Porphyrin metabolism in congenital erythropoietic porphyria

Thesis

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PORPHYRIN METABOLISM IN
CONGENITAL ERYTHROPOIETIC PORPHYRIA

A thesis submitted in support of candidature for the degree of Doctor of Philosophy in the Open University.

by
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in collaboration with
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Author number: P1.22476
Date of submission: 1992
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ABSTRACT

Meso-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I and peroxycetic acid uroporphyrin I have been isolated from the urine and plasma of patients with congenital erythropoietic porphyria (CEP) by high-performance liquid chromatography and characterized by liquid secondary ion mass spectrometry and chemical properties. The physico-chemical properties of these compounds have been studied.

The hydroxy- and peroxycetic acid- uroporphyrin I derivatives are the true metabolites of uroporphyrinogen I in vivo and their presence in urine and plasma is a common feature of CEP. The absence of these derivatives in duodenal aspirate and faeces suggests that they are of erythropoietic origin.

The mechanism of formation of the hydroxy- and peroxycetic acid- uroporphyrin I has been investigated. Peroxyacetic acid uroporphyrin I is formed from uroporphyrinogen I in the presence of $\text{H}_2\text{O}_2$ and iron while the hydroxylated uroporphyrin I derivatives are most probably produced by hydroxyl radicals generated during the formation of peroxycetic acid uroporphyrin I. Destruction of porphyrins is found in the same reaction and can be prevented by desferrioxamine, indicating that it is due to hydroxyl radicals.

The formation of peroxycetic acid- and hydroxy- uroporphyrin I derivatives are uroporphyrinogen I concentration dependent. These derivatives can only be formed when uroporphyrinogen I is accumulated to a certain concentration (approx. 1-2 $\mu$M) and the formation is then proportional to the uroporphyrinogen I concentration. The peroxylation reaction has been shown to take place only at the acetic acid side-chains of porphyrinogen and not at the propionic acid side-chains. The peroxylation reaction can therefore take place whenever a porphyrinogen with an acetic acid substituent is accumulated.

Oral charcoal therapy failed to reduce the porphyrins accumulated in vivo in a patient with CEP. Uroporphyrin I, the major porphyrin accumulating in CEP, was not excreted into bile and interruption of the enterohepatic circulation by binding porphyrins onto charcoal therefore does not benefit CEP. It may, however, be effective in the treatment of hepatic porphyrias in which the accumulated porphyrins are mainly excreted via the gut lumen.
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ABBREVIATIONS

AIP: acute intermittent porphyria
ALA: 5-aminolaevulinic acid
ALA-S: 5-aminolaevulinic acid synthase
ALA-D: 5-aminolaevulinic acid dehydrase
BDS: base deactivated silica
CEP: congenital erythropoietic porphyria
Copro: coproporphyrin
Copro'gen: coproporphyrinogen
Copro'gen-O: coproporphyrinogen oxidase
DES: desferrioxamine
DHM-A: dihydroxymaleic acid
DMF: dimethylformamide
DMSO: dimethylsulphoxide
EDTA: ethylenediaminetetraacetic acid
EHP: erythrohepatic protoporphyria
HCP: hereditary coproporphyria
HMB: hydroxymethylbilane
HMB-S: hydroxymethylbilane synthase
HPLC: high-performance liquid chromatography
Km: Kinetic constant, or Michaelic constant
LSIMS: liquid secondary ion mass spectrometry
MHA: methaemalbumin
NADPH: β-nicotinamide adenine dinucleotide phosphate (reduced form)
NMR: nuclear magnetic resonance spectroscopy
PAAU: peroxyacetic acid uroporphyrin I
PBG: porphobilinogen
PCT: porphyria cutanea tarda
Proto: protoporphyrin
Proto'gen: protoporphyrinogen
Proto'gen-O: protoporphyrinogen IX oxidase
RBC: red blood cells
SOD: superoxide dismutase
3,4-TCB: 3,4,3',4'-tetrachlorobiphenyl
Tris: Tris(hydroxymethyl) aminoethane
TLC: thin-layer chromatography
Uro: uroporphyrin
Uro'gen: uroporphyrinogen
Uro'gen III-S: uroporphyrinogen III synthase
Uro'gen-D: uroporphyrinogen decarboxylase
VP: variegate porphyria
CHAPTER 1 INTRODUCTION

1.1. Haem Biosynthetic Pathway

1.1.1. Porphyrins and the haem biosynthetic pathway

Porphyrins are fully conjugated macrocycles consisting of four pyrrole rings linked by four methine bridges, designated as \( \alpha, \beta, \gamma \) and \( \delta \) (Fig. 1.1). The four pyrrole rings A, B, C and D allow eight side-chain substituents to be attached at positions 1 to 8. The pattern of side chain substitution determines the physical and chemical properties of each individual porphyrin. Porphyrins exhibit intense red fluorescence when exposed to long wavelength ultraviolet light near the 400 nm region due to the conjugated double-bond structure. Porphyrinogens are the reduced form of porphyrin with 6 more protons. It does not fluoresce under UV light and porphyrinogens are easily oxidized back to the corresponding porphyrin in the presence of oxygen.

Porphyrinogens, not porphyrins (except protoporphyrin), are the intermediates of the biosynthetic pathway which lead to the formation of haem, an iron-chelated tetrapyrrole. Haem is the prosthetic group for a number of proteins including haemoglobin, myoglobin, catalase, peroxidases, cytochromes P-450, etc. Without haem, most essential biochemical pathways in the body could not function. Haem biosynthesis takes place in all cells that contain functioning mitochondria, mainly in the haemopoietic tissues and liver. Adult humans synthesize about 0.45 mmol of haem each day. Approximately 80% of haem synthesis occurs in bone
Fig. 1.1. Structures of Porphyrinogens and Porphyrins
marrow where it is utilized for the production of haemoglobin. Most of the remaining haem synthesis occurs in the liver for the production of cytochrome P-450, catalase and various mitochondrial cytochromes (Elder, 1983). The pathway for haem biosynthesis in liver is the same as that in the bone marrow and other haem-forming tissues of the body. Although the intermediates of the pathway are identical, it has not been confirmed that the enzymes which catalyze these steps are structurally the same in various tissues. Thus, the properties of the enzymes in liver may differ from those in the bone marrow and other haem-forming tissues (Bloomer & Bonkovsky, 1989).

The haem biosynthetic pathway is a series of irreversible reactions, consisting of eight discrete enzyme-catalyzed steps (Fig. 1.2.). The first and the last three steps take place in mitochondria while the rest occur in the cytosolic compartment of the cells. The first step is the formation of 5-aminolaevulinic acid (ALA) from succinyl-coenzyme A and glycine catalyzed by ALA synthase (ALA-S). The activity of ALA-S is the lowest in the pathway and is tightly controlled by the end product, haem. It is the rate-limiting enzyme of the pathway. The ALA formed passes from the mitochondria into the cytoplasm of the cell where two molecules of ALA condense to form the mono-pyrrole porphobilinogen (PBG) catalyzed by PBG synthase (PBG-D). The next two steps act together to polymerize four molecules of PBG to form the cyclic tetrpyrrole, uroporphyrinogen III, catalyzed by two independent and sequential cytosolic
Fig. 1.2. Haem Biosynthetic Pathway
enzymes. The first enzyme, hydroxymethylbilane synthase (HMB-S), also called PBG deaminase catalyzes the condensation of four PBG in a head-to-tail manner to form a symmetrical linear tetrapyrrole, hydroxymethylbilane (HMB). This is a highly unstable compound. The second enzyme, uroporphyrinogen III synthase (Uro'gen III-S) catalyzes its cyclization and rearrangement to uroporphyrinogen III, the first porphyrinogen in the pathway. The mechanism of rearrangement has not been fully elucidated. A spiral intermediate has been proposed (Battersby et al., 1978; 1979). In the absence of Uro'gen III-S, HMB spontaneously cyclizes to uroporphyrinogen I. Uro'gen I and III are isomers, both have four acetic acid and four propionic acid side chain substituents on the four pyrrole rings. Uro'gen I is a symmetrical structure, while Uro'gen III has its ring D inverted by the enzyme. Only Uro'gen III is the intermediate of the haem biosynthetic pathway and is utilized to form haem. Normally excess Uro'gen III-S activity is present, which greatly favours the formation of the type III isomer. In contrast to Uro'gen III, Uro'gen I and its metabolite, coproporphyrinogen (Copro'gen) I, are not biologically active and accumulate only under certain pathological conditions. The next step is the last step of the pathway in the cytoplasm. Uroporphyrinogen decarboxylase (Uro'gen-D) catalyzes the sequential removal of the four carboxyl groups of the acetic acid side-chains substituents in Uro'gen I and III to form Copro'gen I and III, through hepta-, hexa- and penta-carboxyl intermediates. Copro'gen I cannot proceed any further in the pathway and is excreted from the body. The
pathway now re-enters the mitochondria. Coproporphyrinogen oxidase (Copro'gen-O), catalyzes the oxidative decarboxylation of Copro'gen III to protoporphyrinogen (Proto'gen) IX by removal of the carboxyl group and of the two protons from the propionic acid groups on rings A and B to form vinyl groups. This step proceeds with the preliminary formation of harderoporphyrinogen, an intermediate with one vinyl group on ring A and a propionic acid group on ring B (Elder et al., 1978). Protoporphyrinogen IX oxidase (Proto'gen-O) catalyzes the oxidation of Proto'gen IX to protoporphyrin IX, the only porphyrin in this pathway, by removing 6 protons to form the highly conjugated double-bond structure. Although Proto'gen IX is readily auto-oxidized to protoporphyrin IX in vitro under aerobic condition at physiologic temperature and pH, an enzyme which catalyzes this oxidation has been identified in yeast, rat liver (Jacobs & Jacobs, 1982), human fibroblast, erythrocytes and leucocytes (Kappas et al., 1983). Chemical evidence has also been obtained for its existence (Jackson et al., 1974; Smith et al., 1976). The last enzyme in this pathway is ferrochelatase, which catalyzes the insertion of ferrous iron into protoporphyrin IX to form haem.

The same pathway is also shared by other important compounds which carry out vitally crucial functions in living systems. Vitamin B_{12}, a cobalt chelated corrin, branches off during the decarboxylation of Uro'gen III, and chlorophyll, a magnesium chelated chlorin, branches out
from protoporphyrin IX (Granick & Beale, 1978).

1.1.2. The regulation of haem biosynthesis

In normal circumstance, the synthesis of porphyrinogens and haem occurs in amount which is just sufficient to provide for the body's metabolic requirement. Only small amount of intermediates (less than 2.5% of the total amount of ALA used for haem) are lost from the pathway due to intracellular oxidation of porphyrinogens to their porphyrins or by diffusion out of cells (Elder, 1982). This balance is achieved by a precise regulation of the haem biosynthetic pathway.

It is suggested that the regulation of the haem biosynthetic pathway has distinct features in different haem biosynthetic tissues (Sassa, 1976; Rutherford et al. 1979; Sassa & Urabe, 1979; Hoffman et al., 1980). In liver, the rate of haem biosynthesis is largely controlled by a negative-feedback effect of haem on ALA-S through three different mechanisms: (1) repression of the synthesis of new enzyme (Whiting & Granick, 1976); (2) interference with the transfer of newly synthesized enzyme from cytosol to mitochondria (Yamauchi et al., 1980); (3) direct inhibition of the enzyme activity (Scholnick et al., 1969). Several authors have proposed that the liver regulatory system is based on the existence of a free hepatic haem pool, in which haem combines with an unidentified aporepressor to adjust the synthesis of ALA synthase. Depletion of the regulatory pool allows ALA synthase to be synthesized at a greater
rate. As the regulatory pool is repleted, synthesis of the enzyme slows (Bloomer & Bonkovsky, 1989). The regulation of the enzyme synthesis by end-product repression makes effective short-term control possible because the turnover of ALA-S is very rapid (Elder, 1983). Other factors also influence hepatic ALA-S activity. The administration of glucose or other carbohydrates decrease the activity (Bloomer & Bonkovsky, 1989), while a wide range of drugs, such as barbiturates, certain steroids and some chemicals increase the activity (Elder & Path, 1982; Bloomer & Bonkovsky, 1989). The activity of ALA-S also appears to be increased as a compensatory effect in most of porphyrias, and this action is responsible for the excessive production of intermediary metabolites proximal to the enzymic blocks in these diseases (Hindmarsh, 1986). Another enzyme, HMB-S, is present at a similarly low activity in liver, which may also exert some control on the pathway. Thus under the conditions of increased ALA production, ALA and PBG accumulate and are excreted in excessive amounts.

In erythroid cells although ALA-S, like in the liver, is under negative feedback control by haem, it does not appear to be the rate controlling enzyme. Ferrochelatase rather than ALA-S may be the rate limiting enzyme (Kappas et al., 1983; Bloomer & Bonkovsky, 1989; Conder et al., 1991). Haem regulates the uptake of iron by reticulocytes, thereby influencing the supply of iron for ferrochelatase (Ponka & Schulman, 1986). In the cell types other than liver and erythroid cells, the control of haem biosynthesis has
been little studied. It seems that the regulation is not similar to that in the liver (Kappas et al., 1983).

Two general features of the pathway in vivo should be noted: 1. The substrate concentrations are far lower than the Michaelic constants of the corresponding enzymes. 2. The activity of HMB-S is much lower than the activities of the enzymes in the remainder of the pathway. Therefore, any HMB formed is likely to be converted immediately to protoporphyrin IX, and unstable porphyrinogen intermediates are therefore protected from oxidation to the corresponding porphyrins and their consequent loss from the pathway. In this case, theoretically, only when an enzyme activity is decreased to well below 50% of normal does substrate accumulate and can the relevant porphyrins be detected (Elder & Path, 1982).

1.2. Porphyrias

Porphyrias are a group of genetic diseases in which enzyme defects in the haem biosynthetic pathway cause excessive accumulation of porphyrins and porphyrin precursors leading to clinical manifestations, mainly neurologic dysfunction and cutaneous photosensitization.

Porphyrias may be classified according to the clinical presentation: acute porphyria associated with acute neurologic manifestations or cutaneous porphyria with skin photosensitization. They may also be classified according to the main source of excessive porphyrin production: hepatic
porphyria, in which the excessive porphyrin is mainly produced in liver or erythropoietic porphyria in which bone marrow is the main site of the excessive porphyrin production (Table 1.1).

1.2.1. Enzyme defects in porphyrias

Haem synthesis in vivo is tightly controlled by haem itself. In spite of the presence of enzyme defects in the haem biosynthetic pathway, haem deficiency is neither a constant nor prominent feature of any type of porphyria, unless there is haemolysis (Elder, 1986). This is guaranteed by the regulation of the biosynthetic pathway: negative feedback control on ALA-S by haem and the far lower concentration of substrates than the Michaelic constant of the corresponding enzymes. When an enzyme activity is defective, the concentration of the corresponding substrate is increased by the negative feedback mechanism to such an extent that it is sufficient to overcome the enzyme defect in order to maintain a normal or near normal rate of the haem synthesis. As its intracellular concentration increases, the substrate may be lost from the pathway by diffusion out the cells or oxidation to porphyrin by molecular oxygen. The porphyrins may be trapped in cells, or excreted into urine or faeces depending on its chemical characteristic or solubility. The accumulation of excessive intermediates in the body may result in neurologic
<table>
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<tr>
<th>Defective Enzyme</th>
<th>Intermediates Accumulated</th>
<th>Disease</th>
</tr>
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<tbody>
<tr>
<td>ALA-Dehydrase (EC 4.2.1.24.)</td>
<td>Large amounts of urinary ALA and coproporphyrin, accompanied by elevated erythrocyte protoporphyrin.</td>
<td>ALA-D Deficiency</td>
</tr>
<tr>
<td>HMB-Synthase (EC 4.3.1.8.)</td>
<td>Large amount of ALA and PBG, accompanied by uroporphyrin isomers due to nonenzymic cyclization of PGB, in the urine.</td>
<td>Acute Intermittent Porphyria</td>
</tr>
<tr>
<td>Uro'gen III-synthase (EC 4.2.1.75.)</td>
<td>Large amount of uroporphyrin I and to a lesser extent of hepta-, hexa-, penta-carboxylic porphyrin I and coproporphyrin I in the urine; faecal coproporphyrin I is greatly increased; erythrocytes contain variable but increased uroporphyrin I and coproporphyrin I.</td>
<td>Congenital Erythropoietic Porphyria</td>
</tr>
<tr>
<td>Uro'gen-Decarboxylase (EC 4.1.1.37.)</td>
<td>Markedly increased uroporphyrin, heptacarboxylic porphyrin and to a lesser extent hexa-, and penta-carboxylic porphyrin and coproporphyrin in urine. Faecal porphyrins</td>
<td>Porphyria Cutanea Tarda</td>
</tr>
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are complex and contain high concentrations of isocoproporphyrin.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Description</th>
<th>Hereditary</th>
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<tbody>
<tr>
<td>Copro'gen-Oxidase</td>
<td>Mainly coproporphyrin III in urine and faeces; ALA and PBG are increased during acute attack and accompanied by increased hepta-, hexa- and penta-carboxylic porphyrins. Protoporphyrin may also be elevated in faeces, but is less than coproporphyrin III.</td>
<td>Coproporphyria</td>
</tr>
<tr>
<td>Proto'gen-Oxidase</td>
<td>Faecal protoporphyrin and to a lesser extent coproporphyrin III are increased, accompanied by X-porphyrin; urinary porphyrin is mainly coproporphyrin; ALA and PBG increased during acute attack.</td>
<td>Variegate Porphyria</td>
</tr>
<tr>
<td>Ferrochelatase</td>
<td>Increased free protoporphyrin in erythrocytes, plasma, bile and faeces.</td>
<td>Erythrohepatic Protoporphyria</td>
</tr>
<tr>
<td>Disease</td>
<td>Acute Neurologic Attacks</td>
<td>Cutaneous Lesions</td>
</tr>
<tr>
<td>---------------------------------------------</td>
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<tr>
<td>ALA-D Deficiency</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Acute Intermittent Porphyria</td>
<td>+</td>
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<tr>
<td>Congenital Erythropoietic Porphyria</td>
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<td>Porphyria Cutanea Tarda</td>
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<tr>
<td>Hereditary Coproporphyria</td>
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<td>Variegate Porphyria</td>
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<tr>
<td>Erythrohepatic Protoporphyria</td>
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disorders, skin lesions, or other symptoms, thus causing porphyrias. Each particular enzyme defect in the pathway, except ALA-S, leads to a certain pattern of intermediates accumulation, to cause specific porphyria (Table. 1.2.). In the case of the defect in ALA-S, the accumulated substrates, succinate and glycine, are intermediates for several biochemical pathways, so conventionally it is not considered as porphyria (Strake et al., 1990). Although the patterns of excreted urinary or faecal porphyrins are adequate for the differentiation of the porphyrias, the assay of enzyme activities may also be necessary, especially in the detection of latent carriers.

The existence of latent porphyria suggests that the enzyme abnormality by itself may not be sufficient to cause expression of the disease. Moreover, biochemical and clinical manifestations in one patient may vary considerably over time, or different individuals may respond differently to the similar enzyme deficiency. Thus, other factors must combine with the enzyme defect to produce biochemical manifestations of the diseases (Elder & Path, 1982; Bloomer & Bonkovsky, 1989). The known factors include increased requirement for haem biosynthesis (Bloomer & Bonkovsky, 1989), certain exogenous chemicals, some drugs, hormones and alcohol.

1.2.2. Clinical manifestation of porphyrias

Porphyrias mainly manifest in two clinical syndromes: (1) acute attacks which manifest as dysfunction of central
peripheral and autonomic nervous system. (2) skin lesions due to photosensitization by increased porphyrin accumulation.

Acute attacks of porphyria occur in ALA-D deficiency porphyria (also called plumboporphyria), acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), and variegate porphyria (VP). Patients with any of these acute porphyrias may generally enjoy good health, but are at the risk of developing severe neurovisceral attacks. The attacks are transient and most commonly followed by complete remission. They may, however, prove fatal. Repeated attacks are common. The attacks are clinically very similar in each of these disorders. The commonest symptom is severe lower abdominal pain, often colicky, lasting for hours to days. Vomiting may occur, and constipation is usual. These reflect the involvement of the autonomic nervous system. Other symptoms and signs are protean and include mental disturbances, muscular weakness which may progress to quadriplegia and respiratory paralysis, hypertension, tachycardia, etc. Residual paralysis, hypertension and renal failure are long-term sequelae. Patients with HCP and VP may also present with cutaneous photosensitization. During an attack, despite the differing sites of the defects in the haem biosynthetic pathway, all patients excrete a massive excess of porphyrin precursors ALA and PBG (except ALA-I deficiency, in which only ALA is increased) in their urine, while during remission the levels of ALA and PBG are usually normal (Elder, 1983; Hindmarsh, 1986; Bloomer & Bonkovsky
Acute attacks are often precipitated by drugs such as barbiturates, sulphonamides, oral contraceptives and enzyme-inducing anticonvulsants and antidepressants. Alcohol consumption, endogenous and exogenous steroid hormonal factors, cigarette smoking, infections and strict dieting or fasting can also trigger acute attacks (Elder, 1983; Moore et al., 1990).

Although there have been many studies on the pathogenesis or mechanism of the neurological dysfunction due to altered haem synthesis, the situation is still not clear. Some hypotheses have been proposed, and two of most attractive ones have been intensively investigated (Bloomer & Bonkovsky, 1989; Moore et al., 1990; Straka, et al., 1990). The first is that the neurologic manifestations are caused by ALA and/or PBG. Either ALA or PBG, or both may be directly neurotoxic. This hypothesis is supported by the fact that acute attacks of porphyria with neurologic dysfunction do not occur unless ALA and PBG are over-produced, and during remission the urinary excretion of ALA and PBG return to normal. ALA may be more important than PBG in this aspect, because in patients with acute attack due to ALA-D deficiency PBG is not over-produced (Laiwah et al., 1987; Bloomer & Bonkovsky, 1989). Also in the patients with lead poisoning where ALA-D is depressed the clinical manifestations resemble AIP. The second is that neurologic manifestations result from haem deficiency. Haem deficiency
in neurons may cause inadequate concentration of mitochondrial cytochromes, resulting in the death of neuron due to serious decrease in synthesis of ATP, or increase the susceptibility of neurons to other injuries including toxic effects. Haem deficiency in hepatocytes plays a key role in the over-production of ALA and PBG. It also alters the metabolism of tryptophan, the precursor of 5-hydroxytryptamin (serotonin), which has a variety of effects on the nervous system. Experiments showed that depletion of hepatic haem in rats reduced hepatic tryptophan pyrrolase activity which caused elevated levels of tryptohan and 5-hydroxytryptophan in the brains of the animals (Litman & Correia, 1986). In human being it was reported that overexcretion of 5-hydroxyindoleacetic acid, the end product of serotonin metabolism was found in a few patients with symptomatic acute porphyria (Ludwig & Epstein, 1961). Therefore haem deficiency may play a role in the pathogenesis of the acute porphyric attack (Laiwah et al., 1987; Bloomer & Bonkovsky, 1989; Moore et al., 1990; Straka et al., 1990).

Photocutaneous lesions of porphyrias do not occur in ALA-D deficiency and AIP, but may be found in all the other porphyrias. In the patients with HCP and VP, photocutaneous lesions are not a constant manifestation, while in the patients with PCT, CEP and EHP, it is virtually always the major clinical symptom. The abnormal skin photosensitivity usually manifests in two distinguishable morphologic patterns. In EHP, the skin lesions is characterized by acute
photo-toxicity reaction in the sun-exposed area. The typical acute reaction begins within several minutes' exposure to sunlight with itching or stinging pain, sometimes followed by crusting and petechiae. Vesicles may occur on the face and hands. After repeated attacks, the skin may become thickened, waxy and pitted with small circular or linear scars, especially over the bridge of the nose, around the mouth, and over the knuckles. Cutaneous fragility, large bullae and mutilation are very rare in EHP. While in other cutaneous porphyrias such as PCT, CEP, VP and HCP, the skin lesions are fundamentally similar, although their severity varies, and develop in a different morphologic array from EHP. Burning, itching, stinging of sun-exposed skin or development of erythema and edema are rare and may only occur in individuals with very large porphyrin accumulations in their skin or plasma, or those subjected to prolonged intense light exposure. The most prominent abnormality is increased fragility of sun-exposed skin, from which the epidermis sheers away with minor trauma to leave raw erosions and blistering eruptions. Subepidermal bullae are frequent, and erosions of various sizes form at the junction of the epidermis with the dermis and heal slowly with crusting, scaring and milia formation. Cumulative damage in severe cases can result in cicatricial deformities of eyelids and lips, acral osteolysis of digits, dystrophic nails, and destruction of the cartilage of the nose and ears. Scaring alopecia, mottled or diffuse hyperpigmentation, and photo-onycholysis with temporary or permanent loss of nails may occur. Hypertrichosis of the
eyebrows, temples, and sides of the face are common. In CEP, the teeth usually show characteristically pink fluorescence with ultraviolet illumination. They may also appear reddish-brown in colour in normal light due to porphyrin deposition (Poh-Fitzpatrick, 1982; Elder, 1983; Bloomer & Bonkovsky, 1989). The pathogenesis or mechanism of the cutaneous lesion and the reason of existing two morphologic patterns of the cutaneous lesion are discussed in Section 1.2.4.

1.2.3. Molecular genetics of porphyrias

All porphyrias are due to enzyme defects in one of the steps which convert ALA to haem and are encoded by a single autosomal gene. Each gene is expressed in all cells which are capable of haem biosynthesis. The only exception is sporadic PCT which is probably not a genetic disease. AIP, PCT, HCP, VP and EHP are inherited as autosomal dominants while ALA-D deficiency and CEP are autosomal recessive traits. Two very uncommon porphyrias are inherited as homozygous autosomal dominant trait: hepatoerythropoietic porphyria (HEP, Uro'gen-D defect) and harderoporphyria (Copro-O defect). Like most other inherited disorders porphyrias also show genetic heterogeneity. Different mutations may produce the same phenotype (Elder, 1986). For example, in AIP which is the most thoroughly studied porphyria at the molecular level, four distinct allelic mutations for HMB-S have been demonstrated (Mustajoki & Desnick, 1985; Desnick et al., 1985). In PCT, a number of different mutations at the Uro'gen-D locus have been identified (Verneuil, 1986). Evidence also shows that some
mutations which decrease the activity of cytosolic enzymes in the liver may not always be found in erythrocytes, examples include some patients with AIP (HMB-S deficiency) and PCT (Uro'gen -D deficiency) (Elder et al., 1980; Desnick et al., 1985). The gene defects in the other porphyrias have not yet been characterized. Suggestions have been made that two distinct genes may encode ALA-S for hepatic and erythropoietic sources (Grandchamp & Nordmann, 1988; Moore et al., 1990). These advances and further studies will help us to improve the understanding of haem biosynthesis and the pathogenesis of the porphyrias. The identification of genetic defects in the porphyrias will also provide a new tool for the most accurate diagnosis of porphyrias, especially in latent carriers.

1.2.4. The possible mechanism of the photosensitivity in porphyrias

Porphyrinogens, the true intermediates of the haem biosynthetic pathway (apart from protoporphyrin), do not induce photosensitivity. However, the accumulated porphyrinogens due to enzyme deficiencies, are susceptible to oxidation to their corresponding porphyrins in presence of oxygen in vivo. The excess porphyrins which are deposited in the epidermal layer or are circulating in the dermal blood vessels absorb light, entering an "excited state". These excited porphyrins tend to return to the ground state by losing their energy through light emission (fluorescence) or transferring electrons to molecular oxygen to form singlet oxygen and possibly other free radicals. Singlet
oxygen in turn damages tissues through several mechanisms including oxidation of membrane lipids, cross-link of proteins, and oxidative damage to nucleic acids, with consequent interference of proper functions of cellular activities or organelles (Bloomer & Bonkovsky, 1989; Elder, 1990; Straka et al., 1990).

Different porphyrins appear to cause distinct photocutaneous symptoms, possibly due to their different physical and chemical properties (Bloomer & Bonkovsky, 1989; Straka et al., 1990). In experimental models, hydrophobic protoporphyrin is shown to accumulate predominantly in mitochondria and inserts into biological membranes, while, in contrast, hydrophilic uroporphyrin accumulates predominantly in lysosomes and is not associated to any significant degree with membranes (Sandbaerg et al., 1982). This may be the reason why there are different photocutaneous symptoms in porphyrias. For example, in erythrohepatic protoporphyria (EHP) where protoporphyrin accumulates, the photocutaneous symptom is characterized by acute erythema, burning and itching of light exposed skin, particularly the face and the back of the hands. In porphyria cutanea tarda where uroporphyrin accumulates, the photocutaneous symptom is characterized by increased fragility of light exposed skin, particularly where bullous lesions and erosions occur (Bloomer & Bonkovsky, 1989; Straka et al., 1990).
1.3. Analytical Techniques for the Study of Porphyrin Metabolism

1.3.1. Spectrophotometry

1.3.1.1. UV absorption spectrometry

Porphyrins display a characteristic UV absorption spectra, consisting of a major band at about 400nm which is called the "Soret" band, and four smaller absorption bands between 500 and 630nm in neutral solvents which are called the "etio" spectrum. The Soret band generally has 10 to 15 times greater absorption (molar extinction coefficient of 2 to $5 \times 10^5$) than the next major band, which occurs at around 500nm. With the formation of a porphyrin dication in diluted aqueous HCl, or the formation of metalloporphyrins, the four small bands of the etio spectrum are replaced by two bands (Smith, 1975; Kappas et al., 1983). The Soret band is therefore, the choice for spectrophotometric determination. The existence of Soret band is due to the fully conjugated nucleus of the macrocycle, therefore, porphyrinogens do not have a Soret band, but show very weak absorption at 220-240 nm (Smith, 1975A; Mauzerall, 1978; Lim et al, 1988).

1.3.1.2. Fluorescent spectrometry

Porphyrins are readily excited by light at around 395-420 nm and emit intense red fluorescence at about 589-620 nm, depending on molecular structure. Spectrofluorometric methods provide much more sensitivity and specificity than UV absorption spectrometry (Kappas et al., 1983).
Porphyrinogens do not fluoresce.

Metalloporphyrins generally fluoresce at shorter wavelength than the corresponding free porphyrins, and need higher energy for excitation. The fluorescence of many metalloporphyrins is strongly solvent-sensitive and therefore excited state energies are solvent-dependent (Hopf & Whitten, 1975). However, not all metalloporphyrins are fluorescent. Haem, protoporphyrin chelated with Fe (II), and porphyrins chelated to Mn, Co, Cu and Ag which are paramagnetic metals do not fluoresce, while those chelated to Zn, Mg and Pb which are diamagnetic metals do (Hopf & Whitten, 1975; Kappas et al., 1983).

1.3.2. Chromatography

1.3.2.1. Thin-layer chromatography

Thin-layer chromatography (TLC) is a very widely used technique for separating porphyrins. The separation is most commonly carried out on silica gel plates with a mixture of polar and non-polar organic solvents such as chloroform, dichloromethane and carbon tetrachloride mixed with ethyl acetate and ethanol as the developing solvents. Practically all of the common classes of porphyrins methyl esters from uroporphyrin to protoporphyrin have been separated by TLC. The porphyrin methyl esters migrate according to the number of carboxyl groups. The lesser carboxyl groups they have, the faster they move on TLC plate. The porphyrin methyl esters separated by TLC are easily recovered by removal of
the adsorbent band from the plate and extracting the porphyrin methyl ester from the supporting material with a solvent mixture slightly more polar than that used for developing the chromatography. The separation can be improved by repeating the development of the plate or by two-dimensional TLC (Elder, 1971A; Fuhrhop & Smith, 1975A). Free porphyrin carboxylic acids can also be separated by TLC, but the developing solvent system is more complex (Smith, 1975B). Although TLC is less suitable to the separation of the porphyrin isomer it is still a useful technique for the screening of porphyrins and for small scale preparative isolation of porphyrin methyl esters.

1.3.2.2. High-performance liquid chromatography

Porphyrin analysis has been greatly improved by the development of HPLC which provides more efficient separation, more accurate identification and quantitation of porphyrins. Porphyrins can be separated either as methyl esters by normal-phase chromatography (also called absorption chromatography) or as free acids by reversed-phase or reversed-phase ion-pair chromatography. The separation of porphyrin methyl esters by normal-phase chromatography was the first attempt of separating porphyrin by HPLC (Cavaleiro et al., 1974) and the principle is similar to that of TLC. The porphyrin methyl esters are sequentially eluted with increasing number of ester side-chains, thus protoporphyrin dimethyl ester is the first and uroporphyrin octamethyl ester the last to be eluted (Lim et al. 1988). Although the resolution, speed of analysis and
reproducibilities have substantially improved with the use of microparticulate (3-10 μm) silicas, and aminopropyl-bonded silica columns, there are disadvantages associated with separation of porphyrin methyl esters by HPLC. Porphyrins have to be converted into esters for analysis. The esterification procedure is time consuming and may lead to undesirable complications. Porphyrin methyl esters may partially hydrolysed on the column and will therefore not be eluted from the silica column and remain undetected. Metal chelates and mixed methyl-ethyl esters may form during the derivatization, which will lead to errors in identification and quantitation (Lim, 1991). However, the esterification procedure provides a much cleaner porphyrin extract (especially from faeces) for HPLC analysis, and the higher capacity of normal-phase columns can be used for preparative separation.

The above problems can be avoided by separating the porphyrins as free acids. Ion-exchange HPLC was tried with little success due to poor column efficiency and resolution (Evans et al., 1975). Reversed-phase ion-pair chromatography achieved the separation of free porphyrins with two to eight carboxylic acid groups, the elution order is the more polar porphyrin, uroporphyrin, first followed by hepta-, hexa-, penta-carboxylic acid porphyrins, coproporphyrin and protoporphyrin. This system, however, has its limitations, such as the limited separation of type I and III isomers (only occurs for coproporphyrins) and the requirement for a higher column temperature and the re-equilibration time
between analyses is long (Rossi & Curnow, 1986).

Reversed-phase HPLC (usually on ODS columns) with aqueous buffer-organic modifier as eluents has shown great potential for porphyrin separation and has provided a very powerful tool for porphyrin analysis. The most commonly used systems are phosphate buffer with methanol or acetonitrile (Englert et al., 1979; Ford et al., 1981; Sakai et al., 1983; Schreiber et al., 1983; Johnson et al., 1988), and ammonium acetate buffer with methanol and/or acetonitrile as mobile phases (Lim et al., 1988). The use of ammonium acetate buffer has many advantages. Ammonium acetate buffer provides the right pH for porphyrin separation. It is highly soluble in aqueous methanol and acetonitrile, allowing high molar concentrations to be used. Therefore, it has high buffering capacity to allow the acid extracts of porphyrin free acids to be injected directly onto the reversed-phase column without damaging it. It is also a good masking agent for residual silanol groups on reversed-phase columns thus improving the resolution and the peak shape. Ammonium acetate is relatively volatile, and can be easily removed if necessary. The retention mechanism is mainly hydrophobic interaction, but ion-pair mechanism may also play a role (Lim & Peters, 1984). The elution order of the porphyrins is from polar to non-polar. Gradient elution using ammonium acetate buffer with a mixture of acetonitrile and methanol as organic modifiers can simultaneously separated porphyrins with two to eight carboxylic acid groups, including the naturally occurring type I and type III isomers (Li et al.,
While using the same system under different isocratic elution conditions, the separation of four coproporphyrin isomers, four penta-carboxylic acid porphyrin III isomers, five penta'gen isomers, six hexa'gen III isomers, and four hepta'gen III isomers can be achieved (Lim, 1991). Uro I,II and III isomers can be completely separated, but the resolution of isomer III and IV still remains unsatisfactory. The use of base deactivated silica (BDS) and polymer-based reversed-phase columns with ammonium acetate-methanol/acetonitrile system can further improve the separation of certain isomers.

1.3.3. Mass spectrometry

Mass spectrometry is a valuable technique which can provide accurate information concerning the molecular mass, molecular composition, and a number of other distinctive features (Smith, 1975A). Thus it has played a very important role in the structure determination of all the new porphyrins discovered in recent years. In this thesis the molecular weights of the new porphyrins are determined by liquid secondary ion mass spectrometry (LSIMS). The principle of LSIMS is that porphyrin methyl esters gain one proton to give a molecular ion (MH+) during the bombardment by caesium ion (Cs+) beam. The ratio of mass to charge (m/z) of this ion gives the exact molecular weight. The naturally occurring porphyrin free acids have very low volatility and cannot be readily analysed directly by MS (Jackson, 1989).
1.4. Aims of Study

The main objective of this thesis is to study in detail porphyrin metabolism in patients with CEP. The following areas are covered:

1. To investigate whether oral charcoal treatment is effective for reducing porphyrin levels in patients with CEP.

2. To isolate, identify and characterize new porphyrin metabolites in the urine and blood of patients with CEP.

3. To elucidate the origin, mechanism of formation and biological significance of the new porphyrin metabolites.
CHAPTER 2
EFFECT OF ORAL CHARCOAL TREATMENT IN A CASE OF
CONGENITAL ERYTHROPOIETIC PORPHYRIA

2.1. Introduction

Congenital erythropoietic porphyria (CEP), also known as erythropoietic porphyria, congenital porphyria or Gunther's disease, is an extremely rare disease. It was the first porphyria to be described in 1874 by Schultz (1874) and one patient with CEP had been studied over 25 years in the early 20th century by Gunther. Since then less than 100 cases have been reported (Marver & Schmid, 1972; Nordmann & Deybach, 1982; Goldberg et al., 1987; Bickers & Patrick, 1987).

Congenital erythropoietic porphyria is transmitted in an autosomal recessive manner. The enzyme defect lies at the level of Uro'gen III-S. The predominant site of the metabolic abnormality is the erythropoietic system. The enzyme defect leads to a highly abnormal accumulation of porphyrins in bone marrow, peripheral blood, and other organs. These reflect the three main manifestations of CEP: chronic photo-dermatitis with photosensitivity, massive porphyrinuria, and haematological abnormalities. The neurological symptoms observed in the acute porphyrias do not occur in this type of porphyria. A similar disease can also occur in bovines (Rimington, 1955; Rimington & With, 1973; Miyagi et al., 1976; Moore et al., 1978).
The onset of CEP usually occurs at birth or during early infancy, but late onset does happen occasionally (Kramer et al., 1965; Weston et al., 1978). The first symptom in infants suggesting CEP is often pink to dark brown staining of the diapers due to large amounts of porphyrins in the urine. The characteristic cutaneous photosensitivity causes the most mutilating skin lesions of the porphyrias. It presents as vesicules or bullae following sun exposure, mainly on the face and the back of the hands. The vesiculae contain a serous fluid which may exhibit a red fluorescence under ultraviolet light. Subepidermal lesions progress to crusted erosion and secondary infections which heal slowly with scarring and either hyperpigmentation or more rarely, hypopigmentation. Repetitive damage and infections may lead to severe mutilation of aural and nasal cartilage, and digits, as well as functional limitation. All exposed or already affected areas of the skin of patients remain more sensitive to slight injury, and a diffuse thickening of the skin is a common feature after several years. Hypertrichosis appears in most patients but alopecia may also occur. Erythrodontia (red-stained teeth) is a common finding in both deciduous and permanent teeth and it is pathognomonic of CEP. The eyes may be affected in the form of severe ulcerations, ectropion or cataract (Kappas et al., 1989).

Haemolysis frequently occurs and it is a striking feature of CEP. In most patients, the anaemia is only slight and is well-compensated by enhanced erythrocyte production.
However, in some cases anaemia may be severe and sometimes life threatening, requiring multiple transfusions (Nordmann & Deybach, 1982). The signs of heightened erythropoiesis including hyperplastic red bone marrow, reticulocytosis, and increased plasma iron turnover may be found. Erythroblasts are found in peripheral blood. The fragility of the erythrocytes is apparently increased, and the life span is often shorter than normal. The erythrocytes fluoresce bright red under ultraviolet light and the bones are reddish-brown, discoloured by accumulated porphyrins. Pronounced splenomegaly is observed in almost every patient (Ippen & Fuchs, 1980; Nordmann & Deybach, 1982).

The urine always contains greatly increased uroporphyrin and to a lesser extent coproporphyrin. Small amounts of hepta-, hexa- and penta-carboxylic acid porphyrins are also excreted. Though the type I isomers predominate, uroporphyrin III is also definitely increased in all patients (Wintrobe et al., 1981). Urinary excretion of 5-aminolevulinic acid and porphobilinogen is all normal. Faeces contain large amount of coproporphyrin I, with only small amounts of uroporphyrin I and protoporphyrin within normal range. The plasma also contains excessive uroporphyrin I and coproporphyrin I. Erythrocytes contain variable but always increased concentrations of uroporphyrin I and coproporphyrin I, and protoporphyrin is usually normal.

The treatment of CEP includes limiting exposure to sunlight, avoiding trauma to the skin, and treatment of skin
infection. β-Carotene and barrier creams can be used to prevent the effects of light on the skin. Transfusion of packed erythrocytes can suppress the endogenous erythropoiesis and reduce the production by negative-feedback control, as well as correct the anaemia. Haematin may be worth trying to avoid the risk of multiple transfusions. Splenectomy can reduce the haemolysis, lessen the porphyrin excretion, and diminish the skin manifestations. There is little evidence, however, for significant long-term improvement. Therefore, no efficient therapy has been established for this disease. (Ippen & Fuchs, 1980; Nordmann & Deybach, 1982; Bloomer & Bonkovsky, 1989).

It has been suggested that oral charcoal may be used to reduce the excessive circulating and tissue porphyrin levels (Pimstone et al., 1987) in HEP and CEP (Tishler & Winston, 1990). The evidence, however, is inconclusive. This chapter describes the detailed study of a case of CEP following treatment with oral charcoal to assess the effectiveness of this form of treatment for CEP.

2.2. Case Report

The patient who is now aged 23 years old, was found to have congenital erythropoietic porphyria at 2 months of age when he presented with persistent neonatal jaundice, haemolysis and cutaneous photosensitivity. There was no history of consanguinity. Erythrocyte Uro'gen III-S activity was reduced to 19% of normal in the subject and to
approximately 50% in both parents and in his sister (Wright & Lim, 1983) consistent with autosomal recessive inheritance and also indicating that the sister was a carrier. He was treated with intermittent blood transfusions and splenectomy was carried out at the age of 7 years. Regular medication also included ascorbic acid, β-carotene, folic acid, penicillin after the splenectomy, and avoidance of sunlight. From the age of 10 years he received regular subcutaneous infusions of desferrioxamine at night. Despite these measures he continued to have extensive photomutilation of the hands and face, with shortening of the digits, and when 20 years old he complained of pain in the spine and sternum and was found to have compression fractures of 2 vertebrae. Treatment of osteoporosis with clodronate (dichloromethylene bisphosphonate) was also carried out (Pullon et al., 1991).

At the time when this study was performed he was also on a more intensive regime of blood transfusions (2-3 units at approximately 4 weeks intervals) which raised the haematocrit from a mean of 0.24 to 0.36%.

2.3. Experimental

2.3.1. Material and reagents

Ammonium acetate, glacial acetic acid, trichloroacetic acid, I₂, concentrated HCl, concentrated H₂SO₄, NaHCO₃, NaOH, ethylenediaminetetraacetic acid (EDTA), Triton X-100, MgCl₂, dimethyl sulfoxide (DMSO), diethyl ether and chloroform were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile and
methanol were HPLC grade from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland). Porphobilinogen and uroporphyrin were from Sigma Chemical Co. (Poole, Dorset). Tris(hydroxymethyl)aminoethane (Tris) was obtained from Boehringer Mannheim GmbH. (F.R.G.).

2.3.2. Charcoal treatment procedure

After an 8-week "run-in" period with his usual maintenance regime, activated charcoal (Medicoal, Lundbeck, Farillon Ltd) was ingested as an aqueous suspension taken through a straw, in 5 divided doses to a total of 50g daily for 3 weeks. Blood samples for porphyrins, haemoglobin, haematocrit, electrolyte and creatinine, and 24 hour urine collections, were obtained weekly, and blood samples were obtained also before and after blood transfusions throughout the 11-week study period. During the first 24 hours of administration of charcoal, blood samples were obtained at 3 hour intervals. Stool samples were obtained weekly.

The quantity of porphyrins and the activity of HMB-S and Uro'gen III-S were analysed by HPLC procedures (Wright & Lim, 1983; Lim et al., 1988), other measurements were done by standard laboratory methods.

2.3.3. Duodenal porphyrins

After an overnight fast, a triple-lumen tube was passed under fluoroscopic control. Isotonic saline (0.15 M) solution adjusted to pH 7 with 0.1 M NaOH and containing $^{14}$C polyethylene glycol (PEG) as a non-absorbable marker
was infused at a rate of 10 ml/min through a port in the proximal duodenum. After equilibration for 1h, duodenal content was aspirated at 1 ml/min from proximal and distal ports 15 cm apart for 3 consecutive 30 min periods. Porphyrin content in the aspirates was measured by HPLC, and levels/min at the distal aspiration port were obtained from the product of porphyrin concentration and flow rates, calculated by established methods based on differences in the concentrations of PEG in the perfusate and aspiration sample (Cooper et al., 1966; Pimstone et al., 1987).

The aspirate was acidified with concentrated HCl (0.5 ml HCl to 5 ml of sample) to dissolve any precipitated calcium salts which absorb porphyrins. After centrifugation the sample was ready for HPLC analysis.

2.3.4. Isolation of erythrocytes and plasma

Heparinized blood, 10 ml, was centrifuged at 2000 g for 10 min. The plasma was transferred to a clean tube. The buffy coat leucocytes was removed and discarded with a Pasteur pipette. The erythrocytes was washed three times with ice cold 0.15 M NaCl and centrifugation at 2000 g for 10 min after each wash. The plasma and packed red cells were stored at -20°C if not used immediately.

2.3.5. Sample preparation for HPLC separation

2.3.5.1. Porphyrins in urine

Urine, 24 h collection, was collected in plastic
container with boric acid as preservative. Random urine samples were collected into universal bottles. The samples were kept at -30°C in the dark until use. All urine samples were treated with concentrated HCl (5 ml per 100 ml urine) to dissolve any precipitated porphyrins and to prevent the formation of metalloporphyrins.

2.3.5.2. Extraction of porphyrins in faeces

Faeces, 25-50 mg, was placed in a graduated centrifuge tube for analysis and a larger sample (approx. 250 mg) was put into a suitable vessel to be dried down for quantitative calculation. Concentrated HCl, 1 ml, was added to the centrifuge tube and vortex-mixed until the particles disintegrated. The sample was allowed to stand for 5 min with occasional vortex-mixing. Then ether, 3 ml, was added and the contents of the tube were thoroughly vortex-mixed to give an emulsion followed by addition of 3 ml of water. To avoid undue degradation of protoporphyrin, the water must be added within 10 min of the acid addition. After centrifugation, the upper ether layer containing chlorophyll derivatives and carotenoids, and the pad of insoluble material at the interface were discarded. The lower acidic aqueous layer contained the porphyrins. This phase was flushed with N₂ for 10-20 min to remove any residual ether which may interfere with the HPLC analysis. The volume was recorded and the solution was now ready for HPLC analysis (Rossi & Curnow, 1986).
2.3.5.3. Direct esterification porphyrins in faeces

Wet faeces (0.5 g) was thoroughly mixed with 5 ml of 10% (v/v) sulphuric acid in methanol. The mixture was refluxed for 20 min or left standing overnight in the dark at room temperature. The mixture was then thoroughly mixed and centrifuged at 2000g for 10 min. The supernatant was transferred to a separating funnel and the residual was washed with 10% sulphuric acid in methanol with centrifugation. The supernatants were combined, and the resulting porphyrin methyl esters were extracted with chloroform. The extractant was washed with saturated aqueous NaHCO₃ and then sufficient water. After filtering through a filter paper, the organic solution was evaporated to dryness at about 40°C under N₂.

The porphyrin methyl esters were either separated by TLC or hydrolysed with 25% HCl for 72 h in the dark at room temperature for HPLC analysis.

2.3.5.4. Extraction of porphyrin from erythrocytes

The packed erythrocytes (25 μl) were lysed by mixing with 75 μl of distilled water in a 10 ml centrifuge tube. The red cell haemolysate was then vortex-mixed for 1 min with 100 μl of 20% TCA/DMSO (1:1, v/v) containing mesoporphyrin as internal standard. The mixture was stood at room temperature for 5 min with occasional vortex-mixing and then centrifuged at 2,000 g for 10 min at 4°C in a CoolSpin. The supernatant was transferred to a clean tube for HPLC analysis.
2.3.5.5. Extraction of porphyrin from plasma

Plasma (100 μl) was thoroughly vortex-mixed for about 2 min with 100 μl of 20% TCA/DMSO (1:1, v/v) containing mesoporphyrin as internal standard in an Eppendorff centrifuge tube. The mixture was centrifuged for 3 min at 10,000g and the supernatant was transferred into a clean tube for HPLC analysis.

2.3.5.6. Assay of the activities of HMB-8 and Uro III-S

The method described by Wright and Lim (1983) was used. Packed RBC, 30 μl, was added to 1.4 ml of 0.05 M Tris-HCl buffer, pH 8.25, containing 74 μM MgCl₂ and 0.1% Triton X-100. After mixing, the solution was heated at 56°C for 1 h to deactivate Uro'gen III-S. The mixture was cooled to room temperature and 3 μl of fresh packed RBC was added. The mixture was pre-incubated at 37°C for 5 min and the reaction was started by adding 50μl of PBG (7.4 μM) as substrate. The incubation was continued for a further 30 min at 37°C in the dark. The reaction was stopped by adding 1.5 ml of 10%TCA containing 0.5% I₂ (w/v). After centrifugation the supernatant was subjected to for HPLC analysis.

2.3.6. High-performance liquid chromatography (HPLC) of porphyrins

A Varian Associates (Walton-on-Thames, Surrey, U.K.) Model 5000 liquid chromatography with a Varian UV-100 variable wavelength detector set at 405 nm and a Perkin-Elmer LS3 fluorescent detector set at excitation 400
and emmision 618 nm, respectively, was used. Samples were injected via a Rheodyne (Cotati, CA, USA) 7125 injector fitted with a 500 µl loop. The separation was carried out on a 250 mm x 5 mm Hypersil-ODS column (Shandon Scientific, Runncorn, Cheshire, U.K.). Unless otherwise stated the mobile phase generally consisted of solvent A: 9% (v/v) of acetonitrile in 1 M ammonium acetate buffer, pH 5.16 and solvent B: 10% (v/v) of acetonitrile in methanol. The elution program varied according to the separations required. The flow rate was 1 ml per min throughout. The elution programme for the quantitation of porphyrins from uro- to copro-porphyrin in urine, plasma and erythrocytes was by linear gradient elution from 0% to 90% solvent B in 30 min, followed by isocratic elution at 90% solvent B for 10 min. The column was re-equilibrated at 0% solvent B for 10 min before the next injection.

The elution program for the quantitation of faecal porphyrins was isocratic elution at 15% solvent B for 10 min; linear gradient elution from 15 to 70% solvent B from 10 to 45 min; linear gradient elution from 70 to 95% solvent B from 45 to 50 min, followed by isocratic elution at 95% solvent B for a further 10 min.

The elution program for the quantitation of duodenal aspirates porphyrins was as follows: time 0 to 20 min, 50% solvent B to 70% solvent B; time 20 to 30 min, 70% solvent B to 95% solvent B; time 30 to 40 min, isocratic elution at 95% solvent B.
2.4. Results and Discussion

Blood transfusion temporarily increased the erythrocyte Uro'gen III-S activity and incompletely suppressed the endogenous erythropoiesis. This led to a decrease of plasma and urinary porphyrins, and also improved the symptoms. This effect required regular transfusion with a haematocrit maintained between 34 and 42%.

Under the usual treatment conditions the activity of erythrocyte Uro'gen III-S was 38-86 nmol Uro III/ml RBC per hour (normal 159-352 nmol Uro III/ml RBC per hour). A rise of the activity was observed immediately after blood transfusion. No significant change was seen during the administration of charcoal (Fig. 2.1).

The total urinary porphyrin excretion determined by HPLC (Fig. 2.2.) was 79-283 μmol/24h under the usual treatment condition for the patient (normal total porphyrins < 0.35 μmol/24h with <0.04 μmol Uro I/24h). The total porphyrins in the plasma (Fig. 2.3.) were 2.5-4.1 μmol/l (0.01-0.02 μmol/l in normal subject) and in the erythrocytes (Fig. 2.4.) ranged from 3.2-10.6 μmol/l (0.4-1.7 μmol/l in normal subjects). The majority (approx. 75%) was Uro I with much less (approx. 15%) of Copro I and small amount of hepta-, hexa- and penta-carboxylic porphyrins (Table 2.1.). In the faeces (Fig. 2.5.) Copro I was the major porphyrin with some Copro III, penta I, a little Uro I and traces of hepta-, hexa-carboxylic acid porphyrins and protoporphyrin
Fig. 2.1. Erythrocyte Uro'gen III-S Activity in Congenital Erythropoietic Porphyria Treated with Blood Transfusion and Oral Charcoal

The average of the activity before charcoal treatment was $67.18 \pm 14.7$ nmol Uro III/ml RBC per hour (mean ± SD) and after charcoal treatment was $65.23 \pm 7.32$ nmol Uro III/ml RBC per hour.
Fig. 2.2. HPLC Separation of Urinary Porphyrins of a Patient with CEP

Peak 1, uroporphyrin I; peak 2, heptacarboxylic acid porphyrin I; peak 3, hexacarboxylic acid porphyrin isomers; peak 4, pentacarboxylic acid porphyrin I; peak 5, coproporphyrin I.
Peak 1, uroporphyrin I;
peak 2, heptacarboxylic acid porphyrin I;
peak 3, hexacarboxylic acid porphyrin isomers;
peak 4, pentacarboxylic acid porphyrin I;
peak 5, coproporphyrin I.

Fig 2.3. HPLC Separation of Plasma Porphyrins of a Patient with CEP.
Fig. 2.4. HPLC Separation of Erythrocyte Porphyins of a Patient with CEP

Peak 1, uroporphyrin I; peak 2, coproporphyrin I.
Fig. 2.5. HPLC Separation of Faecal Porphyrins of a Patient with CEP
Table 2.1. Porphyrin Levels in Urine, Plasma and Erythrocytes in a Patient with Congenital Erythropoietic Porphyria

<table>
<thead>
<tr>
<th></th>
<th>Urine (μmol/24h)</th>
<th>Plasma (μmol/l)</th>
<th>RBC (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Range</td>
<td>&lt; 0.35</td>
<td>0.01-0.02</td>
<td>0.4-1.7</td>
</tr>
<tr>
<td>Total Porphyrin</td>
<td>79-283</td>
<td>2.5-4.1</td>
<td>3.2-10.6</td>
</tr>
<tr>
<td>Porphyrin (% of Total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uro I</td>
<td>75</td>
<td>66</td>
<td>83</td>
</tr>
<tr>
<td>Copro I</td>
<td>15</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Hepta to penta</td>
<td>8</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
IX. A series of new porphyrins were also found in the urine and plasma, which will be discussed in later chapters. The changes in 24h urinary porphyrin excretion suggested a delayed inverse relationship of the urinary porphyrins following the rise in haematocrit following blood transfusion, consistent with reduced porphyrin synthesis when endogenous erythropoiesis was reduced. This relationship was not, however, as distinct as previously reported (Piomelli et al., 1986), and was not found during the administration of charcoal (Fig. 2.6.). A small decrease in plasma and erythrocytes porphyrin levels were seen during the first 24 h of charcoal administration particularly in the first three samples at 3 h intervals (Fig. 2.7.), but this was not detectable in samples collected during the following 3 weeks (Fig. 2.8.). The pattern of variability of plasma porphyrin levels paralleled the findings in the urine with no indication of reduction due to charcoal. Compliance with charcoal ingestion was excellent from inspection of stool specimens.

The porphyrin pattern in the duodenal aspirates is similar to faecal porphyrin. More than 95% of the total porphyrins were coproporphyrin I, with small amount of coproporphyrin III, very little pentacarboxylic porphyrin I and III, and traces of uroporphyrin I making up the rest (Table 2.2.). Three unknown peaks which were very small appeared before and after coproporphyrin isomers (Fig. 2.9). The porphyrin concentrations obtained in consecutive 30 min
Fig. 2.6. Urinary Porphyrin Excretion in Congenital Erythropoietic Porphyria Treated with Blood Transfusions and Oral Charcoal

The peaks of the haematocrit plot indicate transfusion of 2-3 unit of packed red cells.
Fig. 2.7. Uroporphyrin I Levels in Plasma and Erythrocytes after Oral Charcoal

Blood samples were obtained before the first dose of charcoal and at 3 hour intervals during the administration of 10g doses at 4 hour intervals to a total of 50g.

The average of erythrocyte uroporphyrin I before charcoal treatment was $6.06 \pm 2.57 \mu\text{mol/l}$ (mean $\pm$ SD) and during the first 25 h after charcoal treatment was $3.93 \pm 0.34 \mu\text{mol/l}$ (mean $\pm$ SD); the average of plasma uroporphyrin I before charcoal treatment was $2.45 \pm 0.57 \mu\text{mol/l}$ (mean $\pm$ SD) and during the first 25 h after charcoal treatment was $2.07 \pm 0.298 \mu\text{mol/l}$ (mean $\pm$ SD).
Fig. 2.8. Plasma Uroporphyrin I Levels in a Case of Congenital Erythropoietic Porphyria Treated with Blood Transfusions and Oral Charcoal

Slight reduction of the high levels of plasma uroporphyrin is apparent in the week following blood transfusions, but there was no further reduction during the 21 days of charcoal administration. The average of the plasma uroporphyrin I before charcoal treatment was 2.45 ± 0.57 µmol/l (mean ± SD) and after charcoal treatment was 2.16 ± 0.32 µmol/l (mean ± SD).
Table 2.2. Porphyrins in Duodenal Aspirates of the Patient with Congenital Erythropoietic Porphyria

<table>
<thead>
<tr>
<th>Total Porphyrin (nmol/l)</th>
<th>Porphyrin Levels* (nmol/min)</th>
<th>Porphyrins (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4-I</td>
<td>4-III</td>
</tr>
<tr>
<td>1. 229</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>2. 100,386</td>
<td>4,206</td>
<td></td>
</tr>
<tr>
<td>3. 20,738</td>
<td>782</td>
<td></td>
</tr>
</tbody>
</table>

*At distal port: porphyrin concentration (nmol/ml) x "flow rate" (ml/min).
Flow rate was calculated as described in Sec. 2.3.3.

1., 2., and 3.: samples collected from three consecutive 30 min periods (see Sec. 2.3.3.).
Fig. 2.9. HPLC Separation of Duodenal Aspirate Porphyrin of a Patient with CEP

Peak 1, pentacarboxylic acid porphyrin I; peak 2, pentacarboxylic acid porphyrin III; peak 4, coproporphyrin I; peak 5, coproporphyrin III; peak 3, 6 and 7, unknown.
samples were consistent with the onset of contraction of the
gallbladder with the peak porphyrin concentration occurring
during the second 30 min period. The quantity in this sample
was so great that coproporphyrin crystallised out on
cooling.

The addition of oral charcoal for 21 days in a dose
close to the level of tolerability failed to reduce
porphyrin levels or to improve this patient's clinical
status. A previous report of benefit sustained over a period
of 9 months in a case of "congenital erythropoietic
porphyria" is not directly comparable since that patient
had deficiency of Uro-D characteristic of porphyria cutanea
tarda (Pimstone et al., 1987) as well as a congenital
dyserythropoietic anaemia (Kushner et al., 1982), and not
classical Gunther's disease. It seems unlikely that major
differences in response depend on the particular brand of
charcoal used, since although differences in binding
affinities can be demonstrated in vitro (Tishler & Winston,
1985), the binding of porphyrins to charcoal in the faeces
is so firm that quantitative extraction is not possible even
under the most rigorous conditions, including the use of
concentrated HCl or in situ methylation. Differences in
response may in part be related to the quantity of charcoal
used and to the frequency of its administration, thus
180g/day of Acta Char was ingested by the patient as
described by (Pimstone et al., 1987). An additional factor
may be the porphyrin load, and it is notable that in the
patient described here the plasma and urinary levels were
higher than in previously reported cases treated with charcoal or hypertransfusion (Table 2.3.), although similar values have previously been reported (Ippen & Fuchs, 1980). More importantly, however, it would seem likely that any benefit of charcoal would depend on the extent of the enterohepatic circulation of porphyrins and thus also on the proportion of hydrophilic porphyrins in the plasma. Pimstone et al. (1987) measured duodenal porphyrin concentration and flow rates after contracting the gallbladder with cholecystokinin, as a measure of hepatic secretion rate, and found that approximately 95% of the porphyrins measured at the ampulla of Vater were reabsorbed 15 cm distally. In the present study the porphyrin level at the ampulla was not measured because the proximal aspiration port was just rostral, but the very high concentrations obtained 15 cm distally make it unlikely that such a high proportion could already have been reabsorbed in this short portion of the gut under our more "physiological" conditions of spontaneous gallbladder function. The values obtained indicate minimum values of 150 μmoles (about 100 mg) of porphyrins excreted into the gut in this 90 min period, compared with 200 mg/24h calculated by Pimstone et al. (1987) for their case. Even on the conservative assumption that there may be only 7 equivalent cycles of gallbladder discharge per 24h, up to 1 mmole or 650 mg of porphyrins would be excreted into the gastrointestinal tract in this patient. From the porphyrin content of his faecal samples (3430 nmoles/g dry weight), of which more than 95% was
Table 2.3. Porphyrin Levels in Congenital Erythropoietic Porphyria

<table>
<thead>
<tr>
<th>Plasma (µmol/l)</th>
<th>Urine (µmol/24h)</th>
<th>Literature Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.56-0.88</td>
<td>23.3-58.3</td>
<td>Piomelli et al. (1986)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.33</td>
<td>18.7-21.0</td>
<td>Pimstone et al. (1987)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.51</td>
<td>96.8</td>
<td>Tishler &amp; Winston (1990)&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.53-4.03</td>
<td>79-283</td>
<td>This case&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.50-6.65</td>
<td>38.5-171*</td>
<td>Ippen &amp; Fuchs (1980)</td>
</tr>
</tbody>
</table>

*: µmol/l; a: transfused; b: oral charcoal.
coproporphyrin I, and total dry weight (25-30 g/24h) reabsorption of up to 90% can be deduced, consistent in this case also with a major degree of enterohepatic circulation of porphyrins. It is notable also that the stool porphyrin concentration reported here is 10 times greater than that found by Tishler & Winston, (1985). Uroporphyrin itself, however, does not reach the gastrointestinal tract in significant amounts and was not detected in duodenal aspirates (Pimstone et al., 1987) or in bile (Mukerji et al., 1985), and lack of significant uroporphyrin excretion in duodenal aspirates was also confirmed in our patient. Binding of porphyrins in the gut lumen, with interruption of their enterohepatic circulation, could favour decarboxylation of uroporphyrinogen, resulting in an increase in less polar porphyrins excreted in bile, with a fall in plasma uroporphyrin. In our case, uroporphyrin accounted for 75% of the plasma porphyrin compared with only 6.9%, or up to 24% including heptacarboxylic porphyrins in the case of Pimstone et al. (1987). These differences could in part explain the apparent lack of response to charcoal in this patient.

The present study does not support previous suggestive findings that oral charcoal may be of value in the symptomatic treatment of congenital erythropoietic porphyria. Oral charcoal given for 31 days in another patient with "probable" congenital erythropoietic porphyria (Tishler & Winston, 1990) reduced the plasma and erythrocyte levels of porphyrin but no clinical benefit was apparent. In
another report of a 12 year old boy with congenital erythropoietic porphyria (Hift et al., 1990), charcoal reduced urine, plasma and erythrocyte porphyrin levels during a 6 months treatment period, but no details were given of any improvement in clinical symptoms.

Clear distinction has not previously been made in the literature between the report of dramatic beneficial effect of charcoal in a single case of congenital erythropoietic porphyria due to deficiency of uroporphyrinogen decarboxylase (Pimstone et al., 1987) and those with the classic deficiency of Uro'gen III-S (Gunther's disease). It may be appropriate to refer to the classical disease as congenital erythropoietic porphyria and to the other as hepatoerythropoietic porphyria. Evidence of clinical benefit of charcoal administration in CEP is not available. The reasons for these differences have been discussed above.

2.5. Conclusions

Oral charcoal was of no value in the symptomatic treatment of a patient with congenital erythropoietic porphyria (Gunther's disease). This was probably because the major porphyrin in plasma, uroporphyrin I which is believed to play an important role in the disorder, was not excreted in bile and interruption of the enterohepatic circulation of the large load of coproporphyrin by binding to charcoal was insufficient to shift the equilibrium from uroporphyrinogen I to coproporphyrinogen I with a resulting decrease in plasma uroporphyrin.
3.1. Introduction

One of the characteristic features of congenital erythropoietic porphyria (CEP) is the excretion of a large amount of uroporphyrin I in the urine. Thin-layer chromatography (TLC) of the porphyrin methyl esters isolated from the urine of CEP patients consistently showed the presence of porphyrins migrating below the major uroporphyrin band (Fig. 3.1). These polar porphyrins, often called sub-uroporphyrins, are thought to be partially hydrolysed porphyrin methyl esters. Analysis of the underivatized urinary porphyrins in these patients by high-performance liquid chromatography, however, still showed the presence of polar porphyrins eluted before uroporphyrin and near the solvent front (Fig. 3.2). These observations would suggest that the polar porphyrins detected by TLC and HPLC are most probably unidentified porphyrin metabolites. This chapter describes in detail the isolation, identification and characterization of new porphyrin metabolites in the urine and plasma of patients with CEP. An attempt has also been made to correlate the presence of these metabolites with uroporphyrinogen III synthase (Uro III-S) activity and the concentrations of porphyrins in the urine and blood of CEP patients.
Fig. 3.1. TLC Separation of Urinary Porphyrin Methyl Esters from a Patient with CEP.
Fig. 3.2. HPLC Separation of Urinary Porphyrin of a Patient with CEP.

Peak 1, uroporphyrin I; peak 2, heptacarboxylic acid porphyrin I; peak 3, hexacarboxylic acid porphyrin isomers; peak 4, pentacarboxylic acid porphyrin I; peak 5, coproporphyrin I.
3.2. Experimental

3.2.1. Materials and reagents

Uro I and III octamethyl esters were from Sigma Chemical Co. (Poole, Dorset, U.K.). Thallic trifluoroacetate was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Ammonium acetate, acetic anhydride, glacial acetic acid, ethylenediaminetetraacetic acid (EDTA), zinc acetate, trichloroacetic acid, I₂, concentrated HCl, concentrated H₂SO₄, NaHCO₃, KOH, NaOH, Ca(OH)₂, metallic sodium, mercury, dimethylsulphoxide (DMSO), carbon tetrachloride, chloroform, dichloromethane, dimethylformamide (DMF), tetrahydrofuran, SO₂ gas and pyridine were Analar grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile, ethyl acetate, ethanol and methanol were HPLC grade from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland, U.K.). Alumina 90 (70-230 mesh) for column chromatography was from E. Merck (Darmstadt, Germany). C₁₈ Bond Elut extraction cartridge was from Analytichem International (Habor City, CA, USA.).

3.2.2. Separation and isolation of the new porphyrin metabolites in the urine of patients with CEP by HPLC

The HPLC system consisted of a Varian Associates (Walton-on-Thames, Surrey, UK) model 5000 pump and a Varian UV-100 variable wavelength detector set at 400 nm. The acidified urine (200 µl portions) was directly injected by a Rheodyne (Cotati, CA, USA) 7125 injection valve fitted
with a 500 µl loop. The separation was carried out on a 25 cm x 25 mm Hypersil-ODS column (Shandon Scientific, Runcorn, Cheshire, UK) by gradient elution. The gradient mixtures were 9% (v/v) acetonitrile in 1 M ammonium acetate buffer pH 5.16 (Solvent A) and 10% (v/v) acetonitrile in methanol (solvent B). The elution program was as follows. Time 0 to 16 min, 0% solvent B (100% solvent A) to 11% solvent B; time 16 to 24 min, isocratic elution at 11% solvent B; time 24 to 38 min, 11% solvent B to 24% solvent B; time 38 to 60 min, 24% solvent B to 60% solvent B and time 60 to 80 min, 60% solvent B to 80% solvent B. The flow rate was 1 ml/min throughout. The column was then re-equilibrated at 0% solvent B for 10 min before injection of next sample. The chromatogram is shown in Fig. 3.3. The peaks 1, 2, 3, 4, 5 and 6 were collected into individual flasks containing little EDTA solid. The acetonitrile and methanol content in the eluates were removed by evaporation at 30-40°C under nitrogen. The purity of the isolated porphyrins was checked by HPLC. Repurification was carried out if necessary.

3.2.3. Solid-phase extraction of the urinary porphyrins

After evaporating the acetonitrile and methanol by heating at 30-40°C under N₂, the individual peak collection was mixed with a equal volume of 0.5 M ammonium acetate buffer, pH 5.16 to further dilute any residual organic solvent. The mixture was loaded under vacuum into a C₁₈ Bond Elut cartridge which had been pre-washed successively with 5 ml of methanol and 3 x 5 ml of 0.5 M ammonium buffer, pH
Fig. 3.3. HPLC Separation of Urinary Porphyrin of a Patient with CEP.
5.16. The cartridge was washed with 2 x 5 ml of 0.5 M ammonium acetate buffer, pH 5.16. The porphyrins retained on the cartridge were then recovered by elution with methanol : acetonitrile (v/v, 1:1) until little UV fluorescence remained on the cartridge. The eluent was evaporated to dryness under nitrogen at 30-40°C.

3.2.4. Esterification of porphyrins

The porphyrins were dissolved in 2 ml of 10% (v/v) conc. $\text{H}_2\text{SO}_4$ in methanol or ethanol and the solution was left standing for 20 h at 4°C in the dark. The mixture was then transferred into a separating funnel containing chloroform (3 ml) and extracted rapidly with 6 ml water. The chloroform layer was washed successively with aqueous saturated sodium bicarbonate solution (5 ml) and water (5 ml). The organic layer was filtered through a 5.5 cm filter paper (Whatman No.1) pre-wetted with chloroform and the filtrate was evaporated to dryness at 30°C under nitrogen.

3.2.5. Acetylation of hydroxylated porphyrins

The method of Jackson et al. (1968) was used for the preparation of meso-acetoxypropoporphyrin III. The porphyrin methyl ester was redissolved in 200 µl of pyridine and 50 µl of acetic anhydride, mixed thoroughly and stood at room temperature for two hours in the dark. The solution was evaporated to dryness at 30-40°C under nitrogen. The residue was analysed by mass spectrometry.
3.2.6. Hydrolysis of porphyrin esters

Porphyrin methyl or ethyl esters were dissolved in 25% of HCl and then hydrolysed in the dark at room temperature for 72 hours. The esters may also be dissolved in conc. HCl and then diluted to 25% with water.

3.2.7. Reduction and aromatization of hydroxylated porphyrins

The free porphyrin was dissolved in 0.01 M KOH, flushed with nitrogen and stoppered. The porphyrin was reduced with 3% (w/w) sodium amalgam until no UV fluorescence was detectable. The resulting solution was transferred into a clean tube, and aromatized by adding an equal volume of a saturated solution of I₂ in 10% trichloroacetic acid. The mixture was allowed to stand in room temperature for 5 min and then analysed by HPLC.

3.2.8. Synthesis of meso-hydroxyuroporphyrin I and III

The procedure used was similar to that described for the synthesis of meso-hydroxycoproporphyrin I (Barnett et al., 1973). Zinc (II) uroporphyrin I and III octamethyl estes were prepared by treating the porphyrin ester in chloroform with zinc acetate in methanol at 50°C for 10 min as described by Fuhrhop & Smith (1975). Any excess zinc acetate was washed away with water at the end of the reaction. The organic layer containing zinc uroporphyrin was collected after filtering through a filter paper pre-wetted with chloroform. The filtrate was evaporated to dryness under nitrogen at 30-40°C.
The resulting zinc (II) uroporphyrin I or III octamethyl ester (app. 2 mg) in 2.5 ml of dichloromethane was mixed with thallic trifluoroacetate (3 mg) in 1 ml of tetrahydrofuran, and stirred for 1 min. Water (50 µl) in tetrahydrofuran (1 ml) was added, and the mixture was stirred for a further 10 min. The solution was treated briefly with SO₂ gas followed by addition of 100 µl concentrated HCl. After being stirred for 5 min the mixture was poured into water (20 ml) and extracted with dichloromethane (10 ml). The organic layer was filtered through a filter paper pre-wetted with chloroform. The filtrate was evaporated to dryness under N₂. The residue was redissolved in chloroform and loaded into a Pasteur pipette half-filled with alumina 90 which was previously washed with chloroform. The column was eluted with chloroform (10 ml) and then 10 ml of chloroform/methanol (1:1, v/v). The chloroform/methanol fraction contained meso-hydroxyuroporphyrin octamethyl ester. The solvent was removed by evaporation under N₂. The residue was hydrolysed with 25% HCl and purified by HPLC as described in Sec. 3.2.6. and 3.2.2., respectively.

3.2.9. Dehydration of the hydroxylated uroporphyrin methyl esters by Heating

The porphyrin methyl ester was dissolved in 0.5 ml dimethylformamide in a Pyrex tube. The solution was flushed with nitrogen and the tube was capped and heated at 135°C for 2 hours in the dark. After cooling to room temperature, the solution was transferred into a
separating funnel containing chloroform (1 ml). The mixture was extracted after water (2 ml) was added. The chloroform layer was passed through a filter paper pre-wetted with chloroform. The filtrate was evaporated to dryness at 30°C under nitrogen. The residue was analysed by mass spectrometry.

3.2.10. Dehydration and decarboxylation of hydroxylated uroporphyrins by heating

The porphyrin free acid was dissolved in 0.5 M HCl in a Pyrex tube, flushed with nitrogen, capped and then heated at 135°C for 30 min or 1 h. After cooling to room temperature, the solution was analysed by HPLC.

3.2.11. Alkaline hydrolysis of peroxyacetic acid uroporphyrin I

Peroxyacetic acid uroporphyrin I was dissolved in 0.1 M KOH, NaOH or Ca(OH)$_2$ and the solution was heated at 30-40°C for 5 min. The pH of the solution was adjusted to between 5-7 and the product was analysed by HPLC.

3.2.12. Separation and isolation of the new porphyrin metabolites in the plasma of patients with CEP by HPLC

The plasma was prepared as described in Sec. 2.3.5.5. For larger scale extraction of the new porphyrin metabolites, the pooled plasma (20 ml) was vortex-mixed with the equal volume of 20% TCA (w/v)-DMSO (v/v, 1:1). After centrifugation the supernatant was collected. The residue was again vortex-mixed with 5 ml of 20% TCA-DMSO (v/v, 1:1),
centrifuged and the supernatant was collected. The pooled supernatants were diluted 1:15 (v/v) with 0.5 M ammonium acetate buffer (pH 5.16) and loaded under suction onto a C\textsubscript{18} Bond-Elut Cartridge. The porphyrins were then extracted as described in Sec. 3.2.3.

The porphyrins were re-dissolved in 0.3 M HCl and separated by HPLC. The HPLC condition and the elution program were the same as described in Sec. 3.2.2. except that any peaks eluted after 30 min were washed out by 95% solvent B. The individual peaks 1-7 (Fig. 3.4) were collected into separate flasks containing little EDTA solid. The organic solvent was removed and the porphyrins were recovered by solid-phase extraction on C\textsubscript{18} Bond Elut cartridges as described in Sec. 3.2.3.

3.3. Spectrophotometry

A Kontron Uvikon 860 spectrophotometer operated in Lambda-scan absorption mode with scanning wavelengths from 250 to 700 nm was used for recording the electronic spectra. Both H (quartz-halogen) and D\textsubscript{2} (deuterium) lamps were on with lamp change at 340 nm. The porphyrin free acids were dissolved in 0.5M HCl for peaks 1-4 and 0.1M HCl for peaks 5 and 6 (Fig. 3.3.). Peak detection and trace area functions were used to identify the exact wavelength of the peaks as required.
Fig. 3.4. HPLC Separation of Plasma Porphyrin of a Patient with CEP.
3.4. Liquid Secondary Ion Mass Spectrometry (LSIMS)

A VG Analytical (Manchester, U.K.) ZAB2-E mass spectrometer, operated at 8 KeV accelerating voltage and fitted with a caesium gun (35 KeV, 0.5 μA emission) was used for LSIMS. Porphyrin esters were dissolved in a small volume (ca. 10 μl) of chloroform:methanol (2:1, v/v) and 1 μl was added to the standard stainless steel LSIMS target probe previously prepared with thioglycerol (1 μl) as liquid matrix. Mass spectra were acquired in the positive ion mode at 30 s/decade using the VG Analytical 11-250J data system in continuous multichannel analysis mode at a resolution of 1500 RP.

Accurate mass measurement was carried out using peak matching at a resolution of 7000 RP. Internal reference masses were produced by adding uroporphyrin I octamethyl ester and aqueous sodium iodide solution to the sample. The protonated and sodiated molecular species ([MH]+ 943.3613 and [MNa]+ 965.3433) were used as reference peaks.

3.5. Results and Discussion

3.5.1. HPLC of polar porphyrins in the urine of a patient with CEP

Fig. 3.2. has clearly shown that there are unknown polar porphyrins eluted before uroporphyrin. The gradient elution programme was therefore modified so that these porphyrins could be separated. The HPLC separation is shown
in Fig. 3.3. There were six unidentified porphyrins, numbered 1-6, detected and their identification and characterization are described in the following sections.

3.5.2. Identification of meso-hydroxyuroporphyrin I

Peak 1 (Fig. 3.3.) had a characteristic pink porphyrin fluorescence. The mass spectra of the methyl and ethyl esters gave the MH⁺ signals at m/z 959 and 1071, respectively (Fig. 3.5. and 3.6.), indicating the presence of eight carboxylic acid groups and an increase of 16 mass unit over Uro octamethyl ester (MH⁺, m/z 943) and octaethyl ester (MH⁺, m/z 1055), respectively. This indicated that the compound is closely related to uroporphyrin and strongly pointed to a substitution of uroporphyrin by a hydroxy group.

To confirm the presence of -OH group, the octamethyl ester of peak 1 was treated with acetic anhydride in pyridine. The acetyl derivative was analysed by LSIMS and a MH⁺ signal at m/z 1001 (Fig. 3.7.), an increase of 42 mass unit, was obtained confirming the presence of a hydroxy group.

Accurate mass measurement of the methyl esters of peak 1, with uroporphyrin I octamethyl ester and aqueous sodium iodide solution as the internal reference, were performed. The methyl ester gave MH⁺ 959.3532, only 1.0 ppm less than the theoretical value of 959.3562 for C₄₈H₅₅O₁₇N₄, the elemental formula of a hydroxylated uroporphyrin.
Fig. 3.5. LSIMS of Meso-Hydroxyuroporphyrin I Methyl Ester
Fig. 3.7. LSIMS of Acetylated Meso-Hydroxyuroporphyrin I Methyl Ester
Assuming that the hydroxylated porphyrin is a uroporphyrin I derivative, there are four possible positions in which an -OH group can be attached to the uroporphyrin I structure (Fig. 3.8.). These are meso-hydroxy-, \( \beta \)-hydroxypropionic acid-, hydroxyacetic acid- and N-hydroxy-derivatives. To identify the position of the hydroxy group in peak 1, the free porphyrin in 0.1 KOH was reduced with 3% (w/w) sodium amalgam followed by aromatization with \( I_2 \). The reaction gave Uro I as the only product when analysed by HPLC and LSIMS. The reaction proved that the hydroxy group was located at the meso-position (Jackson et al., 1968) and established that the porphyrin was the type I isomer. The structure is shown in Fig. 3.8.

That the assigned structure was correct was confirmed by synthesis of meso-hydroxyuroporphyrin I by means of the thallic trifluoroacetate method (Barnett et al., 1973) described for the preparation of oxophlorins. The synthetic meso-hydroxyuroporphyrin I had exactly the same retention time as peak 1 under various HPLC conditions. It gave the same molecular weights for its methyl ester and acetoxy derivative as the natural peak 1 when analysed by LSIMS. When the synthetic meso-hydroxyuroporphyrin I was reduced with 3% Na/Hg followed by aromatization with \( I_2 \) it also gave Uro I as the only product. This further proved that peak 1 is meso-hydroxyuroporphyrin I.
Fig. 3.8. Structures of Hydroxylated Uroporphyrin I Derivatives

I, meso-hydroxyuroporphyrin I; II, meso-hydroxyuroporphyrin III; III, N-hydroxyuroporphyrin I; IV, β-hydroxypropionic acid uroporphyrin I; V, hydroxyacetic
3.5.3. Identification of β-hydroxypropionic acid uroporphyrin I

Peak 2 (Fig. 3.3.) also has a characteristic porphyrin fluorescence. The mass spectrum of the methyl ester showed a MH\(^+\) signal at m/z 959 and that of the ethyl ester showed a MH\(^+\) signal at m/z 1071. This was similar to the meso-hydroxyuroporphyrin I and indicated that the compound also had eight carboxylic acid groups and was probably an isomer of meso-hydroxyuroporphyrin I. An increase of 16 mass unit over Uro octamethyl ester (MH\(^+\), m/z 943) and octaethyl ester (MH\(^+\), m/z 1055) suggested substitution by a -OH group. The methyl ester gave an accurate mass of MH\(^+\) 959.3544, 1.9ppm less than the theoretical value of 959.3562 for C\(_{48}\)H\(_{55}\)O\(_{17}\)N\(_4\), the elemental formula of a hydroxylated uroporphyrin. Acetylation of the methyl ester gave a MH\(^+\) signal at m/z 1001 when analysed by LSIMS. An increase of 42 mass confirmed the presence of a -OH group.

Peak 2 (Fig. 3.3.) was eluted after meso-hydroxyuroporphyrin I. Since in reversed-phase HPLC uroporphyrin I eluted before uroporphyrin III, it is possible that peak 2 is the type III isomer of meso-hydroxyuroporphyrin I. Meso-hydroxyuroporphyrin III was therefore synthesized and analysed by HPLC. It was eluted immediately after meso-hydroxyuroporphyrin I (Fig. 3.9.) and was clearly resolved from peak 2. This rules out the possibility that peak 2 is the type III isomer of meso-hydroxyuroporphyrin I. There are four possible isomers of meso-hydroxyuroporphyrin III. Under the HPLC conditions
Fig. 3.9. HPLC Separation of Synthetic Meso-Hydroxyuroporphyrin I and III.

Peak 1, meso-hydroxyuroporphyrin I; peak 2, meso-hydroxyuroporphyrin III; peak 3, Uro I; peak 4, Uro III; peak 5, late eluting peaks.
used, no separation of these type III isomers was achieved.

Since peak 2 is not meso-hydroxyuroporphyrin III, the other three hydroxylated uroporphyrin I structures (Fig. 3.8) have to be considered. Reduction of peak 2 with Na/Hg followed by re-oxidation with I$_2$ gave a signal of Uro I (MH$^+$ 943), a dehydration product (MH$^+$ 941) with substantially unchanged material (MH$^+$ 959) when the crude reaction product was analysed by LSIMS (Fig. 3.10.). This suggested that the compound was derived from Uro I and the $-$OH group was situated in a position which was easily dehydrated. The most probable structure is $\beta$-hydroxypropionic acid uroporphyrin I (Fig. 3.8.), since only a $-$OH group on the $\beta$-position of propionic acid can be easily dehydrated. A $-$OH group on the pyrrole N position is unstable while a $-$OH group on the acetic acid function cannot be dehydrated. The methyl ester of peak 2 was subjected to dehydration by heating in dimethylformamide (DMF) and the product analysed by LSIMS. The product gave a MH$^+$ signal at m/z 941 (959-18) (Fig. 3.11.) clearly showing dehydration of the parent compound. The porphyrin free acid of peak 2 was also subjected to dehydration by heating. It gave two products when analysed by HPLC (Fig. 3.12). Analysis by LSIMS showed that apart from the dehydration product (MH$^+$ 941) a decarboxylation derivative was also found. This derivative has a MH$^+$ signal at m/z 883 and a possible structure is shown in the reaction scheme in Fig. 3.13. The above HPLC, chemical and LSIMS evidences all pointed to peak 2 being $\beta$-hydroxypropionic acid uroporphyrin I.
Fig. 3.10. LSIMS of The Reaction Product Following Reduction With Na/Hg And Aromatization With I₂ Of β-Hydroxypropionic Acid Uroporphyrin I
Fig. 3.11. LSIMS of Dehydration Product of β-Hydroxypropionic Acid Uroporphyrin I Methyl Ester
Fig. 3.12. HPLC Separation of Dehydration and Decarboxylation of β-Hydroxypropionic Acid Uroporphyrin I

Peak 1, β-hydroxypropionic acid uroporphyrin I; peak 2, dehydration product of β-hydroxypropionic acid uroporphyrin I; peak 3, dehydration and decarboxylation product of β-hydroxypropionic acid uroporphyrin I.
Fig. 3.13. Reaction Scheme of Dehydration and Decarboxylation β-Hydroxypropionic Acid Uroporphyrin I
3.5.4. Identification of hydroxyacetic acid uroporphyrin I

Peak 3 (Fig. 3.3.) has the characteristic porphyrin fluorescence. The mass spectra of the methyl and ethyl esters showed MH\(^+\) signals at m/z 959 and 1071, respectively, similar to those of meso-hydroxy- and \(\beta\)-hydroxypropionic acid-uroporphyrin I. This compound must be therefore also a hydroxylated uroporphyrin. Accurate mass measurement for the methyl ester gave MH\(^+\) 959.3557, 0.5 ppm less than the theoretical value of 959.3562 for C\(_{48}\)H\(_{55}\)O\(_{17}\)N\(_4\), the molecular formula of a hydroxylated uroporphyrin. Acetylation of the methyl ester gave a MH\(^+\) signal at m/z 1001 when analysed by LSIMS. An increase of 42 mass unit corresponded to an acetoxy derivative and confirmed the presence of a \(-OH\) group. Thus peak 3 is an isomer of meso-hydroxy- and \(\beta\)-hydroxypropionic acid-uroporphyrin I. Reduction with Na/Hg followed by re-oxidation with I\(_2\) gave only a trace of Uro I and largely unchanged material when the product was analysed by HPLC and LSIMS. The formation of Uro I showed that this compound was also derived from Uro I.

The methyl ester of peak 3 was subjected to dehydration by heating in dimethylformamide (DMF) and then analysed by LSIMS and HPLC. No dehydration product was detected. This indicated that the \(-OH\) group was substituted at a position which was difficult or not possible for it to be dehydrated. The only possible structure for this to occur is with the hydroxy group located on an acetic acid substituent.
To further confirm that peak 3 cannot be dehydrated the porphyrin free acid was subjected to heating in 0.5 M HCl and the product analysed by HPLC and LSIMS. HPLC analysis showed that two products were formed (Fig. 3.14) and both compounds had an identical MH⁺ signal at m/z 901 (Fig. 3.15. & 3.16.). This indicated decarboxylation of the hydroxylated uroporphyrin I without dehydration, confirming that the -OH group is located on the acetic acid function. The structures of the two decarboxylated hydroxyacetic acid uroporphyrin I, hydroxyacetic acid heptacarboxylic porphyrin I, are shown in Fig. 3.17. Since Uro I is a symmetrical molecule there can be only one hydroxyacetic acid Uro I derivative and there are two possible ways of decarboxylating the hydroxyacetic acid Uro I. The decarboxylation products are expected to form in a ratio of 2:1 as clearly shown by the HPLC analysis.

3.5.5. Identification of peroxyacetic acid uroporphyrin I

Peak 4 is different from the above three hydroxylated uroporphyrin I derivatives in that it has a greenish-yellow colour instead of the purple-brown colour, but like the hydroxylated derivatives it gave a characteristic red porphyrin fluorescence.

The mass spectrum of the methyl ester gave a MH⁺ signal at m/z 945 (Fig. 3.18.), an increase of 2 Da over uroporphyrin octamethyl ester. There are four possible structures in which the MH⁺ signal is m/z 945. These are
Fig. 3.14. HPLC Separation of Decarboxylation Hydroxyacetic Acid Uroporphyrin I

Peak 1, hydroxyacetic acid uroporphyrin I; peak 2 & 3, decarboxylation products of hydroxyacetic acid uroporphyrin I.
Fig. 3.15. LSIMS of Hydroxylated Heptacarboxylic Acid Porphyrin I Methyl Ester
Fig. 3.17. Structures of Hydroxyheptacarboxylic Acid Porphyrin I Isomers
Fig. 3.18. LSIMS of Peroxyacetic Acid Uroporphyrin I Methyl Ester
phlorin, thioacid-, peroxyacetic acid- and peroxypropionic acid-uroporphyrin I (Fig. 3.19).

A phlorin structure is easily oxidised to the corresponding porphyrin by various oxidizing agents, for example, air, iodine or chloranil (Woodward, 1962). Since peak 4 was very stable under aerobic condition and could not be oxidised to the porphyrin by I$_2$ (freshly prepared iodine saturated in 10% TCA), the phlorin structure can be ruled out.

The ethyl ester of peak 4 showed the MH$^+$ signal at m/z 1043 (Fig. 3.20.), representing an increase of seven methylene units over methyl ester. This clearly showed that the compound possessed seven and not eight carboxylic acid groups. Thus one of the side-chain carboxylic acid groups of uroporphyrin has been modified and the thioacid, peroxyacetic acid and peroxypropionic acid structures are all possible.

Since it is possible to differentiate between a thioacid and a peroxyacid structure by high resolution mass spectrometry, the accurate mass of the methyl ester was measured. The accurate mass of the methyl ester gave a MH$^+$ signal at m/z 945.3416, very close (+1.0 ppm) to the theoretical value of 945.3406 for C$_{47}$H$_{53}$O$_{17}$N$_4$, the elemental formula of a peroxyacid uroporphyrin. For a
Fig. 3.19. Possible Structures of Uroporphyrin I Derivatives with MH+ at m/z 945
Fig. 3.20. LSIMS of Peroxyacetic Acid Uroporphyrin I Ethyl Ester
or a phlorin structure (C_{48}H_{57}O_{16}N_{4})", the measured mass units were +19.8 ppm and -37.6 ppm of the theoretical values, respectively. Peak 4 was therefore established as a peroxyacid uroporphyrin I.

Acetylation of the methyl ester with pyridine and acetic anhydride was achieved only with difficulty. The mass spectrum of the acetylated product showed a MH^+ 987 signal (Fig. 3.21.) but substantial starting material (MH^+ 945) remained. This is in contrast to the hydroxylated derivatives of uroporphyrin I which were easily acetylated and is consistent with a peroxyacid structure.

To confirm that peak 4 is peroxyacid uroporphyrin, this compound was treated by dilute alkali, such as 0.1M KOH and Ca(OH)₂. The compound was completely converted into Uro I which was identified by HPLC and mass spectrometry. Peak 4 was therefore clearly peroxyacid uroporphyrin I. Further confirmation was obtained by heating the compound in dilute HCl in the dark when Uro I was formed as the only product.

Peroxylation of a carboxylic acid group may take place on either an acetic acid or a propionic acid group. The final structural assignment requires nuclear magnetic resonance (NMR) spectroscopy. Since the compound was not present in sufficient quantity in the urine, insufficient sample had been isolated for NMR studies. At present, the
Fig. 3.21. LSIMS of Acetylated Derivative of Methyl Esters of Peroxyacetic Acid Uroporphyrin I Methyl Ester
peroxyacetic acid uroporphyrin I structure is favoured. An attempt to isolate an analogous coproporphyrin derivative in the urine and faeces of the patients with hereditary coproporphyria containing a high concentration of coproporphyrin was unsuccessful. This indicated that the peroxylolation reaction \textit{in vivo} may take place only on acetic acid group and not on propionic acid group. The studies on the mechanism of formation of peroxyacid porphyrins in Chapter 5 have, however, confirmed the peroxyacetic acid structure.

3.5.6. Identification of hydroxyheptacarboxylic acid porphyrins in the urine of patients with CEP

Fig. 3.3. has clearly shown that there were two other peaks, 5 and 6, eluted between uroporphyrin and heptacarboxylic acid porphyrin. The mass spectra of the methyl esters and ethyl esters of these two compounds were identical, with the MH$^+$ signal at m/z 901 and 999 (Fig. 3.22.), respectively. This is an increase of 16 Da over the methyl and ethyl esters, respectively, of heptacarboxylic acid porphyrin (MH$^+$, m/z 885 and 983) indicated that they were hydroxylated heptacarboxylic porphyrins. When peak 5 and 6 were subjected to reduction with Na/Hg followed by oxidation with saturated I$_2$ in 10% TCA, no significant change was found as analysed by HPLC. These closely resembled peak 2, \(\beta\)-hydroxypropionic acid uroporphyrin I and peak 3, hydroxyacetic acid uroporphyrin I.
Fig. 3.22. LSIMS of Hydroxylated Heptacarboxylic Acid Porphyrin Methyl and Ethyl Ester
Since the compounds were not converted to heptacarboxylic porphyrin I, they are most probably and $\beta$- and $\alpha$-hydroxypropionic acid hydroxyacetic acid heptacarboxylic porphyrin I. Peak 6 was confirmed as hydroxyacetic acid heptacarboxylic acid porphyrin I by heating hydroxyacetic acid uroporphyrin I when a compound with identical retention time to peak 6 was formed (Fig. 3.23.). Peak 5 is most probably $\beta$-hydroxypropionic acid heptacarboxylic porphyrin I as when $\beta$-hydroxypropionic acid uroporphyrin I was heated and analysed by HPLC a peak corresponding to peak 5 was detected together with its dehydration and decarboxylation products (Fig. 3.24.).

3.5.7. Identification of hydroxy- and peroxyacetic acid-uroporphyrin I derivatives in the plasma of patients with CEP

The HPLC separation of porphyrins in the plasma of patients with CEP is shown in Fig. 3.4.. There were seven peaks eluted before Uro I. Peaks 1 and 4 had not been identified because they were not found in the urine of the patients with CEP and sufficient amounts for isolation and positive identification were not available. Peak 8 was the large amount of uroporphyrin I present in the plasma.

Peak 2 co-eluted with synthetic meso-hydroxyuroporphyrin I under all HPLC conditions. It was therefore identified as meso-hydroxyuroporphyrin I. This was confirmed by sodium amalgam reduction of the compound followed by aromatization with iodine, which eliminated
peak 1, hydroxyacetic acid uroporphyrin I;
peak 2 & 3, decarboxylation products of
hydroxyacetic acid uroporphyrin I.
Peak 2 had the same retention time
as (b) peak 6.

peak 1, meso-hydroxyuroporphyrin I
peak 2, B-hydroxypropionic acid uroporphyrin I
peak 3, hydroxyacetic acid uroporphyrin I
peak 4, peroxyacetic acid uroporphyrin I
peak 5, B-OH propionic acid-7COOH porphyrin I
peak 6, hydroxyacetic acid-7COOH porphyrin I

Fig. 3.23. HPLC Separation of Decarboxylated Products of Hydroxyacetic Acid
Uroporphyrin I (a) Comparing with Urinary Porphyrin of a Patient with CEP (b)
peak 1, $\beta$-OH propionic acid uroporphyrin I
peak 2, dehydration product
peak 3, decarboxylation product
peak 4, dehydration and decarboxylation product
Peak 3 had the same retention time as (b) peak 5.

peak 1, meso-hydroxyuroporphyrin I
peak 2, $\beta$-hydroxypropionic acid uroporphyrin I
peak 3, hydroxyacetic acid uroporphyrin I
peak 4, peroxyacetic acid uroporphyrin I
peak 5, $\beta$-OH propionic acid-7COOH porphyrin I
peak 6, hydroxyacetic acid-7COOH porphyrin I

Fig. 3.24. HPLC Separation of the Decarboxylated Products of $\beta$-Hydroxypropionic Acid Uroporphyrin I (a) Comparing with Urinary Porphyrin of a Patients with CEP (b)
the hydroxyl group to give uroporphyrin I.

Peak 3 had exactly the same retention time as authentic meso-hydroxyuroporphyrin III and therefore was identified as meso-hydroxyuroporphyrin III. This assignment was confirmed by reduction of the compound followed by re-oxidation which gave uroporphyrin III as the only product.

Peak 5 had identical chromatographic and chemical behaviour to that of β-hydroxypropionic acid uroporphyrin I isolated from the urine of patients with CEP and was therefore assigned as β-hydroxypropionic acid uroporphyrin I. This compound was easily dehydrated by heating in diluted HCl to give the monoacrylic derivative as previously described (Sec. 3.5.3.).

The chromatographic and chemical properties of peak 6 were identical to those of hydroxyacetic acid uroporphyrin I isolated from the urine of patients with CEP. On heating in dilute HCl no dehydration product was formed, indicating that the hydroxyl group was attached to an acetic acid group and therefore difficult to dehydrate. Some partial decarboxylation products were formed instead, as described in Section 3.5.4. Thus it was identified as hydroxyacetic acid uroporphyrin I.

Peak 7 had the characteristic greenish-yellow colour with a typical red porphyrin fluorescence under UV illumination, similar to peroxyacetic acid uroporphyrin I.
isolated from the urine of patients with CEP. It gave uroporphyrin I when reacted with 0.1 M KOH or Ca(OH)\textsubscript{2} and was stable under acidic conditions. It also had the same retention time under all conditions as peroxyacetic acid uroporphyrin I isolated from the urine from CEP patients. This peak was therefore identified as peroxyacetic acid uroporphyrin I.

3.5.8. Stability of hydroxy- and peroxyacetic acid-porphyrins

*Meso*-hydroxyuroporphyrin I was reasonably stable in acid and no change was observed when dissolved in 5 M HCl for 10 min in the dark. It was stable in weak alkaline, e.g. 0.1 M KOH, but was less stable in stronger alkaline solutions. When the compound was dissolved in 0.7 M KOH for 10 min in the dark, less than half of the original material was detected by HPLC. When it was dissolved in 0.85 M KOH for 10 min in the dark, the colour of the solution turned into greenish-yellow and lost its UV fluorescence. The resulting solution was analysed by HPLC and nothing was detected by the fluorescent detector. This indicated that under strong alkaline condition the highly conjugated macrocycle of the porphyrin was opened.

*Meso*-hydroxyuroporpyrin I was not very stable to heat. When it was heated at 135°C for 30 min in the dark, the compound was reduced to about 1/3 of the original amount when analysed by HPLC. A late eluting peak was formed which was detected by UV-VIS but not by fluorescent detection.
When it was heated at 135°C for 1 h, nothing was detected with the fluorescent detector and the late eluting peak detected by the UV-VIS detector was increased. This also indicated ring opening on prolonged heating of the compound.

β-Hydroxypropionic acid-, hydroxyacetic acid uroporphyrin I, β-hydroxypropionic acid- and hydroxyacetic acid heptacarboxylic acid porphyrins were stable in both acid and alkaline solutions, in contrast to meso-hydroxyuroporphyrin I.

Peroxyacetic acid uroporphyrin I was stable in acid but was hydrolysed to uroporphyrin I in alkaline solution. It was also unstable to heat. When it was heated at 135°C in dilute HCl in the dark for 30 min, Uro I was found and this was increased with increasing time of heating.

3.5.9. Electronic absorption spectra of hydroxy- and peroxyacetic acid- porphyrins

Spectrophotometric determination showed that meso-hydroxy-, β-hydroxypropionic acid-, hydroxyacetic acid uroporphyrin I, β-hydroxypropionic acid heptacarboxylic acid porphyrin I and hydroxyacetic acid heptacarboxylic acid porphyrin I in acid solution had characteristic visible absorption spectra of porphyrin dication which resembled Uro I (Fig.3.25-30). They all had one major Soret band and two satellite bands with a weaker band as a shoulder attached to the first one.
Fig. 3.25. Electronic Absorption Spectra of Uroporphyrin I
Fig. 3.26. Electronic Absorption Spectra of Meso-Hydroxyuropporphyrin I
Fig. 3.27. Electronic Absorption Spectra of β-Hydroxypropionic Acid Uroporphyrin I
Fig. 3.28. Electronic Absorption Spectra of Hydroxyacetic Acid Uroporphyrin I
Fig. 3.29. Electronic Absorption Spectra of 3-Hydroxypropionic Acid Heptacarboxylic Acid Porphyrin
Fig. 3.30. Electronic Absorption Spectra of Hydroxyacetic Acid Heptacarboxylic Acid Porphyrin
The Soret band of *meso*-hydroxyuroporphyrin I is 410nm, longer than Uro I (405 nm) which is consistent with a substitution attached to the conjugated macrocircle ring (Williams & Fleming, 1987). The Soret bands of β-hydroxypropionic acid- and hydroxyacetic acid uroporphyrin I are 405.5 and 405 nm, respectively, very close to Uro I.

The spectrum of peroxyacetic acid uroporphyrin I is different from Uro I (Fig.3.31.). The Soret band was split and the two major satellite bands at about 540-620 nm were replaced by a single band at the wavelength of 619.0 nm.

The electron absorption spectra showed that the Soret Bands of β-hydroxypropionic acid- and hydroxyacetic acid-heptacarboxylic acid porphyrins were at 404.5 and 403.5nm, respectively. The slightly shorter wavelength than uroporphyrin is consistent with the loss of a carboxylic acid group (Williams and Fleming, 1987). Compared with the Soret Band of *meso*-hydroxy-, β-hydroxypropionic acid- and hydroxyacetic acid- uroporphyrin I, these two compounds are very different from *meso*-hydroxyuroporphyrin I but are close to β-hydroxypropionic acid- and hydroxyacetic acid- uroporphyrin I. The Soret band of β-hydroxypropionic acid heptacarboxylic acid porphyrin had slightly longer wavelength than the hydroxyacetic acid heptacarboxylic acid porphyrin which resembled the observed longer wavelength of β-hydroxypropionic acid uroporphyrin I than hydroxyacetic acid uroporphyrin I. This further confirms that peak 5 (β-hydroxypropionic acid heptacarboxylic acid porphyrin I) in
Fig. 3.31. Electronic Absorption Spectra of Peroxyacetic Acid Uroporphyrin I
Table 3.1. Wavelength of the Porphyrin Dications in Diluted HCl

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Soret Band</th>
<th>Satellite Band I</th>
<th>Satellite Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>URO I</td>
<td>405 nm</td>
<td>551 nm</td>
<td>592 nm</td>
</tr>
<tr>
<td>meso-OH Uro I</td>
<td>410 nm</td>
<td>555 nm</td>
<td>613 nm</td>
</tr>
<tr>
<td>β-OH propionic acid Uro I</td>
<td>405.5 nm</td>
<td>552 nm</td>
<td>593.5 nm</td>
</tr>
<tr>
<td>OH-acetic acid Uro I</td>
<td>405 nm</td>
<td>551 nm</td>
<td>593 nm</td>
</tr>
<tr>
<td>CH₂-COOOH Uro I</td>
<td>394.5 &amp; 406.5 nm</td>
<td>619 nm</td>
<td></td>
</tr>
<tr>
<td>β-OH propionic acid-7COOH porphyrin</td>
<td>404.5 nm</td>
<td>551 nm</td>
<td>593 nm</td>
</tr>
<tr>
<td>OH-acetic acid-7COOH porphyrin</td>
<td>403.5 nm</td>
<td>550.5 nm</td>
<td>592.5 nm</td>
</tr>
</tbody>
</table>
Fig. 3.3. is the metabolite of β-hydroxypropionic acid uroporphyrin I, and that peak 6 (hydroxyacetic acid heptacarboxylic acid porphyrin I) is the metabolite of hydroxyacetic acid uroporphyrin I.

The absorption wavelengths of uroporphyrin I, meso-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I, peroxycetic acid uroporphyrin I, β-hydroxypropionic acid heptacarboxylic acid porphyrin and hydroxyacetic acid heptacarboxylic acid porphyrin are listed in Table 3.1.

3.6. Conclusions

_Meso_-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I, peroxycetic acid uroporphyrin I, β-hydroxypropionic acid heptacarboxylic acid porphyrin I and hydroxyacetic acid heptacarboxylic porphyrin I have been isolated from the urine of patients with CEP and characterised by HPLC, chemical behaviour and LSIMS. The hydroxy- and peroxycetic acid uroporphyrin I derivatives have also been identified in the plasma of these patients together with a little _meso_-hydroxyuroporphyrin III. These compounds are most probably derived from uroporphyrin I or uroporphyrinogen I which accumulated in large quantity in patients with CEP due to uroporphyrinogen III synthase deficiency. The origin and mechanism of formation of these new porphyrin metabolites have been investigated and are described in the following chapters.
APPENDIX

Meso-hydroxyuroporphyrin I, \( \beta \)-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I and peroxyacetic acid uroporphyrin I were isolated and characterised mainly from the urine of one patient with confirmed CEP. The clinical history and biochemical findings of the patient are as follows.

The patient (MD) was a 23 years old man, who was diagnosed as congenital erythropoietic porphyria at 2 months of age when he presented with persistent neonatal jaundice, haemolysis and cutaneous photosensitivity. There was no history of consanguinity. Erythrocyte Uro'gen III-S activity was reduced to 19% of normal in the subject and to approximately 50% in both parents and in his sister (Wright & Lim, 1983) consistent with autosomal recessive inheritance and also indicating that the sister was a carrier. He was treated with intermittent blood transfusions and splenectomy was carried out at the age of 7 years. Regular medication also included ascorbic acid, \( \beta \)-carotene, folic acid, penicillin after the splenectomy, and avoidance of sunlight. From the age of 10 years he received regular subcutaneous infusions of desferrioxamine at night. Despite these measures he continued to have extensive photomutilation of the hands and face, with shortening of the digits, and when 20 years old he complained of pain in the spine and sternum and was found to have compression fractures of 2 vertebrae. Treatment of osteoporosis with clodronate (dichloromethylene bisphosphonate) was carried out (Pullon et al., 1991). At the time when this study was performed he was also
on a more intensive regime of blood transfusions (2-3 units at approximately 4 weeks intervals).

Biochemical investigation over eight weeks revealed that the activity of erythrocyte Uro'gen III-S was 38-86 nmol Uro III/ml RBC per hour. Urinary porphyrin excretion was 79-283 μmol/24h with 75% of Uro I, 15% of Copro I, 8% of hepta to penta-carboxylic acid porphyrins dominated in type I isomers and 1.6% of uroporphyrin I derivatives. Plasma porphyrin was 2.5-4.1 μmol/l with 66% of Uro I, 14% of Copro I, 18% of hepta to penta-carboxylic acid porphyrins and 0.6% of uroporphyrin I derivatives. Erythrocyte porphyrin was 3.2-10.6 μmol/l with 83% of Uro I and 17% of Copro I, other porphyrins were not detectable.

After the uroporphyrin I derivatives were identified in the urine of MD, their existence in the urine of another four patients with CEP was investigated. The HPLC chromatograms of urinary porphyrin of three out of the four patients showed identical patterns with the patient MD. In the urine of the fourth patient (CB), only traces of meso-hydroxyuroporphyrin I and β-hydroxypropionic acid uroporphyrin I were found. Further analysis showed that the activity of Uro'gen III-S was 75.1 nmol/ml RBC per h, the porphyrin level in his urine was 3.732 μmol/24h and in his plasma was 26.485 nmol/l, both dominated in Uro I together with some Copro I. Therefore uroporphyrin I excretion in patient CB was much lower than that in patient MD. This indicated that the existence of uroporphyrin I derivatives was closely related to the level of uroporphyrin I in vivo which was be discussed in more detail in Section 6.3..
CHAPTER 4
THE ORIGIN OF HYDROXY- AND PEROXYACETIC ACID- PORPHYRINS IN CEP

4.1. Introduction

Meso-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I and per oxyacetic acid uroporphyrin I have been identified in the urine and plasma of patients with CEP. Their origin, however, is unknown. It is possible that these compounds are formed artifactly in the blood or urine due to the relatively high concentration of Uro I present in these body fluids, particularly in stored samples. It is equally possible that they are true metabolites derived from Uro I or Uro'gen I in vivo by an oxidation process. This chapter investigates the source and the origin of hydroxy- and per oxyacetic acid porphyrins in CEP.

4.2. Experimental

4.2.1. Materials and reagents

Ammonium acetate, glacial acetic acid, trichloroacetic acid, I₂, concentrated HCl, NaHCO₃, NaOH, ethylenediaminetetraacetic acid (EDTA), NH₄Cl, sucrose, KHCO₃, ascorbic acid, Triton X-100, MgCl₂, dimethyl sulphoxide (DMSO), diethyl ether and chloroform were AnalAr grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn
Chemicals (Walkerburn, Borders, Scotland, U.K.).
Tris(hydroxymethyl)aminoethane (Tris) was from Boehringer Mannheim GmbH. (F.R.G.). Heparin was from Paines & Byrne Ltd. (Greenford, UK). Porphobilinogen, uroporphyrin, coproporphyrin, glutathione, dithiothreitol and Dextran (MW 500,000), were from Sigma Chemical Co. (Poole, Dorset).

4.2.2. HPLC of porphyrins in clinical samples and reaction mixtures

A Varian Associates (Walton-on-Thames, Surrey, U.K.) Model 5000 liquid chromatography with a Varian UV-100 variable wavelength detector set at 405 nm and a Perkin-Elmer LS3 fluorescent detector set at excitation 400 and emission 618 nm, respectively, was used. Samples were injected via a Rheodyne (Cotati, CA, USA) 7125 injector fitted with a 500 μl loop. The separation was carried out on a 250 mm x 5 mm Hypersil-ODS column (Shandon Scientific, Runncorn, Cheshire, U.K.). Unless otherwise stated the mobile phase generally consisted of solvent A: 9% (v/v) of acetonitrile in 1 M ammonium acetate buffer, pH 5.16 and solvent B: 10% (v/v) of acetonitrile in methanol. The elution program varied according to the separations required. The flow rate was 1 ml/min throughout. The elution programs were as follows.

1. For the quantitation of meso-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I and peroxyacetic acid uroporphyrin I in the urine and plasma:
Time 0 to 16 min, 0% solvent B (100% solvent A) to 11% solvent B; time 16 to 24 min, isocratic elution at 11% solvent B; time 24 to 38 min, 11% solvent B to 24% solvent B; time 38.1 to 48 min, isocratic at 95% solvent B to wash the column.

For complete profile of the porphyrins, run the gradient from 0 to 38 min as above, then from time 38 to 60 min, 24% solvent B to 60% solvent B and time 60 to 80 min, 60% solvent B to 80% solvent B. The column was then re-equilibrated at 0% solvent B for 10 min before injection of next sample.

2. For the analysis of porphyrins in duodenal aspirates and faeces:

Isocratic elution at 15% B for 10 min; linear gradient elution from 15 to 70% B from 10 to 45 min; linear gradient elution from 70 to 95% B from 45 to 50 min, followed by isocratic elution at 95% B for a further 10 min.

3. For measurement of the production of peroxyacetic acid uroporphyrin I in the incubation mixture of red cell haemolysates with Uro'gen I or PBG as substrate:

Isocratic elution at 15% solvent B for 30 min, followed by isocratic elution at 95% for 10 min. Re-equilibrate the column at 15% solvent B for 10 min before the next injection.

4. For investigation of hydroxylated uroporphyrin in
the reaction mixture of red cell haemolysates with Uro'gen I as substrate:

   Time 0 to 30 min, 0% solvent B to 3% solvent B; time 30 to 40 min, 3% solvent B to 9% solvent B; time 40 to 50 min, 9% solvent B to 20% solvent B; time 50.1 min, 95% solvent B to elute late eluting peaks.

5. For analysis of the incubation mixture of red cell haemolysates with heptacarboxylic acid porphyrinogen I as substrate:

   Isocratic elution at 19% solvent B for 34 min, followed by 95% solvent B for 10 min to wash the column.

6. For analysis of reaction mixture of red cell haemolysates with pentacarboxylic acid porphyrinogen I as substrate:

   Time 0 to 40 min, 15% solvent B to 40% solvent B; time 40.1 min 95% solvent B for 5 min to wash the rest.

7. For analysis of reaction mixture of red cell haemolysates with Copro'gen I as substrate:

   Isocratic elution at 55% solvent B.

4.2.3. Preparation of blood sample

   Erythrocytes and plasma were prepared as described in Sec. 2.3.4.

   Leucocytes were prepared by erythrocytes sedimentation and NH₄Cl buffer lysis (Cuttes, 1970). Heparinized blood, 20
ml, was added to an equal volume 3% (w/v) Dextran solution (MW 500,000) in 0.15 M NaCl, mixed and centrifuged at 2100 g for 10 min at 4°C. The supernatant was removed with a Pasteur pipette. The leucocyte layer was pipetted into a clean tube. Any contaminated red blood cells (RBC) were lysed by adding 7-8 ml of ice-cold NH₄Cl buffer pH 7.4 (8.3g NH₄Cl, 1g KHCO₃ and 1ml of 0.1 mol/l EDTA per litre) and standing on ice for 7 min. After centrifugation, the supernatant was decanted and the leucocytes were washed thrice with ice cold saline. If the RBC contamination was not completely eliminated, NH₄Cl buffer lysis could be repeated. The leucocytes were then resuspended in 0.25 M sucrose containing 0.05 M Tris-HCl buffer, pH 7.4 and 5 units of heparin per 20 ml of blood. The leucocytes were lysed by freezing and thawing thrice before use. If not used immediately, the leucocytes were stored at -30°C.

4.2.4. Incubation of porphyrinogen with red cell haemolysates

Uro'gen I, Uro I, hepta'gen I, penta'gen I, Copro'gen I and Copro I were used as substrates, respectively. Packed RBC, 15 μl, were pre-incubated with 1.4 ml of 0.05 M Tris-HCl buffer, pH 8.25, containing 74 μM MgCl₂ and 0.1% Triton X-100 at 37°C in the dark for 5 min. The reaction was started by adding 50 μl of porphyrinogen and continuously incubated for a further 10 min. The reaction was stopped by adding 1.5 ml of 10% TCA containing 0.5% I₂. After mixing and centrifugation the supernatant was injected into the HPLC for analysis.
Parallel blanks were prepared by 1. using boiled RBC; 2. using incubation buffer instead of RBC; and 3. using incubation buffer instead of porphyrinogen. When porphyrinogens other than Uro'gen were incubated with RBC, 10% TCA containing I_2 / DMSO (v/v, 1:1) was used to stop the reaction and extract the porphyrins.

4.2.5. Incubation of porphobilinogen (PBG) with red cell haemolysates

Method 1. Packed RBC 30 µl, were added into 1.4 ml of 0.05 M Tris-HCl buffer, pH 8.25, containing 74 µM MgCl_2 and 0.1% Triton X-100. After mixing, the mixture was heated at 56°C for 1 h to deactivate Uro'gen III-S. The mixture was cooled to room temperature and 3 µl of fresh unheated RBC were added. The mixture was pre-incubated at 37°C for 5 min and then the reaction was started by adding 50µl of PBG (8.3 µg) as the substrate. The incubation was continued for a further 30 min at 37°C, in the dark. The reaction was stopped by adding 1.5 ml of 10%TCA containing 0.5% I_2. After centrifugation the supernatant was ready for HPLC analysis.

Method 2. Packed RBC 30 µl, were added into 1.4 ml of 0.05 M Tris-HCl buffer pH 8.25, containing 74 µM MgCl_2 and 0.1% Triton X-100. The mixture was heated to 56°C for 1 h, cooled to room temperature and pre-incubated at 37°C for 5 min. The mixture then incubated with PBG as described in method 1 without further addition of fresh red cells.
4.2.6. Incubation of uroporphyrinogen I with red cell haemolysates in presence of reducing agents

1. Addition of reducing agents in the incubation buffer:

Reducing agents glutathione, ascorbic acid and dithiothreitol were dissolved in Tris-HCl buffer, pH 8.25, containing 74 μM MgCl₂ and 0.1% Triton X-100, respectively. The concentrations of each reducing agent in the buffer were as follows: glutathione 0.5, 1, 2, and 3 mM; ascorbic acid 0.5, 1, 2, 3, 50, 100 and 300 mM; and dithiothreitol 10 mM (pH was adjusted to 6.8).

Packed RBC, 15 μl, were pre-incubated with 1.4 ml of 0.05 M Tris-HCl buffer pH 8.25, containing 74 μM MgCl₂, 0.1% Triton X-100 and various concentrations of glutathione, ascorbic acid or dithiothreitol, respectively, at 37°C in the dark for 5 min. The reaction was started by adding 50 μl of 80 μM uroporphyrinogen I and continuously carried out as described in Sec. 4.2.4.

2. Addition of reducing agents in the Uro'gen I solution:

Uro'gen I, 160 μM, was reduced with 0.5% Na/Hg and then diluted with equal volume of Tris-HCl buffer, pH 8.25, containing 74 μM MgCl₂, 0.1% Triton X-100, and glutathione (1, 2, 4, 6 and 8 mM) or dithiothreitol (2, 4, 6, 8 and 10 mM), respectively.
Packed RBC, 15 µl, were pre-incubated with 1.4 ml of 0.05 M Tris-HCl buffer pH 8.25, containing 74 µM MgCl$_2$, 0.1% Triton X-100 at 37°C in the dark for 5 min. The reaction was started by adding 50 µl of 80 µM uroporphyrinogen I prepared as above and continuously carried out as described in Sec. 4.2.4. The final concentrations of glutathione in the reaction mixture were 0.017, 0.034, 0.068, 0.102 and 0.137 mM; and dithiothreitol were 0.034, 0.068, 0.102 and 0.137 and 0.170 mM.

4.2.7. Incubation of uroporphyrinogen I with red cell haemolysates in presence of carbon monoxide

Packed RBC, 15 µl, were added into 4 x 1.4 ml of 0.05 M Tris-HCl buffer pH 8.25, containing 74 µM MgCl$_2$, 0.1% Triton X-100 in a sealed tube (vol. 4 - 4.5 ml). Two of the tubes were equilibrated with 80% of carbon monoxide in oxygen for 2 min with shaking. The other two tubes contained 80% nitrogen in oxygen. The tubes were pre-incubated for 5 min in the dark at 37°C. Then the reaction was started by injecting 10 µl of Uro'gen I (15 nmol) into each of the tubes with a syringe and continuously incubated for further 10 min. The reaction was stopped by addition of 1.5 ml of 10% TCA containing 0.5% I$_2$, again, through a syringe. After mixing and centrifugation, the supernatant was injected into the HPLC for analysis.

4.2.8. Incubation of uroporphyrinogen I with plasma and leucocytes

Leucocytes prepared as described in Sec.4.2.3. were
divided into two portions, one was used in the reaction mixture and the other was boiled for blank control. Plasma, 100 μl, was also boiled for blank control.

Plasma, 100 μl, or leucocytes isolated as described above was pre-incubated with 1.4 ml of 0.05 M Tris–HCl buffer, pH 8.25, containing 74 μM MgCl₂ and 0.1% Triton X-100 at 37°C in the dark for 5 min. The reaction was started by adding 50 μl of 80 μM uroporphyrinogen I and continuously carried out as described in Sec. 4.2.4.

4.3. Results and Discussion

4.3.1. Source of hydroxy- and peroxyacetic acid-uroporphyrin I

In order to find out whether the accumulation and excretion of hydroxy- and peroxyacetic acid uroporphyrin I derivatives in plasma and urine is a common feature of patients with CEP, urine and plasma of CEP patients with different degree of clinical and biochemical manifestations were analysed. Fresh urine samples were analysed to eliminate the possibility that these compounds were derived artifactly in stored samples.

In CEP patients where urinary Uro I levels were high (78-283 μmol / 24 h) hydroxy- and peroxyacetic acid uroporphyrin I derivatives were easily detected. In one patient who excreted much lower levels of urinary uroporphyrin I (2.28-4.22 μmol/24h) only traces of meso-
hydroxyuroporphyrin I and β-hydroxypropionic acid
uroporphyrin I were found and hydroxyacetic acid
uroporphyrin I and peroxyacetic acid uroporphyrin I were
hardly detectable. These results show that the presence of
hydroxy- and peroxyacetic acid- uroporphyrin I derivatives
in the urine is a common feature of CEP.

Since hydroxy- and peroxyacetic acid- uroporphyrin I
derivatives must be formed by an oxidation process, they may
originate from the oxidation of uroporphyrinogen I by air
when they are excreted out of the body in the urine.
Oxidation of uroporphyrinogen I in the dark by air and with
I₂, however, produced no hydroxy- and peroxyacetic acid-
derivatives. The urine of patients with acute intermittent
porphyria during acute attack when high concentrations of
PBG are excreted were also analysed. Again no hydroxy- nor
peroxyacetic acid- uroporphyrin I derivatives were detected.
Since PBG in the urine will condense to form
uroporphyrinogens which then oxidised to form uroporphyrins,
it again shows that simple aerial oxidation of
uroporphyrinogen to porphyrin is not responsible for the
production of hydroxy- and peroxyacetic acid- uroporphyrins.
Hydroxy- and peroxyacetic acid- uroporphyrin I derivatives
were also undetectable in the duodenal aspirates and faeces
of the patient with CEP who excreted relatively large amount
of these compounds in the urine. This indicated that
hydroxy- and peroxyacetic acid- uroporphyrin I derivatives
were not formed in the liver of CEP patients and they
therefore are likely to be of erythropoietic origin since
these compounds were detected in the plasma of patients with CEP. To test this hypothesis the reaction of Uro'gen I with erythrocytes from CEP and normal subjects was investigated in detail in order to find out whether in the presence of an excess of Uro'gen I hydroxylation and peroxylation reactions could take place.

4.3.2. Reaction of uroporphyrin I with red cell haemolysates

When uroporphyrin I was incubated with red cell haemolysates no hydroxy- nor peroxyacetic acid- derivatives were found. These derivatives must be therefore derived from uroporphyrinogen I rather than Uro I.

4.3.3. Reaction of uroporphyrinogen I with red cell haemolysates

When uroporphyrinogen I was incubated with red cell haemolysates, a peak with the same retention time as the natural peroxyacetic acid uroporphyrin I was detected under various HPLC conditions (Fig. 4.1.). This peak was collected, purified and analysed by LSIMS as described in chapter 3. The methyl and ethyl esters gave MH⁺ signals at 945 and 1043, respectively (Fig. 4.2. & 4.3.), identical to the peroxyacetic acid isolated from the urine of patients with CEP. To confirm this, the free porphyrin in this fraction was treated with 0.1M KOH when it was completely converted into UroI. Thus this peak must also be peroxyacetic acid uroporphyrin I. Hydroxylated uroporphyrin I derivatives were also formed in small quantity (Fig. 4.1). These will be discussed in more detail in Chapter 5. The above results
Fig. 4.1. HPLC Separation of Porphyins in a Reaction Mixture of Uroporphyrinogen I with Red Cell Haemolysates

Peak 1, meso-hydroxyuroporphyrin I; peak 2, β-hydroxypropionic acid uroporphyrin I; peak 3, peroxyacetic acid uroporphyrin I; peak 4, uroporphyrin I; other peaks have not been investigated.
Fig. 4.2. LSIMS of Peroxyacetic Acid Uroporphyrin I Methyl Ester Isolated from a Reaction Mixture of Uro'gen I with Red Cell Haemolysates
Fig. 4.3. LSIMS of Peroxyacetic Acid Uroporphyrin I Ethyl Ester Isolated from a Reaction Mixture of Uro'gen I with Red Cell Haemolysates
clearly show that in the presence of excess of Uro'gen I, reactions occur in the red cells which convert Uro'gen I into its hydroxy- and peroxyacetic acid derivatives.

To further confirm that the formation of peroxyacetic acid uroporphyrin I was from uroporphyrinogen I, PBG was used as the substrate in the incubation mixture with red cell haemolysates. In the absence of Uro'gen III-S hydroxymethylbilane (HMB) cyclises non-enzymically to Uro'gen I. Thus when heated red cell haemolysates was incubated with PBG, a substantial amount of Uro'gen I was generated. This reacted with red cell haemolysates giving a peak with the exactly the same retention time as the natural peroxyacetic acid uroporphyrin I (Fig. 4.4.). This proved that the peroxyacetic acid uroporphyrin I was derived from Uro'gen I by reaction with red cells.

In the presence of Uro III-S, HMB is converted to Uro'gen III. Thus when unheated red cell was added to the incubation mixture with PBG, four peaks which eluted before Uro I were detected (Fig. 4.5.). The same four peaks were obtained when Uro'gen III was incubated with red cell haemolysates. LSIMS analysis of the methyl and ethyl esters showed that they were peroxyacetic acid uroporphyrins with the MH⁺ signals at m/z 945 and 1043, respectively. Treatment of each of these compounds with 0.1 M KOH converted them into Uro III. They were therefore the four type III isomers of peroxyacetic acid uroporphyrin. Since Uro III is an unsymmetrical compound it is expected to rise to four
Fig. 4.4. HPLC Separation of Porphyrins in a Reaction Mixture of PBG with Heated Red Cell Haemolysates

Peak 1, peroxyacetic acid uroporphyrin I; peak 2, uroporphyrin I; other peaks are not identified.
Fig. 4.5. HPLC Separation of Porphyrins in a Reaction Mixture of PBG with Unheated Red Cell Haemolysates

Peak 1, peroxyacetic acid uroporphyrin I + III; peak 2 - 4, peroxyacetic acid uroporphyrin III isomers; peak 5, uroporphyrin I; peak 6, uroporphyrin III; other peaks are not identified.
peroxyacetic acid derivatives. One of the peroxyacetic acid uroporphyrin III isomers co-eluted with the type I isomer. These results show that red cells can convert both excess Uro'gen I and III into the peroxyacetic acid derivatives.

In control experiments where boiled red cells were incubated with Uro'gen I, very little peroxyacetic acid uroporphyrin I was formed, indicating that the denatured protein lost its ability to catalyse the reaction.

4.3.4. Comparison of the formation of peroxyacetic acid uroporphyrin I with red cell haemolysates from normal subject and patient with CEP

Red cell haemolysates from a normal person and from a patient with CEP were investigated. When 30 µl of heated red cell haemolysates were incubated with PBG the peroxyacetic acid uroporphyrin I generated were similar in both the normal and CEP reaction mixtures. When unheated red cell haemolysates were used, however, the peroxyacetic acid uroporphyrin I peak in the reaction mixture of CEP was only slightly decreased and three very small type III isomers of peroxyacetic acid uroporphyrins were detected. In the reaction mixture of unheated normal red cells, the peroxyacetic acid uroporphyrin I peak was reduced to about one third of that with heated red cells but the type III isomer peaks were much larger than those in the reaction mixture with the erythrocytes from the patient with CEP (Table 4.1.).
Table 4.1. Comparison of the Formation of Peroxyacetic Acid Uroporphyrin I and III with Red Cell Haemolysates from Normal Subject and Patient with CEP

<table>
<thead>
<tr>
<th>Subject</th>
<th>I</th>
<th>III-1</th>
<th>III-2</th>
<th>III-3</th>
<th>I+III</th>
<th>III-1</th>
<th>III-2</th>
<th>III-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>150</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>52</td>
<td>18</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>CEP</td>
<td>158</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>109</td>
<td>12</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

I: peroxyacetic acid uroporphyrin I.
III: peroxyacetic acid uroporphyrin III.
Unit: peak height in mm.

Note: One of the type III isomers co-eluted with the type I isomer. Assuming that roughly equal proportions of the four type III isomers are formed, the unheated red cells of normal subject and CEP patient should produce about 19 and 13 mm of the fourth isomer, respectively.
When unheated red cell haemolysates were incubated with PBG, the HMB generated by HMB-S was used by Uro'gen III-S as substrate to generate Uro'gen III. In the patient with CEP where Uro'gen III-S is defective, Uro'gen III production is less than the spontaneous cyclisation of HMB to uroporphyrinogen I. Since the formation of peroxyacetic acid uroporphyrin was proportional to the Uro'gen concentration in the reaction mixture (Sec. 4.3.5.2.), more peroxyacetic acid uroporphyrin I was therefore produced by the red cells of the patient with CEP and more type III isomers by the normal red cells which synthesised more uroporphyrinogen III.

4.3.5. Optimization of reaction conditions for the formation of peroxyacetic acid uroporphyrin I

4.3.5.1. Relationship between the volume of red cell haemolysates and the production of peroxyacetic acid uroporphyrin I

To determine the optimum volume of red cell haemolysates for the production of peroxyacetic acid uroporphyrin I, fixed concentration of Uro'gen I (final conc. 16.9μM) was incubated with various amount of red cell haemolysates. The result showed that at this concentration of Uro'gen I peroxyacetic acid uroporphyrin I production was linear to the volume of red cell haemolysates up to 15μl as shown in Fig. 4.6.
Fig. 4.6. Relationship Between Haemolysates Volume and the Formation of Peroxyacetic Acid Uroporphyrin I (PAAU)

This is the typical result of five experiments.
4.3.5.2. Relationship between the concentration of Uro'gen I and the production of peroxyacetic acid uroporphyrin I

To investigate the optimum concentration of Uro'gen I used in the reaction mixture for the production of peroxyacetic acid uroporphyrin I, a fixed volume of red cell haemolysates (15μl) was incubated with various concentration of Uro'gen I. The result showed that the production of peroxyacetic acid uroporphyrin I was linear to the concentration of Uro'gen I up to 10μM. The rate of the formation then slowed down and levelled off at 15μM (Fig. 4.7.). The failure of calculating the kinetic constant (Km) indicated that the formation of peroxyacetic acid uroporphyrin I is not due to an enzymic reaction.

4.3.5.3. Relationship between incubation time and production of peroxyacetic acid uroporphyrin I with red cell haemolysates

When Uro'gen I was incubated with red cell haemolysates, the production of peroxyacetic acid uroporphyrin I was very rapid and reached the maximum value within one minute. There was no significant difference in the formation of peroxyacetic acid uroporphyrin I when incubation was carried out between 1 and 20 min. Incubation longer than 20min, however, caused a slow decrease in peroxyacetic acid uroporphyrin I production as shown in Fig. 4.8.. When incubation was carried out for 12 h and 18 h, the amount of peroxyacetic acid uroporphyrin I detected was about 30% of that after 10 min incubation. Uro I recovered was also reduced, but to a much lesser extent. No additional
Fig. 4.7. Relationship Between the Concentrations of Uro'gen I and the Production of Peroxyacetic Acid Uroporphyrin I (PAAU) in Red Cell Haemolysates

This is the typical result of three experiments.
Fig. 4.8. Relationship Between Incubation Time and the Formation of Peroxyacetic Acid Uroporphyrin I (PAAU) and Uro I Recovery with Red Cell Haemolysates
This is the mean of four experiments.
peaks were detected. Addition of EDTA (1mM) into the incubation buffer did not affect the result.

4.3.5.4. pH optimum for the production of peroxyacetic acid uroporphyrin I

When 15 μM of red cell haemolysates was incubated with Uro'gen I (final Conc. 2.7 μM), the maximum production of peroxyacetic acid uroporphyrin I was at approximate pH 8 as shown in Fig. 4.9. At pH above 9, the amount of peroxyacetic acid uroporphyrin I detected decreased rapidly. There are two possible reasons for this observation. The first is that the formation of peroxyacetic acid uroporphyrin I was inhibited, the second is that peroxyacetic acid uroporphyrin I was not stable under alkaline conditions and tended to convert into Uro I spontaneously under these conditions (Sec. 3.5.5.). This will be discussed in next chapter.

4.3.5.5. Relationship between incubation temperature and the formation of peroxyacetic acid uroporphyrin I

Uro'gen I (final Conc. 2.7μM) was incubated with 15μl of red cell haemolysates at various temperature. The result surprisingly showed that the maximum amount of peroxyacetic acid uroporphyrin I was detected at 0-10°C. At higher incubation temperatures the yield of peroxyacetic acid uroporphyrin I was lower as shown in Fig. 4.10.. The reason for this behaviour was not clear.
Fig. 4.9 pH Optimum for the Production of Peroxyacetic Acid Uroporphyrin I
This is the mean of eight experiments.
Fig. 4.10. Relationship Between Incubation Temperatures and the Amount of Peroxyacetic Acid Uroporphyrin I (PAAU)

This is the mean of two experiments.
4.3.6. Effect of reducing agents on the formation of peroxyacetic acid uroporphyrin I

Since peroxyacetic acid uroporphyrin I must be formed by an oxidation process experiments were carried out to determine whether reducing agents can inhibit its production.

The reducing agent glutathione was used for this investigation. Uro'gen I (final Conc. 2.7 μM) was incubated with 15 μl of red cell haemolysates in buffer containing 0.5, 1, 2 and 3 mM of reduced glutathione, respectively. The results clearly showed that glutathione inhibited the formation of peroxyacetic acid uroporphyrin I by more than 60% (Table 4.2.). Addition of glutathione into the Uro'gen I solution to give a much lower final concentration of 0.017, 0.034, 0.068, 0.102 and 0.137 mM in the reaction mixture also inhibited the formation of peroxyacetic acid uroporphyrin I (Table 4.3.).

Other reducing agents, such as ascorbic acid and dithiothreitol, were also tested for inhibition with similar positive results. The formation of peroxyacetic acid uroporphyrin I was inhibited by the presence of both ascorbic acid and dithiothreitol.
Table 4.2. Effect Of Glutathione In The Reaction Mixture On The Formation Of Peroxyacetic Acid Uroporphyrin I (PAAU)

<table>
<thead>
<tr>
<th>Glutathione (mM)</th>
<th>PAAU produced (nmol/ml RBC)</th>
<th>Percentage of Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.0</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>3.85</td>
<td>32.1</td>
</tr>
<tr>
<td>1</td>
<td>3.26</td>
<td>27.2</td>
</tr>
<tr>
<td>2</td>
<td>3.41</td>
<td>28.4</td>
</tr>
<tr>
<td>3</td>
<td>4.60</td>
<td>38.3</td>
</tr>
</tbody>
</table>

This is the typical result of two experiments.
Table 4.3. Effect Of Glutathione In Uro'gen I Solution On The Formation Of Peroxyacetic Acid Uroporphyrin I

<table>
<thead>
<tr>
<th>Glutathione (mM)</th>
<th>PAAU produced (nmol/ml RBC)</th>
<th>Percentage of Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.2</td>
<td>100</td>
</tr>
<tr>
<td>0.017</td>
<td>10.7</td>
<td>95.9</td>
</tr>
<tr>
<td>0.034</td>
<td>10.4</td>
<td>92.7</td>
</tr>
<tr>
<td>0.068</td>
<td>7.88</td>
<td>70.5</td>
</tr>
<tr>
<td>0.102</td>
<td>7.79</td>
<td>69.7</td>
</tr>
<tr>
<td>0.137</td>
<td>7.97</td>
<td>71.3</td>
</tr>
</tbody>
</table>

This is the typical result of two experiments.
4.3.7. Inhibition of the production of peroxyacetic acid uroporphyrin I by carbon monoxide

The principal component in red cell haemolysates is haemoglobin. To investigate whether haemoglobin is involved in the formation of peroxyacetic acid uroporphyrin I, red cell haemolysates, 15μl, were saturated with 80% CO in O₂ prior to the reaction followed by incubating with Uro'gen I in a sealed tube. The formation of peroxyacetic acid uroporphyrin I under this condition was reduced to nearly 50% of that produced in the sealed tube with the same volume of 80% N₂ in O₂. This result indicated that haemoglobin played a very important role in the formation of peroxyacetic acid uroporphyrin I, which will be discussed in Chapter 5.

4.3.8. Reaction of Uro'gen I with plasma and leucocytes

Hydroxylated and peroxyacetic acid uroporphyrin I have been identified in the plasma. To investigate the source of these derivatives, plasma (100 μl) was incubated with Uro'gen I and compared with the parallel blanks using boiled plasma or water instead of plasma. In the incubation mixture with fresh plasma peroxyacetic acid uroporphyrin I was detected, but the quantity formed was about 10% of that in the incubation mixture with 30 μl of RBC and Uro'gen I (2.7 μM). Leucocytes (isolated from 10 and 20ml of blood) were also incubated with Uro'gen I. However, the peroxyacetic acid uroporphyrin I detected in the reaction mixture with boiled leucocytes was 16% more than that with unboiled leucocytes but was less than that with plasma. The HPLC
separation profiles of both reaction mixtures with plasma and leucocytes were qualitatively similar to that with red cell haemolysates. These results further confirm the requirement of haemoglobin for the production of peroxycacetic acid uroporphyrin I.

4.3.9. Formation of peroxycacetic acid heptacarboxylic acid porphyrinogen I in red cell haemolysates

To investigate whether heptacarboxylic acid porphyrinogen I can similarly form a peroxycacetic acid derivative, it was incubated with 15μl of red cell haemolysates at 37°C in the dark for 10min. Since there was no peroxycacetic acid heptacarboxylic acid porphyrin found in the urine of patients with CEP, the products formed in the reaction mixture were compared with the three reaction blanks with boiled RBC, incubation buffer instead of RBC and without heptacarboxylic acid porphyrinogen I. Seven peaks were found in the reaction mixture which were essentially absent in the blanks (Fig.4.11.). To isolate these peaks in sufficient quantities for LSIMS characterization, the reaction mixtures were pooled and the porphyrins were extracted by C_{18} cartridge before HPLC separation. Each peak was collected individually, repurified, and esterified for LSIMS analysis.

The MS spectra of peaks 5, 6 and 7 gave the same MH$^+$ signal at m/z 887 (Fig. 4.12.). A 2 Da over the heptamethyl ester of heptacarboxylic acid porphyrin (MH$^+$, m/z 885) clearly suggested that these three peaks were peroxycacetic acid
Fig. 4.11. HPLC Separation of Hydroxylated and Peroxyacetic Acid Heptacarboxylic Acid Porphyrins Formed in a Reaction Mixture of Hepta'gen I and Red Cell Haemolysates

Peak 4, 6-hydroxypropionic acid heptacarboxylic acid porphyrin I; peak 5 – 7, peroxyacetic acid heptacarboxylic acid porphyrin I isomers.
Fig. 4.12. LSIMS of Peroxyacetic Acid Heptacarboxylic Acid Porphyrin Formed in a Reaction Mixture of Hepta'gen I and Red Cell Haemolysates
analogues of heptacarboxylic acid porphyrin I. Assuming that, like peroxyacetic acid uroporphyrin I, peroxylation took place at the acetic acid function then, there are three possible peroxyacetic acid derivatives which can be formed from heptacarboxylic acid porphyrinogen I (Fig. 4.13.)

Peak 4 gave the MH\(^+\) signal at m/z 901 (Fig. 4.14.), an increase of 16 Da unit over the methyl ester of heptacarboxylic acid porphyrin. The molecular weight suggested that this peak was the hydroxylated heptacarboxylic acid porphyrin. This peak had exactly the same retention time as the \(\beta\)-hydroxypropionic acid heptacarboxylic acid porphyrin I found in the urine of patients with CEP under two different HPLC elution conditions. It is therefore identified as the \(\beta\)-hydroxypropionic acid heptacarboxylic acid porphyrin. Peaks 1-3 did not give an identifiable MS signal.

4.3.10. Formation of peroxyacetic acid pentacarboxylic acid porphyrinogen I in red cell haemolysates

Like heptacarboxylic acid porphyrinogen I, pentacarboxylic acid porphyrinogen I was also incubated with 15\(\mu\)l of RBC in the dark at 37°C for 10min. The reaction mixture was again compared with the blanks (pentacarboxylic acid porphyrinogen incubated with boiled RBC, incubation buffer instead of RBC or pentacarboxylic acid porphyrinogen I). There were 7 peaks which were obviously present in larger amount than those in the blanks, but in relatively small quantities and less than that produced by
Fig. 4.13. Structures of Type I Peroxyacetic Acid Heptacarboxylic Acid Porphyrin Isomers
Fig. 4.14. LSIMS of β-Hydroxypropionic Acid Heptacarboxylic Acid Porphyrin Formed in a Reaction Mixture of Hepta'gen I and Red Cell Haemolysates
heptacarboxylic acid porphyrinogen I. It seems that porphyrinogens with fewer carboxylic acid groups are more difficult to convert to peroxyacid form. To increase the yield different pH (7.0, 7.5 and 8.25) were investigated. The pH used, 8.25, in the present reaction, however was found to be optimum.

The relatively low rate of conversion made the investigation difficult. The reaction mixtures were pooled, extracted by C18 cartridge and then separated by HPLC (Fig. 4.15). The peaks were collected and repurified by HPLC. They were esterified and analysed by LSIMS. Peaks 1-3 were insufficient for LSIMS analysis and peaks 5-7 did not give an identifiable MS signal. Peak 4 gave a MH⁺ signal at m/z 771 (Fig. 4.16), which was 2 Da more than the methyl ester of pentacarboxylic acid porphyrin. This indicated that the compound was peroxyacetic acid pentacarboxylic acid porphyrin I.

4.3.11. Attempted formation of peroxyacetic acid coproporphyrinogen I in red cell haemolysates

Coproporphyrinogen I was also incubated with red cell haemolysates at different pH and substrate concentrations. The reaction mixture was compared with the parallel blanks (Coprop'gen I incubated with boiled RBC, incubation buffer instead of RBC or Coprop'gen I), no significant difference was found between the reaction and blank mixtures. Therefore no peroxyacid or hydroxylated product was formed under all reaction conditions (Fig. 17). This would suggest that the
Fig. 4.15. HPLC Separation of Porphyrins in a Reaction Mixture of Penta'gen I with Red Cells Haemolysates

Peak 4, peroxyacetic acid pentacarboxylic acid porphyrin I; peak 8,
Fig. 4.16. LSIMS of Peroxyacetic Acid Pentacarboxylic Acid Porphyrin in the Reaction Mixture of Penta'gen I and Red Cell Haemolysates
Fig. 4.17. HPLC Separation of Porphyrins in a Reaction Mixture of Copro'gen I with Red Cells Haemolysates
production of a peroxyacid derivative requires an acetic acid function and since coproporphyrinogen has four propionic acid groups and no acetic acid group, no product was formed. This is also consistent with the fact that as the numbers of acetic acid groups decreased from uroporphyrinogen to hepta- and penta-carboxylic acid porphyrinogen, peroxylation was progressively more difficult. This also confirmed that the assignment of peroxyacetic acid uroporphyrin I in Chapter 3 was correct.

4.4. Conclusions

The hydroxylated and peroxylated uroporphyrin derivatives found in the urine and plasma of patients with CEP are true metabolites of uroporphyrinogen I and are a common feature of CEP. These derivatives were not found in the urine of AIP, thus ruling out the possibility that they were formed artifactly due to the high concentration of uroporphyrin in the urine. The formation of the peroxyacetic acid uroporphyrin I in the incubation mixture of red cell haemolysates with uroporphyrinogen I, but not with uroporphyrin, as substrates confirmed that these derivatives were formed in vivo from uroporphyrinogen. The failure to detect these derivatives in duodenal aspirates and faeces from the patients with CEP and the faeces from HCP indicated that in CEP these derivatives are of erythropoietic origin. The failure of formation of a peroxyacid coproporphyrinogen suggested that peroxylation can only take place in the presence of an acetic acid group, and confirms the assignment of the peroxyacetic acid uroporphyrin I structure.
Incubation of PBG with red cell haemolysates from patient with CEP produced a much higher proportion of peroxyacetic acid uroporphyrin I compound to normal red cells. This reaction may be used in conjunction with the Uro'gen III-S assay for the diagnosis of CEP. The inhibition by reducing reagents on the formation of peroxyacetic acid uroporphyrin I indicated that the formation of these derivatives was due to an oxidation mechanism. The detailed mechanism of the formation of these derivatives is discussed in the next Chapter.
CHAPTER 5
MECHANISMS OF THE FORMATION OF HYDROXYLATED AND
PEROXYACID UROPORPHYRIN DERIVATIVES

5.1. Introduction

In the last chapter the in vitro formation of meso-hydroxy-, β-hydroxypropionic acid-, hydroxyacetic acid-
uropherin I and peroxyacetic acid uroporphyrin I
derivatives from uroporphyrinogen I by incubation with red
cells haemolysates was described. The results indicated that
an oxidation process was involved. To identify which
oxidizing species is responsible, several chemicals and in
vitro systems which generate various active oxygen species,
mainly free radicals, were tested. The term "free radical"
refers to any species that has one or more unpaired
electrons (Halliwell and Gutteridge, 1985A). This chapter
describes experiments carried out to investigate the
possible mechanisms of formation of hydroxylated and
peroxylated uroporphyrin I derivatives.

5.2. Experimental

5.2.1. Material and reagents

Ammonium acetate, glacial acetic acid,
trichloroacetic acid, I₂, concentrated HCl, KCl, NaOH,
ammonium iron (III) sulphate, KOH,
ethylenediaminetetraacetic acid (EDTA), Triton X-100,
MgCl₂, di-sodium hydrogen orthophosphate dihydrate,
potassium dihydrogen orthophosphate, hydrogen peroxide,
FeCl₃, dimethyl sulfoxide (DMSO), xanthine oxidase (Grade IV), xanthine (sodium salt), were from BDH Chemicals (Poole, Dorset). Acetonitrile and methanol were HPLC grade from Rathburn Chemicals (Walkerburn, Borders, Scotland). Human albumin, allopurinol, haemin, dihydroxymaleic acid (DHM-A), β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), uroporphyrin and coproporphyrin were from Sigma Chemical Co. (Poole, Dorset). Tris(hydroxymethyl)aminoethane (Tris) was from Boehringer Mannheim GmbH. (F.R.G.). Desferrioxamine mesylate (DES) was from Ciba Laboratories (Horsham, West Sussex). 3,4,3',4'-tetrachlorobiphenyl (3,4-TCB) was from Ultra Scientific, Hope, RI.

5.2.2. Incubation of Uro'gen I with liver microsomes

5.2.2.1. Preparation of liver microsomes

Chick-embryo microsome induced by 3,4-TCB stimulates the NADPH-dependent oxidation of uroporphyrinogen (De Matteis et al., 1988). Chick liver microsomes were given by Dr De Matteis and prepared as described by De Matteis et al. (1988). Chick embryos, 16 days of age, of the Rhode Island Red x White Rock strain, were obtained from Orchard Farm, Pinner, Middx., UK. Liver microsomes were prepared 24 h after a single dose of 3,4-TCB (145 μg/embryo) dissolving in 10 μl of 1,4-dioxan given by injection through the air sac into the fluids surrounding the embryo. The livers were perfused in situ by injecting 5 ml of ice-cold phosphate-buffered saline (pH 7.3) through the heart. The microsomes
were then obtained by differential centrifugation as described by Unseld & De Matteis (1978)., washed with 1.15% (w/v) KCl and stored frozen (at -70°C) as pellet overlaid with 0.5 ml of the KCl solution, until use.

5.2.2.2. Reaction procedure

Microsomes, equivalent to 454 pmol of cytochrome P-450 in 1.15% (w/v) KCl, were pre-incubated in 0.1 M Hepes buffer, pH7.4, containing 2 mM EDTA and with or without 175 μg of NADPH in 50 μl 1.15% (w/v) KCl and 1 μg of 3,4-TCB in 5 μl DMSO in a final volume of 3.48 ml for 5 min at 28°C in the dark. The reaction was started by adding 20 μl of Uro'gen I (15 nmol). After mixing the incubation was continued for further 30 min under the same condition. The reaction was stopped by addition of 3.5 ml of 10% TCA containing 0.5% I₂. After centrifugation, the supernatant was analysed by HPLC.

Parallel blanks were prepared as follows: reagent blank by adding 1.15% (w/v) KCl instead of microsomes; zero time blank by adding 10% TCA containing 0.5% I₂ before addition of Uro'gen I. The reaction mixture of red cell haemolysates with Uro'gen I was also performed as a reference to compare the formation of peroxyacetic acid uroporphyrin I from different systems.

5.2.3. Incubation of Uro'gen I with Tris-HCl buffer

Uro'gen I, 20 μl (15 nmol), was incubated with 1.4 ml of 50 mM Tris-HCl buffer, pH 8.25, containing 74 μM
MgCl\(_2\)·6H\(_2\)O and 0.1% Triton X-100, either at 28°C in the dark or at room temperature under illumination of a 60W bulb within 12 inches of diameter for various time. At the end of the incubation the reaction mixture was either directly injected into HPLC or mixed with equal volume of 10% TCA containing 0.5% I\(_2\).

5.2.4. Incubation of Uro'gen I with haematin, methaemalbumin and dihydroxymalic acid in Tris-HCl buffer

Methaemalbumin (MHA) was prepared as described by Tenhunen et al. (1968). The stock solution (2.5 mM) was obtained by dissolving 13mg of haemin in 2.5 ml of 0.1 M NaOH containing 12 mg of Tris base. This solution was mixed with 5ml of 2% human albumin and the pH was adjusted to 7.4 with 1M HCl. This solution was kept frozen at -30°C until use. This stock solution was diluted to 250 µM with H\(_2\)O just before use.

Haematin was prepared by dissolving haemin in 0.01 M KOH to a stock solution of 2.5 mM. This solution was kept at -30°C. The stock solution was diluted by 0.01 M KOH to 250 µM just before use.

Dihydroxymaleic acid (DHM-A), also called dihydroxyfumaric acid, was freshly prepared on the day of the experiment. DHM-A was dissolved in the incubation buffer to make 10 mM solution. This solution was kept on ice and was diluted to 1 mM with incubation buffer before use.
Uro'gen I (15 nmol) was added to an incubation mixture in a total volume of 1.4 ml containing 0.05 M Tris-HCl buffer, pH 8.25, 74 μM MgCl₂, 0.1% Triton X-100, 1 mM EDTA and either 10 nmol of MHA, 10 nmol of haematin, or 300 nmol of DHM-A, respectively. This mixture was incubated either in the dark at 28°C or under illumination by an electric bulb (60 W) within 12 inches of diameter at room temperature for various time intervals. At the end of the incubation the reaction mixture was either injected into HPLC directly for analysis or mixed with an equal volume of 10% TCA containing 0.5% I₂ to oxidise any remaining porphyrinogen before HPLC analysis.

5.2.5. Incubation of Uro'gen I in xanthine-xanthine oxidase system

Xanthine-xanthine oxidase system is an oxidizing system which generates superoxide, hydrogen peroxide and hydroxyl radical sequentially. Xanthine, 26.1 mg was dissolved in approximately 3 drops of 1 M NaOH followed by 5 ml of distilled water with gentle heating. The concentration of the solution was 29.98 mM. Xanthine oxidase, 150 μl (24.3 mg protein/ml, 0.17 unit/mg) was added into 10 ml of 0.1 M Tris-HCl buffer, pH 7.4, to make up a solution of 0.062 unit/ml.

KH₂PO₄-Na₂PO₄ buffer (0.1 M), pH 7.4 or 0.1 M Tris-HCl buffer, pH 7.4, were preincubated with 0.0087 unit/ml of xanthine oxidase and 57 μM of Fe (ferric) as ammonium sulphate salt in the dark at 28°C for 5 min. Then Uro'gen I
30 nmol was added followed by addition of 4 mM xanthine and the reaction mixture was incubated at 28°C in the dark for further 30 min. The final volume of the reaction mixture was 3 ml. The reaction was stopped by adding 3 ml of 10% TCA containing 0.5% I\textsubscript{2}. After vortexed mixing, the mixture was centrifuged and the supernatant was analysed by HPLC.

5.2.6. Incubation of Uro'gen I with hydrogen peroxide/Fe-EDTA system

H\textsubscript{2}O\textsubscript{2}, 30% (w/v), was diluted to desired concentration with 0.1 M Na-K-phosphate buffer containing 1mM EDTA, pH 7.4.

Fe-EDTA was prepared as follows. FeCl\textsubscript{3}, 4 mM, was made by dissolving 27.80 mg in 25.06 ml of H\textsubscript{2}O. EDTA, 5.13 mM, was made by dissolving 26.20 mg in 13.72 ml of H\textsubscript{2}O. Then equal volumes of the two solutions were mixed together and incubated at 37°C for 3 h. Desferrioxamine mesylate (DES), 0.5 g, was dissolved in 7.6 ml of H\textsubscript{2}O to make up a stock solution of 100 mM.

H\textsubscript{2}O\textsubscript{2}, 85 μl (of the desired concentration), was preincubated with 0.1 M Na-K-phosphate buffer containing 1 mM EDTA, pH 7.4, at 28°C in the dark for 5 min. The reaction was started by addition of 20 μl (15 nmol) of Uro'gen I followed by 100 μl of Fe-EDTA. The total incubation volume was 3 ml. The incubation was continued under the same condition for 30 min. Then the reaction was stopped by adding 3 ml of 10% TCA containing 0.5% I\textsubscript{2}.

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5.2.7. Analysis and isolation of the reaction products by HPLC

The HPLC system consisted of a Varian Associates (Walton-on-Thames, Surrey, UK) model 5000 pump and a Varian UV-100 variable wavelength detector set at 400 nm. Samples were directly injected by a Rheodyne (Cotati, CA, USA) 7125 injection valve fitted with a 500 μl loop. The separation was carried out on a 25 cm x 25 mm Hypersil-ODS column (Shandon Scientific, Runcorn, Cheshire, UK) by either isocratic or linear gradient elution. The gradient mixtures were 9% (v/v) acetonitrile in 1 M ammonium acetate buffer pH 5.16 (Solvent A) and 10% (v/v) acetonitrile in methanol (solvent B). The flow rate was 1 ml/min throughout.

1. For measurement of the production of peroxyacetic acid uroporphyrin I:

Isocratic elution at 15% solvent B for 30 min, followed by isocratic elution at 95% for 10 min. The column was re-equilibrated at 15% solvent B for 10 min before next injection.

2. For analysis of the whole porphyrin profile:

Time 0 to 16 min, 0% solvent B (100% solvent A) to 11% solvent B; time 16 to 24 min, isocratic elution at 11% solvent B; time 24 to 38 min, 11% solvent B to 24% solvent B; time 38 to 60 min, 24% solvent B to 60% solvent B and time 60 to 80 min, 60% solvent B to 80% solvent B. The column was then re-equilibrated at 0% solvent B for 10 min.
before injection of next sample. For investigation of the porphyrins eluted before Uro I, the gradient was stopped at 38.1 min and the column was then washed with 95% solvent B for 10 min.

3. For investigation of hydroxylated uroporphyrin I in the reaction mixture:

   Time 0 to 30 min, 0% solvent B to 3% solvent B; time 30 to 40 min, 3% solvent B to 9% solvent B; time 40 to 50 min, 9% solvent B to 20% solvent B; time 50.1 min, 95% solvent B to wash late eluting peaks.

4. For analysis of the hydroxylated and peroxylated derivatives of Copro'gen I:

   Isocratic elution at 55% solvent B.

5.3. Results and Discussion

5.3.1 Formation of peroxycetic acid uroporphyrin I in a liver microsomal system induced by 3,4-TCB

   Microsomes isolated from the liver of chick embryos which were pre-treated with 3,4-TCB in vivo could greatly stimulate the oxidation of Uro'gen in the presence of NADPH and 3,4-TCB (Sinclair et al., 1987; De Matteis et al., 1988). Since the conversion of a carboxylic acid group of uroporphyrinogen to peroxyacid group is an oxidative reaction, this microsomal system was used to investigate possible formation of peroxycetic acid uroporphyrin I.
However, the formation of peroxyacetic acid uroporphyrin I was very poor under these conditions, though Uro'gen I was oxidised to uroporphyrin I as monitored by spectrophotometer at 405 nm in the end of the incubation. Prolonging the incubation time to 5 h made no difference to the amount of peroxyacetic acid uroporphyrin I formed. Under the same conditions, erythrocytes produced much more peroxyacetic acid uroporphyrin I than microsomes but gave a much lower recovery of Uro I (Table 5.1.). It was also noticed that unlike the oxidation of uroporphyrinogen which could be greatly stimulated by 3,4-TCB (De Matteis et al., 1988), 3,4-TCB had little though significant effect on the formation of peroxyacetic acid uroporphyrin I. This result indicated that this microsomal system was not active in formation of peroxyacetic acid uroporphyrin I, and the reason will be discussed later.

5.3.2. Effect of light on the formation of peroxyacetic acid uroporphyrin I

When Uro'gen I was incubated in Tris-HCl buffer at pH between 7.4 and 8.2 in the dark there was virtually no peroxyacetic acid uroporphyrin I detected, despite the addition of 10% TCA containing 0.5% I₂ to oxidize the porphyrinogens. This incubation mixture was always used as reagent blank. When the incubation was carried out under the illumination of light, however, significant amount of
Table 5.1. Formation of Peroxyacetic Acid Uroporphyrin I by Chick-Embryo Liver Microsomes

<table>
<thead>
<tr>
<th></th>
<th>PAAU formed</th>
<th>Yield(%)</th>
<th>Uro I recovered</th>
<th>Recovery(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>0.003</td>
<td>0.02</td>
<td>11.8</td>
<td>78.77</td>
</tr>
<tr>
<td>MC+NADPH</td>
<td>0.006</td>
<td>0.04</td>
<td>9.78</td>
<td>65.23</td>
</tr>
<tr>
<td>MC+3,4-TCB</td>
<td>0.007</td>
<td>0.05</td>
<td>9.75</td>
<td>64.98</td>
</tr>
<tr>
<td>MC+NADPH+3,4-TCB</td>
<td>0.017</td>
<td>0.11</td>
<td>10.2</td>
<td>67.84</td>
</tr>
<tr>
<td>RBC</td>
<td>0.311</td>
<td>2.07</td>
<td>7.11</td>
<td>47.41</td>
</tr>
</tbody>
</table>

MC: microsomes.
PAAU: peroxyacetic acid uroporphyrin I.
Yield: peroxyacetic acid uroporphyrin I formed as a percentage of the starting material Uro'gen I (15 nmol).
Recovery: Uro I detected in the reaction mixture as a percentage of the starting material Uro'gen I (15 nmol).
Unit for PAAU formed and Uro I recovered: nmol/incubation.
Results were obtained with addition of 0.5% I₂ in 10% TCA.
This is the mean of eight experiments.
peroxyacetic acid uroporphyrin I was formed. Though the maximum production of peroxyacetic acid uroporphyrin I in this incubation mixture did not differ from that with an incubation mixture of red cell haemolysates with Uro'gen I, the rate of the formation was much slower and virtually no peroxyacetic acid uroporphyrin I was detected within the first 30 min of incubation. The formation of peroxyacetic acid uroporphyrin I increased rapidly from 30 to 40 min together with a rapid increase of oxidation of Uro'gen I to Uro I. The products of peroxyacetic acid uroporphyrin I and Uro I reached their maxima between 50 to 60 min and then declined gradually as shown in Table 5.2.

Overnight incubating under light illumination led to the disappearance of peroxyacetic acid uroporphyrin I and a nearly 50% loss of Uro I comparing with incubation for 1h. Addition of EDTA (1mM) to the incubation buffer did not stop the loss.

It was also found that the addition of a tiny amount of Uro I to the reaction mixture could speed up the formation of peroxyacetic acid uroporphyrin I though it did not enhance the total amount of the product formed.

It is well known that under the illumination with light and in the presence of oxygen, porphyrin can absorb light and enter a higher electronic excitation state. This excited porphyrin then transfers the energy onto the $O_2$ molecule and thereby creates "excited oxygen" such as singlet $O_2$
(Halliwell & Gutteridge, 1985A) and superoxide (Bickers & Pathak, 1987). In some cases the sensitized porphyrin may react with oxygen to yield hydrogen peroxide or with water to form hydroxyl radicals (Bickers & Pathak, 1987).

Thus the formation of peroxyacetic acid uroporphyrin I from Uro'gen I under light illumination can be explained as follows. Uro'gen I undergoes oxidation to Uro I spontaneously in the presence of oxygen. Under illumination with light the uroporphyrin I formed becomes excited and reacts with oxygen to form some sort of active oxygen species. These active oxygen species may in turn react with the Uro'gen I in the reaction mixture to produce peroxyacetic acid uroporphyrin I and also accelerate the oxidation of the Uro'gen I at the same time. There were several aspects of the formation of peroxyacetic acid uroporphyrin I in buffer alone under light which support this hypothesis: (1) no peroxyacetic acid uroporphyrin I was produced in the first 30 min, (2) the appearance of Uro I preceded that of peroxyacetic acid uroporphyrin I, (3) the addition of Uro I could accelerate the formation of peroxyacetic acid uroporphyrin I. This too indicates that there may be free radicals involved in the formation of peroxyacetic acid uroporphyrin I.
Table 5.2. Time Course for the Formation of Peroxyacetic Acid Uroporphyrin I from Uro'gen I Incubated with Buffer Alone under Light Illumination

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>PAAU Formed</th>
<th>Yield (%)</th>
<th>Uro I Recovered</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.28</td>
<td>1.89</td>
</tr>
<tr>
<td>30</td>
<td>0.024</td>
<td>0.16</td>
<td>2.04</td>
<td>13.6</td>
</tr>
<tr>
<td>35</td>
<td>0.044</td>
<td>0.29</td>
<td>3.77</td>
<td>25.1</td>
</tr>
<tr>
<td>40</td>
<td>0.182</td>
<td>1.21</td>
<td>8.97</td>
<td>59.8</td>
</tr>
<tr>
<td>50</td>
<td>0.209</td>
<td>1.4</td>
<td>9.40</td>
<td>62.7</td>
</tr>
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<td>60</td>
<td>0.195</td>
<td>1.3</td>
<td>10.9</td>
<td>72.5</td>
</tr>
<tr>
<td>75</td>
<td>0.18</td>
<td>1.2</td>
<td>11.4</td>
<td>75.8</td>
</tr>
<tr>
<td>90</td>
<td>0.149</td>
<td>0.99</td>
<td>10.7</td>
<td>71.3</td>
</tr>
<tr>
<td>105</td>
<td>0.136</td>
<td>0.91</td>
<td>10.1</td>
<td>67.6</td>
</tr>
<tr>
<td>120</td>
<td>0.134</td>
<td>0.89</td>
<td>10.6</td>
<td>70.9</td>
</tr>
<tr>
<td>180</td>
<td>0.114</td>
<td>0.76</td>
<td>9.77</td>
<td>65.1</td>
</tr>
</tbody>
</table>

Yield and Recovery: see Table 5.1.

Unit: nmol/incubation

Results were obtained without addition of 10% TCA containing 0.5% I$_2$ at the end of the reaction.

This is the mean of four experiments.
5.3.3. The effect of haematin, methaemalbumin and dihydroxymaleic acid on the formation of peroxyacetic acid uroporphyrin I

The result obtained above pointed to the possibility of a free radical reaction. What was the specific reactive oxygen species? The role of ferrous iron in promoting free radical reaction which catalyzes oxidation of Uro'gen has been widely studied (De Matteis, 1988; Wood & Calas, 1989; Mukerji & Pimstone, 1990). Iron could initiate a free-radical chain reaction pathway involving superoxide radical and hydroxyl radical (Mukerji & Pimstone, 1990). In addition, the major component in the erythrocytes which could react with Uro'gen I to produce peroxyacetic acid uroporphyrin I may be haem, the protoporphyrin chelated with iron. Thus haematin (PFe$^{3+}$OH) and methaemalbumin (MHA, haematin complex with albumin to increase the solubility) were chosen for the investigation of the involvement of Fe. Dihydroxymaleic acid (DHM-A), which generates superoxide anion and H$_2$O$_2$ chemically, was also employed to test the involvement of these species.

Various amounts of haematin (5, 10 and 25 nmol), MHA (5, 10, 25 and 50 nmol) and DHM-A (50, 100, 150, 200, 250, 300 and 400 nmol) were incubated with 15 nmol of Uro'gen I for different time interval (10 min, 30 min, 1 h, 1 h 30 min and 2 h) in the dark at 28°C or under the illumination of light (60W bulb, within 12 inches of diameter) at room temperature. It was found that 10 nmol (final Conc. 7 μM in the reaction mixture) of haematin or MHA, and 300 nmol
(final Conc. 0.2 M in the reaction mixture) of DHM-A gave the better yield of peroxyacetic acid uroporphyrin I. Therefore, 7 µM for both haematin and MHA and 0.2 M for DHM-A were used for this study.

When the incubation was carried out in the dark, despite the addition of haematin, MHA or DHM-A, both the oxidation of Uro'gen I to Uro I and the formation of peroxyacetic acid uroporphyrin I were very poor. Prolonging the incubation time did not significantly affect the result. Addition of 10%TCA containing 0.5% I₂ at the end of the incubation to ensure the oxidation of any peroxyacetic acid uroporphyrinogen I produced did not increase the peroxyacetic acid uroporphyrin I detected, though it greatly increased the Uro I recovered as shown in Table 5.3.. The different values of peroxyacetic acid uroporphyrin I in Table 5.3. (a) and (b) were most probably due to the oxidation of the porphyrinogen by I₂.

When the incubation was carried out under light illumination, significant amount of peroxyacetic acid uroporphyrin I could be detected at 30 min in all incubation mixtures containing MHA, haematin or DHM-A. However, when incubated for 1 h MHA and haematin gave a similar amount of peroxyacetic acid uroporphyrin I to that produced by incubating Uro'gen I with buffer alone while DHM-A gave a
Table 5.3. Effect of Haematin, MHA and DHM-A on the Formation of Peroxyacetic Acid Uroporphyrin I in the Dark

(a) without the addition of 10%TCA containing I₂ at the end of the incubation:

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>PAAU formed</th>
<th>Uro I recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&lt; 0.001</td>
<td>0.120</td>
</tr>
<tr>
<td>30</td>
<td>&lt; 0.001</td>
<td>0.470</td>
</tr>
<tr>
<td>60</td>
<td>&lt; 0.001</td>
<td>1.008</td>
</tr>
<tr>
<td>92</td>
<td>0.004</td>
<td>1.402</td>
</tr>
<tr>
<td>120</td>
<td>0.006</td>
<td>2.150</td>
</tr>
<tr>
<td>DHM-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>&lt; 0.001</td>
<td>0.672</td>
</tr>
<tr>
<td>60</td>
<td>0.006</td>
<td>3.258</td>
</tr>
</tbody>
</table>

This is the typical result of two experiments.
Table 5.3. Effect of Haematin, MHA and DHM-A on the Formation of Peroxyacetic Acid Uroporphyrin I in the Dark

(b) with the addition of 10% TCA containing $I_2$ at the end of the incubation:

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>PAAU formed</th>
<th>Uro I recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.027</td>
<td>12.4</td>
</tr>
<tr>
<td>180</td>
<td>0.023</td>
<td>10.8</td>
</tr>
<tr>
<td>MHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.023</td>
<td>9.45</td>
</tr>
<tr>
<td>Haematin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.025</td>
<td>10.2</td>
</tr>
<tr>
<td>DHM-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.026</td>
<td>10.8</td>
</tr>
<tr>
<td>120</td>
<td>0.026</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Uro'gen I: 15 nmol/incubation

Unit: nmol/incubation

This is the typical result of two experiments.
Table 5.4. Effect of Haematin, MHA and DHM-A on the Formation Of Peroxyacetic Acid Uroporphyrin I under the Illumination of Light

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Buffer</th>
<th>MHA</th>
<th>Haematin</th>
<th>DHM-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P5 produced</td>
<td>Uro I recovered</td>
<td>P5 produced</td>
<td>Uro I recovered</td>
</tr>
<tr>
<td>30</td>
<td>0.021</td>
<td>2.09</td>
<td>0.056</td>
<td>4.97</td>
</tr>
<tr>
<td>60</td>
<td>0.268</td>
<td>9.25</td>
<td>0.234</td>
<td>10.6</td>
</tr>
<tr>
<td>MHA</td>
<td>&lt; 0.001</td>
<td>0.20</td>
<td>0.066</td>
<td>4.96</td>
</tr>
<tr>
<td>30</td>
<td>0.069</td>
<td>6.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.255</td>
<td>11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haematin</td>
<td></td>
<td></td>
<td>0.141</td>
<td>7.48</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHM-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Uro'gen I: 15 nmol/incubation.
Unit: nmol/incubation.
Results were obtained without addition of 10% TCA containing I₂ in the end of the incubation.
lower yield as shown in Table 5.4. Addition of 10% TCA containing I₂ at the end of 1 h incubation did not make much difference in the formation of peroxyacetic acid uroporphyrin I and the oxidation of Uro'gen I to Uro I. It seemed that MHA, haematin or DHM-A alone could not produce peroxyacetic acid uroporphyrin I but could speed up the rate of the reaction. Superoxide dismutase was added into one of the incubation mixtures with DHM-A and Uro'gen I. The result showed that instead of stopping the reaction, it actually increased the product formation from 0.141 to 0.208 nmol/incubation, the same value as incubating Uro'gen I in buffer alone in the presence of light. Superoxidase dismutase catalyses the dismutation of O₂⁻ to H₂O₂. This suggests that H₂O₂ may be involved in the formation of peroxyacetic acid uroporphyrin I and that the spontaneous dismutation of O₂⁻ to H₂O₂ may not be vigorous enough for the formation of a significant amount of peroxyacetic acid uroporphyrin I.

5.3.4. The formation of peroxyacetic acid uroporphyrin I from xanthine-xanthine oxidase system

A classical oxidation system, the xanthine – Xanthine oxidase system, was also employed to investigate the formation of peroxyacetic acid uroporphyrin I. Xanthine – xanthine oxidase system can generate superoxide anion (O₂⁻) in the first place, followed by H₂O₂ and then hydroxy radical (·OH) in the presence of iron. The reaction scheme is as follows:

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Allopurinol inhibits the catalytic activity of xanthine oxidase; superoxide dismutase can protect against the effect of $O_2^-$; catalase can protect against the effect of $H_2O_2$; and mannitol, DMSO, benzoate can protect against the effect of $\cdot OH$. Thus using the above reagents it is hoped that the identity of the active oxygen species responsible for the formation of peroxyacetic acid uroporphyrin I may be clarified.

In the first experiment with 15nmol of Uro'gen I per incubation it was found that xanthine was essential for the formation of peroxyacetic acid uroporphyrin I. Iron could enhance the formation of peroxyacetic acid uroporphyrin I but to a much less extent than that in the oxidation of Uro'gen in a similar system (Francis & Smith, 1988). The formation of peroxyacetic acid uroporphyrin I increased with increasing incubation time as shown in Table 5.5. This was
different from the time course of incubating Uro'gen I with red cell haemolysates or in buffer alone under light illumination. Increasing the concentration of xanthine did not give a higher yield of peroxyacetic acid uroporphyrin I. Increasing the concentration of xanthine oxidase to above 0.0078 unit/ml increased the formation of peroxyacetic acid uroporphyrin I, but led to an extra peak in the reaction mixture when analysed by HPLC. This extra peak was increased with the increase of the concentration of xanthine-xanthine oxidase, and may interfere with the detection of peroxyacetic acid uroporphyrin I. Thus 30 min incubation with xanthine oxidase 0.0087 unit/ml, xanthine 4 mM and Fe (ferric iron ammonium sulphate salt) 57 μM (final concentration in the incubation mixture) was chosen as the standard procedure for the rest of the experiments. Under these conditions, the relationship between Uro'gen I concentration in the reaction mixture and the formation of peroxyacetic acid uroporphyrin I was investigated. The result showed that the formation of peroxyacetic acid uroporphyrin I increased linearly with the concentration of Uro'gen I up to 30 nmol (final conc. 10 μM) as shown in Fig. 5.1. Therefore 30 nmol of Uro'gen I was chosen for the rest of the studies.

To make sure that in this system the catalytic activity of xanthine oxidase was required for the formation of peroxyacetic acid uroporphyrin I, various concentration of allopurinol were added to the reaction mixture. The result clearly showed that allopurinol greatly inhibited the
Table 5.5. Formation of Peroxyacetic Acid Uroporphyrin I from Xanthine -Xanthine Oxidase System

<table>
<thead>
<tr>
<th>Additional Components</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>X.O.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fe</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uro'gen I</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>xanthine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Inc.Time</td>
<td>10 30 60</td>
<td>10 30</td>
<td>10 30</td>
<td>10 30</td>
</tr>
<tr>
<td>PAAU</td>
<td>0.032 0.050 0.104</td>
<td>0.010 0.008</td>
<td>0.023 0.032</td>
<td>0.006 0.008</td>
</tr>
<tr>
<td>Uro I</td>
<td>9.27 9.27 9.54</td>
<td>11.8 12.8</td>
<td>13.37 11.41</td>
<td>14.3 15.0</td>
</tr>
</tbody>
</table>

X.O.: xanthine oxidase  
Inc. Time: incubation time (min)  
Uro'gen I: 15 nmol/incubation  
Unit: nmol/incubation  
This is the typical result from five experiments
Fig. 5.1. Relationship Between the Concentration of Uro'gen I and the Production of Peroxycetic Acid Uroporphyrin I in Xanthine-Xanthine Oxidase System

PAAU (nmol/incubation) ■

Concentration of Uro'gen I (JM)

Uroporphyrin I Recovered (nmol/incubation) ▲
formation of peroxyacetic acid uroporphyrin I and the formation was decreased as the concentration of allopurinol increased (Table 5.6).

To find out which reactive oxygen species was responsible for the formation of peroxyacetic acid uroporphyrin I, various amount of superoxide dismutase were added to the reaction mixture. Low concentration of superoxidase dismutase inhibited the formation of peroxyacetic acid uroporphyrin I, but as the concentration increased, inhibition was weaker instead of stronger as shown in Table 5.7.

Superoxide dismutase is responsible for dismutating $O_2^-$ to $H_2O_2$. Since higher concentration of superoxidase dismutase did not cause a progressive inhibition, it is possible that the products resulting from the activity of superoxidase dismutase, either $H_2O_2$ or a further metabolite $'OH$, is responsible for the formation of peroxyacetic acid uroporphyrin I.

\[
\text{SOD} \quad \text{Fe}^{2+} \\
O_2^- \rightarrow H_2O_2 \rightarrow 'OH
\]

Thus the scavenger of $H_2O_2$, catalase, was added to the xanthine - xanthine oxidase system. The results showed that catalase did inhibit the formation of peroxyacetic acid.
Table 5.6. Effect of Allopurinol on the Formation of Peroxyacetic Acid Uroporphyrin I

<table>
<thead>
<tr>
<th></th>
<th>1 (Control)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopurinol (mM)</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>PAAU (nmol/incubation)</td>
<td>0.065</td>
<td>0.027</td>
<td>0.020</td>
<td>0.014</td>
</tr>
<tr>
<td>% of Control</td>
<td>100</td>
<td>41.8</td>
<td>31.0</td>
<td>21.7</td>
</tr>
<tr>
<td>Uro I recovered</td>
<td>16.8</td>
<td>18.1</td>
<td>19.2</td>
<td>19.0</td>
</tr>
<tr>
<td>(nmol/incubation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uro I recovery (%)</td>
<td>56</td>
<td>60.3</td>
<td>64</td>
<td>63.3</td>
</tr>
</tbody>
</table>

Uro'gen I: 30 nmol/incubation.
Table 5.7. Effect of Superoxide Dismutase on the Formation of Peroxyacetic Acid Uroporphyrin I

<table>
<thead>
<tr>
<th></th>
<th>1(Control)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD(unit/ml)</td>
<td>0</td>
<td>5</td>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>PAAU</td>
<td>0.131</td>
<td>0.053</td>
<td>0.051</td>
<td>0.075</td>
</tr>
<tr>
<td>% of Control</td>
<td>100</td>
<td>40.1</td>
<td>39.3</td>
<td>57.3</td>
</tr>
<tr>
<td>URO I recovered</td>
<td>19.6</td>
<td>23.9</td>
<td>24.8</td>
<td>24.2</td>
</tr>
<tr>
<td>Uro I recovery (%)</td>
<td>65.3</td>
<td>79.7</td>
<td>82.7</td>
<td></td>
</tr>
</tbody>
</table>

Uro'gen I: 30 nmol/incubation.

Unit of PAAU and Uro I recovered: nmol/incubation.

This is the mean of the duplicate results of one experiment.

In another similar experiment 50 unit/ml of SOD was used and the
uroporphyrin I, and as its concentration increased the inhibition was also increased as shown in Table 5.8. Addition of both superoxide dismutase (50 unit/ml) and catalase (1500 unit/ml) together to xanthine - xanthine oxidase system gave 52% peroxyacetic acid uroporphyrin I of control reaction mixture, did not further increase the inhibition.

To investigate whether \( \cdot \text{OH} \) was involved in the formation of peroxyacetic acid uroporphyrin I, the scavenger, mannitol was added to the system at various concentrations. The result showed that it had no significant effect on the formation of peroxyacetic acid uroporphyrin I (Table 5.9.). This suggests that hydroxyl radical is not responsible for the formation of peroxyacetic acid uroporphyrin I.

5.3.5. Formation of Peroxyacetic Acid Uroporphyrin I by \( \text{H}_2\text{O}_2 \)

To make sure the \( \text{H}_2\text{O}_2 \) was responsible for the formation of peroxyacetic acid uroporphyrin I, different concentrations of commercial \( \text{H}_2\text{O}_2 \) were reacted with Uro'gen I in presence of Fe-EDTA as described in Sec. 5.2.6. The result showed that at low concentration of \( \text{H}_2\text{O}_2 \) no peroxyacetic acid uroporphyrin I was produced, and as the concentration increased the formation of peroxyacetic acid uroporphyrin I also increased up to 2.5 mM. When 25 mM of
### Table 5.8. Effect of Catalase on the Formation of Peroxyacetic Acid Uroporphyrin I

<table>
<thead>
<tr>
<th></th>
<th>1 (Control)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (unit/ml)</td>
<td>0</td>
<td>300</td>
<td>1500</td>
<td>3000</td>
</tr>
<tr>
<td>PAAU</td>
<td>0.073</td>
<td>0.051</td>
<td>0.038</td>
<td>0.034</td>
</tr>
<tr>
<td>% of Control</td>
<td>100</td>
<td>69.7</td>
<td>52.4</td>
<td>46.9</td>
</tr>
<tr>
<td>Uro I recovered</td>
<td>16.7</td>
<td>20.7</td>
<td>21.6</td>
<td>21.7</td>
</tr>
<tr>
<td>Uro I recovery (%)</td>
<td>55.6</td>
<td>69</td>
<td>72</td>
<td>72.3</td>
</tr>
</tbody>
</table>

*Uro'gen I:30 nmol/incubation*

*Unit of PAAU and Uro I recovered: nmol/incubation*

*This is the typical result of two experiments.*
Table 5.9. Effect of Mannitol on the Formation of Peroxyacetic Acid

<table>
<thead>
<tr>
<th></th>
<th>1(Control)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol(Mm)</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>PAAU (nmol/incubation)</td>
<td>0.076</td>
<td>0.071</td>
<td>0.073</td>
<td>0.073</td>
<td>0.077</td>
</tr>
<tr>
<td>% of Control</td>
<td>100</td>
<td>94</td>
<td>96.7</td>
<td>96.1</td>
<td>1.01</td>
</tr>
<tr>
<td>URO I(nmol/incubation)</td>
<td>21.4</td>
<td>22.1</td>
<td>22.3</td>
<td>22.8</td>
<td>24.3</td>
</tr>
<tr>
<td>% of URO I recovery</td>
<td>71.4</td>
<td>73.8</td>
<td>74.5</td>
<td>75.8</td>
<td>80.9</td>
</tr>
</tbody>
</table>

Uro'gen I: 30nmol/incubation.

This is the mean of three experiments.
H$_2$O$_2$ was used the formation of peroxyacetic acid uroporphyrin I was similar to that produced at 2.5 mM, but at this concentration the porphyrins formed in the reaction mixture began to decrease soon after the reaction was stopped. At concentration higher than 25 mM, almost no peroxyacetic acid uroporphyrin I was detected, and Uro I recovery was only about 30%. No peaks other than Uro I were detected by UV-vis detector at 405 nm or a fluorescent detector set at Ex. 400 nm and Em. 618 nm. Omission of Fe-EDTA which acted as a catalyst (De Matteis, 1988; Aust et al., 1985) in H$_2$O$_2$/Fe-EDTA system caused very little formation of peroxyacetic acid uroporphyrin I as shown in Table 5.10. Addition of catalase tremendously inhibited the formation of peroxyacetic acid uroporphyrin I (0.009 nmol/incubation formed) and gave more than 80% recovery of Uro I. This suggested that the formation of peroxyacetic acid uroporphyrin I is indeed due to H$_2$O$_2$.

An iron chelator, as well as a scavenger of \cdot OH, desferrioxamine (DES) was added to the H$_2$O$_2$/Fe-EDTA system. Its effect on the formation of peroxyacetic acid uroporphyrin I was in opposite directions depending on the concentration in the reaction mixture. At low concentration the formation of peroxyacetic acid uroporphyrin I was almost completely abolished, but as the concentration increased the inhibition decreased as shown in Table 5.11. No satisfactory explanation for these findings can as yet be
Table 5.10. Effect of H$_2$O$_2$ and Fe-EDTA on the Formation of Peroxyacetic Acid Uroporphyrin I

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (µM)</th>
<th>Fe-EDTA (68.4µM)</th>
<th>PAAU</th>
<th>Uro I Recovered</th>
<th>Uro I Recovery(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>+</td>
<td>0.003</td>
<td>26.6</td>
<td>88.7</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>0.019</td>
<td>25.4</td>
<td>84.7</td>
</tr>
<tr>
<td>250</td>
<td>+</td>
<td>0.073</td>
<td>19.9</td>
<td>66.3</td>
</tr>
<tr>
<td>2500</td>
<td>+</td>
<td>0.100</td>
<td>16.2</td>
<td>54.0</td>
</tr>
<tr>
<td>25000</td>
<td>+</td>
<td>0.106</td>
<td>15.0</td>
<td>50.0</td>
</tr>
<tr>
<td>250000</td>
<td>+</td>
<td>0.006</td>
<td>10.3</td>
<td>34.3</td>
</tr>
<tr>
<td>2500</td>
<td>-</td>
<td>0.016</td>
<td>29.6</td>
<td>98.7</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0.010</td>
<td>32.4</td>
<td>108</td>
</tr>
</tbody>
</table>

Uro'gen I: 30nmol/incubation.

Unit of PAAU and Uro I recovered: nmol/incubation.

This is the typical result of two experiments.
Table 5.11. Effect of Desferrioxamine on the Formation of Peroxyacetic Acid Uroporphyrin I and the Recovery of Uro I

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (mM)</th>
<th>DES (mM)</th>
<th>Fe-EDTA (68.4μM)</th>
<th>PAAU</th>
<th>Uro I</th>
<th>Uro I recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2.5</td>
<td>0</td>
<td>+</td>
<td>0.082</td>
<td>16.9</td>
<td>56.2</td>
</tr>
<tr>
<td>2. 2.5</td>
<td>0.1</td>
<td>+</td>
<td>0.005</td>
<td>26.3</td>
<td>87.8</td>
</tr>
<tr>
<td>3. 2.5</td>
<td>1</td>
<td>+</td>
<td>0.021</td>
<td>15.3</td>
<td>50.9</td>
</tr>
<tr>
<td>4. 2.5</td>
<td>10</td>
<td>+</td>
<td>0.117</td>
<td>27.9</td>
<td>93</td>
</tr>
<tr>
<td>5. 2.5</td>
<td>10</td>
<td>-</td>
<td>0.018</td>
<td>32.9</td>
<td>109.7</td>
</tr>
<tr>
<td>6. 0</td>
<td>10</td>
<td>+</td>
<td>0.011</td>
<td>37.7</td>
<td>125.5</td>
</tr>
<tr>
<td>7. 25</td>
<td>10</td>
<td>-</td>
<td>0.011</td>
<td>34.8</td>
<td>115.9</td>
</tr>
</tbody>
</table>

Uro'gen I: 30nmol/incubation.

Unit of PAAU and Uro I recovered: nmol/incubation.

This is the typical result of two experiments.
provided and further work is needed. At a concentration of 10 mM, DES could, however protect the loss of porphyrins against the high concentration of H₂O₂. One reaction mixture containing 25 mM of H₂O₂ was analysed a day after stopping the reaction. It showed that the peroxyacetic acid uroporphyrin I had disappeared completely and only 10.8 nmol out of original 30 nmol of Uro'gen I was detected. While in the parallel reaction mixture with 10 mM of desferrioxamine, 0.163 nmol/incubation of peroxyacetic acid uroporphyrin I and 27.2 nmol/incubation of Uro I were detected.

Uroporphyrin I was also treated with the H₂O₂/Fe-EDTA system under various reaction conditions. No peroxyacetic acid derivative could be detected by HPLC. This again showed that the peroxyacetic acid derivative was derived from uroporphyrinogen I rather than uroporphyrin I.

5.3.6. Disappearance of porphyrins during the formation of peroxyacetic acid uroporphyrin I

It was observed that incubating red cell haemolysates with Uro'gen I in the dark overnight and incubating Uro'gen I in buffer under light illumination overnight led to a significant disappearance of all porphyrins. Addition of EDTA (1mM) to the incubation buffer did not stop the loss, which indicated that the loss was not due to the formation of metalloporphyrin. The loss of the porphyrins could be abolished by oxidation with I₂ in 10% TCA after the reaction has processed for 30 min (with red cell haemolysates) or 1 h (with incubation buffer alone). In the xanthine - xanthine
oxidase system, the oxidation of Uro'gen I to Uro I at the end of the 30 min incubation was recorded by UV-vis spectrophotometry at 405 nm before addition of I₂ in 10% TCA. The result showed that the loss of porphyrins was closely related to the formation of peroxyacetic acid uroporphyrin I and the oxidation of Uro'gen I to Uro I by the enzyme. In the reaction mixture in which most of the Uro'gen I had oxidized to Uro I before the addition of I₂ in 10% TCA, the most formation of peroxyacetic acid uroporphyrin I and the least recovery of Uro I were detected after the addition of I₂. In the H₂O₂/Fe-EDTA system, this loss was even more significant, rapid and related to the concentration of H₂O₂, the presence of Fe-EDTA and to some extent to the formation of peroxyacetic acid uroporphyrin I. The result in Sec. 5.3.5. clearly showed that without either H₂O₂ or Fe-EDTA in the reaction mixture the recovery of Uro I would be near 100% (Table 5.10.). When 30nmol of Uro I was incubated with H₂O₂ (2.5 mM) and Fe-EDTA (68.4 μM) in which no peroxyacetic acid uroporphyrin I was formed, also 100% recovery of Uro I was obtained. However, when the incubation mixture contained 30nmol of Uro'gen I under the exact same condition there was only a 56.2% recovery of Uro I. In contrast, when the incubation mixture with 30 nmol of Uro'gen I contained 10mM of desferrioxamine, despite the higher yield of peroxyacetic acid uroporphyrin I gained, 93% recovery of Uro I was obtained (Table 5.11.). This strongly suggested that the reaction between Uro'gen I and H₂O₂/Fe-EDTA gave two reactions: one was the oxidation of the carboxylic acid group of Uro'gen I to form peroxyacetic acid.
uroporphyrin I, the other the formation of hydroxy radical which was responsible for the loss of porphyrins.

The disappearance of porphyrin during porphyrinogen oxidation by $H_2O_2$/Fe-EDTA system and hypoxanthine/xanthine oxidase/Fe-EDTA system had been reported (De Matteis, 1988; Francis & Smith, 1988), in both systems hydroxy radical was suggested to be involved in the decomposition of porphyrins. This was consistent with the results we obtained above.

5.3.7. Attempted formation of peroxyacid coproporphyrinogen I by the action of $H_2O_2$

To investigate whether the corresponding peroxyacid analogue could be formed by treating corproporphyrinogen I or coproporphyrin I with $H_2O_2$/Fe-EDTA system, the porphyrin or porphyrinogen (final Conc. 10 μM) was added to the incubation with 25 mM of $H_2O_2$ and 68.4 μM of Fe-EDTA. The reaction was carried out under exactly the same conditions as described in Sec. 5.2.6. When the reaction mixture was analysed by HPLC no peroxyacid coproporphyrin derivative was found. This again proved that the presence of an acetic acid function is essential for the formation of the peroxyacid derivative.

5.3.8. Mechanisms of formation of peroxyacetic acid and hydroxylated uroporphyrins

The results in Sec. 5.3.5. have clearly shown that the formation of peroxyacetic acid uroporphyrin I is due to $H_2O_2$ in the presence Fe-EDTA. Considering the formation of
peroxyacetic acid uroporphyrin I with red cell haemolysates, the major component in RBC is haemoglobin. Haemoglobin consists of four molecules of haem combined with one molecule of globin through the fifth coordinating ligand of Fe. The function of haemoglobin is cooperative binding and effective delivery of oxygen. When haemoglobin is in the deoxy state, the iron has five ligands and is a Fe(II) complex. Upon oxygen binding to the sixth coordination site, the iron becomes Fe(III) superoxide complex and the structure of haemoglobin is changed which leads to high-affinity to oxygen (Ryan & Aust, 1992). The conversion of Fe (III) superoxide complex to Fe(III) complex and superoxide radical is a reversible reaction. In the presence of porphyrinogen as electron donator the superoxide radical could abstract a hydrogen atom from the porphyrinogen and start a chain reaction in which $O_2^-$ is a propagating species as follows:

$$\text{PH}_6 + 3O_2^- + \text{Fe (III)} \rightarrow P + 3H_2O_2 + \text{Fe (II)}$$

Here PH$_6$ and P are the reduced and oxidised forms of porphyrins (Mukerji & Pimstone, 1990). The $H_2O_2$ may then react with porphyrinogen in the presence of iron to form peroxycetic acid uroporphyrin I. Fe (III) may be reduced by $O_2^-$ and the resulting Fe(II) may then react with $H_2O_2$ to produce ·OH which may lead to the formation of the hydroxylated uroporphyrin I derivatives and the loss of porphyrins.
When red cell haemolysates were boiled, the haemoglobin was denatured and the ability of combining with oxygen was impaired. Thus when the boiled red cell haemolysates were incubated with Uro'gen I the formation of peroxyacetic acid uroporphyrin I was reduced. pH can also affect the affinity of haemoglobin to oxygen significantly. At pH lower than 7.2 the structure of haemoglobin is modified which reduces its affinity to oxygen. While at high pH peroxyacetic acid uroporphyrin I is easily converted into Uro I. Thus the optimal pH range for the formation of peroxyacetic acid uroporphyrin I with red cell haemolysates described in Sec. 4.3.13. can be explained.

The oxygen content in plasma is dependent on the dissolved oxygen and not by any chemical binding. The content of oxygen in plasma is therefore much less than that in RBC (less than 4%). Iron in plasma is mainly transferrin in which two ferric iron are combined with one molecule of protein. It is not involved in the oxidation of biomolecules. But "free" iron may be generated and oxidative stress would ensure (Ryan & Aust, 1992). Thus in the presence of Uro'gen I the formation of peroxyacetic acid uroporphyrin I could happen and much lower yield than that with red cell haemolysates may be expected.
In leucocytes the main oxidation system is as follows:

\[
\text{NAD(P)H oxidase} \\
2O_2 + \text{NAD(P)H} \longrightarrow \text{NAD}^+(P) + 2O_2^- + H^+
\]

\[
\text{SOD} \\
O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2
\]

\[
\text{peroxidase} \\
H_2O_2 \longrightarrow H_2O + O_2
\]

This oxidation procedure is triggered by the phagocytosis of leucocytes. The main intracellular ion in leucocytes is potassium. This may explain why little product was formed when incubating uroporphyrinogen I with leucocytes.

In liver microsomes, the oxidation system is cytochrome P-450. It is suggested that main oxidizing species produced by the microsomes is \(H_2O_2\) (De Matteis, 1988). When Uro'gen I was incubated with 3,4-TCB induced chicken embryos microsomes, however, only the oxidation of Uro'gen I to Uro I was observed and hardly any peroxyacetic acid uroporphyrin I was produced. This may be due to the fact that the microsomes system was not sufficiently vigorous in producing \(H_2O_2\) for the formation of peroxyacetic acid uroporphyrin I. The liver cytochrome P-450 is an oxidation chain which usually transported the electron along the chain and passed the electron onto the substrate. Therefore the oxidation procedure may be different and may not able to promote the
formation of peroxyacetic acid uroporphyrin I.

It is known that peroxyacid can be formed by direct oxidation of carboxylic acids with 30 to 98% hydrogen peroxide in the presence of mineral acid as a catalyst (Plesnicar, 1978). In our study reacting H$_2$O$_2$ with porphyrinogens which containing acetic acid group(s) did form peroxyacid. When reacting H$_2$O$_2$ with porphyrinogens which contained only propionic acid groups or with porphyrins, no peroxyacid formation was observed. This may be due to the electron interaction within the porphyrin molecule. In porphyrin state the highly conjugated aromatic ring withdraws the electron cloud from the side chains to the porphyrin nucleus while in the porphyrinogen state the side chains, especially acetic acid chain, may themselves become the electron withdrawing group. In propionic acid chains the carboxylic acid group may be too far from the porphyrin nucleus, so it is more difficult to withdraw the electron cloud as efficiently as acetic acid group. Thus to form peroxyacid function from the propionic acid group of porphyrinogen or from porphyrin may require a much higher concentration of H$_2$O$_2$. However, this will lead to the destruction of porphyrin ring. This may explain why a peroxyacid derivative cannot be formed from coproporphyrinogen or uroporphyrin.

In all reaction systems which produced peroxyacetic acid uroporphyrin I, there were two peaks which had the same retention time as meso-hydroxyuroporphyrin I and β-
hydroxypropionic acid uroporphyrin I in the incubation mixtures. There was also a trace of hydroxyacetic acid Uro I detected. These two peaks also eluted out at the same positions of natural meso-hydroxyuroporphyrin I and \( \beta \)-hydroxypropionic acid uroporphyrin I by expanding the elution program. That they were meso-hydroxyuroporphyrin I and \( \beta \)-hydroxypropionic acid uroporphyrin I was confirmed by co-injection with authentic compounds into HPLC.

The peak height of meso-hydroxyuroporphyrin I was similar in the incubation mixtures of Uro'gen I with red cell haemolysates, xanthine - xanthine oxidase and \( \text{H}_2\text{O}_2/\text{Fe-EDTA} \), but smaller in the incubation mixture of Uro'gen I with buffer alone or with buffer containing haematin, methaemalbumin and dihydroxymaleic acid. The peak height of \( \beta \)-hydroxypropionic acid uroporphyrin I was generally very small in all incubation mixtures, except that of Uro'gen I with \( \text{H}_2\text{O}_2/\text{Fe-EDTA} \) in which this peak was 10 times higher than in the other systems. The relationship between the peak height of these two peaks and the formation of peroxyacetic acid uroporphyrin I has not been fully investigated. In the system of \( \text{H}_2\text{O}_2/\text{Fe-EDTA} \), however, it was certain that as the concentration of desferrioxamine increased, the peak height of these two peaks were decreased.

The existence of meso-hydroxyuroporphyrin I and \( \beta \)-hydroxypropionic acid uroporphyrin I in the reaction mixture in which the peroxyacetic acid uroporphyrin I was formed was proved by HPLC and LSIMS as described in Chapter 3. Thus the
generation of peroxyacetic acid uroporphyrin I also produced the hydroxylated uroporphyrins. This was consistent with the observation that when hepta- and penta-carboxylic acid porphyrinogen I were incubated with red cell haemolysates not only peroxyacid but also hydroxylated derivatives were formed. It is not clear whether the formation of these hydroxylated uroporphyrins was by the same mechanism as the formation of peroxyacetic acid uroporphyrin I. It is likely, however, that these hydroxylated uroporphyrins were formed by a free radical mechanism involving hydroxy radical generated during the oxidation of Uro'gen I to peroxyacetic acid uroporphyrin'gen I. The increased concentration of desferrioxamine resulting in decreased formation of β-hydroxypropionic acid uroporphyrin I suggested that their formation was most probably due to the reaction of Uro'gen I with hydroxy radical.

5.4. Conclusions

The hydroxylated and peroxyacetic acid uroporphyrin I were generated in vitro with various oxidizing systems, and had been confirmed that they were similar compounds to those detected in the urine from patients with CEP. The formation of peroxyacetic acid uroporphyrin I was suppressed by catalase but unaffected by mannitol, indicating that $H_2O_2$ was responsible for the production. The formation of meso-hydroxy- and β-hydroxypropionic acid- uroporphyrin I was decreased by desferrioxamine, suggesting that $·OH$ was responsible for the production of hydroxylated porphyrins. The disappearance of porphyrins during the formation of
peroxyacetic acid uroporphyrin I was also abolished by desferrioxamine, indicating that \cdot OH is responsible for the porphyrin destruction. The failure of the formation of peroxyacetic acid uroporphyrin I from uroporphyrin I and peroxyacid coproporphyrin I from coproporphyrinogen and coproporphyrin I by red cells haemolysates and \( H_2O_2 \) confirmed that peroxylation can occur only at porphyrinogen stage and only at acetic acid group. The possible mechanism of the formation of hydroxylated and per oxyacetic acid uroporphyrin I in erythrocytes and the possible reason why no peroxyacetic acid uroporphyrin I was formed from Uro I and its analogue from coproporphyrinogen were proposed.
CHAPTER 6
GENERAL DISCUSSION AND FUTURE DEVELOPMENT

6.1. Charcoal Therapy in CEP and Possible Alternative Treatment

The administration of oral charcoal to a patient with CEP failed to reduce plasma porphyrin level or to improve his clinical status. This is because approximately three quarters of excess porphyrins produced in erythropoietic system are uroporphyrin I. This highly hydrophilic porphyrin is favourably excreted into urine, and is hardly detectable in the duodenal aspirates and faeces of the patient. Charcoal given orally may be effective in interfering with the enterohepatic circulation of porphyrins by preventing the enteral absorption of porphyrins excreted into the intestinal lumen (Israel & Dayton, 1984; Mukerji et al., 1985; Pimstone et al., 1987; Morton et al., 1988; Tishler & Winston, 1990.). The charcoal adsorption of porphyrins in the gut lumen may increase the flux of free porphyrins from plasma to the lumen, where the porphyrins are entrapped by the charcoal and then excreted in the faeces (Pimstone et al., 1987). Therefore, although the oral charcoal treatment was ineffective in this case of CEP, it may be beneficial to the hepatic porphyrias in which the liver is the major site of production and accumulation of the excess porphyrins. These porphyrins will diffuse or be excreted into bile and thus efficiently eliminated by binding onto charcoal. Erythrohepatic protoporphyria may also benefit from charcoal adsorption therapy as the highly hydrophobic protoporphyrin
can only be excreted into bile and eliminated through the intestine.

Other therapies for CEP also have limited efficacy which include limiting exposure to sunlight, where barrier creams and β-carotene may be of some benefit, and treatment of skin infections (Nordmann & Deybach, 1982; Poh-Fitzpatrick, 1982; Bloomer & Bonkovsky, 1989). Erythrocyte transfusions can correct the anaemia and reduce haemolysis. In particular the transfusions inhibit endogenous erythropoiesis, decreasing the production of uroporphyrin and the derived porphyrin metabolites (Nordmann & Deybach, 1982). In our patient erythrocyte transfusions also increased the erythrocyte Uro'gen III-S (Chapter 2), presumably due to the exogenous Uro'gen III-S which may also play a role in reducing the porphyrins accumulated. It was reported that long-term treatment with high-level transfusions led to complete suppression of symptoms in one case of CEP in which the iron overload was well mitigated by multiple infusions of deferrioxamine (Piomelli et al., 1986). However, it is well known that multiple transfusions can be harmful. The possible ill-effects of blood transfusion include haemolytic transfusion reactions, non-haemolytic febrile reactions, allo-immunization, plasma and urticarial reactions, effect of bacterial contamination and pyrogens and transmission of disease (Gunson, 1983). To avoid the dangers of multiple transfusions, haematin was proposed as an alternative to erythrocyte transfusions (Watson et al., 1974). Haem exerts a negative feedback
repression on the haem biosynthetic pathway and leads to a reduction of porphyrin synthesis (Rank et al., 1990). However the effect is short-lived and does not achieve clinical relief (Nordmann & Deybach, 1982, Rank et al., 1990). Chloroquine may reduce erythrocyte fragility (Ippen & Fuchs, 1980) and thus may reduce haemolysis (Moore et al. 1990). Splenectomy may also mitigate the haemolysis, reduce porphyrin excretion and diminish skin manifestation, but in general there seems to be little evidence for specific and long-term improvement (Nordmann & Deybach, 1982; Bloomer & Bonkovsky, 1989). Replacement therapy, such as substitution of the defective enzyme, genetic modifications and reparation of the impaired gene may be the solution. These therapies, however, are not possible at the present time.

6.2. Distribution and Metabolism of Hydroxylated and Peroxyacid Uroporphyrin Derivatives in vivo

Meso-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I and peroxyacetic acid uroporphyrin I have been identified in the urine and plasma from patients with CEP. In contrast these uroporphyrin I derivatives were not found in the duodenal aspirates and faeces of the patient with CEP whose urine contained relatively large amount of these compounds. This indicated that in the patients with CEP these uroporphyrin derivatives are derived from the erythropoietic system, the major site of the defective enzyme and where the excessive uroporphyrin I are produced and accumulated. In one patient
with CEP the plasma and urinary porphyrin levels were measured over 51 days with an interval of 4-7 days. The percentages of hydroxy- and peroxyacetic acid- uroporphyrin I derivatives of the total porphyrins in both plasma and urine were calculated. The results showed that in plasma the uroporphyrin I derivatives were $0.6 \pm 0.07\%$ (mean $\pm$ SD) of the total porphyrins while in the urine uroporphyrin I derivatives were $1.6 \pm 0.39\%$ (mean $\pm$ SD). The more than two fold higher level of these uroporphyrin I derivatives in urine than that in plasma indicated that the formation of these derivatives may lead to increased excretion of the excess uroporphyrin I. This may be a mechanism of the excretion of excess uroporphyrin I.

The existence of $\beta$-hydroxypropionic acid- and hydroxyacetic acid-heptacarboxylic acid porphyrin I and the absence of meso-hydroxy- and peroxyacetic acid-heptacarboxylic acid porphyrin I, and the related analogues derived from hexa- and penta-carboxylic acid porphyrin I are very interesting. In CEP the heptacarboxylic acid porphyrin I accumulated is much less than uroporphyrin I which may not be enough to promote the formation of hydroxylated derivatives from heptacarboxylic acid porphyrinogen I. Therefore, it is very likely that the hydroxylated heptacarboxylic acid porphyrin I derivatives are the metabolites of the corresponding uroporphyrinogen I derivatives. Once the porphyrins are hydroxylated, they also tend to be excreted into urine due to their hydrophilicity and further decarboxylation will thus be less likely.
Therefore no hydroxylated hexa- and penta-carboxylic acid porphyrin I were found in CEP urine. *Meso*-hydroxy- and peroxyacetic acid- heptacarboxylic acid porphyrin I were not detected in the urine of CEP. The most likely reason is that the corresponding uroporphyrin I derivatives are less stable. *Meso*-hydroxyuroporphyrin I may be easily ring opened, while peroxyacetic acid uroporphyrin I is easily converted to uroporphyrin I. The conversion of a peroxyacid group to a carboxylic acid is a reduction reaction, therefore an oxidation reaction may occur simultaneously which may play a role in the pathogenesis of CEP. However, these need further investigation.

6.3. Formation of Hydroxy- and Peroxyacetic Acid-Porphyrins *In Vivo*

The present study has shown that peroxyacetic acid porphyrins and hydroxylated porphyrins are derived from the corresponding porphyrinogens which have acetic acid group(s) under one of the following three conditions: 1. the presence of erythrocytes; 2. light illumination; and 3. the presence of $\text{H}_2\text{O}_2$ and iron. The formation of peroxyacetic acid uroporphyrin I *in vitro* is proportional to the concentration of uroporphyrinogen I. *In vivo* higher concentrations of uroporphyrin I derivatives are found in the patients with CEP whose urinary uroporphyrin I was greatly excessive but much less in the patient with CEP whose urinary uroporphyrin I level is much lower (2.28-4.22 μmol/24h). This indicated that any enzyme defect which led to the accumulation of porphyrinogens with side-chain acetic acid substituents may
induce the formation of these derivatives. Thus these derivatives can be formed not only in CEP but also in PCT in which Uro'gen-D defect lead to the accumulation of uroporphyrin I and III isomers, heptacarboxylic acid porphyrin (dominated by isomer III) and to a lesser extent coproporphyrin, hexa-, penta-carboxylic acid porphyrins and typically isocoproporphyrin. In fact the hydroxylated and peroxycetic acid uroporphyrin, heptacarboxylic acid porphyrin and pentacarboxylic acid porphyrin derivatives have been isolated and identified from the patients with PCT (Luo, 1992). In CEP the metabolic defect is expressed in the erythropoietic system, and the excessive porphyrins are mainly distributed in the bone marrow and the peripheral blood. The porphyrin content in the spleen is frequently but variably increased and may bear a relationship to the rate of RBC destruction by this organ. In the liver porphyrin level is usually not greatly increased with occasional exceptions (Kappas et al., 1983). Thus the highly elevated hydrophillic uroporphyrin I and its hydroxylated and peroxycacid uroporphyrin derivatives tend to be excreted out in the urine. In PCT, on the other hand, the predominant site of the metabolic abnormality is the liver where uroporphyrinogens and heptacarboxylic acid porphyrinogens are accumulated due to the Uro'gen-D defect. The corresponding peroxyacetic acid- and hydroxy- derivatives may be generated because certain amount of H$_2$O$_2$ is continuously being formed in the liver (Halliwell and Gutteridge, 1985B), which together with the existence of excessive porphyrinogens and iron lead to the production of
these derivatives. The derivatives and the side product, hydroxyl radicals, may play a role in the liver damage. Due to the particular location and the function of the liver, the derivatives together with the highly accumulated porphyrins may partially circulated in the blood stream and excreted into urine according to their polarity or partially diffused into ductus biliferi due to their high concentration and undergo the enterhepatic circulation to be excreted in the faeces. The substitution of hydroxy- or peroxyacid- group onto the side chains of uroporphyrinogen or hepta-, hexa- and penta-carboxylic acid porphyrinogens may affect the action of Uro'gen-D and result in the formation of unusual decarboxylation products. These derivatives, especially the β-hydroxypropionic acid derivatives, are structurally possible to be converted to the isocoproporphyrin series which are commonly found in PCT (Elder & Chapman, 1970; Elder, 1971B; 1972). This may explain why isocoproporphyrins are only found in PCT.

6.4. Possible Inhibition of Enzyme Activities by the Hydroxy- and Peroxyacetic Acid- Uroporphyrin Derivatives

In CEP the enzyme defect is Uro'gen III-S and its activity is usually decreased to about half or one third of normal values in erythrocytes (Kappas et al. 1983). There invariably is also an absolute increase in the excretion of Uro III, although much less than the type I isomer (Miyagi et al., 1976; Ippen & Fuch, 1980; Nordmann & Deybach, 1982). It was suggested that though the primary abnormality in CEP was the deficiency of Uro'gen III-S, there may be a
secondary increase of the activities of ALA-S and/or HMB-S, perhaps as a result of feedback depression (Moore et al., 1978; Nordmann & Deybach, 1982; Kappas et al., 1983). The overproduction of the uroporphyrin I was due to the imbalance between HMB-S and Uro'gen III-S activities (Nordmann & Deybach, 1982; Bloomer & Bonkovsky, 1989). Some studies found that in cultured fibroblasts from patients with CEP, the activity of Uro'gen III-S was significantly decreased but no uroporphyrin I and coproporphyrin I were accumulated. Thus it was suggested that the activity of Uro'gen III-S was lower in all tissues, but only in erythropoietic tissue the rate of porphyrin synthesis was high enough to exceed the reduced capacity of the residual Uro'gen III-S (Romeo et al., 1970; Nordmann & Deybach, 1982).

The isolation and characterization of the hydroxy- and peroxyacetic acid- uroporphyrin derivatives provides an explanation for the elevated Uro III level in CEP. The uroporphyrin derivatives and/or the hydroxyl radical generated during the formation of these derivatives may inhibit the activity of Uro'gen-D causing the accumulation of Uro I, III, and their decarboxylated metabolites. It is known that peroxyacid is a very powerful oxidizing reagent which can oxidize various organic compounds (Plesnicar, 1978). In vitro experiments with Uro'gen I as the substrate under various reaction conditions always produced peroxyacetic acid uroporphyrin I as the major product among other derivatives. Though β-hydroxypropionic acid
uroporphyrin I was isolated and identified in the reaction mixture, the amount was less than 10% of that of peroxyacetic acid uroporphyrin I under most of the conditions studied. The exception was the reaction with \( \text{H}_2\text{O}_2 / \text{Fe-EDTA} \) system where the \( \beta \)-hydroxypropionic acid uroporphyrin I was about 70% of peroxyacetic acid uroporphyrin I. There was always a trace of hydroxyacetic acid uroporphyrin I in the reaction mixtures and sufficient compound had not been obtained for the identification. In the urine of CEP the peroxyacetic acid uroporphyrin I was always present in the smallest amount among the uroporphyrin I derivatives. This indicated that the peroxyacetic acid uroporphyrin I was not only produced but might also be consumed in vivo. Thus it is possible that peroxyacetic acid uroporphyrin I may oxidize the Uro'gen-D or impair its function in some way. Hydroxyl radicals which may be generated during the formation of the hydroxy- and peroxyacetic acid- uroporphyrin I derivatives are also a very powerful oxydizing reagent may also play a role in the inhibition of Uro'gen-D. It is also possible that peroxyacetic acid uroporphyrin I and hydroxyl radical may oxidize porphyrinogens and result in the accumulation of porphyrins.

It has been suggested that in uroporphyria the block in uroporphyrinogen metabolism may arise, at least in part, from the oxidation of uroporphyrinogens to the corresponding porphyrins (Heikel et al., 1958; Ferioli et al., 1984; Mukerji et al., 1984; Sinclair et al., 1986). Later, a
clearly defined suggestion was put forward that an inhibitor or an oxidised derivative of porphyrin(ogen) was produced from the oxidation of the porphyrinogen which may inhibit the activity of Uro'gen-D (De Matteis et al., 1988; Lambrecht et al., 1990). One experiment gave the evidence that incubating uroporphyrinogen III with the hydroxyl radical-producing systems hypoxanthine/xanthine oxidase/Fe-EDTA and H₂O₂/Fe-EDTA caused a modest but consistent inhibition of Uro'gen-D activity (Francis & Smith, 1988). These results are consistent with the present study and emphasised the need for investigating the potential of the hydroxy- and peroxyacetic acid uroporphyrin I derivatives as inhibitors of Uro-D.

6.5. Cutaneous Photosensitivity in CEP

Cutaneous photosensitivity is the major clinical problem in CEP. The skin lesions of CEP was not distinguishable from PCT but are very different from those of EHP. This difference is usually thought to be the result of the differing polarities of the porphyrins and the differing affinities of porphyrins to cell components. However the detection of hydroxy- and peroxyacetic acid uroporphyrin I derivatives and the understanding of the mechanism of their formation may allow alternative explanations. In both CEP and PCT, especially in CEP, the plasma porphyrin(ogen) levels are increased and may deposit in the skin. When these compounds are illuminated by light, the oxidised porphyrins may catalyse the reaction of porphyrinogens to form the peroxyacetic acid- and hydroxy-
porphyrin derivatives with the generation of hydroxyl radicals which may in turn damage the skin. Thus the photosensitivity may not only be due to the excited porphyrins but also to the production of hydroxy- and peroxyacetic acid- porphyrin derivatives and hydroxyl radicals. The absence of acute photosensitivity is consistent with the light catalysed formation of the uroporphyrin derivatives from uroporphyrinogen incubated with the incubation buffer alone. Exposure to light destroyed porphyrins in the area illuminated by light (Runge & Watson, 1962; Malina et al., 1978), in agreement with the finding that porphyrins disappeared on prolonging the exposure to light of the incubation mixtures of uroporphyrinogen I and buffer. The above suggested that in CEP and PCT the hydroxy- and peroxyacetic acid porphyrin derivatives themselves, and/or the process of formation of these derivatives may be responsible at least in part for the skin damage caused by light.

6.5. Further Studies

6.5.1. Further Studies of the Mechanism of the Formation of Hydroxy- and Peroxyacetic Acid- Uroporphyrin I Derivatives

The $H_2O_2/Fe$-EDTA chemical system may involve different reaction mechanisms. (1), $H_2O_2$ and $Fe^{3+}$ act similarly to a peroxidase and result in porphyrinogen oxidation (Mason, 1987); (2), If $Fe^{3+}$ is to some extent reduced by the porphyrinogen (De Matteis, 1988) or by $H_2O_2$ (Aust et al., 1985), Fenton reaction will occur resulting in the formation
of ·OH (De Matteis, 1988) and consequent formation of hydroxylated uroporphyrin I derivatives and destruction of porphyrins; (3), iron-oxygen complex may be formed in this system (Ryan & Aust, 1992) which may play a role in the formation of hydroxy- and peroxyacetic acid- uroporphyrin I derivatives. It requires further investigation to clarify which mechanism is responsible for the formation of hydroxy- and peroxyacetic acid- uroporphyrin I derivatives.

It is proposed that the formation of peroxyacetic acid uroporphyrin I is due to \( \text{H}_2\text{O}_2 \) and not hydroxyl radical, because the addition of hydroxyl radical scavengers, mannitol into the xanthine-xanthine oxidase system and high concentration of desferrioxamine into the \( \text{H}_2\text{O}_2/\text{Fe-EDTA} \) system, did not prevent the formation of peroxyacetic acid uroporphyrin I. This, may however, not be a strong enough evidence to confirm the conclusion, especially at lower concentration of desferrioxamine the formation of peroxyacetic acid uroporphyrin I was inhibited. Thus other hydroxyl radical scavengers such as benzoate and DMSO may be used to further prove that the hydroxyl radical does not involve the formation of peroxyacetic acid uroporphyrin I but does involve the formation of hydroxylated uroporphyrin I derivatives.

6.5.2. Isolation and Identification of Novel Components in the Urine and Plasma from Patients with CEP

A small peak in front of hydroxyacetic acid uroporphyrin I in the urine (Fig. 3.3.) and peak 1 and 4 in
the plasma (Fig. 3.4.) of a patient with CEP have not been identified. These components displayed pink fluorescence, therefore they are most probably also porphyrins. The isolation and characterisation of these compounds require further work.

6.5.3. Biological Significance of Hydroxy- and Peroxyacetic Acid- Uroporphyrin I Derivatives

Evidences for the inhibition of Uro-D by these compounds have not yet been obtained. They should be isolated or synthesised in sufficient quantity for in vitro studies on whether they inhibit erythrocyte and hepatic Uro-D activities. The role of these compound in liver damage and in cutaneous photosensitivity also require further investigation.
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