
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</tr>
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<td>( \text{CH}_7 )</td>
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<td>3.58</td>
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<tr>
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</tr>
<tr>
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<td>n.a.</td>
</tr>
<tr>
<td>Distal methylene</td>
<td>3.32 (6 &amp; 7)</td>
<td>3.30 (6 &amp; 7)</td>
<td>3.23 (7)</td>
<td>3.26 (6)</td>
</tr>
<tr>
<td>( \text{N-CH}_3 )</td>
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<td>-4.50</td>
<td>-4.45</td>
<td>-4.44</td>
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</tbody>
</table>

* not enough data to distinguish between resonances
* n.a. = not assigned
### Table 5.3: Assignments of the four zinc-complexed regioisomers of N-ethylprotoporphyrin.

<table>
<thead>
<tr>
<th>Group</th>
<th>δ/ppm</th>
<th>δ/ppm</th>
<th>δ/ppm</th>
<th>δ/ppm</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>(N_A)-EtPP</td>
<td>(N_B)-EtPP</td>
<td>(N_C)-EtPP</td>
<td>(N_D)-EtPP</td>
</tr>
<tr>
<td>(H_\text{F})</td>
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<td>10.42</td>
<td>10.38</td>
<td>10.29</td>
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<tr>
<td>(H_\text{G})</td>
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<td>10.38</td>
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<td>10.29</td>
</tr>
<tr>
<td>(H_\text{C})</td>
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<td>10.17</td>
<td>10.36</td>
<td>10.36</td>
</tr>
<tr>
<td>(H_\text{D})</td>
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<td>10.22</td>
<td>10.22</td>
<td>10.33</td>
</tr>
<tr>
<td>2Vi (int)</td>
<td>8.06</td>
<td>8.26</td>
<td>8.23x</td>
<td>8.21x</td>
</tr>
<tr>
<td>4Vi (int)</td>
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<td>8.04</td>
<td>8.23x</td>
<td>8.21x</td>
</tr>
<tr>
<td>2Vi (term)</td>
<td>6.27x</td>
<td>6.32</td>
<td>6.29x</td>
<td>6.25x</td>
</tr>
<tr>
<td>4Vi (term)</td>
<td>6.21x</td>
<td>6.22</td>
<td>6.29x</td>
<td>6.25x</td>
</tr>
<tr>
<td>proximal</td>
<td>4.37 (complex multiplet)</td>
<td>4.37 (complex multiplet)</td>
<td>4.40 (complex multiplet)</td>
<td>4.35 (complex multiplet)</td>
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<tr>
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<td>3.65</td>
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<tr>
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<td>3.74</td>
<td>3.61</td>
<td>3.65</td>
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<tr>
<td>7-OMe</td>
<td>3.74</td>
<td>3.74</td>
<td>3.64</td>
<td>3.62</td>
</tr>
<tr>
<td>Distal</td>
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<td>(3.32 (6 &amp; 7))</td>
<td>(3.29 (7))</td>
<td>(3.31 (6))</td>
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<tr>
<td>methylene</td>
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<td>(5.10) (quartet)</td>
<td>(5.11) (quartet)</td>
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<td>(-1.85) (triplet)</td>
<td>(-1.86) (triplet)</td>
<td>(-1.83) (triplet)</td>
</tr>
</tbody>
</table>

* This sample was not completely free of \(N_C\)-EtPP (Zn)

* not enough data to distinguish between resonances

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5.3.6. Specific Assignment of the $^1$H NMR Spectra of the Free-Base Regioisomers of N-Methylprotoporphyrin and N-Ethylprotoporphyrin.

Regions of the NOESY spectra of the $\text{N}_B$, $\text{N}_C$, $\text{N}_D$ and $\text{N}_A$ regioisomers of N-methylprotoporphyrin are reproduced in Figures 5.8. - 5.11. The difficulty in assigning them lies in the greater flexibility of the free-base compared to the zinc-complexed form of a porphyrin, as indicated by two sets of resonances for both the 2 and 4 internal vinyl and the 6 and 7 proximal methylene groups in all regioisomers.

Each free-base regioisomer will be considered in turn. The figures representing each regioisomer all have the same format; plot (a) features important NOEs from the meso protons and plot (b) features these NOEs again (at the far left of the figure) but also shows NOEs from the 2 and 4 internal and terminal vinyl proton resonances, as well as NOEs from the 6 and 7 proximal methylene groups. It must be remembered that, in each and every regioisomer, no matter which ring is N-alkylated, $\text{H}_\alpha$ will show NOEs to $^2\text{Vi}$ and $^3\text{CH}_3$, $\text{H}_8$ will show NOEs to $^4\text{Vi}$ and $^5\text{CH}_3$, $\text{H}_7$ will show NOEs to 6 and 7 proximal methylene groups and $\text{H}_5$ will show NOEs to $^1\text{CH}_3$ and $^8\text{CH}_3$.

The $\text{N}_B$ regioisomer (Figure 5.8.) will be considered first as it has non-overlapping meso protons. Looking at plot (a) it is immediately apparent that one meso proton shows NOEs to $^2\text{Vi}$ and $^3\text{CH}_3$, $\text{H}_8$ will show NOEs to $^4\text{Vi}$ and $^5\text{CH}_3$, $\text{H}_7$ will show NOEs to 6 and 7 proximal methylene groups and $\text{H}_5$ will show NOEs to $^1\text{CH}_3$ and $^8\text{CH}_3$.

The NOEs seen from $\text{H}_5$ must be to $^1\text{CH}_3$ and $^8\text{CH}_3$. Identification of which is which comes from referring to plot (b). Here it is seen that the methyl resonance identified by the most high-field of the two NOEs in question shows NOEs to internal and terminal vinyl resonances. Only $^1\text{CH}_3$ (of the two) can be expected to show NOEs to a vinyl group, and
Fig. 5.8. Portions of the NOESY spectrum of free-base Nβ-methylprotoporphyrin (~7.36mM in CDCl₃).
Details and explanation of assignments are given in the text. Acquired with a data matrix of 1K x 512 over 512 increments of 32 scans. Relaxation delay (D₁) = 3s and mixing time (D₉) = 1s. Both dimensions had zero-filling and a gaussian window function (GB = 0.1 and lb = ′10Hz) applied prior to fourier transform.

a) Region showing NOE connectivities between meso protons (9.80-10.10ppm) and methyl (3.20-3.64ppm), proximal methylene (4.22 and 4.43ppm) and distal methylene (3.26 and 3.29ppm) group resonances.

b) Region showing NOE connectivities between meso protons (9.80-10.10ppm), internal vinyl (8.16 and 7.83ppm), terminal vinyl (5.96-6.26ppm), proximal methylene (4.43 and 4.22ppm) and methyl (3.20-3.64ppm) and distal methylene (3.26 and 3.29ppm) group resonances.
that vinyl group must be $^2$Vi. The methyl resonance identified by the lower-field of the two NOEs in question must therefore be $^8$CH$_3$ and indeed this methyl resonance shows an NOE to a proximal methylene group, which both verifies the $^8$CH$_3$ assignment (as only $^8$CH$_3$ (of the two) can be expected to show an NOE to such a group) and also allows assignment of the 7 proximal methylene group. Assignment of the $^3$CH$_3$ and $^5$CH$_3$ groups follows in much the same way. One of these methyl resonances shows NOEs to terminal vinyl protons (which must be of $^4$Vi) and a meso proton. The methyl resonance must be attributable to $^3$CH$_3$ and therefore the meso proton at lowest field is distinguished as H$_\alpha$. The last unassigned meso proton at 9.90ppm is therefore assigned to H$_\beta$, both by exclusion principles and by its showing NOEs to an internal vinyl resonance (of $^4$Vi, not shown) and a methyl group, which must be $^5$CH$_3$. The methyl group identified by these NOEs does indeed itself show an NOE to a proximal methylene resonance which can therefore be assigned to the 6 proximal methylene group.

The most important result from this assignment is that the 6 proximal methylene resonance (which is on a non-alkylated ring C) as well as the $^4$Vi resonances (which are on the alkylated ring B) are moved to higher field relative to the 7 proximal methylene (on ring D) and $^2$Vi (on ring A) resonances respectively.

The $N_C$ regiosomer of free-base N-MePP will be considered next (see Figure 5.9.). As can be seen from the figure, the sample used for this experiment was not completely pure. Assignments can still be made, however. Once again, in plot (a), the meso proton (H$_\beta$) showing NOEs to two methyl groups (which must be $^1$CH$_3$ and $^8$CH$_3$) is the starting point for assignment. Reference to plot (b) identifies that one of these two methyl resonances (the one at lower field) shows NOEs to vinyl resonances, which immediately identifies both the $^1$CH$_3$ and $^2$Vi resonances. The methyl group at higher field of the two under study is therefore $^8$CH$_3$ and the NOE it shows to a proximal methylene group must be to the 7
Fig. 5.9. Portions of the NOESY spectrum of free-base N₄-methylprotoporphyrin (1.03mM in CDCl₃). Details and explanation of assignments are given in the text. Acquired with a data matrix of 1K x 512 over 512 increments of 32 scans. Relaxation delay (D1) = 3s and mixing time (D9) = 1s. Both dimensions had zero-filling and a gaussian window function (GB = 0.1 and λb = 10Hz) applied prior to fourier transform.

a) Region showing NOE connectivities between meso protons (9.94-10.10ppm) and methyl (3.18-3.75ppm), and proximal methylene (4.25 and 4.05ppm) group resonances.

b) Region showing NOE connectivities between meso protons (9.94-10.10ppm), internal vinyl (8.34 and 8.18ppm), terminal vinyl (6.10-6.39ppm), proximal methylene (4.25 and 4.05ppm) and methyl (3.18-3.75ppm) and distal methylene (3.24 and 2.76ppm) group resonances.
proximal methylene. NOEs are also seen from the $^4\text{Vi}$ resonances to a methyl group. The 
*meso* proton at lowest field gives an NOE to this same methyl group, indicating that the 
methyl group in question must be $^2\text{CH}_3$ and the *meso* proton must be $^6\text{H}_2$. The last, 
unassigned methyl group is therefore $^5\text{CH}_3$, verified by an NOE from this resonance to a 
proximal methylene resonance (which must be the 6 proximal methylene) and also by the 
fact that a *meso* proton (which must be $^6\text{H}_2$) shows NOEs both to it and the the $^4\text{Vi}$ internal 
viny resonance. The $^6\text{H}_1$ proton is, as always, immediately identified as it shows NOEs to 
both $^6$ and $^7$ proximal and distal methylene resonances.

The most important result from this assignment is that the $^4\text{Vi}$ resonances (which are on a 
non-alkylated ring B), as well as the 6 proximal and distal methylene resonances (which are 
on the alkylated ring C) are shifted to higher field relative to the $^2\text{Vi}$ (on ring A) and 7 
proximal and distal methylene resonances (on ring D), respectively.

From analysis of the results so far, it would seem that a general pattern is emerging, i.e. that 
$N_2$-alkylation causes resonances of moieties on rings B and C to move to higher field 
whereas $N_C$-alkylation causes resonances of moieties on rings C and B to move to higher 
field. This is unlike the situation with zinc-complexed $N$-alkylated porphyrins, where it is 
resonances of groups on the alkylated ring and the one opposite which experience high-field 
shifts. With the free-base porphyrins studied here the bias would appear to be for high-field 
shifts of groups on the N-alkylated ring and the ring beside it, in the sense that, if a 
propionate-substituted ring is alkylated, moieties on the the vinyl-substituted ring next to it 
will experience shifts, or conversely, if a vinyl-sustituted ring is alkylated, it will be moieties 
on the propionate-substituted ring beside it that will experience shifts. The $N_D$ regiosomer 
shall be considered next to see if such a hypothesis is valid (see Figure 5.10.).
Again assignment starts with the $H_5$ proton. Of the two NOEs seen from this proton resonance to methyl resonances, it is the methyl resonance at lower field of the two that shows NOEs to internal and terminal vinyl resonances, identifying this methyl resonance as $1CH_3$ and the vinyl resonances as those of $2Vi$. $8CH_3$ therefore accounts for the other NOE from $H_5$. An NOE from $8CH_3$ to a proximal methylene group identifies the $7$ proximal methylene resonance. NOEs from a methyl resonance to $4Vi$ resonances identifies the $3CH_3$ resonances and consequently the $H_\alpha$ resonance. $H_\beta$ and $H_\gamma$ are then readily identified, $H_\gamma$ because it shows NOEs to proximal methylene groups and $H_\beta$ because it shows NOEs to a methyl resonance ($5CH_3$) and an internal vinyl resonance ($4Vi$, not shown).

The important result from this assignment is that the $2Vi$ group resonances (which are on a non-alkylated ring A) as well as the $7$ proximal and distal methylene groups (on the alkylated ring D) are moved to higher field relative to the $4Vi$ resonances (on ring C) and the $6$ proximal and distal methylene resonances (on ring D) respectively. So it appears, yet again that it is groups on the ring beside the alkylated ring, as well as on the alkylated rings that experience high-field shifts in the free-base froms of the N-alkylated porphyrins under study. It also appears that, if a vinyl-substituted ring is N-alkylated, it will be groups on the propionate-substituted ring next to it that also experience shifts to high-field and vice-versa.

In no instance do groups on both propionate rings C and D or vinyl-substituted rings A and B show high-field shifts. These results now allow assignment of the $NA$ regioisomer (see Figure 5.11.).

The difficulty here is immediately apparent as the $H_5$ meso proton is superimposed on another meso proton resonance. All three NOEs seen from these superimposed peaks must be to methyl resonances, two of which must be $1CH_3$ and $8CH_3$. The only methyl resonance of these three to show NOEs to vinyl groups is the one to highest field and so this methyl resonance is assigned to $1CH_3$ and the associated vinyl resonances to $2Vi$. The NOEs from
Fig. 5.10. Portions of the NOESY spectrum of free-base N7-methylprotoporphyrin (0.98mM in CDCl3). Details and explanation of assignments are given in the text. Acquired with a data matrix of 1K x 512 over 512 increments of 32 scans. Relaxation delay (D1) = 3s and mixing time (D9) = 1s. Both dimensions had zero-filling and a gaussian window function (GB = 0.1 and lb = 10Hz) applied prior to fourier transform.

a) Region showing NOE connectivities between meso protons (9.91-10.09ppm) and methyl (3.17-3.71ppm), and proximal methylene (4.20 and 4.02ppm) group resonances.

b) Region showing NOE connectivities between meso protons (9.91-10.09ppm), internal vinyl (8.19 and 8.31ppm), terminal vinyl (6.19-6.36ppm), proximal methylene (4.20 and 4.02ppm) and methyl (3.17-3.71ppm) and distal methylene (3.20 and 2.76ppm) group resonances.
Fig. 5.11. Portions of the NOESY spectrum of free-base $N_A$-methylprotoporphyrin (1.22mM in CDCl$_3$). Details and explanation of assignments are given in the text. Acquired with a data matrix of 1K x 512 over 512 increments of 32 scans. Relaxation delay (D1) = 3s and mixing time (D9) = 1s. Both dimensions had zero-filling and a gaussian window function (GB = 0.1 and lb = 10Hz) applied prior to fourier transform.

a) Region showing NOE connectivities between meso protons (9.85-10.11 ppm) and methyl (3.31-3.70 ppm), proximal methylene (4.50 and 4.30 ppm) and distal methylene (3.32 ppm) group resonances.

b) Region showing NOE connectivities between meso protons (9.85-10.11 ppm), internal vinyl (8.23 and 7.92 ppm), terminal vinyl (6.04-6.39 ppm), proximal methylene (4.50 and 4.30 ppm) and methyl (3.31-3.70 ppm) and distal methylene (3.32 ppm) group resonances.
a methyl resonance to the other (4Vi) vinyl resonances therefore allow assignment of $^3\text{CH}_3$ and also the H$_{\text{meso}}$ meso proton. $^5\text{CH}_3$ and $^8\text{CH}_3$ cannot be distinguished without recourse to the chemical shift arguments discussed above, on the basis of which the 2Vi resonances are expected to come at higher field since they are on the alkylated ring A; this is indeed observed. The 7 proximal methylene resonance can be expected to come at higher field as well, because it is situated on the propionate substituted ring beside the alkylated ring. Assuming the 7 proximal methylene does come at higher field, the resonance to which it shows an NOE can be assigned to $^8\text{CH}_3$, whereas the 6 proximal methylene resonance shows an NOE to a methyl resonance which can be assigned to $^5\text{CH}_3$, thus completing all assignments for each regioisomer.

The most important overall finding is the effect that N-alkylation has on the chemical shift values of certain groups in the free-base form. This form has received little study because of the generally acknowledged (Smith et al., 1982) greater difficulty of both working with this form and making definitive assignments. The assignment strategy used here has also been applied to the N-ethylprotoporphyrin free-base regioisomers and it appears that the same general trend holds true for this N-alkylporphyrin as well. Tables 5.4. and 5.5. show the completed assignments of the four regioisomers of free-base N-methylprotoporphyrin and N-ethylprotoporphyrin.
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<tr>
<th>Group</th>
<th>$\delta$ / ppm $\text{N}_A\text{MePP}$ [free]</th>
<th>$\delta$ / ppm $\text{N}_B\text{MePP}$ [free]</th>
<th>$\delta$ / ppm $\text{N}_C\text{MePP}$ [free]</th>
<th>$\delta$ / ppm $\text{N}_D\text{MePP}$ [free]</th>
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<td>10.07</td>
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<td>10.00</td>
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<tr>
<td>$2^\text{Vi}$ (int)</td>
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<td>8.16</td>
<td>8.34</td>
<td>8.19</td>
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<td>$4^\text{Vi}$ (int)</td>
<td>8.23</td>
<td>7.83</td>
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<td>8.31</td>
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</tr>
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<td>6.11</td>
<td>6.23</td>
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</tr>
<tr>
<td>$4^\text{Vi}$ (term)</td>
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<td>5.98$^x$</td>
<td>6.10</td>
<td>6.36</td>
</tr>
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<td>4.50 ($^6$)</td>
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<td>4.25 ($^7$)</td>
<td>4.20 ($^6$)</td>
</tr>
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<td>4.22 ($^6$)</td>
<td>4.05 ($^6$)</td>
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<tr>
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<td>$7^\text{OMe}$</td>
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<td>2.76 ($^7$)</td>
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</tr>
<tr>
<td>N-CH$_3$</td>
<td>-4.61</td>
<td>-4.60</td>
<td>-4.72</td>
<td>-4.73</td>
</tr>
</tbody>
</table>

$x$ not enough data to distinguish between resonances
<table>
<thead>
<tr>
<th>Group</th>
<th>$\delta$ / ppm</th>
<th>$\delta$ / ppm</th>
<th>$\delta$ / ppm</th>
<th>$\delta$ / ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N_{A-EtPP}$</td>
<td>$N_{A-EtPP}$</td>
<td>$N_{A-EtPP}$</td>
<td>$N_{A-EtPP}$</td>
</tr>
<tr>
<td></td>
<td>free</td>
<td>free</td>
<td>free</td>
<td>free</td>
</tr>
<tr>
<td>$H_{10}$</td>
<td>10.15</td>
<td>10.17</td>
<td>10.04</td>
<td>10.12</td>
</tr>
<tr>
<td>$H_{9}$</td>
<td>9.95</td>
<td>10.10</td>
<td>10.06</td>
<td>10.06</td>
</tr>
<tr>
<td>$H_{8}$</td>
<td>9.80</td>
<td>9.83</td>
<td>9.90</td>
<td>10.10</td>
</tr>
<tr>
<td>$H_{5}$</td>
<td>10.04</td>
<td>9.90</td>
<td>9.90</td>
<td>10.10</td>
</tr>
<tr>
<td>$2^{\text{Vi}}$ (int)</td>
<td>7.86</td>
<td>8.18</td>
<td>8.24</td>
<td>8.22</td>
</tr>
<tr>
<td>$4^{\text{Vi}}$ (int)</td>
<td>8.14</td>
<td>7.86</td>
<td>8.11</td>
<td>8.35</td>
</tr>
<tr>
<td>$2^{\text{Vi}}$ (term)</td>
<td>5.96</td>
<td>6.20</td>
<td>6.20</td>
<td>6.14</td>
</tr>
<tr>
<td>$4^{\text{Vi}}$ (term)</td>
<td>6.340</td>
<td>6.12</td>
<td>6.10</td>
<td>6.00</td>
</tr>
<tr>
<td>proximal</td>
<td>4.43 (6)</td>
<td>4.41 (7)</td>
<td>4.27 (6)</td>
<td>4.29 (6)</td>
</tr>
<tr>
<td>methylene</td>
<td>4.23 (7)</td>
<td>4.22 (6)</td>
<td>4.20 (7)</td>
<td>4.16 (7)</td>
</tr>
<tr>
<td>$1^{\text{CH}}$</td>
<td>3.27</td>
<td>3.57</td>
<td>3.63</td>
<td>3.68</td>
</tr>
<tr>
<td>$3^{\text{CH}}$</td>
<td>3.60</td>
<td>3.21</td>
<td>3.46</td>
<td>3.79</td>
</tr>
<tr>
<td>$5^{\text{CH}}$</td>
<td>3.62</td>
<td>3.47</td>
<td>3.07</td>
<td>3.52</td>
</tr>
<tr>
<td>$8^{\text{CH}}$</td>
<td>3.59</td>
<td>3.57</td>
<td>3.37</td>
<td>3.30</td>
</tr>
<tr>
<td>6-OMe</td>
<td>3.66</td>
<td>3.68</td>
<td>3.66</td>
<td>3.71</td>
</tr>
<tr>
<td>7-OMe</td>
<td>3.65</td>
<td>3.69</td>
<td>3.72</td>
<td>3.64</td>
</tr>
<tr>
<td>Distal</td>
<td>3.26</td>
<td>3.24</td>
<td>3.22 (7)</td>
<td>3.17 (6)</td>
</tr>
<tr>
<td>methylene</td>
<td>2.71 (6)</td>
<td>2.78 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-CH$_2$CH$_3$</td>
<td>$-5.08$</td>
<td>$-4.93$</td>
<td>$-5.12$</td>
<td>$-4.93$</td>
</tr>
<tr>
<td>N-CH$_2$CH$_3$</td>
<td>$-2.32$</td>
<td>$-2.22$</td>
<td>$-2.35$</td>
<td>$-2.23$</td>
</tr>
</tbody>
</table>
5.3.7. Comparison of the $^1$H NMR Spectra of the Free-Base and Zinc-Complexed Regioisomeric Pairs of N-propyl and N-butylprotoporphyrin.

It was not possible to separate individual regioisomers of N-1-propylprotoporphyrin (N-PrPP) and N-1-butylprotoporphyrin (N-BuPP) by the techniques used. Instead, these porphyrins were separated into regioisomeric pairs, $N_{A+B}$ and $N_{C+D}$. The spectra of the free-base and zinc-complex forms of N-PrPP and N-BuPP regioisomeric pairs are shown in Figures 5.12 and 5.13, respectively. The high-field regions of the spectra are of particular interest and so are shown in Figure 5.14. The spectra are not of good quality, which precludes assignments, but their analysis still leads to some interesting observations:

1) the spectra of the zinc-complexed forms are much clearer than those of the free-base forms in all cases,

2) the behaviour of the zinc-complex forms of N-PrPP and N-BuPP is similar to the behaviour displayed by the N-MePP and N-EtPP regioisomers. In the spectra of the $N_{A+B}$ regioisomers, two distinct internal vinyl resonances and only one distal methylene resonance are seen, whereas in the spectra of the $N_{C+D}$ regioisomers only one internal vinyl resonance and two distal methylene resonances appear,

3) the behaviour of the free-base forms of N-PrPP and N-BuPP differs from that of N-MePP and N-EtPP, where distinct resonances are seen for both the internal vinyl and distal methylene resonances. The behaviour of the free-base forms of N-PrPP and N-BuPP approaches that of the zinc-complexed form already described above, which is consistent for all the N-alkylporphyrins considered in this study.
Fig. 5.12. Spectra of the regioisomeric pairs of N-1-propylprotoporphyrin. (N_{A+B} = 4.90mM and N_{C-D} = 2.58mM in CDC_{13}). Regions from low to high field are: meso protons, internal and terminal vinyl, proximal methylene, methyl/methoxy singlets and distal methylene protons.

Fig. 5.13. Spectra of the regioisomeric pairs of N-1-butylprotoporphyrin. (N_{A+B} = 0.20mM and N_{C-D} = 0.42mM in CDC_{13}). Regions from low to high field are: meso protons, internal and terminal vinyl, proximal methylene, methyl/methoxy singlets and distal methylene protons.
It would appear then, that differences seen in the spectra of the two forms of N-MePP (one carbon atom) and N-EtPP (two carbon atoms) are markedly less pronounced when the alkyl group is extended to a propyl (3 carbon atoms) or a butyl (4 carbon atoms) moiety. Such an observation indicates that the free-base and zinc-complex forms must be more similar in these instances. This is not an unreasonable statement, as it can be quite simply visualised that a larger N-alkyl group can in no way be accommodated directly in the plane of the pyrrole ring nitrogens. The N-alkylated ring can therefore be expected to come out-of-plane to a considerable degree leaving the other three pyrrole rings to be in the same plane. The N-alkylated ring must be situated such that considerable room is available for coordination of zinc to the other three nitrogen atoms, as resonances associated with the N-alkylated ring experience little or no shift to high-field on zinc-complexation. This is unlike the situation in N-MePP where coordination of zinc to the porphyrin centre would appear to physically obstruct the N-alkyl group such that greater distortion of the alkylated ring is incurred (as indicated by a shift to high-field of resonances associated with the alkylated ring).

Marked differences in the spectra are seen, however, in the high-field regions plotted in Figure 5.14. The CH$_2$ protons of the N-alkyl groups linked directly to a pyrrole ring nitrogen come at the most high-field in all spectra (in the range ~4.92-~5.16ppm). This is expected as a consequence of having to lie directly in, or over, the centre of the porphyrin ring current. More interesting, however, is the difference in positions of the other N-alkyl-associated resonances when free-base and zinc-complex forms are compared. In both N-PrPP and N-BuPP the other resonances experience shifts on zinc-complexation. The effects are slight for the terminal methyl resonances and, in the case of N-BuPP, the methylene next to the terminal methyl, in both zinc-complex and free-base forms, varying from a shift of 0.04ppm to high-field in the case of N$_{A+3}$PrPP, to a shift of 0.12ppm to low-field in the case of N$_{D+3}$BuPP. The methylene group adjacent to the N-CH$_2$ methylene in both cases, however, experiences very marked shifts which are indicated in Figure 5.14. by dashed lines.
Fig. 5.14. High-field regions of the spectra of N-1-propylprotoporphyrin and N-1-butylprotoporphyrin (low field regions in figures. 5.12. and 5.13.). The movement of resonances attributable to the N-alkyl groups (as indicated by dashed arrows) is discussed in the text.
This particular methylene resonance experiences a shift of 0.62ppm to low-field in $N_{A+B}\text{-PrPP}$ on zinc-complexation, and a shift to low-field of 0.56ppm in $N_{C+D}\text{-PrPP}$. In N-butylprotoporphyrin the shift is 0.83ppm to low-field in $N_{A+B}\text{-BuPP}$ and 0.60ppm in $N_{C+D}\text{-BuPP}$. It would therefore appear that coordination of a zinc atom to the porphyrin centre has a local effect only, centred on this methylene group. It may well be that some sort of orbital overlap is occurring between the d-orbitals of the zinc atom and the $sp^3$ orbitals of this methylene group, which affects the shielding of the methylene protons such that they resonate at lower field. A shift to low-field is usually explained in terms of decreased ring current, but this is unlikely as, even though N-alkylation will cause a decrease in ring current, this resonance is the only resonance experiencing such a shift.
5.4. Results of $^{13}$C NMR Studies of N-alkyl Porphyrins and Related Compounds.

5.4.1. Comparison of the $^{13}$C Spectra of Free-Base and Zinc-Complexed Protoporphyrin IX and N-Methylprotoporphyrin, N$_C$-Regioisomer.

The $^{13}$C spectra of the zinc and free-base forms of protoporphyrin and N$_C$-MePP are compared in Figure 5.15. No marked differences are seen between spectra of the free-base (a) and the zinc-complex (b) forms of protoporphyrin. The near symmetry of the molecule is highlighted by the presence of single resonances for all similar carbon atoms in both the 6 and 7 propionate methyl ester groups and the 2 and 4 vinyl groups. For example, the 6 and 7 distal methylene carbons give resonances coincident at 36.8ppm in the free-base form and 37.05ppm in the zinc-complex form. The chemical shift difference for these particular carbon resonances (and all others) between the two forms is not marked. In both the free-base and zinc-complex forms the methyl carbons are split into two groups, with $^1$CH$_3$ and $^3$CH$_3$ (coincident at $\sim$12.5ppm in both forms) coming to low field of $^5$CH$_3$ and $^8$CH$_3$ (coincident at $\sim$11.5ppm in both forms). The meso carbon resonances are clearly distinguished in both forms, giving rise to four distinct resonances in the range 97.1-96.9ppm. Furthermore, in the much more clearly resolved spectrum of the zinc-complex of protoporphyrin, distinct resonances are seen for the $C_\alpha$ atoms (those carbon atoms one bond removed from the pyrrole nitrogen atoms) and the $C_\beta$ atoms (those carbon atoms at positions 1-8 of the porphyrin periphery). $C_\alpha$ resonances lie in the range 147.9-141.4 and $C_\beta$ resonances in the range 138.3-136.2ppm.

Interesting observations can be made on comparison of the $^{13}$C spectra of the two forms of the N-alkylporphyrin with the spectra of the two forms of protoporphyrin;
Fig. 5.15. Comparison of the broad-band proton-decoupled carbon spectra of a) free-base protoporphyrin (5 mM in CDCl₃ recorded at 300 MHz), b) zinc-complexed protoporphyrin (~5 mM in CDCl₃ recorded at 500 MHz), c) free-base N₃-methylprotoporphyrin (7.36 mM in CDCl₃ recorded at 300 MHz) and d) zinc-complexed N₃-methylprotoporphyrin (~7.36 mM in CDCl₃ recorded at 500 MHz). Chemical shifts are referenced to chloroform at 7.69 ppm. P=pyridine.
1) the 6 and 7 proximal methylene, distal methylene and propionate ester carbons now give rise to two distinct resonances (though the methyl carbon atoms of both esters still give rise to only one resonance),

2) the 2 and 4 internal and terminal vinyl carbon atoms now also give rise to distinct resonances,

3) the methyl carbon resonances are no longer split into two groups, but instead give four distinct resonances, and

4) the four meso carbon resonances now cover a larger spectral range.

Such a qualitative inspection of the $^{13}$C spectra does suggest that small changes in $^{13}$C chemical shift of some porphyrin resonances are experienced as result of N-alkylation of a porphyrin. It was therefore decided to try and assign the $^{13}$C spectra of the zinc and free-base forms of N$_2$-MePP (via the already assigned proton spectrum) using the proton-detected HMQC experiment.

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Unfortunately, the $C_{ii}$ and $C_B$ resonances cannot be compared as they are barely resolved in the N-allylporphyrin spectra.
5.4.2. Use of Proton-Detected Heteronuclear Multiple Quantum Coherence (HMQC) Spectroscopy to Aid Assignment of the $^{13}$C Spectra of Zinc and Free-Base N-Methylprotoporphyrin, NC Regioisomer.

The proton-detected HMQC® spectra of the zinc and free-base forms of NC-MePP are reproduced in Figures 5.16. and 5.17. respectively. Correlations between the proton and carbon spectra are made as indicated in the figure, by linking an already assigned proton resonance in F2 to a carbon resonance in F1. As correlations are only seen between protons directly attached to carbon, the unknown carbon resonance is thus defined and assigned. All assignments made in this way are given in Table 5.7. The spectrum of free-base NC-MePP shown in Figure 5.16 was acquired without proton decoupling and so estimations of $^{1}J_{CH}$ coupling constants can be made for this compound and those of interest are given in Table 5.6.

Table 5.6. Some $^{1}J_{CH}$ coupling constants for the free-base NC regioisomer of N-methylprotoporphyrin compared to those of compounds with known hybridisation states.

<table>
<thead>
<tr>
<th>Carbon Atom Involved</th>
<th>$^{1}J_{CH}$ / Hz</th>
<th>Probable Carbon Hybridisation State</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂C=CH₂</td>
<td>124.9</td>
<td>$sp^3$</td>
</tr>
<tr>
<td>CH=CH₂</td>
<td>156.4</td>
<td>$sp^2$</td>
</tr>
<tr>
<td>C=CH</td>
<td>249.0</td>
<td>$sp$</td>
</tr>
<tr>
<td>C₆H₆-H₁</td>
<td>159.0</td>
<td>$sp^2$ (?)</td>
</tr>
<tr>
<td>C₆H₆-H₂</td>
<td>156.6</td>
<td>$sp^2$ (?)</td>
</tr>
</tbody>
</table>

A relationship that exists between the size of $^{1}J_{CH}$ coupling and the hybridisation state of the carbon atom involved allows inclusion of the third column. As hybridisation state is an

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The experimental method used is described in Chapter 3 (NMR : Experimental Methods and Theory.)
indication of chemical structure it was hoped to use coupling constants to probe for structural change around the porphyrin macrocycle. The $J_{1CH}$ coupling constants given are difficult to interpret; the methene carbon atoms of a planar porphyrin would be expected to exhibit $J_{1CH}$ coupling constants corresponding to an sp$^2$ hybrid state, and for ethane this is a value of 156.4 Hz. Two of the methene carbons of the N$_C$-MePP alkylated porphyrin display similar coupling constants ($J_{C\alpha-H\alpha} = 156.6$ Hz and $J_{C\gamma-H\gamma} = 156.6$ Hz). The coupling constant of the $C\beta$ methene carbon is slightly larger ($J_{C\beta-H\beta} = 159.0$ Hz) which implies increased electron density at this particular carbon atom of the porphyrin macrocycle, whereas the coupling constant of the $C\delta$ methene carbon is slightly less ($J_{C\delta-H\delta} = 154.1$ Hz) which implies decreased electron density at this particular carbon atom.

It must now be considered that a free-base planar porphyrin can exist in total of six tautomeric forms depending on the pathway taken by the $\pi$-electrons of the porphyrin ring (Simpson, 1949; Neuberger & Scott, 1952). The effect of N-alkylation must be to make some of these pathways less energetically viable. Some resonance forms will therefore be more utilised than others. The overall effect must therefore be a change in the electron distribution around the porphyrin macrocycle which results in the changes of electron densities seen at the $C\beta$ and $C\delta$ meso carbon positions. These effects are difficult to translate into terms of structural change of the porphyrin macrocycle. However, the chemical shift values given in Table 5.8. do confirm the proposals put forward on inspection of the one dimensional $^{13}$C spectra, ie. that small changes in $^{13}$C chemical shift are incurred on N-alkylation of a porphyrin. Assignments made for the zinc-complexed form of N$_C$-MePP (for spectrum see Figure 5.17) also indicate that small changes in chemical shift are apparent when comparing zinc-complexed protoporphyrin and the zinc-complexed N$_C$-MePP. In all instances, these changes are of the order of a few ppm at most.
Fig. 5.16. Relevant portions of the $^1$H-detected HMQC spectrum of free-base $N_C$-methylprotoporphyrin (7.26mM in CDCl$_3$), acquired without decoupling. Details of assignments are given in the text and table 5.7. Relaxation delay ($D_1$) = 3s, and $1/2J$ (where $J$ = mean one-bond coupling, $D_2$) = 3.7ms. Data matrix was 1k x 256 and was zero-filled and a gaussian window function applied ($GB = 0.07$, $lb = -4$Hz) prior to fourier transform.
Fig. 5.17. Relevant portions of the proton-detected HMQC spectrum of zinc-complexed Nε-methylprotoporphyrin (~7.26mM in CDCl₃) acquired with decoupling. Details of assignments are given in the text and table 5.7. Relaxation delay (D₁) = 3s and 1/2J (where J = mean one bond coupling, D₂) = 3.7ms. Data matrix was 1K x 256 and was processed with zero-filling and a gaussian window function (GB = 0.07, LB = -4Hz) prior to Fourier transform.
The origin of these small changes in $^{13}$C chemical shift lies in the difference of relative contributions to shielding of a $^{13}$C nucleus when compared to a proton. As mentioned in section 3.1.1., the shielding of nucleus can be split into diamagnetic, paramagnetic and interatomic shielding effects. For isolated protons the diamagnetic term is the single most important contributor, whereas for protons forming part of an aromatic macrocycle the interatomic effects are also very important as they form the basis of the ring-current induced shift of which many examples have already been described. A $^{13}$C nucleus, however, has more electrons such that it has a filled 2s shell and a partially filled 3p shell. The most important contribution to the $^{13}$C chemical shift is therefore the paramagnetic term. Therefore any ring-current induced shift effects are likely to be only very small, especially when compared to the $^{13}$C spectral range of a few hundred ppm and, in most instances, will be overshadowed by other factors of stronger influence (Gunther & Schmickler, 1975).

Stereochemical factors have been found to be the major contributor to the $^{13}$C chemical shift, and this is an observation of some importance here. It is highly likely that the the small changes seen in $^{13}$C chemical shift for the same groups between the non-alkylated and N-alkylated forms of the porphyrin considered here are monitoring deformations of the porphyrin macrocycle.
Table 5.7: \(^{13}C\) assignments of the zinc-complex and free-base forms of protoporphyrin IX and the \(\text{N}^\text{c}\) regioisomer of N-methylprotoporphyrin.

<table>
<thead>
<tr>
<th>Group (carbon atom has to have protons attached to be observed)</th>
<th>(\Delta C) Chemical Shifts for PP-DME FREE-BASE (ppm)</th>
<th>(\Delta C) Chemical Shifts for (\text{N}^\text{c})-MePP Isomer-free BASE (ppm)</th>
<th>(\Delta C) Chemical Shifts for (\text{N}^\text{c})-MePP Isomer ZINC-COMPLEX (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methine carbons:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\alpha)</td>
<td>97.8 (97.3(\delta))</td>
<td>97.62 (98.20)</td>
<td>98.19</td>
</tr>
<tr>
<td>(\beta)</td>
<td>97.2 (96.7)</td>
<td>97.12 (97.52)</td>
<td>103.25</td>
</tr>
<tr>
<td>(\gamma)</td>
<td>95.9 (95.4)</td>
<td>95.63 (96.07)</td>
<td>100.84</td>
</tr>
<tr>
<td>(\delta)</td>
<td>96.9 (96.3)</td>
<td>96.83 (97.19)</td>
<td>97.32</td>
</tr>
<tr>
<td>pyrrole ring carbons:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| C<sub>α</sub> | not seen (143.5) | 147.89 (148.57), 147.44 (148.21), 147.17 (147.93), 146.99 (147.72), 146.41 (147.08), 145.82 (146.38), 136.09 & 136.27 (136.41 & 136.86) | 141.40
| C<sub>β</sub> (1.3) | not seen (137.6) | 147.89 (148.57), 147.44 (148.21), 147.17 (147.93), 146.99 (147.72), 146.41 (147.08), 145.82 (146.38), 136.09 & 136.27 (136.41 & 136.86) | not seen |
| C<sub>γ</sub> (2.4) | not seen (135.9) | 136.27 & 136.49 (137.02), 136.49 & 136.73 (136.86) | 137.07, 138.32, 138.59 |
| C<sub>δ</sub> (3.8) | not seen (135.9) | 136.27 & 136.49 (137.02), 136.49 & 136.73 (136.86) | 139.27, 139.39, 140.15, 140.56, 140.65 |
| C<sub>ε</sub> (6.7) | not seen (135.9) | 136.27 & 136.49 (137.02), 136.49 & 136.73 (136.86) | 139.27, 139.39, 140.15, 140.56, 140.65 |
| methyl carbons: | | | |
| \(1^C\)CH<sub>3</sub> | 12.5 (12.5) | 12.57 (12.86) | 12.55 |
| \(2^C\)CH<sub>3</sub> | 12.5 (12.5) | 12.57 (12.86) | 12.42 |
| \(3^C\)CH<sub>3</sub> | 11.5 (11.5) | 11.49 (11.74) | 11.57 |
| \(4^C\)CH<sub>3</sub> | 11.5 (11.5) | 11.44 (11.74) | 11.57 |
| vinyl carbons: | | | |
| \(2^C\)=CH<sub>2</sub> | 130.0 (129.8) | 130.78 (131.03) | 130.59 |
| \(3^C\)=CH<sub>2</sub> | 130.0 (129.8) | 130.70 (131.03) | 129.74 |
| \(4^C\)=CH<sub>2</sub> | 120.6 (120.0) | 118.72 (118.95) | 120.17 |
| \(5^C\)=CH<sub>2</sub> | 120.6 (120.0) | 118.65 (118.95) | 121.69 |
| \(6^C\)=CH<sub>2</sub> | 120.6 (120.0) | 118.65 (118.95) | 122.10 |
| N=CH<sub>2</sub> | - | - | 32.42 |
| 6 & 7 methyl propionate carbons: | | | |
| \(1^C\)=CH<sub>2</sub>COOCH<sub>3</sub> | 21.7 (21.7) | 21.79 (22.14) | 21.60 (5) & 21.95 (7) |
| \(2^C\)=CH<sub>2</sub>COOCH<sub>3</sub> | 36.8 (36.8) | 37.01 (37.34) | 35.45 (5) & 36.90 (7) |
| \(3^C\)=CH<sub>2</sub>COOCH<sub>3</sub> | 173.3 (172.8) | 173.62 (173.48) | not assigned |
| \(4^C\)=CH<sub>2</sub>COOCH<sub>3</sub> | 51.5 (51.5) | 51.47 (51.51) | 51.57 (68) |

* Chemical shifts quoted to two decimal places because of the greater clarity of this spectrum.

@ Chemical shift values quoted in brackets are taken from Smith et al., 1982 for comparison.

° Use of the proton-detected HMQC experiment only leads to assignment of carbon atoms with attached protons; other assignments have been made by comparison of the one-dimensional \(^{13}C\) spectra.

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5.4 Discussion.

All the data collected and described in this chapter indicate that the free-base form of an N-alkylporphyrin is much more flexible and distorted than both the corresponding zinc-complexed N-alkylporphyrin and also protoporphyrin, the parent compound from which the N-alkylporphyrins used in this study are derived. Comparison of NMR spectra of the free-base and zinc-complex forms of protoporphyrin showed little difference, but the differences in the proton spectra of the free-base and zinc-complex forms of N-methylprotoporphyrin and N-ethylprotoporphyrin are marked. Resonances of very similar chemical groupings no longer come at the same chemical shift values, and, as chemical shift value is dependent on magnetic environment, it can be assumed that these resonances belong to protons which have changed their position relative to the porphyrin ring current, i.e. that distortion of the porphyrin has occurred.

In an attempt to more quantitatively assess the movement of these informative resonances as N-alkylation and metalation processes occur, Tables 5.8-5.11 have been drawn up. In these the reference chemical shift value is the chemical shift value for the given group in either the free-base or zinc-complexed form of protoporphyrin as relevant. The upfield shift when on the alkylated ring is then the difference between the chemical shift value of the given group when on the alkylated ring and this reference value. For example, $^1$CH$_3$ comes at 3.31 ppm when it is on the alkylated ring in N$_A$-methylprotoporphyrin. The upfield shift it experiences when on the alkylated ring is therefore 3.71 (reference value) - 3.31 (chemical shift value when on the alkylated ring) = 0.40 ppm. In the same way, the upfield shift when opposite the alkylated ring is the difference in chemical shift value, relative to protoporphyrin, for a given group situated on the ring opposite the alkylated ring. Likewise the last column of the table represents the differences (relative to protoporphyrin) in chemical shift value for given groups when situated on rings either side of the alkylated one. For example, looking at Table
5.8. for the free-base N-methylprotoporphyrins, $^1\text{CH}_3$ experiences a shift of 0.15ppm to high-field when ring B is alkylated and a shift of 0.14ppm to high-field when ring D is alkylated.

This assessment of relative shifts in chemical shift values incurred on N-alkylation and zinc-complexation lends weight to the qualitative arguments already presented for differences between

a) the N-alkylated free-bases (relative to protoporphyrin), and

b) the N-alkylated zinc-complexes (relative to protoporphyrin).

Table 5.8. Shifts of the moieties of N-methylprotoporphyrin free-base regioisomers relative to free-base protoporphyrin.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Reference Chemical Shift Value (ppm)</th>
<th>Upfield Shift when on Alkylated Ring (ppm)</th>
<th>Upfield Shift when Opposite Alkylated Ring (ppm)</th>
<th>Upfield Shift when Beside Alkylated Ring (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1\text{CH}_3$</td>
<td>3.71</td>
<td>0.40</td>
<td>-0.04</td>
<td>0.15 (B)</td>
</tr>
<tr>
<td>$^2\text{Vi}$ (int)</td>
<td>8.30</td>
<td>0.38</td>
<td>-0.04</td>
<td>0.13 (B)</td>
</tr>
<tr>
<td>$^3\text{CH}_3$</td>
<td>3.70</td>
<td>0.50</td>
<td>-0.07</td>
<td>0.05 (A)</td>
</tr>
<tr>
<td>$^4\text{Vi}$ (int)</td>
<td>8.30</td>
<td>0.47</td>
<td>-0.02</td>
<td>0.07 (A)</td>
</tr>
<tr>
<td>$^5\text{CH}_3$</td>
<td>3.61</td>
<td>0.44</td>
<td>0.05</td>
<td>0.15 (B)</td>
</tr>
<tr>
<td>6 (prox)</td>
<td>4.42</td>
<td>0.38</td>
<td>-0.07</td>
<td>0.21 (B)</td>
</tr>
<tr>
<td>6 (dis)</td>
<td>3.29</td>
<td>0.54</td>
<td>0.03</td>
<td>0.03 (B)</td>
</tr>
<tr>
<td>7 (prox)</td>
<td>4.42</td>
<td>0.41</td>
<td>-0.02</td>
<td>0.13 (A)</td>
</tr>
<tr>
<td>7 (dis)</td>
<td>3.29</td>
<td>0.53</td>
<td>0.02</td>
<td>-0.03 (A)</td>
</tr>
<tr>
<td>$^8\text{CH}_3$</td>
<td>3.62</td>
<td>0.46</td>
<td>-0.02</td>
<td>-0.08 (A)</td>
</tr>
</tbody>
</table>
Table 5.9. Shifts of the moieties of N-methylprotoporphyrin zinc-complexed regioisomers relative to free-base protoporphyrin.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Reference Chemical Shift Value (ppm)</th>
<th>Upfield Shift when on Alkylated Ring (ppm)</th>
<th>Upfield Shift when Opposite Alkylated Ring (ppm)</th>
<th>Upfield Shift when Beside Alkylated Ring (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1CH₃</td>
<td>3.73</td>
<td>0.14</td>
<td>0.15</td>
<td>0.04 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03 (D)</td>
</tr>
<tr>
<td>2Vi (int)</td>
<td>8.38</td>
<td>0.25</td>
<td>0.21</td>
<td>0.15 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12 (D)</td>
</tr>
<tr>
<td>3CH₃</td>
<td>3.73</td>
<td>0.19</td>
<td>0.07</td>
<td>0.03 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.11 (C)</td>
</tr>
<tr>
<td>4Vi (int)</td>
<td>8.38</td>
<td>0.32</td>
<td>0.13</td>
<td>0.11 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.20 (C)</td>
</tr>
<tr>
<td>5CH₃</td>
<td>3.64</td>
<td>0.23</td>
<td>0.06</td>
<td>0.05 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.02 (D)</td>
</tr>
<tr>
<td>6 (prox)</td>
<td>4.44</td>
<td>0.22</td>
<td>0.07</td>
<td>0.10 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05 (D)</td>
</tr>
<tr>
<td>6 (dis)</td>
<td>3.31</td>
<td>0.38</td>
<td>0.00</td>
<td>0.01 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06 (D)</td>
</tr>
<tr>
<td>7 (prox)</td>
<td>4.44</td>
<td>0.21</td>
<td>0.10</td>
<td>0.07 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.15 (C)</td>
</tr>
<tr>
<td>7 (dis)</td>
<td>3.31</td>
<td>0.41</td>
<td>0.01</td>
<td>0.00 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.08 (C)</td>
</tr>
<tr>
<td>8CH₃</td>
<td>3.64</td>
<td>0.15</td>
<td>0.04</td>
<td>-0.00 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.01 (C)</td>
</tr>
</tbody>
</table>

Table 5.10. Shifts of the moieties of N-ethylprotoporphyrin free-base regioisomers relative to free-base protoporphyrin.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Reference Chemical Shift Value (ppm)</th>
<th>Upfield Shift when on Alkylated Ring (ppm)</th>
<th>Upfield Shift when Opposite Alkylated Ring (ppm)</th>
<th>Upfield Shift when Beside Alkylated Ring (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1CH₃</td>
<td>3.71</td>
<td>0.44</td>
<td>0.08</td>
<td>0.14 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03 (D)</td>
</tr>
<tr>
<td>2Vi (int)</td>
<td>8.30</td>
<td>0.44</td>
<td>0.06</td>
<td>0.12 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07 (D)</td>
</tr>
<tr>
<td>3CH₃</td>
<td>3.70</td>
<td>0.49</td>
<td>-0.09</td>
<td>0.10 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.24 (C)</td>
</tr>
<tr>
<td>4Vi (int)</td>
<td>8.30</td>
<td>0.44</td>
<td>-0.05</td>
<td>0.16 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19 (C)</td>
</tr>
<tr>
<td>5CH₃</td>
<td>3.61</td>
<td>0.44</td>
<td>0.05</td>
<td>0.15 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19 (D)</td>
</tr>
<tr>
<td>6 (prox)</td>
<td>4.42</td>
<td>0.23</td>
<td>-0.01</td>
<td>0.21 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.13 (D)</td>
</tr>
<tr>
<td>6 (dis)</td>
<td>3.29</td>
<td>0.58</td>
<td>0.03</td>
<td>0.04 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07 (D)</td>
</tr>
<tr>
<td>7 (prox)</td>
<td>4.42</td>
<td>0.26</td>
<td>0.02</td>
<td>0.19 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.23 (C)</td>
</tr>
<tr>
<td>7 (dis)</td>
<td>3.29</td>
<td>0.52</td>
<td>0.05</td>
<td>0.03 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07 (C)</td>
</tr>
<tr>
<td>8CH₃</td>
<td>3.62</td>
<td>0.46</td>
<td>-0.02</td>
<td>-0.08 (A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.13 (C)</td>
</tr>
</tbody>
</table>
Table 5.11. Shifts of the moieties of N-ethylprotoporphyrin zinc-complexed regioisomers relative to free-base protoporphyrin.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Reference Chemical Shift Value (ppm)</th>
<th>Upfield Shift when on Alkylated Ring (ppm)</th>
<th>Upfield Shift when Opposite Alkylated Ring (ppm)</th>
<th>Upfield Shift when Beside Alkylated Ring (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$CH$_3$</td>
<td>3.73</td>
<td>0.16</td>
<td>0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>$^2$Vi (int)</td>
<td>8.38</td>
<td>0.32</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>$^3$CH$_3$</td>
<td>3.64</td>
<td>0.21</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>$^4$Vi (int)</td>
<td>8.38</td>
<td>0.34</td>
<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td>$^5$CH$_3$</td>
<td>3.64</td>
<td>0.20</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>6 (prox)</td>
<td>4.44</td>
<td>0.03</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>6 (dis)</td>
<td>3.31</td>
<td>0.38</td>
<td>-0.06</td>
<td>-0.01</td>
</tr>
<tr>
<td>7 (prox)</td>
<td>4.44</td>
<td>0.03</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>7 (dis)</td>
<td>3.31</td>
<td>0.37</td>
<td>-0.01</td>
<td>-0.06</td>
</tr>
<tr>
<td>$^8$CH$_3$</td>
<td>3.64</td>
<td>0.15</td>
<td>0.04</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Shifts for moieties on the N-alkylated ring are much more pronounced (0.26 - 0.58ppm) in the free-base regioisomers of N-methylprotoporphyrin and N-ethylprotoporphyrin than in the corresponding zinc-complexed regioisomers (0.14-0.38ppm). Also, in the free-base regioisomers, the moieties on the ring opposite the alkylated one experience little ring-current induced shift effects, whereas the moieties on the rings neighbouring the alkylated one display considerable ring-current-induced shift effects. This would suggest that all four rings of the free-base porphyrin are in different environments relative to each other, and relative to their positions in the planar protoporphyrin.

With the zinc-complexed forms of N-methylprotoporphyrin and N-ethylprotoporphyrin, the shifts of moieties on rings neighbouring the alkylated one are much less marked, though significant upfield shifts are seen for groups on vinyl-substituted rings when they are
opposite an alkylated ring. This analysis would suggest that the alkylated ring, and the one opposite to a lesser extent in some instances, are in different environments relative to each other, the two neighbouring rings perhaps being in the same plane. This is expected because of the assignment strategy used, but this analysis, it must be remembered, is referenced to protoporphyrin and so is informative because it highlights distinctions between the non-alkylated and N-alkylated forms.

As the study stands, it would appear that ring-current-induced chemical shift effects can be indicative of structural change within the N-alkylenporphyrins. More information could be gained on extension of these tables to include N-alkylenporphyrins with extended alkyl groups, but at this time, not enough of these compounds are available for further study. The proton spectra of N-1-propylprotoporphyrin and N-1-butylprotoporphyrin, however, indicated little difference between the free-base and zinc-complex forms of these compounds, except for one significant shift of the methylene resonance adjacent to the N-CH₂ group of the N-alkyl group (N-CH₂-CH₂-) in both N-propylprotoporphyrin and N-butylprotoporphyrin. Otherwise the spectra of the free-base forms approached the behaviour of the spectra of the zinc-complex forms. A study of the shifts relative to protoporphyrin would therefore be interesting to see.

A study of shift effects on the ¹³C nucleus would also be desirable, but the data presented here is not extensive enough to draw up similar tables of data. Differences in ¹³C chemical shift value have been observed however (see table 5.7). An attempt to probe porphyrin structure via carbon-proton coupling constants indicated only that the meso carbons are not readily apparent as sites of structural change, though slight changes in coupling constants have been observed. The most useful positions of the carbon skeleton to probe for structural change would be the Cₛ (and to a small extent the Cₚ) carbons of the pyrrole rings. These resonances are, however, either indistinct or not observed in the ¹³C spectra obtained for
the zinc-complexed and free-base forms of N\textsubscript{C}-methylprotoporphyrin, indicating that further experiments would require running for longer times, with more sample.
6. Ferrochelatase: Purification and Inhibition Analysis

6.1. Ferrochelatase: A Brief Overview

6.1.1. Introduction

Ferrochelatase (protohaem ferrolyase, EC 4.99.1.1.) is the last enzyme of the liver haem biosynthetic pathway and is responsible for inserting ferrous iron into protoporphyrin to form haem. It has been found in a variety of animal tissues and cells such as bovine liver (Taketani et al., 1982, Dailey et al., 1983), chicken erythrocytes (Hanson et al., 1984), human liver (Matthews-Roth et al., 1987) and rat liver (Taketani et al., 1981). The enzyme forms an intrinsic part of the inner mitochondrial membrane and in bovine liver mitochondria it has been confirmed to be on the matrix side of this membrane (Harbin et al., 1985).

The enzyme can also be isolated from the yeast Saccharomyces cerevisiae (Camadro et al., 1988), plants such as spinach (Jones, 1968) and the bacteria Rhodopseudomonas sphaeroides and Spirillum itersonii (Dailey, 1977; Dailey, 1982). In plants it is bound to the chloroplast membrane and in bacteria it is associated with the cytoplasmic membrane.

6.1.2. Enzyme Purification.

Ferrochelatase was not purified to homogeneity until 1981 due to the difficulty of stabilising the enzyme during the purification procedure. Taketani and Tokunaga, using rat liver, overcame instability by using a buffer system containing dithiothreitol and glycerol (1981). The enzyme also aggregates (Yoneyama et al., Mazanowska et al., 1969); this problem was overcome by use of blue sepharose chromatography. Once the enzyme was bound to the gel matrix it was not released on elution with non-ionic detergents such as Triton X-100 and Tween 20 even in the presence of high salt at 1.5M sodium chloride. Porphyrin or nucleotide
co-factors were also unable to free the enzyme. It was found, however, that enzyme removal could be brought about by high concentrations of sodium cholate with 1.5M sodium chloride, implying that the enzyme has a highly hydrophobic nature and that the blue dye did not act as a substrate analogue.

The same system was then applied to bovine liver (Taketani et al., 1982), and ferrochelatase was purified to apparent homogeneity as judged by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The procedure involved solubilization with sodium cholate, ammonium sulphate fractionation and blue sepharose CL-6B chromatography. The molecular weight of the purified protein was 42.5kD when measured by SDS-PAGE, but a molecular weight of approximately 200kD is obtained by sepharose 6B gel filtration.

Dailey and Fleming (1983) also purified the enzyme from bovine liver with an increased yield and 2000-fold purification by using a Reactive Red column in tandem with the Blue Sepharose CL-6B column. The molecular weight of approximately 40kD was quoted for both SDS-PAGE and column chromatography on Sepharose CL-6B. Both estimations were in the presence of 0.5% sodium cholate, which seemed to inhibit enzyme aggregation.

A detailed account of the ferrochelatase purification procedure using blue Sepharose is included in the methods section of this chapter, as its attempted purification was an integral part of the work undertaken for this thesis.

6.1.3. Ferrochelatase from other sources.

The gene (Hem 15) which encodes for ferrochelatase from Saccharomyces cerevisiae has been isolated by functional complementation of a Hem 15 mutant (Labbe-Bois, 1990). Following on from this work a baculovirus expression vector has been constructed (Eldridge
et al, 1992). Using this system approximately 25 µg of protein/ml of culture were gained. Purification on a Pharmacia Hi-trap blue affinity column resulted in pure ferrochelatase with kinetic parameters similar to those described for the wild-type enzyme from *Saccharomyces cerevisiae*.

The Japanese group have isolated the cDNA encoding mouse ferrochelatase from a mouse erythroleukaemia (MEL) cell cDNA library in λgt 11 expression vector by immunoscreening with a polyclonal antibody (Taketani et al, 1990). The cDNA allowed for expression of active ferrochelatase by transfected cell cultures.

The same group have also isolated the cDNA encoding human ferrochelatase from a human placenta cDNA library in bacteriophage λgt 11 by screening with a radiolabelled fragment of mouse ferrochelatase cDNA. The human enzyme showed 88% sequence homology to mouse ferrochelatase and 46% sequence homology to the yeast enzyme (Nakahashi et al, 1990).

As a result of all these efforts, mouse and human ferrochelatases have now been engineered into an *E. coli* expression vector (Dailey et al, 1994 (a)) and the expressed recombinant proteins purified and characterised (Dailey et al. 1994 (b)). Interestingly, the human recombinant protein has an [2Fe-2S]++ iron-sulphur cluster formed by 30 amino acids at its carboxy-terminus. The iron-sulphur cluster has been found to be necessary for enzyme activity in this protein and so is proposed to have some stabilising role. The cluster however, is not found in the yeast or bacterial ferrochelatase enzymes. Another group has also published a protocol for the overexpression of mammalian ferrochelatase in *E. coli* (Ferreira 1994(a)) and have also characterised the enzyme as a metalloenzyme with a [2Fe-2S] cluster (Ferreira et al, 1994(b)).
6.1.4. Kinetic Studies on Ferrochelatase

The most detailed kinetic characterisation of ferrochelatase to date (Dailey and Fleming, 1983), was performed using purified bovine liver enzyme. N-methylprotoporphyrin, previously thought to be an irreversible inhibitor of ferrochelatase, was found to be a reversible competitive inhibitor ($K_i = 7 \text{nM}$) with respect to one of the normal enzyme substrates, protoporphyrin and a non-competitive inhibitor with respect to ferrous iron (the other normal enzyme substrate). Haem, one of the products, is a non-competitive inhibitor with respect to iron. These findings led to the proposal of a sequential Bi-Bi kinetic model for ferrochelatase, with ferrous iron binding prior to porphyrin binding and haem being released prior to the release of two protons. The kinetic scheme is outlined below in Figure 6.1.

**Figure 6.1.** Kinetic scheme of the action of ferrochelatase (taken from Dailey and Fleming, 1983). $E =$ enzyme, $M_2^+ =$ divalent metal cation, $Fe^2+ =$ ferrous iron, $NMPP =$ N-methylprotoporphyrin, $PP =$ protoporphyrin and $2H =$ 2 hydrogen atoms.

6.1.5. Ferrochelatase Activity Related to Porphyrin Structure

**Planar Porphyrins**

A study of the effect of porphyrin structure on its ability to be utilised by ferrochelatase has been made (Honeybourne et al, 1975) and the important findings are shown in Table 6.1.
The best rates of metalloporphyrin synthesis are achieved using porphyrin 1 as a substrate. This porphyrin has no substituents at positions 1-4 but does have propionate groups at positions 6 and 7. Rates of metalloporphyrin synthesis with deuteroporphyrin, mesoporphyrin and protoporphyrin are progressively less when compared to this porphyrin. Ferrochelatase activity is also lowered on movement of the propionate groups: movement of the 6-propionate to the 5 position (as in porphyrin 2), and additional movement of the 7-propionate to the 8 position (as in porphyrin 3) lowers, and then causes complete loss of metalloporphyrin synthesis activity of the enzyme. The study shows, therefore, that ferrochelatase has a hydrophobic site capable of binding porphyrin substrates with hydrophobic substituents at positions 1, 2, 3 and 4, though such moieties do not appear to enhance ferrochelatase activity. Also shown, is that the propionate side-chains at positions 6 and 7 clearly play a crucial role in porphyrin binding and utilisation.


<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Substituents</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H H H H Me Pr Pr Me</td>
<td>5.0</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>H H H H Me Me Pr Me</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>H H H H Me Pr Me Pr</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>DeuteroIX</td>
<td>Me H Me H Me Pr Pr Me</td>
<td>4.0</td>
<td>1.4</td>
</tr>
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<td>MesoIX</td>
<td>Me Et Me Et Me Pr Pr Me</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>ProtoIX</td>
<td>Me Vi Me Vi Me Pr Pr Me</td>
<td>0.8</td>
<td>0.11</td>
</tr>
<tr>
<td>ProtoXIII</td>
<td>Vi Me Vi Me Vi Pr Pr Me</td>
<td>1.4</td>
<td>0.25</td>
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</tbody>
</table>
N-alkylated Porphyrins

The structure of N-alkylated porphyrins has also been investigated with regard to their ability to inhibit ferrochelatase (de Matteis et al, 1985). It has been found that:

a) 2 propionate side-chains are still important for porphyrin binding,
b) only N-alkylated substrates of ferrochelatase cause inhibition, suggesting that N-alkylation is not the only prerequisite for inhibition to occur,
c) regioisomeric type and the size of the N-alkyl group have an effect on inhibition efficiency, as the $N_{C+D}$ regioisomers of N-propylprotoporphyrin are much less effective inhibitors of ferrochelatase than the $N_{A+B}$ regioisomers of the same, whereas the $N_{A+B}$ and $N_{C+D}$ regioisomers of N-methylprotoporphyrin and N-ethylprotoporphyrin appear to have similar inhibitory activities,
d) substituents at the 2 and 4 positions may be important in N-alkylporphyrin binding, as N-methyl derivatives of deuteroporphyrin and protoporphyrin are more effective ferrochelatase inhibitors than the N-methyl derivative of mesoporphyrin, which lacks hydrophobic groups at these positions.

Biologically-derived N-alkylporphyrins

The N-alkylporphyrins formed on administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) analogues, especially those with extended N-alkyl groups, have also received a lot of interest. Work been carried out with both chick embryo liver cell culture and the rat. A summary of the various DDC analogues used, the N-alkylporphyrins formed and their ferrochelatase inhibitory activity is given in Table 6.2.

<table>
<thead>
<tr>
<th>DDC analogue</th>
<th>N-alkylporphyrin formed from DDC</th>
<th>Studied in Rat or chick embryo liver cell culture</th>
<th>Ferrochelatase Inhibitory Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Butyl DDC</td>
<td>N-Butyl PPIX</td>
<td>Both</td>
<td>YES</td>
</tr>
<tr>
<td>4-Pentyl DDC</td>
<td>N-Pentyl PPIX</td>
<td>Both</td>
<td>YES</td>
</tr>
<tr>
<td>4-Hexyl DDC</td>
<td>N-Hexyl PPIX</td>
<td>Both</td>
<td>YES</td>
</tr>
<tr>
<td>4-Isobutyl DDC</td>
<td>N-Isobutyl PPIX</td>
<td>Both</td>
<td>NO</td>
</tr>
</tbody>
</table>

Studies in the rat also showed that the $N_{A+B}$ regioisomers of a particular N-alkylporphyrin are more inhibitory than the $N_{C+D}$ regioisomers of the same. Thus the lack of inhibition caused by the 4-isobutyl DDC analogue was explained in terms of the low proportion of $N_{A+B}$ regioisomer found in the resulting N-isobutyl protoporphyrin (McCluskey et al, 1989). This work was also extended, however (Kimmett et al, 1992), to show that biologically derived $N_{A+B}$ N-alkylporphyrins are more inhibitory than the corresponding $N_{A+B}$ synthetically made N-alkylporphyrins, a finding finally explained when viewed in terms of relative proportions of enantiomers as well as regioisomers. A 50:50 ratio of $N_{A,N_{B}}$ regioisomers of N-ethylprotoporphyrin is made synthetically, whereas a 80:20 ratio is found in 4-ethyl DDC derived N-ethylprotoporphyrin. The biological $N_{A}$ regioisomer (and perhaps also the epi-$N_{B}$ enantiomer which is very similar after rotation through 180°), was found to be five times as potent as the $N_{B}$ regioisomer. This would indicate that the biologically-derived N-alkylporphyrins tend to be of preferred enantiomeric type for maximal inhibition of ferrochelatase.
6.1.6. The Role of Residues in Porphyrin Binding

Dailey's group have carried out work which investigates the effect of chemical modification of particular amino acid residues on the activity of the ferrochelatase enzyme. Bovine ferrochelatase was used to study the role of arginyl residues in porphyrin binding to the enzyme. Modification of the cationic arginyl, but not lysyl residues resulted in enzyme inactivation. In this instance a porphyrin (2,4-disulphonate deuteroporphyrin) which is a competitive inhibitor of the bovine ferrochelatase was able to afford protection of the enzyme from inactivation. This study therefore indicated that arginyl residues seemed to play an important role in porphyrin binding at the ferrochelatase active site (Dailey & Flemming, 1986).

Another study with bovine ferrochelatase found that the enzyme is rapidly inactivated by sulphydryl group specific reagents, in a manner that suggested that modification of a single sulphydryl group was sufficient to inactivate the enzyme. Moreover, the enzyme was protected from inactivation by ferrous iron, one of ferrochelatases's normal substrates. This suggested the involvement of sulphydryl groups in binding iron at the active site (Dailey, 1984).

6.1.7. Models for the Ferrochelatase Active Site.

Two models have been put forward for the active site of ferrochelatase that encompass all the data already described concerning the nature of the normal substrates and N-alkylporphyrins that are able to interact with ferrochelatase.

The proposal put forward by Ortiz de Montellanos' group (Ortiz de Montallano et al, 1986; see Figure 6.2., (a)) suggests that the porphyrin substrate is drawn into the active site by
Fig. 6.2. Two models proposed for the active site of ferrochelatase. The models differ in the orientation of the porphyrin molecule. Ortiz de Montellanos' group (a) favour a model in which the carboxylic acid groups extend into the active site, whereas Daileys' group (b) propose that the carboxylic acid groups extend outward from the active site into the solvent (taken from Ortiz de Montellano et al, 1986 and Dailey, 1986 respectively).
hydrogen bonding or electrostatic interactions between its carboxylic acid groups and residues in the active site. In this orientation the pyrrole rings C and D are thought to be bound in a sterically constrained region of the active site that disallows gross N-alkylation of these rings. Rings A and B are proposed to be in a relatively open region of the active site which is able to accommodate larger N-alkylsubstituents on these rings.

The alternative proposal comes from Dailey's group (Dailey, 1985; see Figure 6.2., (b)). In this model the propionyl carboxyls of the porphyrin extend outward from a 'pocket' in a fashion similar to the situation in many cytochromes and globin molecules. When the porphyrin substrate is in place at the enzyme active site, the carboxyl groups are proposed to undergo charge-charge interactions with arginine residues at the 'pocket' entrance. Once bound the porphyrin undergoes distortion to facilitate iron insertion, followed by a return to planarity and release of haem from the active site. During the metallation process 2 hydrogen ions are displaced by the iron atom that is now positioned in the centre of the porphyrin ring.

The ability of an N-alkylporphyrin to inhibit ferrochelatase is proposed to be limited by the size and shape of the enzyme's active site. The inherent distorted nature of an N-alkylporphyrin, as proven by X-ray crystallography (Mclaughlin, 1974; Lavallee et al, 1978; Lavallee, 1987), has been proposed to be similar to that of the transition state of a normal porphyrin when undergoing distortion during metal insertion. As enzymes have a higher affinity for transition states than normal substrates, this theory is capable of explaining the tight-binding, but reversible, competitive inhibition of N-methylprotoporphyrin over the normal substrate protoporphyrin.
6.2. Statement of Aims

The aim of this part of the project was to purify ferrochelatase from an animal source, with a view to conducting NMR studies of the enzyme, both on its own and with substrates and inhibitors. Pure enzyme would also lead the way to kinetic studies of the enzyme with substrate and inhibitors. As it transpired, isolation of the enzyme was not entirely successful and so experiments were devised to try and determine the cause of this problem. Also the limited stability and low yields of enzyme precluded NMR or kinetic studies. Instead, inhibition studies using solubilised enzyme and synthetic N-alkylporphyrins were carried out.
5.3. Methods

5.3.1. Introduction

Rat liver was the enzyme source used throughout this project, rodent liver being opted for to keep in line with the NMR work already done on the N-GIPP adduct isolated from mouse liver. The protocol used initially, however, was that described for isolating bovine ferrochelatase. The reported improved yield and bigger size of the bovine ferrochelatase protocol over that of the rat enzyme was of considerable importance, as it was hoped that large quantities of enzyme would be isolated for NMR studies. The purification procedure described in these pages is, however, a modified version of the two protocols; this modified procedure came about due to the lack of success in following the bovine protocol alone. As these difficulties could be perhaps be due to unknown, but important differences between rat and bovine ferrochelatase and therefore viable purification procedures, an attempt was made to revert to the rat protocol whilst retaining the scaled-up size of the bovine protocol.

6.3.2. General Comments

All operations were carried out at 4°C or on ice. Buffers (see below) are kept at this temperature. Dithiothreitol (DTT) and phenylmethysulphonylfluoride (PMSF) were added to buffers just prior to use as their half-lives in aqueous environments are short. PMSF was added from a stock solution in ethanol.
Homogenisation Buffer
10mM Tris/acetate pH 8.1
0.25M Sucrose

Buffer A
20mM Tris/acetate pH 8.1
1mM Dithiothreitol
10μg/ml PMSF
20% Glycerol

Buffer 1
Buffer A + Solubilising Buffer A
1% Sodium Cholate 10% Sodium Cholate
0.1M KCl 1M KCl

Buffer 2
Buffer A + 1% Triton X100
0.5M KCl

Buffer 3
Buffer A + 1% Triton
1M KCl

Buffer 4
Buffer A + 0.5% Sodium Cholate
1M KCl

Buffer 5
Buffer A + 1% Sodium Cholate
1.5M KCl

6.3.3. Preparation of Rat Liver Mitochondria

Batches of mitochondria were prepared in the following manner. Approximately 1000g of liver (from ~100 rats of body mass in the range 180-200g), were made into a 20% w/v homogenate with homogenisation buffer (20 seconds in an Ultraturax or 1min in a Waring blender). The homogenate was then spun down (20min, 900g) to sediment nuclei and cell debris. Supernatants were spun (20min, 10 000g) to sediment mitochondria. Supernatants from this step were then respun to ensure all possible mitochondria are collected. At this stage the mitochondria could be stored at ~70°C if necessary.
6.3.4. Preparation of Sub-Mitochondrial Particles

The mitochondria were resuspended in homogenisation buffer and placed in a vessel contained in an ice/salt water bath and then disrupted by sonication using a Branson 250 Sonicator at 10s intervals (over 2mins, Power = 3, Output = 40W at 50%). The suspension was then spun (20mins, 10 000rpm) to bring down any undisrupted mitochondria. The supernatant from this step was then spun (90mins, 100 000g) to bring down the sub-mitochondrial particles, which were resuspended in a minimal amount of Buffer A only and stored at -70°C to await further processing.

6.3.5. Solubilisation of Ferrochelatase

Ferrochelatase was solubilised from the mitochondrial membrane by adding solubilising buffer (which contains sodium cholate as the active solubilising agent) and Buffer A such that the final concentration of sub-mitochondrial particles was 40 mg/ml and the final concentration of sodium cholate was 1% w/v. The mixture was then gently stirred for 3hrs in the dark at 4°C and then centrifuged (90mins, 100 000g) to bring down the membranes and leave solubilised ferrochelatase enzyme in the supernatant.

6.3.6. Ammonium Sulphate Precipitation

The solubilised enzyme solution was precipitated using saturated ammonium sulphate solution (pH 8.1). The volume (V) of saturated ammonium sulphate solution (in ml) which had to be added to 100ml of solution of initial saturation S1 to produce a final saturation S2 is given by the equation;
V = \frac{100(S2 - S1)}{1-S2}

The solubilised enzyme was first taken to 35% saturation and then stirred for 15 mins followed by centrifugation (15 mins, 16 000g). The pellet was discarded and saturated ammonium sulphate solution added to the supernatant to bring the mixture to 55% saturation. The stirring and centrifugation procedures were repeated, but this time the pellet was saved and resuspended in a minimal amount of Buffer 2, ready to be applied to the column.

6.3.7. Column Chromatography on Blue Sepharose CL-6B

Blue Sepharose CL-6B (Pharmacia) was prepared from freeze-dried gel according to instructions received with the gel. This basically involved ensuring that all additives were freed from the gel before use by washing thoroughly, first with distilled water and then with the appropriate buffer. The column was made from glass (2.5 x 30cm) fitted with Whatman adaptors and was packed with blue sepharose CL-6B washed thoroughly with Buffer 2. Once packed the column was also equilibrated with this buffer. Buffers were pumped through using a peristaltic pump. Between purifications the column itself was washed through with ~4 bed volumes of 8M Urea before re-equilibrating with Buffer 2. The column was linked up to a fraction collector programmed to collect 10ml samples of eluate, at a flow rate of 20ml/hr.

The ammonium sulphate fraction was applied to the column directly and washed on with a further 50ml of Buffer 2. The buffer was then changed to Buffer 3 (containing Triton X100 to bring off unwanted proteins) and 500ml pumped through. The buffer was then changed to a cholate-containing buffer (number 4) in preparation for bringing off ferrochelatase. The column was washed with 150ml of this buffer before changing to Buffer 5 which contains
high salt and high cholate to elute ferrochelatase. All fractions were assayed both for ferrochelatase activity and protein content.

6.3.8. Pyridine Hemochromogen ; An Activity Assay For Ferrochelatase

The activity of ferrochelatase was monitored throughout the purification procedure using the method devised by Porra and Jones (1963) with the modifications of Cole et al (1979). The following reagents are placed in the main chamber of a Thunberg tube on ice :-

- Tween 80 (0.3ml, 1% v/v)
- Mesoporphyrin (with hydrolysed methyl esters, 120μl, 1mM)
- Ethanol (0.3ml)
- 0.2M Tris/Acetate (1.5ml, pH 8.2 @ 37°C)
- Dithiothreitol (60μl, 0.2M)
- Palmitic acid (140μl, 60mM)
- Iron sulphate (120μl, 1M)

The aliquot of enzyme preparation to be assayed is placed in the side-arm of the tube and made up to 800μl with appropriate buffer. Oxygen is then removed from the system and replaced with nitrogen using a manifold, to ensure that the reaction proceeds anaerobically.

The tubes are incubated for 5mins in the dark at 37°C. The reaction is started by tipping the contents of the side-arm into the main chamber of the Thunberg tube and mixing. The total reaction volume is 6.36ml. The reaction is allowed to proceed for 30mins and is stopped by addition of iodoacetamide (0.5ml, 0.4M) and cooling on ice.
The spectrophotometric step involves quantitation of haem as its pyridine haemochromogen. Pyridine (1.5ml) and sodium hydroxide (1.16ml, 0.5M) are added to the reaction mixture which is then split between two cuvettes. Potassium ferricyanide (50μl, 0.1% w/v solution) is added to one cuvette to oxidise the haem. A few grains of sodium dithionite are added to the other cuvette to keep haem in its reduced state.

A reduced - oxidised difference spectrum is then recorded from 600 - 500nm and the amount of haem formed during the reaction period calculated using

\[ \varepsilon_{\text{mM}}^{547} \cdot \varepsilon_{\text{mM}}^{531} = 21.7 \text{mM}^{-1} \text{cm}^{-1} \]

and

\[ \text{nmol haem formed / 30mins} = \frac{\Delta A \times 1000 \times 6.36}{21.7} \]

### 6.3.9. Protein Estimation in the Presence of Detergent

The dithiothreitol and detergent-containing buffers of the ferrochelatase purification interfere with protein estimation by the method of Lowry et al (1951). In instances where detergent is present the procedure of Peterson (1977) was used instead. This involves a deoxycholate-trichloroacetic acid (DOC-TCA) precipitation step to remove substances that otherwise interfere with the protein estimation procedure. Protein samples are made up to 1ml with distilled water and sodium deoxycholate in water (100μl, 0.15%) added. The mixture is left for ten minutes and then trichloroacetic acid (100μl, 72%) added. The samples are then centrifuged (3000rpm, 15min). Any liquid is discarded and the precipitated protein allowed to dry. Protein is then estimated as according to Lowry (Lowry et al, 1951) but in the presence of sodium dodecylsulphate (SDS), which prevents the usual precipitate formation resulting from non-ionic and cationic detergents (Markwell et al, 1978).
6.3.10. Protein Estimation in the Absence of Detergent

When detergent and dithiothreitol are not present, proteins were estimated either using the method of Lowry (1951) or using the Pierce BCA assay (Smith et al, 1985).

6.3.11. Gel Electrophoresis

Preparation and running of gels

SDS-PAGE gels were prepared as follows: Gel plates were cleaned with methanol and assembled in the cassettes of Biorad Mini-Protean gel apparatus used. A line was marked 20mm down from the upper edge of the smaller glass plate. The running gel was prepared by mixing together the reagents required (as shown in the following table) in a 100ml Buchner flask. The mixture was then degassed for 10 minutes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>% gel required (quantities shown in ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5%</td>
</tr>
<tr>
<td>Ultra-Clean H₂O</td>
<td>39</td>
</tr>
<tr>
<td>30% acrylamide / 0.8% bis-acrylamide</td>
<td>20</td>
</tr>
<tr>
<td>1.5M Tris⁺-HCl, pH 8.8</td>
<td>20</td>
</tr>
<tr>
<td>10% SDS*</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Polymerisation was initiated by adding 400µl of 10% ammonium persulphate and 60µl of TEMED® with quick mixing. The still-liquid gel was then transferred to a 20ml syringe fitted with a 19G needle and injected into the gel cassettes up to the level of the marked line. The gel was overlaid with butan-2-ol to exclude air and accelerate polymerisation and left to set at room temperature.

*N,N,N',N'-tetramethylethylene diamine
After setting the butanol was washed off with several rinses of distilled water. Excess water was drained off and a comb inserted. The stacking gel was then prepared by mixing the following reagents in a 100ml buchner flask:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity of reagent required (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra-Clean H2O</td>
<td>28</td>
</tr>
<tr>
<td>30% acrylamide / 0.8% bis-acrylamide</td>
<td>8.5</td>
</tr>
<tr>
<td>0.5M Tris* -HCl, pH 6.8</td>
<td>12.5</td>
</tr>
<tr>
<td>10% SDS*</td>
<td>0.5</td>
</tr>
</tbody>
</table>

After degassing for 10 minutes 0.5ml ammonium persulphate and 50ul TEMED® ® were added, the components mixed and transferred to a syringe as before. The remaining space in the gel cassette was filled, being careful to exclude air bubbles. The gels were then left to set.

The sample loading buffer was 10% SDS containing 10% sucrose and ca. 1% bromophenol blue. Tank buffer was kept as a 5x stock and contained 7.5g Tris, 36g glycine and 2.5g SDS made up to 500ml with water. The stock was kept at room temperature and was diluted just before use with ultra-clean water. Gels were run at approximately 150V.

**Staining with coomassie blue**

Completed gels were stained with 0.25% Coomassie Blue in 50% MeOH, 10% acetic acid in distilled water for up to an hour. They were then placed in quick destain (50% MeOH, 10% acetic acid in distilled water) for approximately half an hour before placing in slow destain (20% MeOH, 10% acetic acid in distilled water). One ferrochelatase preparation could not

---

* Tris(hydroxymethyl)aminomethane
* sodium dodecyl sulphate
* N,N,N',N'-tetramethylthlenediamine
be visualised by Coomassie Blue staining and so the more sensitive silver staining protocol was used.

**Staining with silver stain (after protocol supplied with Sigma kit)**

The gel was fixed with 30% absolute ethanol / 10% glacial acetic acid for 3x20 minutes and then washed with 300ml of distilled water for 3x10 minutes. The gel was then washed with silver equilibration solution (containing 0.5ml silver concentrate in 100ml distilled water) for 30 minutes. A first wash of 5-8 minutes was made with 150ml of developer solution (containing 50g Na₂CO₃ and 0.4ml formaldehyde). The second wash with the same solution was watched carefully, trying to achieve the darkest protein bands with as little yellowing and darkening of the gel as possible. The reaction was stopped by washing for 5 mins in a 1% glacial acetic acid solution (~100ml). The gel was then washed in distilled water (300ml for 3x10 minutes) and then reducer solution for 3x10 minutes. The reducer solution was supplied as three components A (containing potassium ferricyanide), B (containing sodium thiosulphate) and C, (containing sodium carbonate). 0.2ml reducer A, 0.4ml reducer B and 0.07ml of reducer C were made up to 150ml and used in the washing procedure. The gel was carefully watched to ensure that, as the background colour faded, the protein bands were not also being excessively destained. The gel was then rinsed under the tap for one minute and stored in distilled water indefinitely.

**6.3.12. Test of Ammonium Sulphate Precipitation Procedure.**

A batch of mitochondria was prepared from 34.2g of liver and processed up to (and including) the cholate solubilisation step. Samples of solubilised enzyme (4 x 1ml at 40 mg/ml) were taken up to 30, 35, 40 and 45% saturation respectively, stirred for 20mins and then centrifuged (16 000g, 15mins). Protein estimates and ferrochelatase activity assays were performed on the pellet and supernatant in each instance.
6.3.13. Incubation of Blue Sepharose CL-6B

Solubilised enzyme (8mg, 0.8ml), was added to two test-tubes containing Blue Sepharose CL-6B (3ml of a suspended mixture). The tubes were gently whirlimixed periodically over twenty minutes and then the sepharose spun down gently. The supernatants were kept, combined and assayed for protein content and ferrochelatase activity. Elution buffers (1ml, as indicated below) were then added and the samples left for 20mins before spinning the sepharose down as before. Supernatants and gels (resuspended in appropriate buffer) were both assayed for protein content and ferrochelatase activity.

<table>
<thead>
<tr>
<th>Test Tube</th>
<th>Elution Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A + 1.5M KCl + 1% Cholate</td>
</tr>
<tr>
<td>2</td>
<td>Buffer A + 1.0M KCl + 1% Cholate</td>
</tr>
</tbody>
</table>


All experiments followed the same general procedure already outlined in the previous section, but in these instances only ferrochelatase activity in the supernatants and shown by the gels was monitored.

The effect of pH in the elution buffer was determined by varying pH as follows:

<table>
<thead>
<tr>
<th>Test Tube</th>
<th>Elution Buffer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A + 1% Cholate + 1.5M KCl</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>Buffer A + 1% Cholate + 1.5M KCl</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>Buffer A + 1% Cholate + 1.5M KCl</td>
<td>10.2</td>
</tr>
<tr>
<td>4</td>
<td>Buffer A + 1% Cholate + 1.5M KCl</td>
<td>11.1</td>
</tr>
</tbody>
</table>

*Buffer A = 20mM Tris/acetate, pH 8.1, 20% glycerol, 1mM DTT, 10μg/ml PMSF*
The effect of salt concentration in the application buffer was determined by varying [KCl] as follows:

<table>
<thead>
<tr>
<th>Test Tube</th>
<th>Application Buffer</th>
<th>[KCl] / M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A + 1% Cholate</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>Buffer A + 1% Cholate</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>Buffer A + 1% Cholate</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>Buffer A + 1% Cholate</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The effect of salt concentration in the elution buffer was determined by varying [KCl] of the elution buffer as follows:

<table>
<thead>
<tr>
<th>Test Tube</th>
<th>Elution Buffer</th>
<th>[KCl] / M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A + 1% Cholate</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>Buffer A + 1% Cholate</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>Buffer A + 1% Cholate</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>Buffer A + 1% Cholate</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The effect of detergent concentration was determined by varying the percentage cholate of the elution buffer as follows:

<table>
<thead>
<tr>
<th>Test Tube</th>
<th>Elution Buffer</th>
<th>% cholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A + 1.5M KCl</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Buffer A + 1.5M KCl</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Buffer A + 1.5M KCl</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Buffer A + 1.5M KCl</td>
<td>4</td>
</tr>
</tbody>
</table>


Solubilised enzyme was prepared on a small scale following the protocol already described. This involved taking a few ml of submitochondrial particles, adding the appropriate amount of solubilising buffer and stirring for 3hrs at 4°C in the dark. After this time, the sample was
centrifuged (90 mins, 100 000g) to leave the solubilised enzyme in the supernatant. The supernatant was then dialysed overnight against approximately 100x its volume of Buffer A. Next day the protein would be estimated (see 6.3.9.) and buffer A added to the solubilised enzyme to give a final concentration of 1 mg of protein per ml. This was then aliquoted (900 µl) into eppendorfs and stored at -70°C prior to use. Each new batch of solubilised enzyme was tested for activity (pyridine haemochromogen) and a note made of the amount of enzyme required to give easily observable activity.

Each inhibition study involved taking eight of the frozen eppendorfs containing solubilised ferrochelatase and placing them on ice to thaw. An inhibitor was then added to the eppendorfs as outlined below, making sure mixing was complete by using a Spinmix. As stock solutions of inhibitor were in methanol, methanol also had to be added to the inhibition experiments to keep a constant concentration.

<table>
<thead>
<tr>
<th>Eppendorf No.</th>
<th>Amount of Inhibitor Added to Sample</th>
<th>μl MeOH Added to Sample</th>
<th>[Inhibitor] in Sample /M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µl 1 mM</td>
<td>-</td>
<td>10^-4</td>
</tr>
<tr>
<td>2</td>
<td>10 µl 1 mM</td>
<td>90</td>
<td>10^-5</td>
</tr>
<tr>
<td>3</td>
<td>100 µl 0.01 mM</td>
<td>-</td>
<td>10^-6</td>
</tr>
<tr>
<td>4</td>
<td>10 µl 0.01 mM</td>
<td>90</td>
<td>10^-7</td>
</tr>
<tr>
<td>5</td>
<td>10 µl 0.01 mM</td>
<td>90</td>
<td>10^-8</td>
</tr>
<tr>
<td>6</td>
<td>10 µl 0.001 mM</td>
<td>90</td>
<td>10^-9</td>
</tr>
<tr>
<td>7</td>
<td>NONE</td>
<td>100</td>
<td>NONE</td>
</tr>
<tr>
<td>8</td>
<td>NONE</td>
<td>100</td>
<td>NONE</td>
</tr>
</tbody>
</table>
The eppendorfs were then incubated for 15 minutes at 37°C in a water bath. The reaction was halted at the end of this time by placing on ice.

An equal amount of enzyme (as determined earlier) was then taken from each eppendorf and the amount of haem formed during the time of the incubation quantitated by the pyridine haemochromogen (see 6.3.8.).

6.3.16. Calculation of kinetic constants

Calculation of a $K_m$ and $V_{max}$ for mesoporphyrin

A $K_m$ was calculated for mesoporphyrin in an experiment in which the concentration of mesoporphyrin included in the pyridine haemochromogen assay was varied to cover a range from 1/5 to 8 times the expected $K_m$ ($K_m$ found for pure rat liver = 27$\mu$M (Taketani and Tokunaga, 1981)). The resulting rates were plotted against substrate concentration and the graph analysed according to Michaelis Menten kinetics to yield values of the $K_m$ and $V_{max}$ for mesoporphyrin.

Calculation of inhibition constants for the N-alkylporphyrins

Inhibition constants were calculated using the method of Dixon (1953). The assumption was made that the mode of binding of all the N-alkylporphyrins investigated would be competitive in line with the $K_i$ of 7$n$M found for the combined regioisomers of N-methylprotoporphyrin (Dailey & Fleming, 1983). As the $K_m$ for mesoporphyrin in the absence of inhibitor had already been determined, it was only necessary to carry out inhibition experiments at one substrate concentration as already described (see 6.3.15). A graph of 1/rate versus inhibitor concentration was then plotted, and a horizontal line drawn
through the point $1/V_{\text{max}}$ on the rate axis. This line cuts the graph obtained in the presence of inhibitor at $-K_i$. 
6.4. Results from Ferrochelatase Purification and Optimisation.

6.4.1. Results from an early purification procedure.

The elution profile obtained from the blue sepharose CL-6B column is shown in Figure 6.4.

Ferrochelatase activity was detected in the fractions eluted from the column with Buffer 5 (containing 1% cholate and 1.5M KCl). Protein and activity yields were low (see Table 6.2.). SDS-PAGE gels run to examine the purity of the isolated enzyme showed at least four or five bands on a gel (see Figure 6.3.). A faint band can just be seen at approximately 43Kd which can be attributed to ferrochelatase.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units / mg protein)</th>
<th>Yield (% activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria (from 1190g rat liver)</td>
<td>11 690</td>
<td>126 909</td>
<td>10.85</td>
<td>-</td>
</tr>
<tr>
<td>Sub-mitochondrial particles</td>
<td>2 592</td>
<td>65 499</td>
<td>22.19</td>
<td>100</td>
</tr>
<tr>
<td>Cholate-solubilised enzyme</td>
<td>2 399</td>
<td>45 794</td>
<td>19.10</td>
<td>70</td>
</tr>
<tr>
<td>Ammonium sulphate fractionation (35-55%)</td>
<td>2 363</td>
<td>36 134</td>
<td>15.30</td>
<td>55</td>
</tr>
<tr>
<td>Fractions from blue sepharose CL-6B column</td>
<td>1.92</td>
<td>1 345</td>
<td>700</td>
<td>2</td>
</tr>
<tr>
<td>Final preparation after concentration and dialysis</td>
<td>0.9</td>
<td>809</td>
<td>898</td>
<td>1.2</td>
</tr>
</tbody>
</table>

This ferrochelatase preparation lost activity on freezing and storing at -20°C, and did not compare well with that achieved by Taketani and Tokunaga (1981). The overall yield of

---

* units of ferrochelatase activity are expressed as nmol haem formed /30 mins
* ammonium sulphate interferes with ferrochelatase pyridine luemochromogen assay
Fig. 6.3. SDS-PAGE gels monitoring the progress of the ferrochelatase purification. Above is a 10% gel showing the stages prior to the blue Sepharose CL-6B column. Below is a 15% gel showing the purity of a freeze-dried sample of a fraction eluted from the column that displayed ferrochelatase activity.

1, 2 & 3 = freeze-dried final enzyme prep after dialysis
Fig. 6.4. Ferrochelatase elution profile from Blue Sepharose CL-6B.
activity is low (1.2% as opposed to 25%) and marked losses in weight and activity are seen at the mitochondrial to submitochondrial processing step and on placing the preparation onto the sepharose blue CL-6B column. It was therefore decided to investigate these steps to try and optimise the overall purification procedure. It was found that continuous sonication (2mins) of a less dilute mitochondrial preparation (about 20mg/ml protein as opposed to 40 mg/ml used previously) gave a better yield of sub-mitochondrial particles from the mitochondria (results not shown).

6.4.2. Results From Verifying Ammonium Sulphate Fractionation Procedure.

It is known (Dailey, 1977; own work) that ammonium sulphate interferes with the pyridine haemochromogen activity assay for ferrochelatase, but even so the loss of approximately 10 000 activity units between the cholate-solubilised step and the ammonium sulphate fractionation seemed excessive.

Figure 6.5. shows the results obtained from an experiment devised to test the recommended ammonium sulphate cuts (35-55%). The graphs clearly show that ferrochelatase activity will not be markedly lost on following the established protocol. The graphs show that, at 35% ammonium sulphate, more protein and ferrochelatase activity resides in the supernatant, whereas at 45% ammonium sulphate, there is more protein and ferrochelatase activity in the pellet. The experiment has therefore verified that ferrochelatase activity is placed on the sepharose column without undue losses, and that the ammonium sulphate cuts are correct.
Fig. 6.5. Graphs showing the effect of ammonium sulphate cuts on a) the distribution of protein between pellet and supernatant and b) the distribution of ferrochelatase activity between pellet and supernatant.
6.4.3. Results of Assaying Gel Matrix for Ferrochelatase Activity.

It was now known that optimal amounts of ferrochelatase were being placed on the blue sepharose CL-6B column. It was also known that the ferrochelatase must stick to the gel because no ferrochelatase activity had been detected coming off the column at any time other than when expected. It was decided to carry out an experiment testing the gel matrix for ferrochelatase activity, as the enzyme could either be stuck to the gel and not released by the elution buffers used, or inactivated at some stage of the purification procedure.

Figure 6.6. shows the results from a simple experiment comparing the specific ferrochelatase activities of the gel matrix and the elution buffer which is meant to release ferrochelatase from the gel matrix. It is clearly seen that the specific activity of the gel matrix is much higher than the specific activity of the elution buffer designated to bring off ferrochelatase. This observation implies that ferrochelatase is not efficiently removed from the gel matrix by the elution buffer (Buffer 5, containing 20mM Tris/acetate, pH 8.1, 20% glycerol, 1mM DTT, 10μg/ml pmsf, 1% cholate and 1.5M KCl).

6.4.4. Results from Experiments Testing the Effects of pH, Salt and Detergent Concentrations on Ferrochelatases Affinity for Sepharose.

These experiments were designed to monitor the effectiveness of buffers with varying pH and salt and detergent concentrations in removing bound ferrochelatase from the sepharose CL-6B gel matrix, as it had been found that ferrochelatase activity appeared to be strongly bound to the column and was not efficiently eluted by the established elution buffer.

Figure 6.7. shows the results obtained from two experiments in which a) the concentration of KCl in the application buffer was varied and b) the concentration of KCl in the elution
Fig. 6.6. Plot showing the marked difference in ferrochelatase activity between the buffer designated to remove ferrochelatase activity from the gel matrix and the gel matrix itself.
buffer was varied. As can be seen from part a) of the figure, increasing the salt concentration of the application buffer to 1.5M KCl causes an increase in the amount of ferrochelatase activity eluted by the elution buffer, which is in accordance with the fact that 1.5M KCl is the salt concentration normally used for elution buffer. Otherwise altering the salt concentration of the application buffer has little effect on the efficacy of removing ferrochelatase activity from the gel matrix, as indicated by the higher ferrochelatase activity of the gel matrix at all salt concentrations. It would appear that ferrochelatase does stick better to the gel at the lower salt concentrations which makes use of a 0.1M KCl application buffer (as outlined in the published protocol) advisable.

The effects of varying the KCl concentration of the elution buffer are shown in part b) of the figure and can be seen to be negligible. The ferrochelatase activities of the gel matrix and the elution buffer are approximately equivalent in the range 1 - 2.5M KCl (1.5M = normal elution buffer) and so changing the salt concentration of the elution buffer will have little effect on removing bound ferrochelatase from the gel matrix.

Figure 6.8. shows the results from two experiments in which a) the pH of the elution buffer was varied and b) the concentration of cholate in the elution buffer was varied.

It can be seen from part a) of the figure that to increase the alkalinity of the elution buffer above pH 9 is disadvantageous, as at higher pH the ferrochelatase activity of the gel matrix far exceeds that of the elution buffer. At the lower pH 7, the activity of the elution buffer does exceed that of the gel, but care is needed as, at pH 6, the buffer becomes a gelatinous-like material, which would complicate purification greatly. The suggested pH of 8.1 lies between these two extremes. Part b) of the diagram is the most interesting as it shows that increasing the cholate concentration of the elution buffer is clearly advantageous, as increasing the % cholate from 1% through to 4% causes increased elution of ferrochelatase activity.
Fig. 6.7. Graphs showing the effects of
a) changing the salt concentration of the application buffer and
b) changing the salt concentration of the elution buffer in attempts
to elute ferrochelatase from Sepharose CL-6B.
Fig. 6.8. Graphs showing the effects of varying a) the pH of the elution buffer, and b) the concentration of cholate in the elution buffer in attempts to elute ferochelatase from sepharose CL-6B.
activity, hand-in-hand with decreased ferrochelatase activity of the sepharose gel matrix. This observation implies that increasing the cholate concentration causes more efficient removal of ferrochelatase from the gel matrix.

6.4.5. Results From Optimised Ferrochelatase Purification.

Figure 6.9. shows the elution profile from blue sepharose CL-6B during a ferrochelatase purification using a 3% cholate buffer as the final elution buffer. Also shown is an SDS-PAGE gel of the final product. Table 6.3. below outlines the various stages of the purification procedure.

Table 6.3. Purification of ferrochelatase from rat liver mitochondria using optimised procedure.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Total Activity (units(^1))</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (units / mg protein)</th>
<th>Yield (% activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-mitochondrial particles</td>
<td>21 613</td>
<td>1 382</td>
<td>15.6</td>
<td>100</td>
</tr>
<tr>
<td>55% ammonium sulphate pellet</td>
<td>11 936</td>
<td>824</td>
<td>14.5</td>
<td>55</td>
</tr>
<tr>
<td>Cholate-solubilised enzyme</td>
<td>19 250</td>
<td>1 050</td>
<td>18.3</td>
<td>66</td>
</tr>
<tr>
<td>Fractions from blue sepharose CL-6B</td>
<td>1 924</td>
<td>3.8</td>
<td>507.6</td>
<td>9</td>
</tr>
</tbody>
</table>

As can be seen the overall yield of activity has been improved (9% versus 1.2%, see table 6.2.). The specific activity of the final sample is, however, less than that achieved previously (507 versus 898 nmol meso utilised/min/mg protein) and, as can be seen from the SDS-

\(^1\) ferrochelatase activity is expressed in units of nmol haem formed / 30 mins
\(^2\) ammonium sulphate interferes with ferrochelatase pyridine haemochromogen assay

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Fig 6.9. Silver-stained SDS-PAGE gel of a freeze-dried sample of a preparation of ferrochelatase eluted from the blue sepharose CL-6B column with a 3% cholate buffer. The elution profile from Sepharose CL-6B using the optimised protocol is also shown.
PAGE gel, the preparation is still not pure. Again, activity was lost on storage at ~20°C after 5 days. These difficulties meant that kinetic studies with pure ferrochelatase were not possible.
6.5. Results From Ferrochelatase Inhibition Studies Using Synthetic N-alkylporphyrin Inhibitors.

6.5.1. Comparison of the Ferrochelatase Inhibitory Activity of N-alkylporphyrins with differing N-alkyl Groups.

A lack of pure ferrochelatase for inhibition studies meant that cholate-solubilised ferrochelatase was used in its place for inhibition studies using synthetic N-alkylporphyrins.

Figures 6.10. - 6.15. are plots showing the ferrochelatase inhibitory activities of
a) the four free-base regioisomers of N-methylprotoporphyrin,
b) the four zinc-complexed regioisomers of N-methylprotoporphyrin,
c) the four free-base regioisomers of N-ethylprotoporphyrin.
d) the four zinc-complexed regioisomers of N-ethylprotoporphyrin,
e) the free-base regioisomeric pairs of N-propylprotoporphyrin and
f) the zinc-complexed regioisomeric pairs of N-propylprotoporphyrin.

Comparison of the free-base figures reveals that the free-base regioisomers of all the N-alkylporphyrins studied, except for $N_{C+D}$ propylprotoporphyrin, have ferrochelatase inhibitory activity.

Comparison of the zinc-complex figures reveals that, whereas the vinyl-substituted $N_A$ and $N_B$ regioisomers of N-methylprotoporphyrin and N-ethylprotoporphyrin are clearly inhibitory, the propionate substituted $N_C$ and $N_D$ regioisomers are less so (an effect more obvious in the N-ethyl rather than the N-methyl instance). The case is less stated for the regioisomeric pairs of N-propylprotoporphyrin. It appears that both the vinyl substituted $N_{A+B}$ and the propionate substituted $N_{C+D}$ fraction of this particular N-alkylporphyrin
Fig. 6.10. Ferrochelatase inhibitory activity of free-base regioisomers of N-methylprotoporphyrin.

Fig. 6.11. Ferrochelatase inhibitory activity of zinc-complexed regioisomers of N-methylprotoporphyrin.
Fig. 6.12. Ferrochelatase inhibitory activity of free-base regioisomers of N-ethylprotoporphyrin.

Fig. 6.13. Ferrochelatase inhibitory activity of the four zinc-complexed regioisomers of N-ethylprotoporphyrin.
Fig. 6.14. Ferrochelatase inhibitory activity of the free-base regioisomeric pairs of N-1-propylprotoporphyrin.

Fig. 6.15. Ferrochelatase inhibitory activity of the zinc-complexed regioisomeric pairs of N-1-propylprotoporphyrin.
inhibit ferrochelatase, but only at high concentrations of inhibitor \((10^{-5} - 10^{-4} \text{M})\), and the \(N_{\text{C=3D}}\) fraction appears never to achieve 100% inhibition.

6.5.2. Determination of Kinetic Constants.

An experiment to determine \(K_m\) and \(V_{\text{max}}\) for mesoporphyrin, the normal enzyme substrate, yielded values of \(17.07 \pm 5.61 \mu\text{M}\) and \(1.39 \pm 0.10\text{nmol mesoporphyrin utilised/min/mg protein}\).

The inhibition data discussed in the previous section was then used in conjunction with the \(V_{\text{max}}\) value for mesoporphyrin to generate inhibition constants for each of the \(N\)-alkylporphyrins under study. These constants should be treated cautiously, as the data used to generate these constants came from the results of inhibition experiments which were only carried out once for each different \(N\)-alkylporphyrin. The inhibition constants determined for each \(N\)-alkylporphyrin are shown in Table 6.4.

The inhibition constant, \(K_i\), is classified as the reciprocal of the enzyme-inhibitor affinity. Therefore the larger the value of \(K_i\), the higher the concentration of inhibitor required to produce a given degree of slowing on the enzyme-substrate reaction. The values for \(K_i\) indicated in the table vary by orders of magnitude.
Fig 6.16. Graph used to determine the $K_m$ and $V_{max}$ for mesoporphyrin, using the pyridine haemochromogen assay as described in section 6.3.16. The rate was measured in nmol mesoporphyrin utilised/30mins/mg protein and the substrate whose concentration was varied was mesoporphyrin. The graph was analysed according to Michaelis Menten kinetics using the Enzfitter software package.
Table 6.4. Original data used for determining the $K_i$ constants of synthetic N-alkylporphyrins which are shown in table 6.5.

<table>
<thead>
<tr>
<th>N-alkylporphyrin</th>
<th>nmol mesoporphyrin utilised /30 mins</th>
<th>nmol mesoporphyrin utilised /30 mins / mg protein (= rate)</th>
<th>$1/$rate</th>
<th>[Inhibitor]/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-MePP A</td>
<td>19.93</td>
<td>1.12</td>
<td>0.90</td>
<td>0</td>
</tr>
<tr>
<td>N-MePP B</td>
<td>18.17</td>
<td>1.01</td>
<td>0.99</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>N-MePP C</td>
<td>5.57</td>
<td>0.31</td>
<td>3.23</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>FREE A</td>
<td>19.64</td>
<td>1.09</td>
<td>0.92</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>FREE B</td>
<td>16.60</td>
<td>9.10</td>
<td>1.10</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>FREE C</td>
<td>16.99</td>
<td>0.94</td>
<td>1.06</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>FREE D</td>
<td>6.74</td>
<td>0.37</td>
<td>2.70</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>FREE E</td>
<td>14.36</td>
<td>0.80</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>FREE F</td>
<td>11.72</td>
<td>0.65</td>
<td>1.54</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>FREE G</td>
<td>12.67</td>
<td>0.70</td>
<td>1.43</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>FREE H</td>
<td>13.78</td>
<td>0.77</td>
<td>1.30</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>FREE I</td>
<td>9.38</td>
<td>0.52</td>
<td>1.92</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>FREE J</td>
<td>2.05</td>
<td>0.11</td>
<td>9.09</td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td>N-EtPP A</td>
<td>15.53</td>
<td>0.86</td>
<td>1.16</td>
<td>0</td>
</tr>
<tr>
<td>N-EtPP B</td>
<td>14.65</td>
<td>0.82</td>
<td>1.22</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>N-EtPP C</td>
<td>12.90</td>
<td>0.72</td>
<td>1.39</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>N-EtPP D</td>
<td>11.43</td>
<td>0.64</td>
<td>1.56</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>N-EtPP E</td>
<td>3.52</td>
<td>0.20</td>
<td>5</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>FREE A</td>
<td>19.64</td>
<td>1.09</td>
<td>0.92</td>
<td>0</td>
</tr>
<tr>
<td>FREE B</td>
<td>19.64</td>
<td>1.09</td>
<td>0.92</td>
<td>$10^{-9}$</td>
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<td>1.01</td>
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</tr>
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</tr>
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<td>$10^{-6}$</td>
</tr>
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<td>1.12</td>
<td>0.89</td>
<td>0</td>
</tr>
<tr>
<td>FREE G</td>
<td>20.22</td>
<td>1.12</td>
<td>0.89</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>FREE H</td>
<td>18.76</td>
<td>1.04</td>
<td>0.96</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>FREE I</td>
<td>12.60</td>
<td>0.70</td>
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</tr>
<tr>
<td>FREE J</td>
<td>4.10</td>
<td>0.23</td>
<td>4.35</td>
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</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<td>$10^{-9}$</td>
</tr>
<tr>
<td>N-EtPP C</td>
<td>22.86</td>
<td>1.27</td>
<td>0.79</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>N-EtPP D</td>
<td>18.76</td>
<td>1.04</td>
<td>0.96</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>N-EtPP E</td>
<td>17.59</td>
<td>0.98</td>
<td>1.02</td>
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</tr>
<tr>
<td>N-EtPP F</td>
<td>10.55</td>
<td>0.59</td>
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</tr>
<tr>
<td>N-EtPP G</td>
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</tr>
<tr>
<td></td>
<td>N-EtPP</td>
<td>D</td>
<td>FREE</td>
<td>ZINC</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>---</td>
<td>------</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-EtPP</td>
<td>15.24</td>
<td>0.85</td>
<td>1.18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.10</td>
<td>0.23</td>
<td>4.35</td>
<td>10^{-6}</td>
</tr>
<tr>
<td></td>
<td>7.32</td>
<td>0.31</td>
<td>3.23</td>
<td>10^{-7}</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>7.62</td>
<td>0.32</td>
<td>3.13</td>
<td>0</td>
</tr>
<tr>
<td>A+B</td>
<td>7.03</td>
<td>0.29</td>
<td>3.45</td>
<td>10^{-8}</td>
</tr>
<tr>
<td>FREE</td>
<td>6.45</td>
<td>0.27</td>
<td>3.70</td>
<td>0</td>
</tr>
<tr>
<td>ZINC</td>
<td>6.45</td>
<td>0.27</td>
<td>3.70</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10.84</td>
<td>0.46</td>
<td>2.17</td>
<td>10^{-9}</td>
</tr>
<tr>
<td>A</td>
<td>11.14</td>
<td>0.46</td>
<td>2.17</td>
<td>10^{-9}</td>
</tr>
<tr>
<td>ZINC</td>
<td>10.84</td>
<td>0.46</td>
<td>2.17</td>
<td>10^{-9}</td>
</tr>
<tr>
<td>B</td>
<td>12.90</td>
<td>0.54</td>
<td>1.85</td>
<td>10^{-9}</td>
</tr>
<tr>
<td>ZINC</td>
<td>12.90</td>
<td>0.54</td>
<td>1.85</td>
<td>10^{-9}</td>
</tr>
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Table 6.5. Inhibition constants determined for the free-base and zinc-complex forms of N-alkylporphyrins with differing N-alkyl groups.

<table>
<thead>
<tr>
<th>N-alkyl porphyrin</th>
<th>Regioisomer</th>
<th>Form</th>
<th>$K_i \pm$ standard error /M</th>
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<tbody>
<tr>
<td>N-Methyl PP</td>
<td>A</td>
<td>FREE</td>
<td>$5.5 \pm 3.2 \times 10^{-9}$</td>
</tr>
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<td>B</td>
<td>FREE</td>
<td>$14.7 \pm 6.4 \times 10^{-8}$</td>
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<td>C</td>
<td>FREE</td>
<td>$7.7 \pm 1.2 \times 10^{-7}$</td>
</tr>
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<td>D</td>
<td>FREE</td>
<td>$16.1 \pm 3.4 \times 10^{-7}$</td>
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<td>N-Ethyl PP</td>
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</tr>
<tr>
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<td>A + B</td>
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</tr>
<tr>
<td>N-PropylPP</td>
<td>C + D</td>
<td>FREE</td>
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<td>ZINC</td>
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</tr>
<tr>
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<td>ZINC</td>
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<tr>
<td>N-Ethyl PP</td>
<td>A</td>
<td>ZINC</td>
<td>$5.7 \pm 2.0 \times 10^{-6}$</td>
</tr>
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<td>ZINC</td>
<td>Does not yield $K_i$ value</td>
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<tr>
<td>N-Propyl PP</td>
<td>A + B</td>
<td>ZINC</td>
<td>$24.8 \pm 6.0 \times 10^{-7}$</td>
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<tr>
<td>N-Propyl PP</td>
<td>C + D</td>
<td>ZINC</td>
<td>Does not yield $K_i$ value</td>
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</table>

* Calculated using the method of Dixon at one substrate concentration ($1/V_{max} = 0.72$).
The $K_i$ values for the free-base forms of both N-methyl and N-ethylprotoporphyrin lie in the range of $10^{-9}$ - $10^{-6}$M. This suggests that, in both cases, all four regioisomers of this form of N-alkylporphyrin are markedly inhibitory, as only small amounts of inhibitor introduced into the enzyme-substrate system are enough to cause a decrease in the normal enzyme-substrate reaction. The free-base N-propyl vinyl-substituted A+B fraction inhibits as much as the least inhibitory of the N-methylprotoporphyrin regioisomers, whereas the N-propyl propionate-substituted C+D fraction does not appear to be inhibitory at the inhibitor concentrations used.

More interestingly, marked differences are seen between the vinyl-substituted $N^\alpha$ and $N^\delta$, compared with the propionate-substituted $N^\chi$ and $N^\varphi$, regioisomers of the zinc-complexed forms of all the N-alkylporphyrins studied. The $N^\alpha$ and $N^\delta$ regioisomers of N-methylprotoporphyrin yield $K_i$ constants of the magnitude $10^{-8}$ and $10^{-7}$M respectively, whereas the $N^\chi$ and $N^\varphi$ regioisomers of the same yield $K_i$ constants of the magnitude $10^{-5}$M. The $K_m$ for mesoporphyrin with enzyme was found to be in the micromolar range. As $K_i$ can be taken as the dissociation constant for the enzyme-inhibitor complex (assuming the inhibitor undergoes no modification by the enzyme) and $K_m$ can be taken as the dissociation constant for the enzyme-substrate complex (assuming that enzyme and substrate are in equilibrium with the enzyme-substrate complex) this would therefore suggest that the enzyme has less affinity for the $N^\chi$ and $N^\varphi$ regioisomers of zinc-complexed N-methylprotoporphyrin than for mesoporphyrin (substrate used under assay condition).

The difference between the $N^\alpha$ and $N^\delta$, compared with $N^\chi$ and $N^\varphi$, regioisomers of the zinc-complexed forms of N-ethyl and N-propylprotoporphyrin is even more marked. Both N-alkylporphyrins have inhibitory $N^\alpha$ and $N^\delta$ regioisomers, but the $N^\chi$ and $N^\varphi$ regioisomers have no inhibitory activity at the inhibitor concentrations used.
6.6. Discussion

The work described in this chapter has covered two main areas
1. the attempted purification of ferrochelatase, and
2. an inhibition analysis of structurally similar N-alkylporphyrins, using cholate-solubilised ferrochelatase

The ferrochelatase purification was never completely successful. In all instances the resultant preparation was not homogeneous and quickly lost activity on storage at ~20°C. The difficulty of purifying (whilst keeping stable) the enzyme is well known (Mazanowska, 1969; Yoneyama et al, 1969; Mailer, 1980) but has to some extent been overcome by using a dithiothreitol/glycerol buffer system (Taketani & Tokunaga, 1981). In the case of the present purification, however, the enzyme was still highly labile, despite these precautions, as evidenced by loss of ferrochelatase activity after storing for 72hrs at ~20°C. This could, of course, be due to the low yield of enzyme which results in very dilute protein solutions. An attempt to concentrate one preparation resulted in severe loss of enzyme (1.9 -> 0.9mg) as ferrochelatase has a tendency to stick to membranes of all sorts with irrecoverable loss of activity (Dailey, 1986). The constant lability and lack of homogeneity of all ferrochelatase preparations undertaken meant that the inhibition analysis of structurally similar N-alkylporphyrins had to be carried out in the presence of cholate. This was far from ideal, as at this stage of the purification protocol there are still very many contaminating proteins. Nevertheless, it was hoped to be able to identify differences between the ferrochelatase inhibitory activities of the various structural analogues.

The N-alkylporphyrins used in the inhibition analysis were N-alkylprotoporphyrins, structures for some of which are shown in Figure 6.16. These differ structurally from the in vivo substrate of ferrochelatase (protoporphyrin IX) in that an N-alkyl group is attached to
Fig. 6.16. Ball and stick representations of a) the $N_B$ regioisomer of $N$-methylprotoporphyrin and b) the $N_3$ regioisomer of $N$-propylprotoporphyrin.
one of the pyrrole ring nitrogens such that the normally planar protoporphyrin ring becomes
distorted. The N-alkylprotoporphyrins differ structurally from mesoporphyrin (the substrate
used in the pyridine haemochromogen assay for ferrochelatase) by an N-alkyl group and the
presence of ethyl groups in place of the vinyl groups found in protoporphyrin. Use of
mesoporphyrin rather than protoporphyrin as substrate was necessary because experiments
using protoporphyrin as substrate gave very low activities, which would make inhibition
effects very difficult to detect (results not shown). This was not unexpected, as
protoporphyrin has been found to give a lower rate (compared with mesoporphyrin) of
metalloporphyrin synthesis in sheep mitochondria (Honeybourne et al, 1979). Furthermore,
pure ferrochelatase from rat, bovine and liver erythrocytes, tested with a range of substrates,
yielded the lowest measured $K_m$ and $V_{max}$ values with protoporphyrin (Dailey, 1986).

The main difference between substrate (be it mesoporphyrin or protoporphyrin) and the N-
alkylporphyrins that is of importance to this study is that the substrate is planar whereas the
N-alkylporphyrins are not. It has been proposed (Dailey et al, 1989), that during normal
catalytic function of the ferrochelatase active site, the normally planar substrate distorts. The
distortion has been attributed to favourable $\pi$-stacking interactions between the porphyrin
and aromatic residues present in the active site, and also to electronic interactions between
the porphyrin nitrogens and an amino acid residue in the active site. This distortion, which
allows insertion of a iron atom into the porphyrin centre before the porphyrin is returned to
planar and released, represents the transition state of the enzyme function. An N-
al kylporphyrin therefore, which is already distorted, will be able to experience all favourable
interactions but will block (by means of its N-alkyl group) the insertion of the iron atom into
the porphyrin centre.

The role of N-alkylporphyrins as transition state analogues has received considerable
support. An antibody elicited to a distorted N-methylporphyrin has been shown to catalyse
metal ion chelation by the planar porphyrin (Cochran & Schultz, 1990). Additionally, it has been shown that the rate of incorporation of copper (II) into a non-planar dodecaphenylporphyrin is much more rapid than the rate of incorporation achieved with a planar tetraphenylporphyrin (Takeda et al, 1992).

The idea behind the inhibition analysis undertaken here was to vary the position and size of the N-alkyl group to see if this had any effect on ferrochelatase inhibition, and also to see if incorporation of a metal ion (zinc (II)) lessened or enhanced any inhibitory effects.

Similar analyses have been carried out previously. Ortiz de Montallano et al, in two separate papers (1980 and 1981) investigated the inhibitory activity of the free-base forms of the four regioisomers of N-methylprotoporphyrin and N-ethylprotoporphyrin. Using the same activity assay as used here, but perhaps an even less pure rat liver preparation, they were able to show that, whereas all four regioisomers of N-methylprotoporphyrin are potent inhibitors of ferrochelatase, the NC and ND regioisomers of N-ethylprotoporphyrin are 30-100 times less potent than the NA and NB regioisomers, both of N-ethylprotoporphyrin and N-methylprotoporphyrin.

A study by de Matteis et al (1982) yielded slightly different results. In their study the ferrochelatase inhibitory activities of the A+B and C+D fractions of N-methylprotoporphyrin were compared, both of the free-base and zinc-complex forms. Their results show that the propionate-substituted C+D fraction of the free-base form of N-methylprotoporphyrin appeared to be slightly less inhibitory than the vinyl-substituted A+B fraction. Incorporation of zinc at the porphyrin centre caused a dramatic loss of ferrochelatase activity in the C+D fraction, whereas activity of the A+B fraction was retained. These observations were confirmed in a succeeding study (de Matteis et al, 1985), where it was also found that the regioisomeric pairs of the free-base form of N-EtPP had similar inhibitory activities (unlike
the findings of Ortiz de Montellano et al., 1981) but, however, that N\textsubscript{C+D} fraction of the free-base form of N-propylprotoporphyrin is markedly less inhibitory than the N\textsubscript{A+B} fraction.

The study conducted here was unable to find any marked differences in the ferrochelatase inhibitory activity of the free-base regioisomers of N-methyl- or N-ethylprotoporphyrin. The activity profiles and K\textsubscript{i} values calculated for the N-methylprotoporphyrin regioisomers suggest that the propionate-substituted N\textsubscript{C} and N\textsubscript{D} regioisomers may be slightly less inhibitory than the vinyl-substituted N\textsubscript{A} and N\textsubscript{B} regioisomers, which would be in keeping with the work of de Matteis et al. The activity profiles and K\textsubscript{i} values calculated for the N-ethylprotoporphyrin regioisomers, however, show very little difference in ferrochelatase inhibitory activity between the N\textsubscript{A} and N\textsubscript{B}, compared with the N\textsubscript{C} and N\textsubscript{D}, regioisomers. This result is not in agreement with the findings of Ortiz de Montellano et al, but agrees with the work of de Matteis et al. Further experiments would certainly be needed to ensure that this finding is not an experimental error. Marked differences in ferrochelatase inhibitory activity are, however, distinctly apparent between the A+B and C+D fractions of N-propylprotoporphyrin, again in keeping with the observations made by de Matteis et al. The A+B fraction is inhibitory, whereas the C+D fraction is never able to achieve 100% inhibition at the inhibitor concentrations used.

There are also clearly significant differences in the inhibitory activity of the zinc-complexed regioisomers of all the N-alkylprotoporphyrins studied. In all cases, the vinyl-substituted N\textsubscript{A} and N\textsubscript{B} regioisomers continue to display ferrochelatase inhibitory activity, whereas the propionate-substituted N\textsubscript{C} and N\textsubscript{D} regioisomers display either:

1. inhibitory activity much lower than the N\textsubscript{A} and N\textsubscript{B} regioisomers, or
2. an inability to ever achieve 100% inhibition of ferrochelatase at the inhibitor concentrations used.
In structural terms this implies that the free-base N-alkylporphyrins are apparently capable of entering the ferrochelatase active site and causing inhibition. The porphyrins cease to be inhibitory only when the N-alkyl group is extended to three carbon atoms and is placed on a propionate-substituted \( \text{N}_C \) or \( \text{N}_D \) pyrrole ring nitrogen.

Incorporation of a zinc atom (as discussed previously) into the porphyrin centre causes the three-non-alkylated rings to be held in the same plane relative to one another, whereas the N-alkylated ring is severely distorted. The ferrochelatase active site appears capable of encompassing such distortion of the vinyl-substituted \( \text{N}_A \) and \( \text{N}_B \) pyrrole rings, as ferrochelatase inhibitory activity is seen even in the \( \text{N}_{A+B} \) fraction of N-propyloprotoporphyrin. It cannot, however, encompass the same distortions at the propionate-substituted \( \text{N}_C \) and \( \text{N}_D \) pyrrole rings, as the \( \text{N}_C \) and \( \text{N}_D \) zinc-complexed regioisomers of all N-alkylporphyrins studied are markedly less inhibitory or cannot achieve complete inhibition of ferrochelatase.

In terms of the active site models proposed for ferrochelatase, either model can accommodate the data presented here. Ortiz de Montellanos' model (1986), which favours an active site in which the porphyrin extends its carboxylic acid groups into a pocket to align with charged amino acid residues, could explain the difference in ferrochelatase activities of propionate versus vinyl-substituted N-alkylporphyrins in terms of the increasing distortion of the porphyrins, assuming that increased size of the N-alkyl group causes increased distortion of the N-alkylated ring. As the N-alkyl group is extended from a methyl, to an ethyl and then to a propyl group, it could be envisioned that the \( \text{N}_C \) or \( \text{N}_D \) alkylated zinc complexes would experience increasingly restricted entry to the active site as the size of the N-alkyl group is increased. Once in the active site charge-charge interactions between the propionate group and the amino acid residue in the active site are necessary for proper alignment of the porphyrin such that iron insertion can take place. This would also be increasingly difficult to
achieve, as the propionate groups, as a result of N-alkylation, are now on pyrrole rings which are no longer in the same plane and so may not be able to form the necessary charge-charge interactions with ease, thus the $N_C$ and $N_D$ regioisomers of the zinc-complexed N-alkylporphyrins studied are inefficient inhibitors of ferrochelatase.

Distortion of the vinyl-substituted $N_A$ and $N_B$ rings in the zinc-complexes will not cause a loss of inhibitory activity because, in this model, the pyrrole rings $A$ and $B$ are in an open area which can more easily accommodate such distortions. As the zinc atom holds the $N_C$ and $N_D$ propionate-substituted rings in the same plane, the necessary charge-charge interactions within the active site can be made but iron insertion can still not take place due to the presence of the N-alkyl group. Thus the $N_A$ and $N_B$ regioisomers of the zinc-complexed N-alkylporphyrins studied are effective inhibitors of ferrochelatase.

A similar argument also holds for the model proposed by Dailey (1986). This model proposes that the carboxylic acid groups of the porphyrin substrate or inhibitor extend outward from the active site. The lack of inhibition displayed by the zinc-complexed $N_C$ and $N_D$ regioisomers can again be explained in terms of improper charge-charge alignments of the propionate groups with amino acid residues at the active site. $N_A$ and $N_B$ regioisomers are better inhibitors because they can form better charge-charge interactions. This model, however, does call for the active site to be large enough to accommodate the N-alkyl group of the inhibitor, a requirement which is not necessary in Ortiz de Montellano's model.

The ferrochelatase inhibitory activity of the free-base regioisomers of all the N-alkylporphyrins studied, excepting $N_{C+D}$ N-propylprotoporphyrin, can be explained by comparing the structure of the free-base to that of the zinc-complex. It has been shown by X-ray crystallography that the structure of a N-alkylmetalloporphyrin is more distorted than that of a corresponding free-base N-alkylporphyrin (see 2.3.). Also the free-base form of a
porphyrin is much more flexible, as there is no metal atom to confer rigidity upon the molecule. The lesser distortion and greater flexibility of the free-base form compared with its zinc-complexed analogue may well explain the inhibitory activity of this form. Though N-alkylation incurs distortion of the N-alkylated ring, the flexibility of the porphyrin may well allow charge-charge interactions to be made even when the N-alkyl group is as large as an ethyl group and situated on a propionate-substituted N_3 or N_5 ring. When the N-alkyl group is extended to a propyl group and placed on rings C or D, however, the distortion of the porphyrin and size of the N-alkyl group must make the necessary interaction more difficult as the free base N_3+D fraction of N-propylprotoporphyrin cannot achieve complete inhibition of ferrochelatase.
7. General Discussion

A general discussion has been included to try and draw together the experimental data of chapter 5, in which the $^1$H NMR spectral characteristics of various N-alkylporphyrins were defined and chapter 6, in which the ferrochelatase inhibitory activity of the self-same N-alkylporphyrins was determined.

To do this, the changes in chemical shift (relative to a planar porphyrin) seen on N-alkylation have to be related to the structure of the porphyrins under study. The work described in chapter 5 supports earlier investigations of other workers (Caughey & Ibers, 1963, Jackson & Drearden, 1973) which indicated that the structural changes incurred on N-alkylation of a normally planar porphyrin are manifested by shifts to high field of proton resonances in the $^1$H NMR spectra of these compounds. It has been seen that the resonances of protons belonging to groups associated with the alkylated ring move to high field relative to the resonances of the protons of equivalent groups on non-alkylated rings. Thus two sets of resonances for a particular group of protons (such as the internal vinyl protons at positions 2 and 4 of the porphyrin periphery, and the distal methylene protons of the propionate groups at positions 6 and 7 of the porphyrin periphery) indicates that the pyrrole rings on which these groups are situated are no longer in similar magnetic environments, or have become misaligned with respect to one another (considering that in protoporphyrin all pyrrole rings lie in the same plane). This observation provides us with a probe for the misalignment of pyrrole rings relative to each other which is easily picked out in the $^1$H NMR spectra of N-alkylprotoporphyrins. In Table 7.1, this probe for non-planarity is linked to the ability of a set of N-alkylprotoporphyrins to inhibit ferrochelatase.

It is known (de Matteis et al, 1985), that, for the porphyrin to be utilised by ferrochelatase, the propionate groups of a porphyrin inhibitor or substrate have to be in the free-acid and not the methyl ester form. It has also been suggested that the alignment
of the two propionate side-chains relative, perhaps, to charged amino acid residues, may also play a crucial role in positioning of a porphyrin at the ferrochelatase active site (de Matteis et al, 1985; Dailey, 1986; Ortiz de Montellano, 1986). The data presented here provides strong evidence that the alignment of the propionate groups, as well as the position of the N-alkyl substituent, is important in determining the inhibitory activity of a particular N-alkylporphyrin.

The position of the N-alkyl substituent shall be considered first. Table 7.1 provides evidence that the free-base and zinc-complex forms of the \( \text{N}_A \) and \( \text{N}_B \) regioisomers of all N-alkylprotoporphyrins studied are, without exception, capable of inhibiting ferrochelatase. Therefore it would appear that, as long as the site of structural change is at the A or B pyrrole rings, the N-alkylprotoporphyrins considered in this study possess the structural characteristics necessary for ferrochelatase inhibition. It can also be deduced from Table 7.1 that it is only some \( \text{N}_C \) and \( \text{N}_D \) regioisomers of the N-alkylprotoporphyrins studied that are incapable of ferrochelatase inhibition. In these porphyrins the site of structural change is at the C or D pyrrole rings. N-alkylation at these rings will cause the pyrrole rings (and the propionate groups on them) to be in different planes and so it may well be that the propionate groups are involved in the observed lack of ferrochelatase inhibition. This aspect shall now be considered.

Equivalent distal methylene protons of the 6 and 7 propionate groups will give only one, superimposed complex multiplet in the \( ^1\text{H} \) NMR spectra of an N-alkylporphyrin. This indicates that the two propionate groups are in the same magnetic environment and also that the pyrrole rings C and D (on which they are situated) are most likely in the same plane. Table 7.1 tells us that all N-alkylprotoporphyrins with one superimposed distal methylene resonance inhibit ferrochelatase, and that this inhibition occurs regardless of the size of the N-alkyl group or the form of the porphyrin (whether free-base or zinc-complex). The inhibition is dependent on the position of the N-substituent, as mentioned already, as only the \( \text{N}_A \) and \( \text{N}_B \) regioisomers have this spectral characteristic and are
capable of inhibition. In structural terms this implies that, when pyrrole rings C and D are in the same plane, the propionate groups are aligned with each other and inhibition of ferrochelatase can occur. It should be noted that this characteristic is also seen in the spectrum of the substrate, protoporphyrin, indicating that this structural feature is important in substrate utilisation as well as enzyme inhibition. This correlates well with active site models which propose that N-alkylporphyrin inhibitors are targeted to the active site because of their tetrapyrrole structure and, once there, are able to position themselves at the active site via charge-charge interactions between the propionate groups and amino acid residues of the protein.

The same porphyrins also have two distinct internal vinyl resonances which implies that pyrrole rings A and B are not in the same plane and the vinyl groups on them must be misaligned relative to one another. Every N-alkylprotoporphyrin with this spectral characteristic is capable of inhibiting ferrochelatase. This observation has two possible explanations: 1) the vinyl groups may actually have an important role in the positioning of a porphyrin at the ferrochelatase active site such that inhibition, as opposed to catalysis, occurs. This is proposed because the substrate (protoporphyrin) has equivalent vinyl groups whereas the vinyl groups of the N-alkylprotoporphyrins capable of ferrochelatase inhibition have two vinyl groups which are misaligned. 2) the active site positions the A and B pyrrole rings of the N-alkylporphyrin in a relatively 'open' area with enough room to accommodate structural change (as discussed already in describing active site models).

Conversely, there is a subset of N-alkylprotoporphyrins in Table 7.1. possessing the spectral characteristics of one superimposed internal vinyl resonance and two distal methylene resonances. All these N-alkylprotoporphyrins are non-inhibitors or, at best, only partial inhibitors of ferrochelatase. This is again regardless of whether the porphyrin is in the free-base or zinc-complex form or the size of the N-alkyl substituent. Again, the position of the N-alkyl substituent is important as, in these instances, it is only NC and
ND regiosomers which possess such characteristics and are also non-inhibitory. The spectral characteristics imply that pyrrole rings A and B in the same plane as each other and pyrrole rings C and D (with the associated propionate groups) are in distinctly different planes relative to each other.

Table 7.1. Relationship between $^1$H NMR spectral characteristics and ferrochelatase inhibitory activity of a set of N-alkylprotoporphyrins.

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Regio-isomer</th>
<th>Form</th>
<th>Number of internal vinyl resonances</th>
<th>Number of distal methylene resonances</th>
<th>Ferro-chelatase inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin</td>
<td></td>
<td>free-base</td>
<td>1</td>
<td>1</td>
<td>substrate</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td></td>
<td>zinc</td>
<td>1</td>
<td>1</td>
<td>substrate</td>
</tr>
<tr>
<td>N-MePP</td>
<td>A</td>
<td>free-base</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>B</td>
<td>free-base</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>C</td>
<td>free-base</td>
<td>2</td>
<td>2</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>D</td>
<td>free-base</td>
<td>2</td>
<td>2</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>A</td>
<td>zinc</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>B</td>
<td>zinc</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>C</td>
<td>zinc</td>
<td>1</td>
<td>2</td>
<td>partial inhibitor</td>
</tr>
<tr>
<td>N-MePP</td>
<td>D</td>
<td>zinc</td>
<td>1</td>
<td>2</td>
<td>partial inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>A</td>
<td>free-base</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>B</td>
<td>free-base</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>C</td>
<td>free-base</td>
<td>2</td>
<td>2</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>D</td>
<td>free-base</td>
<td>2</td>
<td>2</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>A</td>
<td>zinc</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>B</td>
<td>zinc</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>C</td>
<td>zinc</td>
<td>1</td>
<td>2</td>
<td>non-inhibitor</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>D</td>
<td>zinc</td>
<td>1</td>
<td>2</td>
<td>non-inhibitor</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>A+B</td>
<td>free-base</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>C+D</td>
<td>free-base</td>
<td>1</td>
<td>2</td>
<td>non-inhibitor</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>A+B</td>
<td>zinc</td>
<td>2</td>
<td>1</td>
<td>inhibitor</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>C+D</td>
<td>zinc</td>
<td>1</td>
<td>2</td>
<td>non-inhibitor</td>
</tr>
</tbody>
</table>

These porphyrins would therefore be unable to make the necessary charge-charge interactions of propionate group with amino-acid residue as the propionate groups are
misaligned. It is also interesting to note that, in these N-alkylprotoporphyrins, the pyrrole rings A and B are in the same plane as is the case with the substrate, protoporphyrin. This structural feature is therefore not the primary feature of a porphyrin which dictates whether or not inhibition or porphyrin utilisation will occur.

These two scenarios, however, do not encompass all of the data provided in Table 7.1. The free-base \( N_C \) and \( N_D \) regioisomers of N-methylprotoporphyrin and N-ethylprotoporphyrin display spectral characteristics of two internal vinyl resonances and two distal methylene resonances. These regioisomers also effectively inhibit ferrochelatase. It has already been stated that all N-alkylprotoporphyrins with misaligned vinyl groups inhibit ferrochelatase, but it is more surprising that an N-alkylprotoporphyrin with misaligned propionate groups can effectively inhibit the enzyme, especially in view of the preceding discussion which would strongly suggest that alignment of the propionate groups is necessary for inhibition to occur.

The \( ^1H \) NMR spectra of regioisomers of both forms of N-propylprotoporphyrin and N-butylprotoporphyrin were compared with those of N-methylprotoporphyrin and N-ethylprotoporphyrin in chapter 5, with the conclusion that, once the N-alkyl group was extended to three carbon atoms or beyond, ready distinctions cannot be made between the spectra of zinc-complex and free-base forms. The behaviour of the free-base form approaches that of the zinc-complex. This was explained in terms of the steric constraints of a larger N-alkyl group which may well cause the N-alkylated ring to come out-of-plane to a considerable degree leaving the other three pyrrole rings to be in the same plane relative to one another.

The greater distinctions between the spectra of the free-base and zinc-complex forms of N-methylprotoporphyrin and N-ethylprotoporphyrin indicate that these compounds are readily capable of coping with conformational change. If the pyrrole rings C and D are required to be in the same plane so that alignment of the propionate groups groups can
occur (as would seem to be the case in view of the preceding discussion), it may well be possible that the free-base N\textsubscript{C} and N\textsubscript{D} regioisomers of N-methylprotoporphyrin and N-ethylprotoporphyrin are capable of undergoing a conformational change which will bring about alignment of the propionate groups to allow inhibition. Such a conformational change is not possible for N-alkylprotoporphyrins with larger N-alkyl groups because of the steric constraints imposed by the larger N-alkyl group and therefore the propionate groups of these free-base N\textsubscript{C} and N\textsubscript{D} regioisomers cannot be brought into alignment and so ferrochelatase inhibition cannot occur.

In conclusion, a combination of the NMR and inhibition data has allowed an insight into the mechanism of inhibition of ferrochelatase by N-alkylporphyrins. The data suggests that the ferrochelatase active site is capable of accepting N-alkylprotoporphyrins with large N-alkyl groups, but only if the N-alkyl group is on pyrrole rings A or B. The active site cannot, however, appear to accommodate groups larger than an ethyl group if the N-alkyl group is on pyrrole rings C or D. N-alkylation of the C or D pyrrole rings also causes the propionate groups to become misaligned. In most instances this results in a porphyrin which is incapable of inhibiting ferrochelatase. There are some instances, however, (the N\textsubscript{A} and N\textsubscript{B} regioisomers of free-base N-methyl- and N-ethylprotoporphyrin) where the NMR data would indicate that the propionate groups are misaligned but the inhibition data indicates that ferrochelatase inhibition still occurs. It is proposed that, in these instances, the small size of the N-alkyl group and the flexibility of the free-base form may allow a conformational change of the porphyrin which will bring the propionate groups into better alignment such that necessary interactions can be made and inhibition will occur.
APPENDIX
Fig. A.1. The structure and nomenclature of N-methylprotoporphyrin dimethyl ester (N-MePP, R = CH₃) and the griseofulvin-protoporphyrin adduct (N-GfPP, R = structure drawn above).
Table A.1. The extinction coefficients of some porphyrins and related compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extinction Coefficients Read at 410nm and 430 nm respectively for free-base or zinc-complexed porphyrins (units = mM⁻¹cm⁻¹)</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-MePP</td>
<td>128</td>
<td>605</td>
</tr>
<tr>
<td>N-MePP[Zn]</td>
<td>145</td>
<td>670</td>
</tr>
<tr>
<td>N-EtPP</td>
<td>120</td>
<td>619</td>
</tr>
<tr>
<td>N-EtPP[Zn]</td>
<td>as for N-EtPP</td>
<td>684</td>
</tr>
<tr>
<td>N-PrPP</td>
<td>as for N-EtPP</td>
<td>633</td>
</tr>
<tr>
<td>N-PrPP[Zn]</td>
<td>as for N-EtPP</td>
<td>698</td>
</tr>
<tr>
<td>N-BuPP</td>
<td>as for N-EtPP</td>
<td>647</td>
</tr>
<tr>
<td>N-BuPP[Zn]</td>
<td>as for N-EtPP</td>
<td>712</td>
</tr>
<tr>
<td>N-GiPP</td>
<td>as for N-MePP</td>
<td>941</td>
</tr>
<tr>
<td>N-GiPP[Zn]</td>
<td>as for N-MePP[Zn]</td>
<td>1003</td>
</tr>
<tr>
<td>PPIX</td>
<td>171</td>
<td>590</td>
</tr>
<tr>
<td>PPIX[Zn]</td>
<td>as for PPIX</td>
<td>653</td>
</tr>
<tr>
<td>Mesoporphyrin</td>
<td>445</td>
<td>594</td>
</tr>
</tbody>
</table>

* Extinction coefficients are taken from Smith, 1975 or Ortiz de Montellano and Kunze, 1981(e).


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