# PORPHYRIA CUTANEA TARDA
# SOME CLINICAL AND BIOCHEMICAL ASPECTS

(Porphyria cutanea tarda, enkele klinische en biochemische aspecten)

PROEFSCHRIFT

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**PREFACE**

It has been known for several years that the disease porphyria cutanea tarda is caused by a disturbance in the synthesis of heme, leading to overproduction and accumulation of porphyrins. These porphyrins have the property of absorbing ultraviolet light in the 400 NM range of the spectrum and transforming this light to red fluorescence. The porphyrins can also be excited to a triplet state, which can induce oxygen radicals (singlet oxygen) and so cause tissue damage.
Clinically the accumulation of porphyrins manifests itself as blistering of the sun-exposed skin and by the excretion of red urine. In the past there has been much discussion over the cause of this disease. Both familial and sporadic occurrence has been described. The crucial role of the enzyme uroporphyrinogen decarboxylase (URO-D) has been reported, being deficient in all investigated tissues in the familial form. It has been assumed that the sporadic form is acquired or that there is a genetic predisposition to damage of the enzyme URO-D only in the liver. Although factors as alcohol, estrogens and iron can influence the manifestations of the disease or trait, these factors are not thought to be responsible for the metabolic defect per se. It is known that environmental toxins such as hexachlorobenzene and dioxin can cause a porphyria similar to PCT; however the presence of these or comparable toxins, has not been looked for in sporadic forms of PCT.
Porphyria cutanea tarda is probably the most common form of human porphyria in the Netherlands.Our first study was stimulated by seeing a number of patients with PCT who gave a history suggesting familial occurrence.
We developed a method that is relatively easy and cheap to perform to detect URO-D deficiency in erythrocytes. Using this method it became possible to confirm the familial nature of PCT in some individuals and to differentiate between the inherited form and the sporadic form of PCT.
The assay was also found to be useful for the detection of asymptomatic individuals. In this study we found in addition to the decrease of activity of uro-D, an increased activity of the enzyme porphobilinogen deaminase (PBG-D) in affected persons. We questioned if this phenomenon could also be found in an experimental porphyria in the rat and if this increase in PBG-D activity was regulated by heme. In experiments with rats we found a decrease in URO-D activity and an increase of PBG-D activity in rat liver tissue after induction of porphyria with hexachlorobenzene (HCB). This increase in PBG-D could not be inhibited by heme. In contrast to delta-aminolevulinic acid synthetase there appeared to be no feedback mechanism that suppresses PBG-D formation in HCB induced porphyria in the rat. We were also able to show that hematin did not reverse the increase of PBG-D activity that is caused by lead and phenobarbital.
The next goal of our research was to study the well known but poorly understood role of iron in the pathogenesis of PCT. We hypothesized that iron could exert its toxic action by catalyzing the process of lipid peroxidation, in which biomembranes and enzymes are damaged. Radicals of drugs and toxins formed by the mixed

function oxygenase system in the liver have been shown to initiate lipid peroxidation.
In the fourth study we showed that an increase in lipid peroxidation occurs simultaneously with the development of porphyria after administration of HCB and iron to rats, and that an iron-deficient state protects against toxicity of HCB. These findings provoked a further study concerning the effect of radical scavengers on the prevention of the combined toxicity of HCB and iron.
However administration of α-tocoferol together with HCB did not prevent the toxicity of this xenobiotic, although it appeared to shorten the time of recovery. The implications of these findings for the pathogenesis of PCT are discussed in this thesis.

## LIST OF ABBREVIATIONS AND OF INTERNATIONAL ENZYME NOMENCLATURE

### Heme Synthesis

| | |
|---|---|
| ALA | - delta-aminolevulinic acid |
| PBG | - porphobilinogen |
| UROgen | - uroporphyrinogen |
| URO | - uroporphyrin |
| HEPTA | - heptacarboxyl-porphyrin |
| HEXA | - hexacarboxyl-porphyrin |
| PENTA | - pentacarboxyl-porphyrin |
| COPROgen | - coproporphyrinogen |
| COPRO | - coproporphyrin |
| PROTOgen | - protoporphyrinogen |
| PROTO | - protoporphyrin |

| | | |
|---|---|---|
| [E.C.2.3.1.37] | ALA-S | - ALA synthetase |
| [E.C.4.2.1.24] | | - ALA dehydratase |
| [E.C.4.3.1.8 ] | PBG-D | - porphobilinogen deaminase |
| [E.C.4.2.1.75] | | - uroporphyrinogen III cosynthetase |
| [E.C.4.1.1.37] | URO-D | - uroporphyrinogen decarboxylase |
| [E.C.1.3.3.3 ] | | - coproporphyrinogen oxidase |
| [E.C.1.3.3.4 ] | | - protoporphyrinogen oxidase |
| [E.C.4.99.1.1] | | - ferrochelatase |

**Porphyrias**

Non Acute Porphyrias

CEP - congenital erythropoietic porphyria
HEP - hepato-erythropoietic porphyria
PCT - porphyria cutanea tarda
EPP - erythropoietic protoporphyria
- harderoporphyria
- chemical-induced porphyria

Acute Porphyrias

AIP - acute intermittent porphyria
- ALA dehydratase deficiency
VP - variegate porphyria
HC - hereditary coproporphyria

Other Abbreviations

PHA - polyhalogenated aromatic
HCB - hexachlorobenzene
TCDD - (2,3,7,8)-tetrachlorodibenzo-p-dioxin

# 1. INTRODUCTION

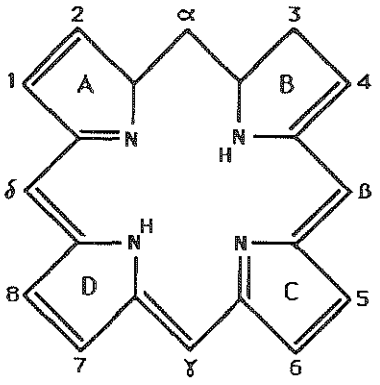
## 1.1. Importance of Porphyrins

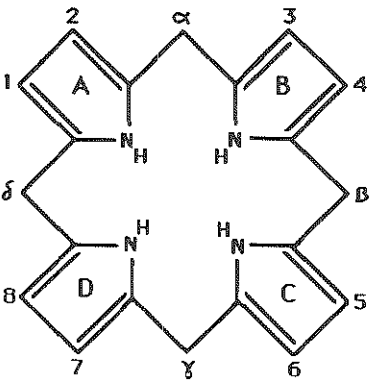
Porphyrins are essential for life. Porphyrins consist of a tetrapyrrolic ring. The ligand-binding sites within this ring permit the binding of metals. The metalloporphyrins have remarkable properties. Iron-containing heme is essential for almost all biological oxidations, and is bound in these circumstances to proteins, thus forming enzymes. The magnesium-containing compounds, the chlorophyls of the green plants, are essential for photoenergetic reactions, in which carbon dioxide and water are transformed into carbohydrate and oxygen under influence of sunlight, and are thus essential for life on earth.
In clinical medicine we encounter porphyrins in the porphyrias, which are disturbances of heme synthesis. In these disturbances both porphyrins and their precursors can accumulate. The porphyrias are relatively rare diseases, that are usually based on an inborn error of metabolism, a genetically-determined enzyme deficiency. However acquired forms of porphyria have been recognized for almost a century, and can be caused by drugs and environmental pollutants like hexachlorobenzene (HCB), tetrachlorodioxin (TCDD) and lead (see Debets and Strik 1979, De Matteis 1978b, , Moore 1980a).
Recently extensive reviews on the subject of porphyrins, heme synthesis and porphyrias have been published (Moore 1987a, Kappas 1989). The basic knowledge of porphyrin structure, heme synthesis and turnover, and porphyrias will be only briefly discussed here. A more extensive review will be given on the subject of porphyria cutanea tarda and on HCB-induced porphyria.

## 1.2. Biochemistry of Porphyrins and Heme

### 1.2.1. Structure of Porphyrins

Porphyrins consist of four pyrrole rings linked by four methylene bridges to form the macrocycle. This is a multiplanar structure to which eight side chains may be affixed. These side chains determine the characteristics of the porphyrins. The four pyrrole rings are designated A,B,C,D, the four methylene bridges alpha, beta, gamma, delta (Fig.1.2.-1). The porphyrin nomenclature originally developed by Hans Fisher is used. A revised nomenclature has been recommended by The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB), but in the litterature almost exclusively the Fisher nomenclature has been used (Kappas 1989).
The normal biologic intermediates in heme or chlorophyl synthesis are porphyrinogens - hexahydroporphyrins in which each methylene bridge is reduced. There are four possible isomers of uroporphyrinogen and coproporphyrinogen, but only isomers I and III occur in nature. Only the III isomers can be used for heme synthesis. Of the fifteen possible varieties of the protoporphyrinogen, only protoporphyrinogen IX occurs in biological systems. The normal excreted




| Position | 1,3,5 | 2,4 | 6 | 7 | 8 |
|---|---|---|---|---|---|
| Uroporphyrin I | acetyl | propionyl | propionyl | acetyl | propionyl |
| Uroporphyrin III | acetyl | propionyl | propionyl | propionyl | acetyl |
| Coproporphyrin I | methyl | propionyl | propionyl | methyl | propionyl |
| Coproporphyrin III | methyl | propionyl | propionyl | propionyl | methyl |
| Protoporphyrin IX | methyl | vinyl | propionyl | propionyl | methyl |

methyl : $-CH_3$ propionyl : $-CH_2-CH_2-COOH$

acetyl : $-CH_2-COOH$ vinyl: $-CH=CH_2$

**Fig 1.2.-1 The chemical structure of porphyrins**

form is porphyrin, the oxidized form of porphyrinogen not utilized for heme synthesis. Uroporphyrin, which contains eight carboxyl groups is hydrophylic and is excreted in the urine. Coproporphyrin (four carboxyl groups) is excreted both in bile and urine. The lipophylic protoporphyrin is excreted solely in the bile.

### 1.2.2. Heme Synthesis.

Heme synthesis is schematically shown in figure 1.2.2-1. Condensation of succinyl co-A and glycine, catalyzed by ALA synthetase, leads to formation of delta-aminolevulinic acid (ALA). The enzyme ALA dehydratase catalyzes formation of the pyrrole porphobilinogen (PBG) from 2 molecules of ALA. Porphobilinogen deaminase (or uroporphyrinogen I synthetase , or hydroxymethylbilane synthetase) forms the tetrapyrrole ring uroporphyrinogen I from four molecules of PBG, by binding them head to tail, to form the tetrapyrrole hydroxymethylbilane, which spontaneously cyclizes to uroporphyrinogen I (Hindmarsh 1986). Under influence of uroporphyrinogen III cosynthetase the isomer III is formed by inversion of the D ring (figure 1.2.2-2). In normal human cells there is an excess of uroporphyrinogen III. Only this isomer III can be utilized in further heme synthesis. From uroporphyrinogen four carboxyl groups are removed step by step by the enzyme uroporphyrinogen decarboxylase (URO-D). Jackson (1976) has shown that the first decarboxylation takes place in the D ring and subsequently the A, B and C ring are decarboxylated. Heptacarboxyl-, hexacarboxyl-, pentacarboxyl-and coproporphyrinogen are formed.
Coproporphyrinogen is oxidatively decarboxylated to protoporphyrinogen IX by coproporphyrinogen oxydase.
Protoporphyrinogen IX is oxidized by removal of six atoms of hydrogen to protoporphyrin IX. The porphyrin ring is now ready for incorporation of ferrous iron by ferrochelatase to form heme.
These enzymatic steps occur at different sites in the mammalian cell. ALA is formed in the mitochondrion, whereas the next steps PBG, uroporphyrinogen and coproporphyrinogen formation take place in cytoplasm.
Coproporphyrinogen returns to the mitochondrion where the final formation of heme is completed. Heme may then return to cytoplasm where it can be bound as a prosthetic group to various enzymes. (De Matteis 1978a).

### 1.2.3. Regulation of Heme Synthesis in the Liver

Sassa and Granick (1970) showed that heme reduced de novo synthesis of ALA-synthethase, induced by a variety of substances in chick embryo liver. At concentrations of 20-50 nM, heme reduces mRNA levels of ALA-S. Polysomes of liver cells of drug-induced, heme-treated animals were translated in vitro. ALA-S, detected by immunoprecipitation, was reduced in the heme-treated animals. It was assumed that heme decreased the amount of translatable mRNA.

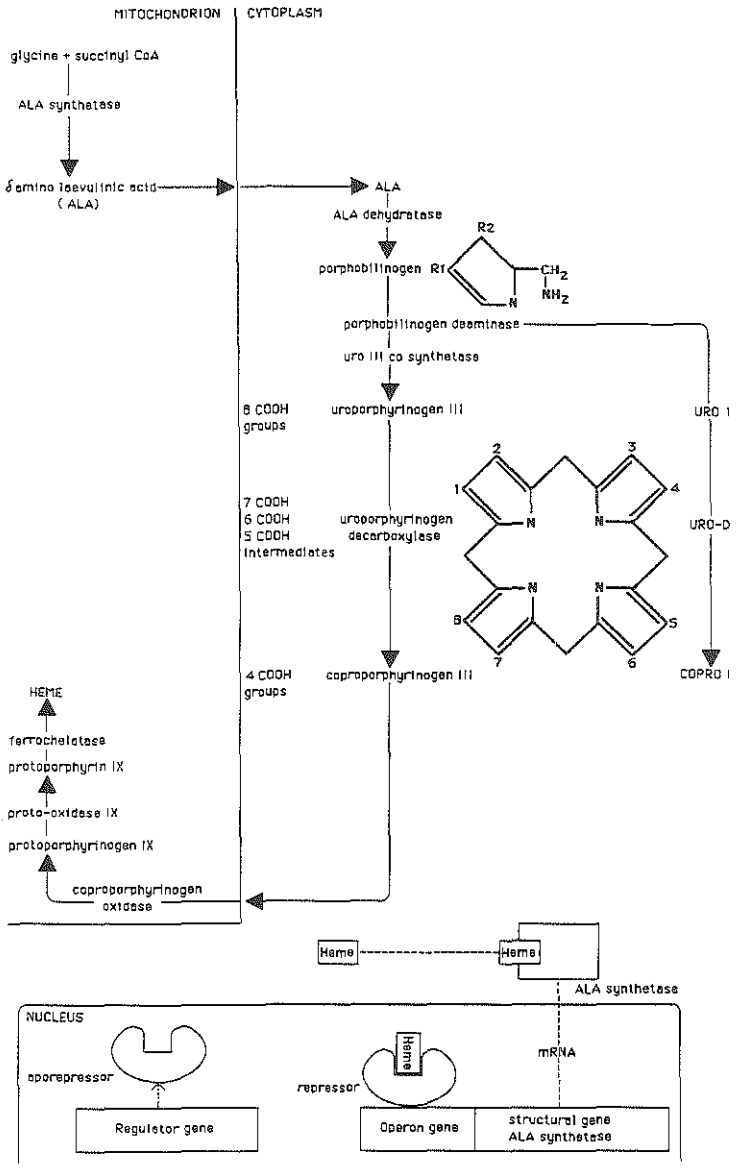



**Fig 1.2.2-1 The synthesis of heme at the cellular level.**

Later direct evidence was obtained by mRNA dot blot hybridization (May 1986).
Originally heme was thought to inhibit the synthesis by inhibiting translation of mRNA into protein ALA-synthetase (Sassa 1970, Tyrrell 1972). However indirect methods and unphysiological amounts of heme were used in these experiments.
Heme is also thought to inhibit transport of ALA-S from the endoplasmatic reticulum to the mitochondrion (Hayashi 1972), Gayatri 1973,May 1986). Others have stated that the apoprotein cytochrome P450 induces translation of ALA-synthetase (Padmanaban 1973).

**1.2.3.1. Biodegradation of heme**
Breakdown of the porphyrin ring at the alpha-position by heme oxygenase is the first step of heme biodegradation. The reaction requires oxygen and NADPH. Biliverdin IX and carbon monoxide are formed. Biliverdin is reduced to bilirubin by biliverdin reductase. Bilirubin is finally conjugated with glucuronic acid and excreted in the bile (Tait 1978).

**1.2.4. Heme transport**

Hemoglobin that is lost from erythrocytes in the circulation will bind to haptoglobin and albumin. These complexes enter the hepatocytes by pinocytosis after binding to membrane receptors. Within the cell these complexes are broken after fusion with lysosomes. Lysosomal heme oxygenase degrades heme. Free heme is bound by hemopexin and albumin. These complexes undergo the same fate as the hemoglobin complexes (Koskelo 1977))

**1.2.5. Occurrence of Heme**

In every cell of the human body, heme-containing enzymes are essential for the respiratory chain (cytochromes A, B and C). The largest stores of heme, however, are the red blood cells (hemoglobin) and the skeletal muscle (myoglobin). In the normal man of 70kg about 500 - 700g of hemoglobin and 35 - 200g of myoglobin are present. Hemoglobin is stable once synthesized and remains in the erythrocyte during the lifespan of the erythrocyte (about 120 days in man). Myoglobin turns over more slowly. The other hemoproteins - cytochromes, catalase, etc. - have much shorter half lives. The cytochromes of the cytochrome P450 have a biphasic survival curve. The larger fraction has an half life of 7 - 10 hours, the smaller fraction 24 - 48 hours. Of the heme that is synthesized in the liver about 70% is required for the formation of cytochrome P450, the rest for other cytochromes and heme-containing enzymes (Tait 1978). Of these latter enzymes cytochrome b5, which is also localized in the microsomes, and catalase which occurs in the peroxisomes, and tryptophan pyrrolase, that is present in the cytosol.
fraction, are the most important (Muller-Eberhard 1985). Radioactive incorporation studies with labeled glycine and delta-ALA show

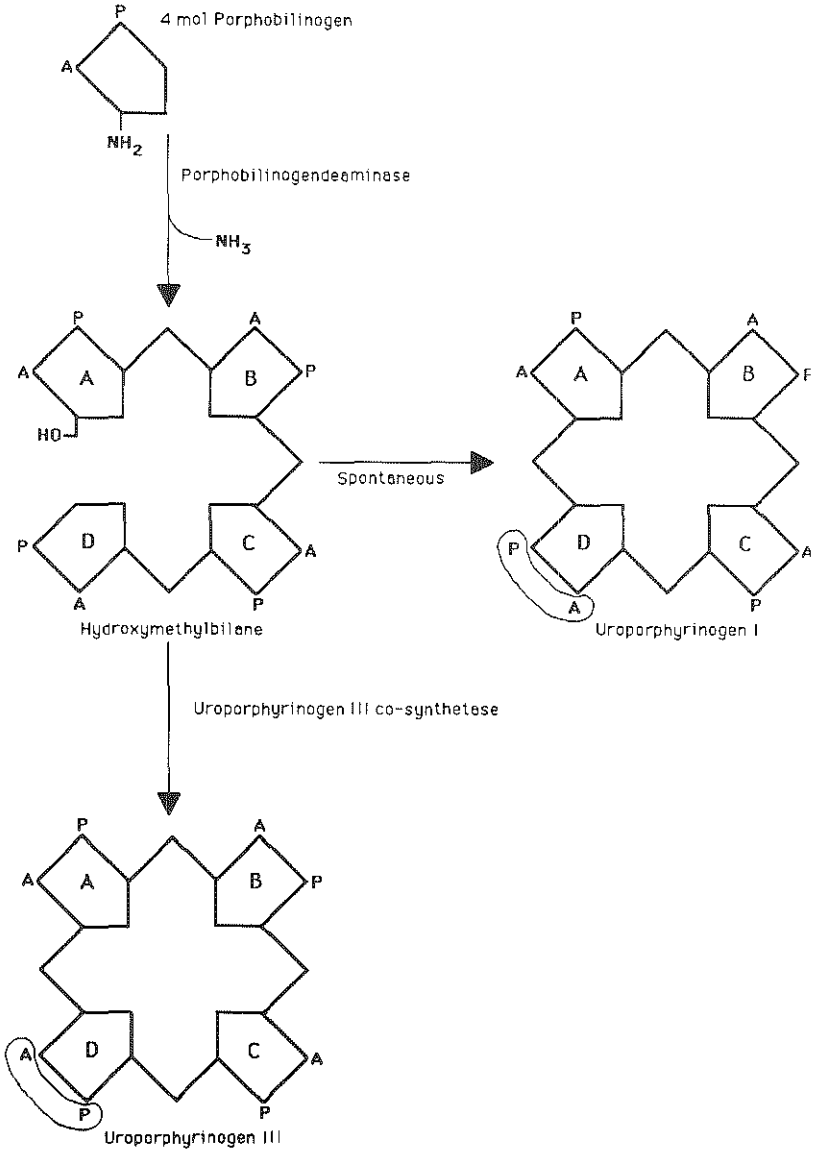



**Fig 1.2.2-2 The synthesis of uroporphyrinogen I and uroporphyrinogen III.**

Table 1.3.1
Clinical presentation of attacks in acute porphyria

| symptoms | % | physical findings | % |
|---|---|---|---|
| abdominal pain | 90 % | tachycardia | 83 % |
| constipation | 80 % | hypertension | 55 % |
| vomiting | 80 % | motor neuropathy | 53 % |
| paresis | 60 % | pyrexia | 38 % |
| pain in limbs | 51 % | bulbar involvement | 18 % |
| pain in back | 50 % | sensory loss | 15 % |
| confused state | 32 % | cranial nerve involvement | 9 % |
| urinary frequency | 30 % | hepatomegaly | 5 % |
| dysuria | 28 % | retinal artery spasm | 5 % |
| abnormal behaviour | 23 % | visual disturbance | 5 % |
| seizures | 12 % | | |
| diarrhoea | 8 % | | |
| stupor | 7 % | | |
| coma | 6 % | | |

(Waldenstrom 1937, Eales 1963, Brodie 1980, Bissell 1985)

that 15 - 20% of formed bilirubin is derived from hepatic heme. Drugs such as barbiturates, estrogens and alcohol and pollutants such as HCB and TCDD, and endogenous substances like steroids and bile acids can induce the production of the mixed function oxygenases (e.g. cytochrome P450) and thus increase the need for heme (Bock 1978, Tait 1978). This will result in an increase of ALA-synthetase activity, by a negative feedback mechanism, as described above. This induction of the mixed function oxygenases is accompanied by proliferation of the smooth endoplasmatic reticulum which contains these enzymes (Bock 1978).

## 1.3. The Porphyrias

Extensive reviews have been published on this subject (Bloomer 1976, Meyer 1978a, Goldberg 1980 , Ibraham 1983, Bissell 1985, Bissell 1987 , Meyer 1987, Moore 1987a, Kappas 1989).
Porphyrias are disorders of heme synthesis, characterized by increased formation and excretion of porphyrins and of porphyrin precursors. Increased porphyrin formation can give rise to a photodermatosis; increased formation of porphyrin precursors ALA and PBG is associated with neurotoxic and psychiatric symptoms, the so-called acute porphyric attack.
The signs and symptoms of the acute porphyric attack are listed in table 1.3-1. Abnormal findings in general laboratory investigations are listed in table 1.3-2.
The acute abdominal pain, that is colicky and accompagnied by constipation and even paralytic ileus is explained by autonomic neuropathy. Fever, tachycardia and hypertension are also explained by autonomic dysregulation. Pain, pareses and absent reflexes are due to peripheral neuropathy, possibly caused by neurotoxic properties

of these porphyrin precursors. This is also confirmed by the finding of demyelination, axonal degeneration of peripheral nerves and degenerative changes in the central nervous system. ALA has been shown to inhibit $Na^+K^+$ dependent ATP-ase, thus inhibiting ion transport across membranes and affecting neuromuscular function (Becker 1971). Accumulation of ALA in the hypothalamus is thought to be responsible for the inappropriate antidiuretic hormone exretion that can occur in the acute porphyric attack. Besides ALA, PBG has also shown to inhibit nerve muscle function (Feldman 1971, Loots 1975). Nevertheless after removal by charcoal perfusion of ALA and PBG in one patient the symptoms of the porphyric attack persisted (Laiwah 1983). In patients with acute porphyrias it has been observed, that symptoms rapidly disappear after i.v. administration of hematin (heme) but the increased secretion of ALA and PBG may persist. (Dhar 1975, Watson 1977, 1978). ALA-S activity decreases after heme administration (Moore 1980b).
In rats with experimental porphyrias it has been found that the heme containing enzyme tryptophan-pyrollase is deficient. This results in increased levels of tryptophan in plasma and brain and finally of serotonin or 5-hydroxytryptamin (Litman 1983, Correia 1987). This studies suggests that tryptophan mediated neurotransmitters may be responsible for the neurologic symptoms. Support is found for this hypothesis in one study in which one family with acute intermittent porphyria is described, in which increased excretion of 5 hydroxy-indolacetic acid, a degradation product of 5-hydroxytryptamine has been found (Ludwig 1961). Another effect of tryptophan consists of inhibition of gluconeogenesis resulting in hypoglycemia. Glucose administration, however, inhibits ALA-S induction (Tschudy 1978, Correia 1987).
Classically the porphyrias are subdivided in the erythropoietic and hepatic forms, depending on the main site of porphyrin overproduction - the bone marrow or the liver. In daily practice it is more convenient to discriminate between acute or chronic (or non-acute) porphyrias. Most porphyrias are caused by an inborn error of metabolism which manifests itself by a decreased activity of one of the enzymes of heme synthesis. Acquired forms have also been recognized. The generally accepted classification of the porphyrias, their abnormalities in porphyrin metabolism and their enzyme deficiencies is given in table 1.3-3 and 1.3-4. Normal values according to Bissell are given in table 1.3-5.
In the so-called acute porphyrias, a secondary increase of ALA-S is found, caused by the decreased production of heme. Certain drugs like barburates induce the mixed function oxygenase system (e.g. cytochromes P450) This provokes a porphyric attack. Under basal conditions normal levels of ALA and PBG may be found in patients wich acute porphyrias. Accumulation of ALA and PBG can occur because the activity of PBG-D does not increase in the acute form, in contrast to the chronic forms PCT and EPP, in which an increased activity of PBG-D has been found (Brodie 1977). PBG-D is the second rate-limiting enzyme. Meyer compared enzyme activities in nmol ALA formed or utilized per gram liver per hour of almost all enzymes of heme synthesis. ALA-S activity and PBG-D activity are about 20 nmol ALA/gr liver /hour, while ALA dehydratase, URO-D and coprooxidase show about 100 times more activity (Meyer,1978b).

Little is known about the control mechanism of PBG-D activity. The capability to raise PBG-D activity is probably the main factor determining the difference between acute and chronic porphyria (Brodie 1977).

general labarotory findings
- leucocytosis 20 %
- proteinuria 8 %
- hyponatremia 8 %
- elevated thyroxine
- elevated thyroxine binding globulin
- elevated cholesterol and L.D.L
- elevated transaminases
- rarely, elevated amylase
- rarely, hypercalcemia

(Brodie 1980, Bissell 1985, Meyer 1987)

**Table 1.3.-2**

The Acute Porphyrias

The acute porphyrias can be divided in two groups: The first is charactarized by the acute porphyric syndrome without skin symptoms, the second by both the acute porphyric syndrome and skin symptoms (see table 1.3-4).Both clinical signs and symptoms and determination of porphyrins and porphyrin precursors in urine and feces-and occasionally in erythrocytes- will lead to the diagnosis. Measurement of enzyme activity is valuable in identifying asymptomatic individuals and in family studies (Elder 1983a, Disler 1984a).

**Acute Intermittent Porphyria (A.I.P.)**
Acute Intermittent porphyria is the most frequent form of acute porphyrias except in South Africa, where variegate porphyria is the main form. A.I.P is a heriditary autosomal dominant disorder, that presents commonly in young adults, with symptoms of the acute porphyric syndrome, associated with production of dark-red urine containing large amounts of PBG and ALA (Brodie 1980). Waldenstrom has described the largest, but not the first series (Waldenstrom 1937). The first description of acute porphyria was presented by Stokvis in the Nederlands Tijdschrift van Geneeskunde in 1889. In retrospect however it is not clear whether the patient Stokvis described had AIP or toxic porphyria. In 1920 Snapper described a patient with AIP, the family of this patient was investigated 20 years later (Snapper 1920, Te Velde 1989).
The deficient enzyme is PBG-D. The severity of the disease can vary remarkably. Recently this disease has been shown to be heterogenous both in enzymatic activity and in immunologic activity of PBG-D (Mustajoki 1981 ,Goldberg 1985, Wilson 1986). The defects at DNA level have also been elucidated (Grandchamp 1989). At this moment

Table 1.3-3

Biochemical abnormalities in porphyrias

| | A.I.P. | ALA-D-def | VP | HC | Pbintox | PCT | HEP | EPP |
|---|---|---|---|---|---|---|---|---|
| red blood cells | | | | | | | | |
| -uro | N | | N | N | N | N | N | N |
| -copro | N | | N | N | N | N | N | (+) |
| -potro | N | | N | N | ++ | N | ++ | +++ |
| urine | | | | | | | | |
| D-ALA | (+++) | +++ | (+++) | (+++) | +++ | N | N | N |
| PBG | (+++) | | (+++) | (+++) | (+) | N | N | N |
| uroporphyrin | ++ | | + | + | (+) | ++++ | +++ | N |
| 7-COOH | | | | | | +++ | ++++ | N |
| coproporphyrin | N | ++ | ++ | ++ | +++ | + | ++++ | (+) |
| feces | | | | | | | | |
| coproporphyrin | (+) | | + | +++ | | ++ | ++ | (+) |
| isocoproporphyrin | | | | | | + | + | - |
| protoporphyrin | (+) | | +++ | + | | (+) | (+) | ++ |

N =normal (+) =increased in some patients only

\+ =increased level (+++)=only during attacks

++ =moderately increased

+++ =markedly increased

(Goldberg '83, Doss '78, Day '82, Lazaro '84, Lim '84, Toback '87)

it is uncertain, if the genetic heterogenity predicts the clinical presentation. PBG-D deficiency does not lead spontaneously to disease, but this can be manifest after induction of heme synthesis. The main triggering factors of an acute porphyric attack are drugs (induction of hepatic heme synthesis, cytochrome P450), infection (which increases cytochrome P450 heme turnover), and menstruation and pregnancy (Brodie 1980 Ibraham 1983). AIP has been reported in some individuals to be associated with a deficiency of 5-α reductase resulting in increased formation of 5-B hydroxy- steroids, which have been reported to be porphyrinogenic. Prevention of attacks especially by avoidance of unsafe drugs and adequate management of infections is essential (Eales 1979, Khanderia 1984, Wilson 1990). Intranasal luteinizing releasing hormone can prevent cyclic attacks (Anderson 1987).
Attacks in AIP (as in other acute hepatic porphyrias) can be treated by carbohydrate-rich feeding (500 g/day), or if indicated i.v. glucose (20 g/hour). The so-called glucose effect on the acute porhyric attack is explained by the decrease of ALA induction. The mechanism is poorly understood (Tschudy 1978). As soon as possible however the i.v. administration of hematin has to be started. The recommended dose of hematin is 4 mg/kg body weight infused over 15 minutes every 12 hrs during 9 to 6 days (Meyer 1987). Because of the instability of hematin fresh solutions have to be made. Aged solutions are less effective and have more influence on coagulation time (Goetsch 1986). Heme arginate is more stable than hematin and does not cause local irritation. This compound is now the usual form of heme administered in acute attacks.

**ALA dehydratase deficiency**
Bird (1979) described a family with ALA dehydratase deficiency in erythrocytes, which inherited as an autosomal disorder. This defect was asymptomatic in this family, but Doss (1982) described two patients with acute porphyria and the same enzyme defect, probably the homozygous form of this enzyme deficiency. In lead intoxication ALA-dehydratase is one of the affected enzymes, but in this condition other enzymes are also involved.

**The acute porphyrias with skin symptoms**

**Variegate porhyria (VP)**
The most important disease in this group is variegate porphyria. This disease is an autosomal dominant disorder which can manifest itself with skin symptoms that are identical to those seen in PCT, and with acute symptoms. This disease has a remarkably high incidence in white South Africans, of 3 per 1000, and has been traced back to a couple which went to South Africa in the 17$^{th}$ century (Dean 1963, Te Velde 1989 ). Brenner (1980) showed that protoporphyrinogen oxydase was deficient in patients with VP. The activity of this enzyme in fibroblasts of patients is 50 % of normal. During attacks both excretion of porphyrin precursors and porphyrins is increased. Between the attack PBG and ALA excretion in urine may

Enzymatic defects of heriditary porphyrias

| -Acute porphyrias (without skin symptoms) | Enzyme defect |
|---|---|
| Acute intermittent porphyria | PBG-deaminase |
| ALA dehydratase deficiency | ALA dehydratase |
| -Acute Porphyrias with skin symptoms | |
| Variegate porphyria | protoporphyrinogen-oxydase |
| Heriditary coproprophyria | coproporphyrinogen-oxydase |
| -Chronic (or non-acute) porphyrias | |
| Congenital erythropoietic porphyria | uroporphyrinogen III-cosynthetase |
| Erythropoietic protoporphyria | ferrochelatase |
| Porphyria cutanea tarda (PCT) | uroporphyrinogen-decarboxylase |
| - Hepato-erythropoietic porphyria (homozygous form of PCT) | uroporphyrinogen-decarboxylase |

(Meyer 87, Rimington 85)

**Table 1.3-4**

be normal but protoporphyrin and to a lesser extent coproporphyrin in the feces are invariably increased. The acute attacks are treated as in A.I.P.

**Hereditary coproporphyria (H.C)**
This disease was first described by Heymans van den Bergh (1928) H.C. is a autosomal hereditary disorder, which is very rare. Only a few families with this autosomal dominant disorder are known (Brodie 1980). Recently a family study of the first patient published by Heymans van den Bergh was performed in which the diagnosis H.C was confirmed (Te Velde 1989).
The deficient enzyme is coproporphyrinogen oxidase (Elder 1976a). The most prominent symptoms are due to the acute porphyric syndrome. About one third of the patients has skin symptoms, and occasionally jaundice is seen, possibly due to intrahepatic cholestasis (Brodie 1980).

Normal values of porphyrin precursors and porphyrins according to Bissel (1985)

| | Urine (24 hr) | Faeces | Erythrocytes |
|---|---|---|---|
| ALA | < 57 µmol (< 7.5 mg) | - | |
| PBG | < 9.0 µmol (< 2.0 mg) | - | |
| Uroporphyrin | 12-60 nmol (0.01-0.05 mg) | trace | |
| - Hepta | trace | | |
| - Hexa | trace | | |
| - Penta | trace | | |
| Coproporphyrin | 76-382 nmol | < 76 nmol/g (< 50 µg/g) | < 23 nmol/l (< 1.5 µg/dl) |
| Protoporphyrin | - | < 215 nmol/g) (< 120 µg/g) | < 1.3 µmol/l (< 75 µg/dl) |

**Table 1.3.-5**

**Other inherited acute hepatic porphyrias**

Some other inherited forms of acute hepatic porphyrias have been described. One study described the combination of PBG-D and protoporphyrinogen oxidase deficiency as seen in AIP and PV respectively (McColl 1985). Day described a family with a combination of biochemical features of PV and PCT , but did not report enzymatic measurements. These patients belonged to a large family of variegate porphyria (Day 1982a). Another five "unclassified "cases have been reported with increased uroporphyrin urinary excretion and increased fecal porphyrin excretion (Poulos 1980). These patients however have not been completely analyzed.

**Disturbance of heme synthesis in hereditary tyrosinemia.**
Hereditary tyrosinemia, which is caused by an autosomal recessive inherited deficiency of fumarylacetoacetate hydrolase, is known to cause acute and chronic liver failure, renal Fanconi syndrome and hepatocellular carcinoma. In up to 42 % (20/48) of the patients neurologic crises have been described resembling those of the acute porphyric attack (Mitchell 1990).
Paralysis or painfull dysesthesia with or without hypertonia are the most frequent features of this attack while weakness, respiratory insufficiency, selfmutilation, vomiting, ileus, diarrhea and hyponatriemia occur less frequently. Fumarylacetoacetate hydrolase deficiency causes, besides tyrosinemia, an increase of succinylace-

tone which inhibits heme synthesis at the level of ALA dehydratase. This explains the increase of ALA urinary excretion which is especially marked during attacks but also is found between them. Chronic neuropathy has been obseved as in AIP with axonal degeneration and secondary demyelination (Mitchell 1990).

**Lead intoxication**

In lead (Pb) intoxication ALA dehydratase-, ferrochelatase- and coproporphyrinogen oxidase activity are decreased (McColl 1980). Reduced activity of other enzymes such as PBG-D has been reported in severe cases (Sassa 1978), but at lower concentrations the enzyme is protected by a pteroylpolyglutamate derivative (Piper 1977). Increased excretion of ALA and coproporphyrin can be found in urine, and increased values of protoporphyrin (zinc protoporphyrin) in the erythrocytes.

Although there is evidence that the activity of ferrochelatase is decreased, some have stated that lead inhibits the intracellular transport of iron, thus allowing the enzymatic formation of zinc protoporphyrin. This is supported by the fact that photosensibility does not develop in lead intoxication in contrast to protoporphyrinuria (Piomelli 1975), in which the enzyme ferrochelatase is deficient, and by the fact that zinc protoporphyrin cannot be formed spontaneously (Labbe 1987).

This proposed inhibition of intracellular iron transport also explains the increased susceptibility of iron deficient patients to lead intoxication (Piomelli 1987). The competition between iron and zinc in relation to ferrochelatase occurs also in absence of lead intoxication, and in iron deficiency an increase of zinc protoporphyrin has been found (Bottomley 1977).

Many signs of lead intoxication can be ascribed to ALA accumulation or heme deficiency. As in other acute porphyric syndromes increased formation of neurotransmitters as 5-hydroxytryptamine because of tryptophan pyrrolase deficiency could play a role. Abdominal pain, constipation and peripheral neuritis are common. In adults encephalopathy is rare. The neurologic signs are thought to be caused by heme deficiency of the nerve cell (Moore 1987b).

The basophilic stippling of erythrocytes seen in lead intoxication is due to damage of cellular elements including ribosomal RNA and mitochondrial fragments (Sassa 1978). The anemia that is seen in chronic plumbism is hypochromic and microcytic. Ringed sideroblasts and erythyroid hypoplasia may be seen in the bone marrow (Bottomley 1977, Sassa 1978).

Other signs of lead intoxication such as the lead line on the gums and increased density on the metaphyseal plate of growing bones are due to local accumulation of lead. Interstitial nephritis is caused by accumulation of lead especially in the proximal tubuli of the kidney. This can cause glucosuria and amino-aciduria (Fanconi's syndrome) and finally renal failure. This is frequently accompanied by hypertension and gouty arthritis -"saturnine gout"- (Graef 1987).

The diagnosis is confirmed by demonstrating increased blood levels of lead and of zinc protoporphyrin in erythrocytes; increased amounts of urine lead, ALA and coproporphyrin can also be found.

Lead intoxication may be caused by tetraethyl lead, an additive to gasoline, lead containing pipes of drinking water, lead based paints on childrens toys, illegally distilled alcohol, lead containing fumes and dust, especially in reconstruction and demolition industries (Pagliuca 1990).

Cutaneous Porphyrias

**Congenital erythropoietic porphyria (CEP)**
This disease was first described by Gunther 1911. Only about 100 patients with this very rare autosomal recessive disease have been described. The disease manifests itself mostly during the first year of life and is characterized by a severe blistering skin disease with scarring, hemolytic anemia, splenomegaly and red urine causing pink nappies in toddlers. Clinically this disease is identical with severe hepato-erythropoietic porphyria (HEP, see 1.5). CEP is characterized by excess accumulation of uroporphyrin I, which is caused by a deficiency of uroporphyrinogen III cosynthetase, is the biochemical characteristic . Accumulation of URO-I in the teeth is responsible for their red fluorescence when exposed to ultraviolet light. This sign is called "erythrodonty" and was formerly thought to be pathognomonic for CEP, but it also occurs in HEP, the homozygous form of PCT (see 1.4.4 and 1.5). In normal daylight the teeth are brown-yellow. Scarring can give rise to severe mutilation.
Hypertrichosis is also a remarkable phenomenon (Meyer 1978a, Magnus 1980). Less severe forms of this disease have been decribed, starting at a later age and without frank hemolysis (Mascaro 1985a).
Two very rare forms of CEP have been descibed with accumulation of erythrocyte coproporphyrin and protoporphyrin respectively (For references see Meyer 1978a). Avoidance of sunlight, the use of sunscreens and topical antibiotics to prevent secundary infection and severe scarring are measures of some help. Splenectomy can temporarily help to reduce hemolysis and so reduce porphyrin formation and phototoxicity (Ibraham 83, Meyer 1978a).
In one patient long-term treatment with blood transfusions, keeping the hematocrit above 33%, suppressed the clinical manifestations of this disease completely and reduced the excretion of uroporphyrin dramatically. By giving desferrioxamine, iron stores could be reduced to acceptable levels (Piomelli 1986).
Charcoal has been used as an oral binder of porphyrins in CEP resulting in a dramatic decrease of plasma porphyrins to normal ranges within 24 hours, while pretreatment plasma porphyrin values were about fifty times the upper limit of normal. Cholestyramine also has a significant effect but is less effective (Mukerji 1985, Pimstone 87, Tishler 88). The water soluble porphyrins appear to undergo a significant enterohepatic cycling.

**Erythropoietic protoporphyria**

Erythropoietic protoporphyria (EPP) is also called erythrohepatic protoporphyria or protoporphyria. It is an autosomal dominant disorder associated with sunlight sensivity usually beginning in

childhood (Meyer 1978a). Patients can complain about a burning sensation ,without remarkable skin abnormalities, but most frequently edema and erythema develop in the sun-exposed areas. Excoriations and circumoral fissures are often present and occasionally purpura, blisters and urticaria are seen (Suurmond 1970). These lesions can heal without scarring in contrast to the laesions seen in PCT, HEP and CEP.
When prolonged sun exposure persists skin thickening occurs, especially of nose, cheeks and proximal finger joints. Small shallow scars at these sites and linear peri-oral scars are seen in the chronic stage (Suurmond 1970)).
Only mild anemia with normal erythropoiesis and iron turnover has been seen in most cases, but occasionally there is a hemolytic anemia caused by protoporphyrin accumulation in erythrocytes (Meyer 1978a, Ibraham 1983).
Chronic liver disease frequently occurs, which is characterized by massive deposition of porphyrin in bile canaliculi, hepatocytes and sinusoidal cell's, accompanied by inflammation and fibrosis. At autopsy the liver is black, firm and shows a fine nodulation. On light microscopy the porphyrins appear as dark brown pigment, in polarized light these deposits show birefrigency with a dark central Maltese cross. Fluorescence microscopy shows a bright red fluorescence of these protoporphyrin deposits.
Chronic liver disease in EPP can progress to cirrhosis, liver failure and death. The increase of protoporphyrin excretion by bile can result in stone formation of this poorly water soluble porphyrin (Bloomer 1976, Meyer 1978a, Morton 1988).
This increase in protoporphyrin excretion is due to ferrochelatase deficiency which has been demonstrated in the bone marrow cells and cultured skin fibroblast of such patients. Activity of 10-25% of normal has been reported in this disease (Romeo 1977, Mascaro 1985a, Deybach 1985). An homozygous form of the disease has also been described in a patient, who had an enzyme activity of only 6.5 % (Deybach 1985).
The diagnosis can be made by measuring protoporphyrin in the feces and in erythrocytes. In iron deficiency anemia and in lead intoxication increased protoporphyria in erythrocytes can also be found, but the protoporphyrin levels are usually lower then those found in EPP. Family studies confirm the diagnosis. Urinary porphyrins are normal in EPP. Acute porphyric attacks do not occur.
The main source of protoporphyrin in EPP is not clear. Some reports claim that bone marrow produces the excess of protoporphyrin (Schwartz 1971), while others mention the liver (Scholnick, 1971). Recently new evidence suggests that at least a variant with mainly erythropoietic overproduction exists (Samuel 1988).
Administration of red blood cell transfusion may lead to improvement of hepatic disease and reduction of porphyrin excretion (Dobozy 1983, Baart de la Faille 1984).
Chenodeoxycholic acid decreases erythrocyte protoporphyrin levels and the excretion of protoporpyrin by an unknown mechanism (Van Hattum 1986). Cholestyramine interferes with the enterohepatic cycle of the porphyrins (Davidson 1973). Iron therapy has shown to reduce erythrocyte protoporphyrin and fecal protoporphyrin excretion, and even to normalize liver function (Gordeuk 1982). It remains

to be established if these forms of therapy improve prognosis of EPP. In case of terminal liver failure liver transplantation has been performed succesfully (Samuel 1988, Starzl 1989).

Experimental porphyria

Several synthetic agents are known to cause porphyria, since Stokvis (1889) described toxic porphyria caused by the hypnotic drug sulfonal. He was able to induce porphyria with this drug in rabbits and dogs. In 1952 the drug Sedormid, allylisopropylacetylcarbamide, which is structurally related to barbiturates was found to cause a acute porphyria-like syndrome both in men and animals.
2-Allyl-2-isopropylacetamide (AIA) is a congener of Sedormid that was subsequently shown to induce rapid accumulataton of porphyrins and porphyrin precursors in the liver of rodents. Following AIA administration, cytochrome P450 is converted to a green pigment. Recently it was shown that this is an N-methyl substituted porphyrin (Marks 1989). The removal of cytochrome P450 hemes results in a hundred-fold increase of ALA-S (Meyer 1978, De Matteis 1978, Smith 1980).
Barbiturates are strong inducers of the MFO system (Cytochrome P450), but are not porhyrinogenic unless inherited or additional toxic factors are involved.
Apart from AIA some other chemicals are of importance in experimental porphyria, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) unrelated to any particular drug, and griseofulvin, a fungicide, induce accumulation of protoporphyrin. During their metabolism N-substituted porphyrins are formed from cytochrome P450 which inhibits ferrochelatase activity (Cole 1984, Marks 1988). Increased protoporhyrin levels have been found in feces and erythrocytes of patients receiving griseofulvine (Smith 1980).
Hexachlorobenzene (HCB) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are polyhalogenic aromatics (PHA's). These strongly porphyrinogenic substances will be discussed in 1.4.10.

## 1.4. Porphyria Cutanea Tarda (PCT)

### 1.4.1. Introduction

Introduction: PCT is caused by a deficiency of the enzyme uroporphyrinogen decarboxylase (URO-D) which can lead to the massive overproduction of porphyrins - mainly uroporphyrin, heptacarboxyl porphyrin and isocoproporphyrin. Tissue accumulation of these porphyrins can give rise to a blistering skin disease of the sun exposed parts. This disease is known to be inherited as an autosomal dominant disorder, but can also be caused by xenobiotics like hexachlorobenzene (HCB) and 2,3,7,3 tetrachlorodibenzo-p-dioxine (TCDD). In the majority of the patients the cause is not known - this form is called sporadic (in contrast to inherited) PCT. URO-D deficieny alone does not necessarily give rise to the development of porphyria, as can be observed with the inherited form. Under basal conditions the activity of URO-D appears sufficient for heme requirements. However substances like alcohol and estrogens, which can induce the mixed function oxygenase system and so increase heme synthesis can provoke porphyria in individuals with inherited URO-D deficiency. Iron can also induce PCT in such individuals and is necessary for the toxic action of HCB. Removal of iron leads to a decrease of the disease activity. The mechanism by which iron works is not known. Several studies on the effect of iron and on URO-D have been performed, but these are not conclusive. Another remarkable feature of PCT is that there is an association with a variety of diseases including chronic hepatitis, hepatoma, systemic lupus erythematosus and lymphoma.

### 1.4.2. History

An excellent review on this subject has been written by Torben With (1975, 1980). The history of the porphyrias starts in the middle of the nineteenth century, when, for the first time, iron-free heme was prepared by Scherer (1841), by chemical treatment of hemoglobin with sulphuric acid. This iron-free heme appeared to fluoresce with a bright red color. This fluorescence was first described by Thudidum (1867) and later by Stokvis (1871). Hoppe-Seyler (1871) introduced the name hematoporphyrin. In 1911 Günther described for the first time a classical case of what we now know as porphyria cutanea tarda. He defined it as photodermatosis with porphyrinuria, without abdominal pain and paresis and called it hematoporphyria chronica. One must realize that in those days an epidemic of acute porphyria (with abdominal pain and paresis) had taken place after the introduction of the sedative, sulphonal and trional. The porphyric attacks induced by these drugs represent the first chemical induced porphyria. In 1913 Meyer-Betz gave himself an intravenous injection of 200mg haematoporphyrin dissolved in 10 ml. NaOH and diluted to 300 ml with physiological saline. During the injection he felt unwell and had pain in the liver region, radiating to the back. A day later, after exposure to the sun he felt pricking and burning of the exposed skin parts. Erythema and edema developed. This photosensitivity lasted for six weeks. He did not repeat his experiment.

Waldenström in 1937 introduced the name Porphyria Cutanea Tarda. The word tarda is mischosen because the disease can occur in all age groups including the very young. With (1975) states that it would have been better to choose the name porphyria cutanea chronica in analogy with Günther. From urine from PCT patients Waldenström crystallyzed a porphyrin, which he supposed to be uroporphyrin-III. Grinstein (1945) however found these crystals to consist of a mixture of uroporphyrin and heptacarboxyl porphyrin. This heptacarboxyl porphyrin is important in the differential diagnosis of porphyria cutanea tarda and porphyria variegata, as was subsequently shown following the introduction of thin-layer chromatography.
Ippen showed in 1961 the beneficial effect of venasection. One year later the porphyrin mobilizing effect of chloroquine was described by Sweeney (1962). In the early sixties an epidemic of porphyria cutanea tarda occured in Turkey (Cam 1959, 1963). The cause of this epidemic was the fungicide hexachlorobenzene. Wheat contaminated by HCB was consumed by a large group of people. The type of porphyria that occurred was both clinically and biochemically similar to porphyria cutanea tarda. From that time the porphyrinogenic qualities of more xenobiotics were recognized. During the recent past the introduction of high pressure liquid chromatography (HPLC) and the finding that URO-D was the deficient enzyme in PCT were the major developments.

### 1.4.3. Clinical Features

Age and sex distribution. Porphyria cutanea tarda can occur at every age, although the adjective tarda suggests it occurs mainly late in life. Waldenström (1957) reviewed several series of PCT from several countries, comprising 96 patients, 76 men and 20 women. The overall age distribution was 26 - 68 years. In a later series of 40 patients 58% of the patients were women (mean age 46,5 years, distribution 26 - 66 years (Grossman 1979). The cause of the shift is explained by increasing ingestion of alcohol and more importantly the use of estrogens by women. This disease can also be found in the very young (Grossman 1979).

Skin Symptoms and Signs.

These have been extensively reviewed by Tio (1956), Grossman (1979) and Harber (1982).

Predelection sites.
The skin lesions in PCT occur in the sun exposed areas. The face, the back of the hands, the V of the neck and, especially in women, the lower legs and feet. The plantar sides of the hand can also be involved (Tsega 1980).

Signs and symptoms (see table 1.4.3-1: signs and symptoms of the skin in PCT ).

**table 1.4.3-1 : signs and symptoms of the skin.**

**History:**
- **photosensitivity often unnoticed.**
- **increased suspectibility to minor trauma especially of the hand.**

**Physical examination:**
- **vesiculae, bullae, milia**
- **hypertrichosis**
- **hyperpigmentation**
- **hypopigmentation**
- **scarring, sometimes with alopecia**
- **sclerodermoid changes :**
  - **thickening of the skin**
  - **pale yellow discoloration**
  - **sclerodactyly**
  - **dystrophic calcification**
  - **with ulceration**

---

The most frequent symptom is increased fragility of the skin. Minor trauma like stretching ones gloves can even cause losening of the skin and blister formation (Tio 1956). These blisters vary in size from vesiculae 1 - 4 mm in diameter to bullae 0.5 - 3 cm. Grossman (1979) found them to occur in 85% of his patients. These blisters often burst and shallow erosions appear that can infect secondarily and heal slowly (Tio 1956). On these erosions crustae are formed. See figure 1.4.3-2

From healing blisters milia arise that are frequently seen, especially on the dorsum of the hand (Figure 1.4.3-2.)
Hypertrichosis occurs in PCT, mainly in the face, in 63% of patients, (Grossman 1979). Usually hairgrowth starts at the upper margins of the cheek and in the periorbital region, initially thin and light hair, which gradually darkens (Figure 1.4.3-4) Most typically hair appears at the upper margins of the eyebrows. The hair then darkens, thickens and increases in number. Men often complain that shaving is more difficult and that the growth pattern of the beard is changed.
Occasionally this hypertrichosis is found on the trunk, arms and legs (Grossman 1979). Extensive hair growth is especially reported in HCB-induced porphyria (Cam 1963).

Hyperpigmentation is seen in 55%. Most typically it occurs in the face as a peculiar mottled brown-black pigmentation in the periorbital and malar region mimicking melasma. It can be more diffuse in the sun exposed areas, or overall, suggesting hemochromatosis, Addison's disease or renal insufficiency. In Ethiopians darkening of the sun exposed skin was seen in all of the patients described by Tsega (1980).

Scleroderma-like changes are seen in 33%. These are characterized by thickening of the skin and pale yellow discoloration. Mostly they are seen on the face, the V of the neck and the hands, but they can also be seen on covered areas of the back and the chest. Rare symptoms are sclerodactyly and dystrophic calcification. In sclerodactyly the skin of the fingers is thickened and tightened. Circulation of the acra can be compromised which can lead to ulceration. Radiologically bone absorption can be seen in the tufts of the distal phalanges (Grossman 1979, Tio 1956). Dystrophic calcification was observed in three patients by Grossman. It developed in longstanding sclerodermoid plaques, especially in the preauriculair area. Spiculae of calcium deposits can be seen in these ulcers. The soft tissue calcification can also be demonstrated radiologically. Scarring alopecia can occur in areas, which are repeatedly involved.

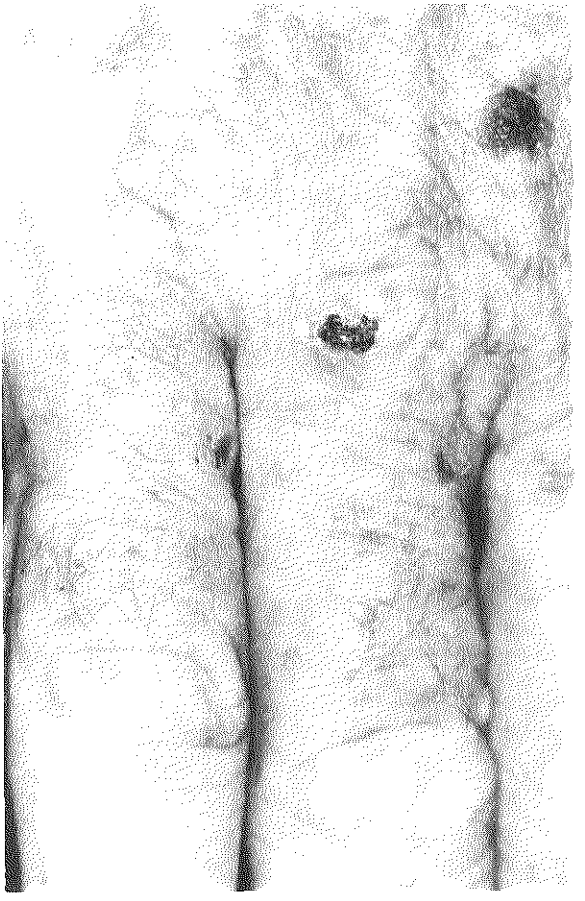


**Fig 1.4.3-1 Close up view of the dorsum of the hand of a patient with PCT erosions with crustae and milia can be seen**

The photophysical properties of porphyrins accumulated in the tissue can also give rise to ocular symptoms. Günther (1911) described a leukoma in congenital erythropoietic porphyria. The most frequent ocular symptoms in the series of Barnes and Boskoff (1952) were blepharochalasis, thickening of the conjunctiva, scarring of the internal and external eyelids, and symblefaron. Phototoxic damage of the sclera and cornea can give rise to ulceration, scarification, scleromalacia and perforation (Sevel 1971). These ulcerations can show red fluorescence under UV light (Chumbley 1977). Ectropion and stenosis of the lacrymal duct as a result of scarring can also complicate PCT (Sober 1981).

### 1.4.4. Biochemical Disturbances

#### 1.4.4.1. Clinical Chemistry

Confirmation of the diagnosis porphyria cutanea tarda begins with the inspection of urine. When porphyrins are present in large amounts, urine may have a red or brown-red color. This red color is also seen in the acute porphyrias, if much PBG is excreted PBG by itself is colorless but it spontaneously polymerizes to tetrapyrroles in urine, especially under acid conditions and if the urine is exposed to light. These tetrapyrroles cause the portwine color of the urine in acute porphyrias (Elder 1980a). If no abnormalities can be seen with naked eye, inspection of the urine with Woods light may reveal a red fluorescence. This fluorescence is intensified after acidification of the urine, or after extraction with amyl-alcohol (or chloroform).

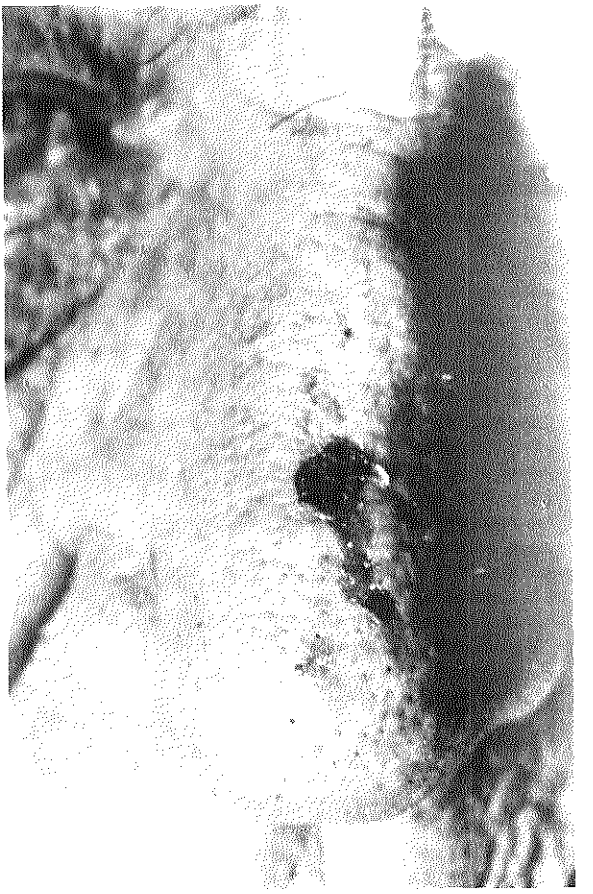


**Fig 1.4.3.-2 Shallow erosion with crustae on the dorsum of the nose**

Quantitative measurement of porphyrins is in fact based on the same principle, extraction of the porphyrins and - using the fluorescent properties of these compounds - fluorometry. Apart from fluorometry, spectrophotometry is also used. Separation of porphyrins can be performed by thin layer chromatography and by HPLC (high pressure liquid chromatography). These techniques are time consuming, partly because of extraction and the necessity of esterification with methanol/$H_2SO_4$, (Elder 1980a).
More recently, HPLC of free porphyrins became possible using a reverse phase column and gradient elution (Meyer 1980, Schreiber 1983). This means a further simplification of this technique. HPLC is also suitable for separation of the isomers I and III (Jackson 1982).

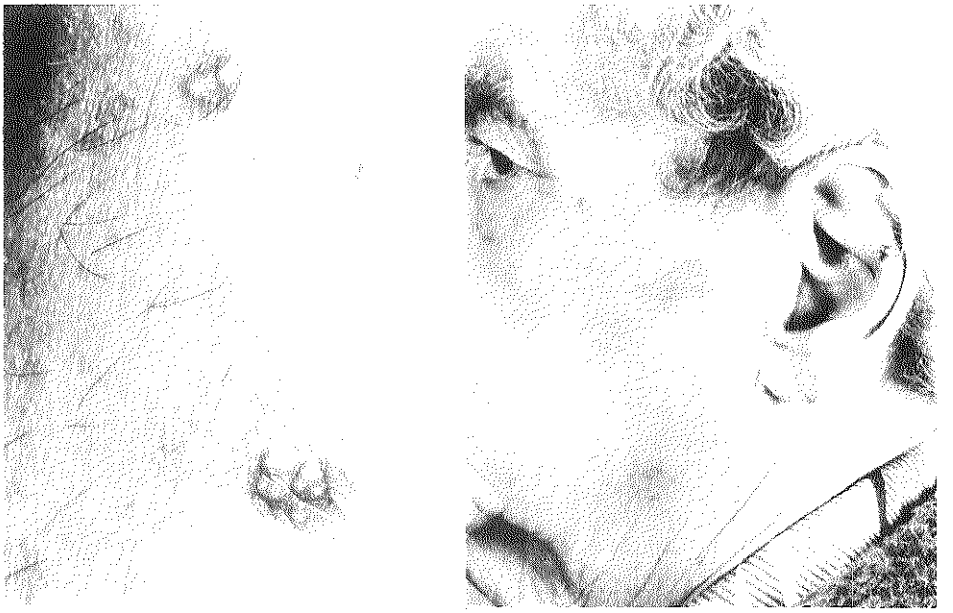


**Fig 1.4.3-3 A cluster of milia on the forearm (left); hypertrichosis on the cheek and the malar bone (right).**

HPLC (and thin layer chromatography) enables us do separate the porphyrins into fractions of uroporphyrin, heptacarboxyl porphyrin, hexacarboxyl porphyrin, pentacarboxyl porphyrin, coproporphyrin and protoporphyrin. The HPLC techniques performed in the laboratory of the Department of Internal Medicine II (University Hospital Dijkzigt, Rotterdam) will be described in chapter 2.
In all detection methods for porphyrins careful handling of the samples is essential. Samples of blood, urine and feces should be kept in the dark. Rapid analysis - within 24 hour - or storage at a -20°degrees of urine and feces is indicated. In the analysis of blood samples removal of hemoglobin is essential, because hemoglobin quenches fluorescences of porphyrins. Naturally occurring pigments and urobilin should be removed before separation of the porphyrins on the HPLC column (Elder 1980a).

Normal values of porphyrins and porphyrin precursor are given by Elder (1980a), who compares several investigators. Normal values according to Bissell (1985) are presented in paragraph 1.3.

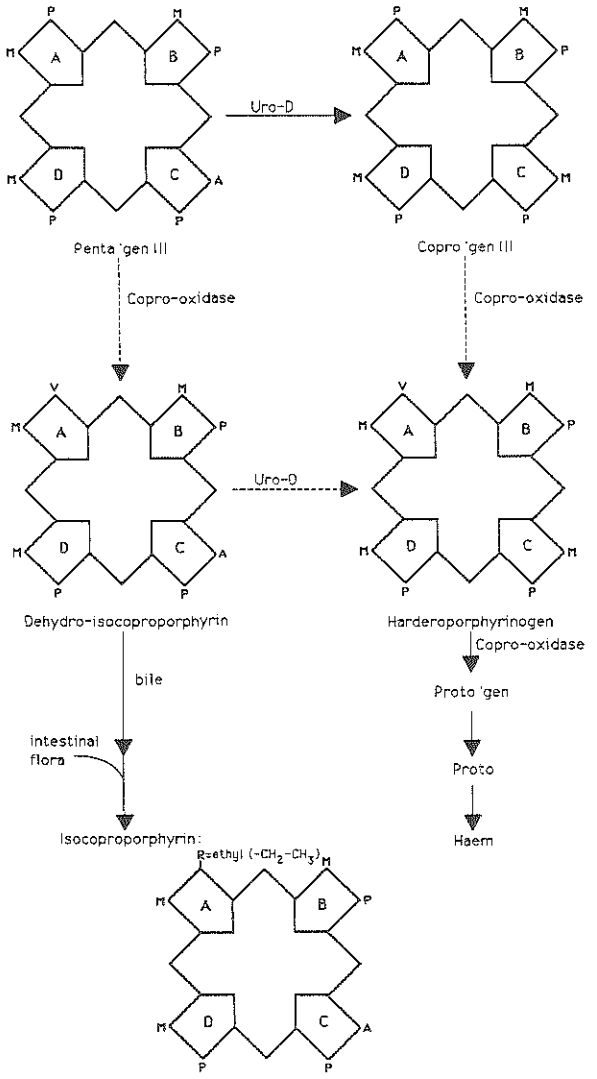



**Fig 1.4.4-1 Alternative pathway for pentacarboxyl porphyrinogen metabolism according to Elder (1977).**

**M = methyl - CH3**
**A = acetyl - CH2-COOH**
**P = proprionyl - CH2-CH2-COOH**
**V = vinyl - CH=CH2**

### 1.4.4.2. Abnormalities in Porphyrin Metabolism in PCT

In PCT there is a characteristic profile of porphyrin overproduction and excretion, that can be explained by the defective enzyme URO-D and normal compensatory heme regulation. (Dowdle 1970, Nacht 1970).
In PCT a marked increase (1000-2000 fold) in uroporphyrin excretion is found. Heptacarboxyl porphyrin and to a lesser extent hepta- en hexa- and coproporphyrin (and total porphyrin) excretion reflects the increased heme synthesis in PCT. The relative amounts of URO, HEPTA, HEXA, PENTA, and COPRO are about : URO : 60%, HEPTA : 25%, HEXA: 3%, PENTA : 3%, and COPRO : 9% of the total.
In feces an increased amount of coproporphyrin is found, mainly consisting of iso-coproporphyrin. A slight increase in protoporphyrin may also be seen (Elder 1977).
The formation of isocoproporphyrin is specific for a URO-D defect. Elder has shown that rat liver copro-oxygenase catalyzes oxidative decarboxylation of the 2-proprionate site of pentacarboxylporphyrinogen III (PENTA'gen III) to form dehydro-isocoprogen which is the precursor of the isocoproporphyrinogen series.
This pathway, that hardly occurs in normals, depends on the ratio of COPRO'gen III and PENTA'gen III. In normal circumstances COPRO'gen III is in excess, so that the the production of the isoCOPRO'gen series is low. In PCT however the decreased activity of URO-D results in low COPRO'gen concentration and a build up of PENTA'gen III levels
Further metabolism of dehydro-isocoprogen III to harderoporphyrinogen (continuing heme synthesis) is also diminished, since this reaction is also catalyzed by URO-D.
PENTA'gen III acts as a competitive inhibitor of COPRO'gen III, further deteriorating heme synthesis.
Dehydro-isocoproporphyrin is excreted both by bile and urine. In the gut isocoproporphyrin and diethyl-isocoproporphyrin are formed under the influence of gut flora (Elder 1975,1977).
The coproporphyrin excreted in urine in PCT also partially consists of dehydro-isocoproporphyrin (Smith 1977).

Isomers

Isomer analysis has shown that in the urine URO was approximately 30% isomer III, HEPTA and HEXA about 90% isomer III and PENTA and COPRO about 50% isomer III. In feces these ratio's are similar. Analysis of liver tissue showed 30% isomer III for URO, and almost 100% isomer III for HEPTA and HEXA. Both PENTA and COPRO consist of 50% isomer III.

Relation of URO-D affinity, Porphyrin and Isomer formation.

The relative affinity of URO'gen I and URO'gen III (as well as the 5,6, and 7 carboxyl porphyrinogens) for URO-D has also been studied. Several authors have shown that URO'gen III is more quickly converted to COPRO'gen III as the I series. URO'gen III has greater affinity to the enzyme than URO'gen I (De Verneuil 1980, 1983a; Smith 1979, 1981).

There are four catalytic sites in the protein URO-D, each for each porphyrinogen, URO'gen, HEPTA'gen, HEXA'gen and PENTA'gen respectively (De Verneuil 1980).
De Verneuil demonstrated that porphyrins with fewer carboxyl groups could inhibit competitively the decarboxylation of the porphyrins with more carboxyl groups, i.e. penta'gen III inhibits decarboxylation of HEPTA- and URO'gen respectively, but reciprocally URO- and HEPTA'gen do not inhibit the decarboxylation of PENTA'gen.
Porphyrins (as opposed to porphyrinogens) also inhibit enzym activity, especially URO and HEPTA (of series I and III) (Smith and Francis 1981).
Doss stated that a secondary coproporphyrinuria can transform to a PCT. This hypothesis is based on pattern analysis of urine porphyrin excretion of PCT patients in various stages of the disease. Four types can be discriminated: A-D (Doss 1971a, 1980, Peschlow 1983).
Type A: COPRO> URO
Type B: URO > COPRO > HEPTA
Type C: URO > HEPTA > COPRO
Type D: overt skin disease, in which total porphyrin excretion exceeds 1200 nmol/24 hour and URO + HEPTA excretion is much higher as COPRO.
In type A URO excretion has to be above normal values. Otherwise one is dealing with a secondary coproporphynuria. This transformation of secondary coproporphyrinuria to uroporphyrinuria can be seen in PCT patients, but - and this is essential - not every secondary coproporphyria transforms in a PCT. Therefore damage or preexistent abnormalitaties in structure or quantity of URO-D are necessary. This will be discussed later.

**1.4.4.3. Uroporphyrinogen Decarboxylase in Sporadic and Familial PCT.**

Uroporphyrinogen decarboxylase (URO-D) is now recognized to be the affected enzyme in PCT, both in the familial and sporadic occurring form, and certain forms of chemical induced porphyria. It's activity is not decreased in other porphyrias (Felsher 1978). It is also known to be the deficient enzyme in hepatoerythropoietic porphyria, which is considered to be the homozygous form of PCT (Elder 1981, Toback 1987).
Most patients with PCT have an negative family history, although familial occurence is often described (Tio 1956, Waldenström 1957, Dehling 1973, Kushner 1976, Te Velde 1976, Topi 1977, Benedetto 1978, De Salamanca 1980a, De Verneuil 1978ab, Blekkenhorst 1978a, Day 1979, Elder 1980b, Strik 1980, Magnin 1982).
PCT in its hereditary form is an autosomal dominant disease. The URO-D gene has been assigned to chromosome 1 (De Verneuil 1984b).
The occurrence of both familial and sporadic PCT, suggest a difference in etiology. In familial PCT an URO-D activity of about 50% of normal has been found in both liver and erythrocytes in patients suffering from this disease (Kushner 1976, Benedetto 1978, De Verneuil 1978ab).

In their relatives URO-D-deficiency was found in erythrocytes in subclinical, but biochemically active disease and latent disease (asymptomatic carrier state).
However families have also been described without a URO-D-deficiency in the erythrocytes (Elder 1980b). About half of the cases of familial PCT have a 50% decrease in URO-D activity in erythrocytes. In 1978 Elder reported that in sporadic PCT, URO-D-deficiency may be demonstrated in liver tissue, but not in erythrocytes (Elder 1978).
In 1982 Elder showed in TCDD-induced porphyria in mice that enzyme activity decreased in their liver, but that immunoreactive URO-D was unchanged. In familial PCT, both catalytic activity of hepatic URO-D as immunoreactivity (also defined as C.R.I.M. - cross reacting immunological material) is reduced to about 50% (De Verneuil 1984a, Sassa 1983, Elder 1985a). This has also been observed in the erythrocytes in familial PCT (Elder 1983b).
In sporadic PCT however catalytic reactivity is reduced, while immunoreactivity is normal or even increased (Elder 1985a).
In clinically overt disease significantly increased excretion of URO in urine was found compared with the group without skin symptoms but with biochemical abnormalities. Also, hepatic catalytic activity of URO-D was significantly decreased in clinical overt disease, compared with the group that showed only biochemical disturbances. Immunoreactivity of URO-D however was increased in sporadic PCT patients especially those with skin lesions, compared with normal controls. Four patients, who were treated with venasection showed normal activity and immunoreactivity of URO-D.
Felsher (1982) found in 4 patients with sporadic PCT, a persistent decrease of hepatic URO-D, even after venasection until remission. In these patients URO-D was not measured in erythrocytes so it is not clear that these patients were suffering from hereditary PCT.
The results of Elder (1985a) indicate that the decrease of URO-D in sporadic PCT is reversible. This brings us to the old hypothesis that URO-D is inactivated by an iron mediated process. This process must leave the enzyme immunologically intact, but damage the reaction site. Under the influence of iron, radicals can be formed and perhaps also highly reactive uroporphyrin-iron complexes that damage the reaction site. This will be discussed later.
That inactivation of URO-D cannot be ascribed to a simple effect of iron or alcohol, factors frequently associated with PCT, is also shown by Felsher (1982) who found normal liver URO-D-activity in most patients with hemochromatosis. In alcoholic disease Felsher found a significant decrease of URO-D-activity but except in two patients not to the level of PCT. Cirrhosis influences the result of URO-D activity measured in liver biopsies, since a significant proportion of the biopsy can consist of collagenous tissue.
The last few years there has been marked progress in the solution of the abnormalities of URO-D in hereditary PCT. First De Verneuil (1984b) showed that the gene that codes for URO-D is located on chromosome 1. Secondly the Paris group compared normal URO-D cDNA and URO-D cDNA of two patients with the homozygous form of PCT, or hepato-eythropoietic pophyria, and was able to find a point mutation G - A at position 860, leading to a glycine to glutamine change in the amino acid sequence at codon 281 (De Verneuil 1986a).

This change has considerable consequences for the protein, making it lose most of its activity. In another study of three different families with hepato-erythropoietic porphyria (HEP), they showed in two patients of the first family a defective URO-D with a half life 12 times shorter than controls (De Verneuil 1986bc). In the only patient of the second family they found a larger molecular weight of URO-D. In the patient of the third family they found a decreased production and an increased degradation of URO-D.
Whether there is a gene defect of URO-D that results in an enzyme more vulnerable to (iron mediated) damage by radicals (e.g. alcohol derived radical intermediates) remains to be established.

### 1.4.5. Other Biochemical considerations

Apart from abnormalities in porphyrin metabolism other biochemical changes can be found in PCT. Most of them can be correlated with liver disease. Increased values of serum gamma glutamyl transpeptidase (GGTP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), are found in 60% to 100 % of patients with PCT (Adjarov 1980).
Elevated values for serum bilirubin and alkaline phosphatase are found in about 30% of the patients. Abnormal BSP retention was seen in 60% of a series of patients, who mostly developed PCT after the use of estrogens (Grossman 1979).
An "abnormal glucose tolerance" has been reported in 60% of patients with PCT (Grossman 1979, Franks 1979). Oral and intravenous glucose tolerance tests were not significantly different from controls in the study of Lisi (1983), who found, however, a hyperinsulinaemic response to intravenous glucose in the PCT group, which they ascribed to the chronic liver disease. It has been suggested that the increased incidence of diabetes mellitus in PCT might be due to iron overload, comparable to the diabetes in hemochromatosis (Cripps 1987). The frequently encountered elevated values of serum iron and ferritin are discussed in 1.4.11.

### 1.4.6. Histology of the liver

There is a spectrum of histological liver abnormalities in PCT, but only one of them seems to be specific : acicular (needle-like) inclusions localized in the cytoplasm of the hepatocytes can be seen in almost every patient with PCT (Waldo 1971 ,Cortes 1980, Kemmer 1983, Fakan 1987).
In some series however these needles have not been mentioned or were seen in a considerably lower proportion of the patients (Biempeca 1974,Grossman 1979, Lefkowitch 1983, Ostrowsky 1984).
Cortes has shown that washing with water of the histological section for a period longer than ten minutes will remove all the needles (Cortes 1980, 1983). In the hematoxylin and eosin stain (H and E) these needles have a brown color and are birefringent under polarized light (See fig.1.4.6-1). After silver staining (Masson-Fontana) these needles become black and lose their birefringence. Ferricyanide gives them a deep blue color and visualizes even those needles not seen in H and E (Fakan 1987). In other stains they cannot be seen except in toluidine blue and sections prepared for

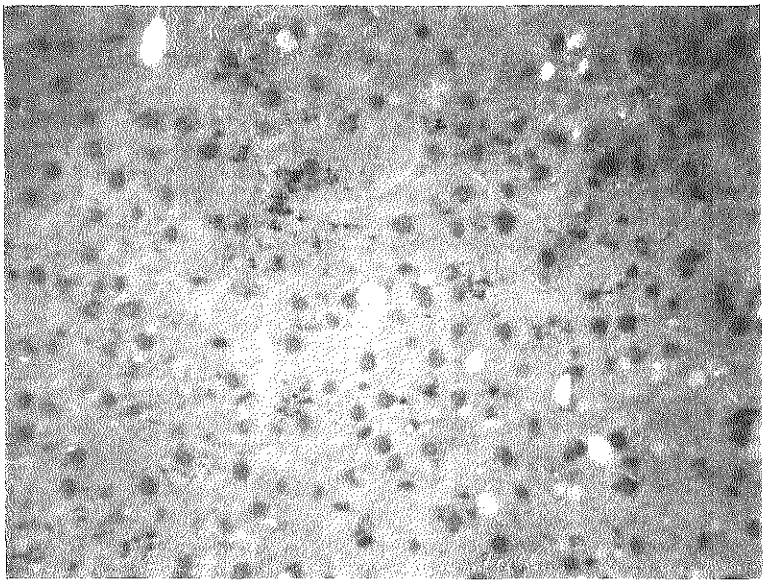


**Fig 1.4.6-1 Birefringent uroporphyrin crystals under polarized light in liver tissue in a patient with a proven PCT (by courtesy of H.A van den Bergen M.D.).**

electron microscopy. These needles are negative for ferrous and ferric iron. These needles have the same structure as crystals of URO I and URO III (Gaidos 1969). They have not been described in any other disease except PCT. Siersema et al (1991) have demonstrated with elektron microscopy that there is a close anatomic relationship between the acicular inclusions and the siderosomes.
Theoretically these crystallized porphyrins can also occur in congenital erythropoietic porphyria and in hepato-erythopoietic porhyria. They have not been observed in EPP, PV and other acute hepatic porphyrias (Bloomer 1976, Mascaro 1985b, Ostrowski 1983).
Another characteristic of almost every patient is autofluorescence of the liver biopsy (Fig.1.4.6-2). After exposure of the sections to U.V. light a red fluorescence is seen in the areas where the needle-like inclusions are situated (Cortes 1980, Lundvall 1969)In protoporphyria (EPP) a birefringent pigment has been decribed as pathognomonic for the disease. A Maltese cross can be seen in this pigment. In the routine H and E sections brown pigment can sometimes be seen in the hepatocytes and bile canaliculi (Bloomer 1976). Apart from the specific changes in liver histology in porphyria cutanea tarda other signs of liver damage have been described.

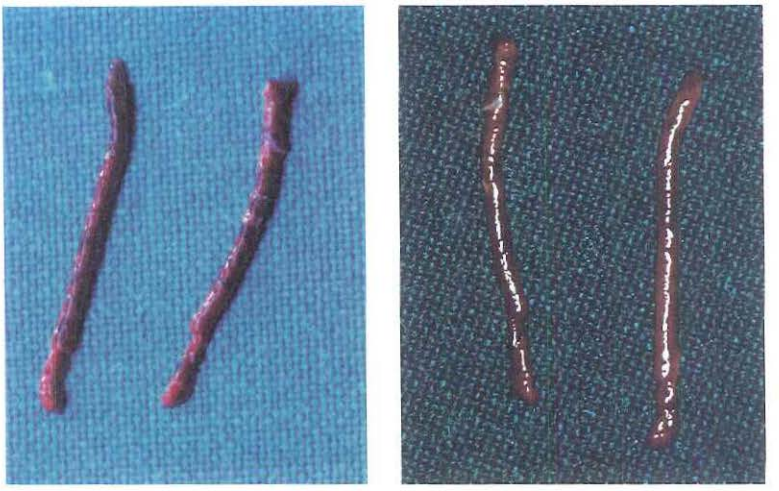


**Fig 1.4.6-2 Autofluorescence of a liver biopsy in Woods light-365 nm (left). The normal aspect is shown at the right side.**

The best documented is the Cortes series (1980). The results of these study are summarized in table 146-1.
Siderosis is seen in up to 91% but is nearly always mild .
Iron is found both in the Kupffer cells and in the cytoplasm and lysosomes of the hepatocytes. Lipofucsin, which like ceroid is a highly oxidized product of fatty acid, is found in more than 77%. The distribution is patchy as it is in the elderly. Mild fatty change is seen in 60% and is focal rather than diffuse or centrilobular as seen in alcoholic liver disease. Hepatocyte hyperplasia is seen in almost every patient. Focal necrosis in the lobuli can occur with formation of acidophilic or Councilman bodies and groups of macrophages containing ceroid and hemosiderin around it. Rarely granulomas are formed around these bodies (Grossman 1979, Cortes 1980).
This lobular inflammation is sometimes reminiscent of that seen in resolving acute hepatitis. Portal mononuclear inflammation (or septal inflammation in case of cirrhosis) was seen in 92%. Periportal inflammation with piecemeal necrosis was seen in 9%.
Lymphoid aggregates surrounding bile ducts have been only described by Cortes (1980) in 43.4 %. This was never associated with bile duct proliferation as in primary or secondary biliary cirrhosis.
Frank cirrhosis occurs in up to 34 %, although some series report a much lower incidence (Chlumska 1979 Lefkowitch 1983).
Cortes demonstrated that there is no significant difference in the incidence of cirrhosis in alcoholic and non-alcoholic porphyric patients. The features decribed are not specific but suggestive for PCT, and should be an indication for the pathologist to look for fluorescence and acicular inclusions.

The mechanism underlying the inflammatory changes is still a matter of debate. One intriguing finding suggests that immune mechanisms play a role. Cytotoxic antibodies against rat liver cells have been demonstrated in the sera of porphyric patients with chronic active hepatitis or cirrhosis but not in sera of porphyric patients without significant liver disease (Baravalle 1983).
Electron microscopically various abnormalities can be found. Besides the typical needles, fat droplets, siderosomes in the cytoplasm, mitochondria of different size and shape with granula inside them have been demonstrated. The endoplasmatic reticulum can show dilatation and signs of destruction. Also lipofucsin bodies can be seen, and autophagous vacuoles containing degenerated structures (Picardi 1972, Kemmer 1983, Biempeca 1974, Ostrowski 1984).

### 1.4.7. Histology of the skin

PCT is a blistering skin disease. The anatomical level of cleavage of the skin is regarded as a key factor for the classification and diagnosis of bullous skin disorders. In older studies only intradermal and subepidermal bullae were discriminated, in more recent studies junctional bullae are also recognized. The split formation in this type of bullae is situated the in the dermal-epidermal junction zone. In dermolytic bullae the level of cleavage is beneath the basal lamina.
In a series of 40 patients with PCT only subepidermal bullae were reported, with characteristic festooning of dermal papillae into its cavity (Grossman 1979). According to Hartshuh (1979), who also performed electron microscopy, blisters that are seen in PCT are not uniform and can be of the epidermal, the junctional and of the dermal type .
In the intradermal type the bulla is surrounded by cells of the basal layer. The basal cell layers close to the blister show remarkable changes. The intercellular space is increased and some basal cells are only connected by small cytoplasmatic bridges. Basal cells sometimes completely lose contact with each other, resulting in vacuoles. In the vicinity of the bulla the number of vacuoles increases. The basal cells also show cytologic changes, with swelling and a loose infrastructure.
The junctional bulla is bordered by the basal lamina at the roof site and by the basal cellayer at the epidermal site. This junctional bulla can change into a dermal bulla, of which the floor and roof is covered with fragments of the basal membrane which is is often multiplicated. This multiplication is a sign of repair of earlier trauma (Hartshuh 1979). In another series, junctional bullae were seen in 4 of 5 and a dermolytic bulla in 1 of 5 patients. Immunofluorescence techniques were used to visualize the location of the basal lamina (Klein 1983).
In the dermis solar degeneration (actinic elastosis) and homogenous hyaline perivascular thickening, that contains periodic acid positive, diastase resistant material are seen (Grossman 1980). Irregular clumps of PAS positive material are seen around these vessels but not in contact with them. These abnormalities are also found in other cutaneous porphyrias as EPP (Kint 1988). With the electron microscope extensive concentric fibrillar reduplications of the

basal lamina of skin vessels have been revealed, and large clumps of fibrillar material scattered through the mid and upper dermis (Caputo 1981, Hartshuh 1979).
With immunopathological techniques IgG deposition has been shown in 13 of 15 patients with PCT in the basal membrane and in all of them in the capillary walls (Kemmer 1988).
Monoclonal antibodies against vitronectin, a glycoprotein wich acts as an inhibitor of the complement system, also show immunoreactivity in the vessel wals of patients with PCT and EPP. It is thought that vitronectin binds to microfibrills (Dahlback 1988).
The sclerodermoid changes that are observed in patients with PCT are the same as in scleroderma (Friedman 1985).

### 1.4.8. Phototoxicity

The first to note the association of sunlight and a severe skin condition, which he called hydroa aestivale (the former name of PCT) which was accompanied by increased excretion of hematoporphyrin was McCall Andersen (1898). Hausmann (1909) was the first to prove that a solution of hematoporphyrin could kill paramecia after exposure to light, but not in the dark. He also studied mice after injecting hematoporphyrin. In the dark they remained healthy but after exposure to light, they developed skin symptoms, redness, edema and necrosis , especially of the hairless skin areas. Blum demonstrated that haematoporphyrin injected into normal human skin and irradiated with filtered light, so that normal sunburn wavelengths (± 300 NM) were excluded, could cause a wheal. He also demonstrated, that an intact circulation was necessary (Blum 1937). After obstruction of the circulation of the fore-arm (when the hematoporhyrin was injected) by a sphygmomanometer such a skin response did not arise. After restoration of the circulation, however a wheal appeared. This was the first evidence supporting the concept that oxygen was a necessary factor for the photodamaging effect of porphyrins.
Later it was found by means of irradation monochromators that a maximum skin response in porphyric patients can be obtained using light with a wavelength of 400 nM (near the Soret band) and - to a lesser extent - with light of the green yellow part of the spectrum, 500 - 600 nM. These wavelengths correspond with absorption spectra of the porphyrins (for review see: Elder 1980a, Magnus 1982, Bickers 1986).
With these monochromators so-called action spectrograms can be obtained. This method is helpful to differentiate porphyria from other photorelated disorders. This method however is not useful to discriminate the various type of porphyrias, as there is a considerable overlap in the absorption spectra of porphyrins.
Evidence for deposition of porphyrins in the skin in porphyria was already found in 1929. Skin fluorescence was seen in the skin sections of patients with porphyria under the fluorescence microscope described by Borst and Köningsdorfer (1929). Quantitative analysis was succesfully performed by several investigators (Van Gog and Schothorst 1973, and Miura 1975), who demonstrated porphyrins in the skin and blister fluid, using thin layer chromatography (Van Gog 1973, Miura 1975, Malina 1978).

Nature and frequency of histological changes in 43 patients (53 biopsies) according to Cortes (1980). No significant differences between alcoholic (23) and non-alcoholic patients (20) were found.

| architecture | no of biopsies |
|---|---|
| preserved | 7 (13%) |
| distorted | 23 (43%) |
| cirrhotic | 18 (34%) |
| hepatocellular carcinoma | 2 ( 4%) |
| no assessment possible | 1 ( 2%) |
| **Other histologic findings** | |
| alcoholic hepatitis | 1 ( 2%) |
| incipient central fibrosis | 8 (15%) |
| interstitial fibrosis | 12 (23%) |
| siderosis | 48 (91%) |
| lipofucsin excess | 41 (77%) |
| nuclear vacuolations | 22 (41%) |
| fatty change | 32 (60%) |
| hepatocellular hyperplasia | 52 (98%) |
| focal lobular necrosis | 48 (91%) |
| acidophilic bodies | 36 (68%) |
| piecemeal necrosis | 5 ( 9%) |
| portal or septal inflammation | 49 (92%) |
| periductal lymphoid aggregates | 23 (43%) |
| arteriolar thickening | 10 (19%) |
| needle-shaped inclusions | 50 (94%) |

Cortes 1980

**Table 1.4.6-1**

### 1.4.8.1. Mechanism of Phototoxicity

Porphyrins are well known photosensitizers. Porphyrinogens do not have these properties. It is not known how the oxidation of porphyrinogens to porphyrins takes place in vivo. Oxidation in the circulation and photo-oxidation in the skin seem to be the most probable pathways.
Porphyrins are thought to be excited by a certain quantum of light. The porphyrins are transformed to the so called triplet state - a very reactive molecule - which can transfer its energy to oxygen. Oxygen radicals are formed in this way (Cannistraro 1978, Bodaness 1977).
These radicals cause tissue damage (Goldstein 1972, Spikes 1975, Girotti 1979). Especially singlet oxygen is thought to be responsi-

ble for tissue damage (Poh-Fitzpatrick 1986). This photodamage is absolutely dependent on oxygen. It does not take place under anaerobic conditions, it is inhibited by singlet oxygen scavengers, it is enhanced by $D_2O$ (Sandberg 1982b). Visible light induces water cleavage and thus the formation of oxygen. This process is sensitized by porphyrins (Borgarello 1981), but heme-containing catalase and riboflavin are also photosensitizing agents (Eisenstark 1987).
In 1981 Lim reported that U.V. radiation with 405 nM light (the absorption spectrum of porphyrins is between 400 and 410 nM) of serum from patients with EPP, resulted in potent chemotactic activity in human polymorphonuclear leukocytes (Lim 1981). Also a decrease of haemolytic titers of complement C5 and a decrease of C3 was found after irradiation. This activation of complement was shown by several other authors in sera of PCT patients after irradiation with U.V. light (Torinuki 1985, Meurer 1985, Pigatto 1986). All authors reported decrease of complement hemolytic activity
and an increase of C3a which is a strong inducer of chemotaxis for polymorphonuclear leukocytes and which causes mast cell degranulation (Roitt 1986). This mechanism can explain the erythema that is seen after U.V. irradiation of the skin, wich precedes a noticible rise of lysosomal enzyme activity for several hours (Volden 1979). The classic concept is, according to Allison (1966), that the skin symptoms arise <u>after</u> photodamage of cells and organelles such as lysosomes. At the present time a significant role for photo-induced complement activation can be claimed. These photodamaging properties of porphyrins have found their clinical use in the treatment of malignant tumors with photodynamic therapy (PDT). Skin metastases of breast tumors have been treated with red light and hemato-porphyrin derivatives. Extensive experience exists at the present time with the local - palliative - treatment of bronchus carcinoma with PDT (Sutedja 1990).
Although porphyrins have similar photochemical properties, there is a marked difference in the skin symptoms of the various porphyrias. Porphyria cutanea tarda, variegate porphyria and heriditary coproporphyria have common features, while erythropoietic protoporphyria has different skin manifestations. Sandberg attributed this to the degree of lipophilicity of porphyrins. Protoporphyrin is lipophilic, uroporphyrin is hydrophilic and coprophyrin is intermediate (Sandberg 1981ab, 1982c).
He performed a series of experiments in which he irradiated subcellular fragments mainly of rat en mice liver cell's. He added exogenous porphyrins to these suspensions or he provoked endogenous porphyrin accumulation by feeding hexachlorobenzene to rats and griseofulvin to mice (Sandberg 1980, 1981ab, 1982abc).
Hexachlorobenzene induces uro- and heptacarboxyl accumulation and griseofulvin induces protoporphyrin accumulation. He found that protoporphyrin mainly damaged mitochondria and mitochondrial enzyme systems (lipophilic structures), while uroporphyrin mainly damaged water soluble compounds like tryptophan- and glutamate dehydrogenase (Sandberg 1982abc, Halnak 1986).
Sandberg found both in HCB-induced porphyria in the rat and in griseofulvin-induced protoporphyria in mice that porphyrins (URO and PROTO) respectively, accumulated in the lysosymes, but in HCB-induced porphyria the accumulated URO and HEPTA caused photodamage

in lysosomes before there was any detectable photodamage to the mitochondria (Sandberg 1982b). In griseofulvin-induced protoporphyria, however, damage of mitochondria was seen before there was any detectable damage of the lysosomes (Sandberg 1981a). Exogenously added protoporphyrin is accumulated in lysosomes as well.
These data are compatible with the lysosome hypothesis of Allison (1966). It is thought that lysosomes take up porphyrins. Under the influence of light the membranes of the lysosomes are damaged, whereafter autolytic enzymes destroy the cells (Torinuki 1980).
The difference between EPP and PCT may be due to the hydrophilic and lipophilic properties of the respective porphyrias. These properties of porphyrins can not explain the marked contrast that exists between erythropoietic protoporphyria and lead intoxication. While both diseases give rise to accumulation of protoporphyrins in the erythrocytes no photosensivity is seen in lead intoxication.
In contrast to EPP, in lead intoxication protoporphyrin does not leak from the red cell (Piomelli 1975). In EPP protoporphyrin is found in plasma, in lead intoxication it is not. This is concordance with the earlier findings that fecal protoporphyrin is not increased in lead intoxication and also that in EPP only a fraction of the erythrocytes are fluorescent, while in lead intoxication a diffuse fluorescence has been found. All these facts can be explained by the hypothesis, that protoporphyrin in lead intoxication forms an insoluble complex with lead or zinc that cannot leak from the red cell.
The skin lesions in VP are similar to those seen in PCT, but they are seen less frequently in VP (Magnus 1980).
That melanin protects also against the phototoxicity of porphyrins has been shown in patients with VP and vitiligo (Westerhof 1981).

## 1.4.9. Chemical-Induced Porphyria Cutanea Tarda

### 1.4.9.1. Chemical-Induced Porphyria in Man

A substance that induces porphyria has to fulfil at least the condition that it inhibits or inactivates one or more enzymes of the heme synthesis pathway, thereby creating the conditions in which porphyrin precursors or porphyrins accumulate. In addition these substances frequently induce heme synthesis by induction of the cytochrome P450 and mixed function oxygenase (MFO) system or lead to cytochrome (heme) destruction. This property aggravates porphyria.
The first chemical induced porphyria was observed by Stokvis (1889) after the introduction of sulphonal and trional. A syndrome was described that we now know to be acute porphyria. Patients suffered from acute abdominal attacks in combination with excretion of massive amounts of porphyrins in the urine, which was burgundy red colored. When the knowledge of the porphyrias increased, the role of chemical substances in the inherited porphyrias became even more clear. The precipitation of an acute attack of porphyria by barbiturates, alcohol and other substances, could be explained by their property to induce the cytochrome P450 and the MFO system (De Matteis 1978a).
The porphyrinogenic properties of the polyhalogenated aromatic hexachlorobenzene became clear in the fifties in Turkey, while the toxicity of the polychlorinated biphenyls were recognized in the sixties (Bleiberg 1964). A porphyria cutanea tarda-like syndrome was described in 1960 in Turkey (Cam 1959,1963 ; Dogramici 1962, 1964). It was caused by hexachlorobenzene (HCB), a fungicide introduced on the market as Chlorable and Surmesam.
HCB was used to decontaminate seed wheat that was infected with the fungus Tilletia triciti. Unfortunately this HCB treated seed wheat was used for human consumption in time of scarcity. The routine procedure was to mix 2 kg fungicide with 1000 kg wheat. The fungicide contained 20% HCB. It was estimated that the daily dose that was taken was 0.05 to 0.2 gram.
Between 1955 and 1959 about 4000 people were affected, especially the younger age group, 83% of the victims were between 2 and 15 years old. All these people developed a porphyria cutanea tarda like-syndrome.
In infants who were breast fed, another syndrome was seen, that was called in Turkish "Pembe Yara" (Pink Sore), which included skin lesions on hands, legs and feet that appeared as papules, which were red en rosy. They developed later to circular plaques with the central parts becoming brown or black, resembling fungus infection (Dean 1961).
These infants also had fevers, diarrhea, and anorexia. Muscle atrophy and convulsions occurred. Enlarged livers and a hyperchromic anemia were seen frequently. Mortality was exceptionally high, the only reported cause was secondary pulmonary infection (Courtney 1979).
The most pronounced features of the PCT-like syndrome were bullae (99%), porphyria (95.4%) associated with brown red urine, weakness (94.7%), hypertrichosis (82.3%), hyperpigmentation (64.9%) and arthritic changes (54.8%). Hepatomegaly was found in most patients during the acute phase (Cripps 1980).

Arthritis is not observed in patiens with PCT not due to HCB. Typically the HCB-associated arthritis is painless with deformation of the proximal and digital interphalangeal joints of the hands. In a follow-up study of Cripps (1980) it appeared that the hands of most young patients remained remarkably small.
Neurological symptoms have been observed too in HCB-induced porphyria: paresthetic neuritis and colicky abdominal pain. In the early studies however no determinations of ALA and PBG were performed. In a follow-up study of Cripps (1984) on some patients increased ALA and PBG values were found, but these increased values did not correlate with neurological signs. However in experimental porphyria induced by HCB in animals increased ALA and PBG values and neurological signs have been well observed (Ockner and Schmid 1961, De Matteis 1961).
Biochemically HCB porphyria is characterized by a marked increase in uroporphyrin excretion and to a lesser extent in urinary and fecal coproporphyrin excretion. Cripps performed two follow up studies in 1980 and 1984 of patients that had active porphyria in the period from 1956-1961. This study in 1984 was done in 204 patients (132 males, 72 females), average age 32.1 years at time of examination. The average age at onset was 10.2 years and the mean duration of the disease was 2.4 years. The most remarkable clinical signs in this series were scars, usually on the cheeks, arms and the dorsa of the hands (85,6 %). The scars were larger than 1 cm. Other signs were hyperpigmentation (71.1%), pinched facies (42.4%), hypertrichosis (47.5%). The most striking clinical features however were painless arthritis (67.1%) and small hands (64.7%).
Sclerodermoid thickening of the skin of the hands with severe shortening of the digits was due to osteoporosis, particularly the terminal phalanx was almost completely reabsorped. Shortness of stature occurred in 44.6% and an enlarged liver was found in 4.4% (in contrast to the acute phase, when almost all patients had an enlarged liver ) A clinically enlarged thyroid was found in 37.3%, which was above the 5% goitre incidence in that area. It was found more frequently in men than in women. Goitre also develops in HCB fed hamsters, not changing $T_4$ levels but significantly depressing $T_3$ levels - changes which possibly reflect chronic disease (Smith 1987).
Neurologic symptoms as weakness, paresthesia and neuritis were seen in about 60% of the patients. Both sensory and motor neuritis were found (Peters 1986a). Slowed nerve conduction is observed in electrophysiological studies (Silbergeld 1987).
A phenomenon that resembles cog-wheeling of patient with Parkinson's disease was found in 30%. Grand mal convulsions were seen only in 1% and may be unrelated to porphyria.
During the period of the study two patients died from liver failure - one a 27 year old male with cirrhosis of the liver, the other a 54 year old female, during treatment of pulmonary tuberculosis. No details were mentioned. Among the total 188 pregnancies that were noted in 42 women, there were 15 fetal deaths. Thirty one children died within a few years after birth, leaving 142 living children. Later studies have shown HCB in the human placenta and in cord blood (Ando 1985), as well as human follicular fluid (Baukloh 1985) suggesting that this substance could reach the oocyte and have a

teratogenic effect. Placental transfer has also been demonstrated in animals and seems to be species dependent (Courtney 1979 , 1985).
The incidence of Pembe Yara in the children of mothers with a HCB intoxication and the incidence of congenital malformations were not mentioned. However the finding of remarkably high levels of HCB in specimens of breast milk of 40 patients, may be the cause of the Pembe Yara syndrome. The mean level was 2.8 p.p.m. This is 140 times the level allowed in cow's milk.
In Cripps' follow up study of 1984, raised uroporphyrin and coproporphyrin was still seen in 17 of 204 patients. In the other patients the excretion was normal or only borderline increased. There was no relation between increased uroporphyrin levels, use of sex steroids and alcohol.
The literature does not mention treatment of HCB-induced porphyria with phlebotomy, although bleeding and iron depletion diminish the porphyrinogenic effect of HCB in the rat (Blekkenhorst 1980).
Peters has treated some patients with chelation with EDTA and reported improvement (Peters 1960, 1986, 1986a). However as can be seen from Cripps' data, spontaneous remission of porphyria can occur (Cripps 1984). Treatment with chloroquine (Vizethum 1980) and the iron chelator desferrioxamine (Wainstok 1986a,1986b) has only been studied in HCB porphyria in rats.

**1.4.9.2. Toxicity of 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) in man**

TCDD is a by-product of certain synthetic herbicides, the burning of house dust, fires of electric transformers and of the defoliant Agent Orange, used in the Vietnam War (Dunagin 1984, Marks 1985, Stehr 1986, Osterloh 1987).
Once introduced into the environment it has been shown in high concentrations of fish from contaminated waters, while also a significant correlation between fish consumption and the serum levels of the consumers of the fish has been demonstrated (Svensson 1991).
Although its potential porphyrinogenic effects were recognized several years ago, overt PCT seems to be not as frequent as previously thought. After the severe contamination of the Seveso area in 1976 only two clinical cases of PCT were described in 60 TCDD exposed individuals and both patients were clearly genetically predisposed. In 22% a secondary coproporphyria and in 5% elevated uroporphyrins were found (Doss 1984,1987). The reports of Bleiberg (1964), and Jirasek (1976) have been used to indicate the porphyrinogenicity of TCDD in humans, but thorough reevaluation of these papers by Jones (1986) showed that it is far from certain that TCDD was the only responsible porphyrinogenic substance. It seems more likely that HCB was a major contaminant of the polyhalogenated aromatics manufactured in these chemical plants.
One other patient with PCT and exposition to TCDD, and also suffering of a sarcoma has been reported (Anonymous 1984a). The main clinical feature of TCDD intoxication is chloracne, not due to the concentration of TCDD in the sebaceous lipid contents but probably due to a proliferative effect on the cutaneous epithelium (Passi

1981). Liver function abnormalities, periferal neuropathy, hyperlipidemia and muscle weakness and depression may occur (Webb 1986). Recently it was shown that cancer mortality of all cancers combined is increased after exposure of TCDD (Fingerhut 1991). Especially bronchogenic cancer and possibly soft tissue tumors are related to exposure to TCDD.

**1.4.9.3. Manifestions of HCB Porphyria in the Rat**

After administration of HCB both neurologic and cutaneous signs have been observed in rats. Ockner and Schmid (1961) fed male rats a diet containing 0.2% HCB. One month later one third of the rats had died after exhibiting tremors, weakness, ataxia and paralysis without evidence of disturbed porphyria synthesis. In the remaining 21 rats increased levels of ALA and PBG were found, but there was only a trace of uroporphyrin and a slight increase of coproporphyrin. De Matteis (1961) induced porphyria in rats with a diet of 0.5% HCB. Taljaard (1972a) found apart from neurological effects also cutaneous lesions, the loss of hair and encrusted hemorrhagic sores. The rats had a marked hepatomegaly. Microscopically there was proliferation of smooth endoplasmatic reticulum and whorl formation. At 33 days no substantial increase in porphyria excretion was seen, after this period a shift to detectable levels of PENTA, HEXA, HEPTA and URO was seen. At 55 days the excretion of URO and HEPTA was more pronounced then of COPRO. In later studies it was shown that the inactivation of uroporphyrinogen decarboxylase not only occurs in hepatic tissue but also in renal and colon tissue (Elder 1976b, San Martin de Viale 1977, Blekkenhorst 1980).
HCB induced porphyria in experimental animals is not a syndrome completely identical with porphyria cutanea tarda. Because both increases in ALA and PBG and neurological symptoms have been observed, HCB-induced porphyria has both signs of an acute and of a chronic hepatic porphyria. Once HCB administration is stopped, porphyria gradually diminishes, as does the HCB content of the liver. After 18 months a normal hepatic URO-D activity is found in the liver and hepatic porphyrin levels have decreased to normal. The HCB content is at that time 0-1% of the observed maximum concentration (Koss 1983).
This indicates that HCB is cleared from the liver and that the damage is related to activity of the enzyme URO-D, to the presence of HCB or its metabolites and is reversible.
This observation is concordant with the finding, that in the majority of patients with HCB porphyria in Turkey, the disease was reversible (Cripps 1984). In mice with an intoxication of TCDD liver URO-D activity remained depressed after regeneration of liver tissue following partial hepatectomy (Smith 1985a).
In experimental animals very high levels of HCB in breast milk were associated with convulsions and death of the neonates. In rats congenital bone malformation can occur (Courtney 1979).
Feeding of HCB during more then 90 weeks results in hepatocelllar carcinomata in 100% of female and 16% of male rats. Liver non-heme iron content was 3-5 times greater in females than in males, but liver tumor incidence did not increase after preloading with iron.
Cytochrome P450 induction in male and female rats is not signifi-

cantly different (Smith 1985b). In other studies thyroid tumors, pheochromocytoma, parathyroid adenoma and hemangioendothelioma have also been found (Kociba 1986).
The various methods by which rats can be made porphyric with HCB are reviewed by Strik (1986).

### 1.4.9.4. HCB, general remarks

HCB (Hexachlorobenzene, $C_6Cl_6$) is a polyhalogenated hydrocarbon (PHA). It is synthesized and does not occur naturally. It is a waste product in the manufacture of chlorinated solvents (often discharged in waste water) and pesticides. HCB is used as a fungicide and is also used in the rubber industry (Courtney 1979). Since the introduction of HCB it has been widely used all over the world. In the environment HCB will adsorb strongly to soil and sediment, and it is poorly soluble in water. From water it will be rapidly volatilized in the air. In water and air slow photolysis is the dominant loss process (Mill 1986). Since it is so poorly metabolized, HCB accummulates in terrestrial and marine animals. Particularly high concentrations have been found in the livers of animals at the end of the biological chain such as predatory birds (Courtney 1979, Vos 1969).
HCB has been demonstrated in cow milk and butter. Eggs, meat and fish have also been found to be contaminated (Courtney 1979), while to a minor extent crops cultivated on contaminated soil can contain HCB as well (Greve 1986). In human fat and breast milk HCB is also present (Niessen 1984, Nyhuis 1986). HCB can already been found in fat of toddlers, and its concentration increases with age (Niessen 84, Ansari 1986). Since the early seventies measures have been taken to prevent further increase of the HCB burden of the environment, and fat, breast milk and cow milk concentrations decreased (Greve 1986, Robinson 1986).

### 1.4.9.5.The pharmacology of HCB

HCB is poorly absorbed when it is administered in water, but 80% is absorbed when given in olive oil (Koss 1975). It is found in every tissue, but concentrations in fat are 80 times greater than in blood (Koss 1975). The distribution in blood, brain, heart, lungs, liver, kidney, testes and fat was studied by Morita and Oishi (1975). After intraperitoneal administration it bypasses the liver, and is transported through the lymph vessels in chylomicrons. After two hours a peak concentration is found in the liver, about ten times as high as in brain, heart and lungs and about three times higher than in kidney and testis. The peak concentration in the liver decreases after 48 hours. After 56 days all organ levels are lower. Relatively high concentrations are found in the lungs and kidneys; the bulk of HCB is found in fat (Morita 1975).
Ingbritsen studied (1982) distribution and excretion of $^{14}C$-HCB using an autoradiographic technique after oral administration of a single dose. In 2 hours HCB was distributed through the body. Peak levels were found after 4 hours in the liver and the brown fat, and at 24 hours in the abdominal and subcutaneous fat. Remarkably the

highest concentrations were not only measured in fat, but also the tne bone marrow, the skin, the Harderian gland, the nasal mucosa, the preputial gland and the intestinal tract. After 90 days high concentrations can only be found in fat, skin, nasal mucosa and preputial gland. Liver concentrations remained high for up to 18 days. The major part of the radioactivity was not evaporable.
The data about the clearance are not consistent. Morita and Oishi suggest a half life of 60 days with an initial half life of 8 - 10 days. However excretion is seen up to 30 years after exposure (Cripps 1984). This is explained by persisting fat stores of HCB.
Koss (1983) found after 18 month only 0.1% of the peak level of HCB in rat liver tissue.
HCB is excreted mostly unchanged in the feces. Koss (1975) found 27% of $^{14}$C-HCB over a period of four weeks in the feces. Seventy percent of the HCB excreted in the feces was unchanged, 30% was excreted as metabolites. Of the original dose 7% was in the urine in 4 weeks, 10% unchanged and 90% as metabolite (Koss 1976). When urine and fecal results are combined about 60% of the chemical is excreted unchanged. Only 3.6% of HCB is excreted via the bile, the bulk of the fecal HCB is excreted by the intestine, especially the colon. The mechanism of intestinal excretion is primarily exudation, not desquamation (Ingebrigtsen 1982, Rozman 1985a,1985b). Intestinal secretion can be stimulated by mineral oil (Rozman 1983) and colestipol (Polin 1986).
In HCB treated rats both phenolic and sulfur containing metabolites, which are formed respectively by oxidation and conjugation by gluthathion transferase, are found. After long term administration the ratio between phenolic and sulfur containing metabolites is 2.8 : 1. After short term administration the ratio is 1.3 : 1. The main phenolic metabolites are pentachlorophenol and tetrachlorohydroquinone.
Sulfur containing metabolites are pentachlorothiophenol and tetra chlorothiophenol (Koss 1976, Debets 1979). Recently Van Ommen (1986) quantitated liver microsomal metabolism of HCB in rats. The main products of microsomal oxidation were pentachlorophenol and tetrachlorohydroquinone (Van Ommen 1985, 1986), and there was extensive covalent binding of metabolites. Ascorbic acid and gluthathione inhibited oxidation, and covalent binding to macromolecules in this in vitro assay.
The thio-group containig metabolites are formed by the cytosolic isoenzymes of glutathione-S-transferase (Vos 1988)

**1.4.9.6. The Mechanism of Induction of Porphyria by HCB and other Polyhalogenated Compounds.**

The main processes that are at present thought to be involved in the porphyrinogenicity of HCB are:
1.Induction of Cytochrome P450 and the mixed function oxygenase system.
2. The inactivation of the enzyme URO-D, which appears to be dependent on oxygen and on the formation of radical intermediates. (Debets 1979).
HCB is a potent inducer of the cytochrome P450 mixed function oxygenase system (MFO). The cytochrome P450 isoenzymes are the termi-

nal oxidases of this hepatic microsomal enzyme system. Most inducers of cytochrome P450 can be divided in two classes typified by phenobarbital (PB) and by 3-methylcholanthrene (3-MC). The type of cytochromes induced by HCB seem to be the same as those observed after induction both with PB and 3-MC (Blekkenhorst 1978b, Debets 1979, Campbell 1983).
Recently more isoenzymes of cytochrome P450 in the rat have been defined immunochemically. The PB type isoenzymes were cytochrome P450b and P450e, while cytochrome P450c and P450d were induced by 3-MC. HCB induces the cytochromes P450b , -c and -d, in contrast to the lower chlorinated benzenes, which induce invariably the PB types (Goldstein 1986). TCDD however is a strong 3-MC-like inducer. The patterns of cytochrome-P450 isoenzymes that are induced seem not to be essential for the porphyrinogenity of a substance as appears from the observation that HCB and TCDD are both porphyrinogenic. However, in mice the oxidation of uroporhyrinogen was shown to be catalyzed by cytochrome P450 IA2. This isoenzyme appeared to be essential for the development of porphyria in this strain of mice. The inducibility of this isoenzyme appears to be inherited (Sinclair 1990).
PB and 3-MC only aggravate porphyria and were believed not to be porphyrinogenic by themselves (Wainstok 1984,Goldstein 1986). Recently Urquhart(1988) demonstrated that 3-MC given together with ALA is porphyrinogenic in mice.
HCB and TCDD induce the following activities of the MFO - arylhydrocarbon hydroxylase (AHH), biphenyl hydroxylase (BPH), ethoxyresorufin-O-deethylase (EROD), and ethoxycoumarin-O-deethylase (ECOD) (Goldstein 1986, Carpenter 1985c).
After administration of HCB two phases in the development of porphyria can be recognized (Koss 1983). When HCB is given induction of cytochrome system is reflected in a 2 - 3 fold increase of ALA synthetase, and a mild coproporphyrinuria, reflecting an increased demand for heme.
In the second phase porphyria develops, accompanied by a 10 - 20 fold increased need for heme, and finally of the enzyme URO-D. Immunogenic properties of the enzyme remain intact, which implies inactivation of the enzyme and not an inhibition of URO-D synthesis (Elder 1982).
The four steps of decarboxylation seem to be affected equally (Rios de Molina 1987). Structural changes in the enzyme have also been demonstrated (Rios de Molina 1986). Recently it was demonstrated that hepatic uroporphyrin increases before URO-D actvity decreases. This is due to the fact that uroporhyrinogen is very susceptible to oxidation (Lambrecht 1988). Oxidative processes play a major role in the pathogenesis of HCB induced porphyria as is pointed out below.
The main hypothesis of the mechanism of inactivation of URO-D following HCB administration is that radical precursors (epoxides) of the phenolic and sulfur containing metabolites inactivate this enzyme (Debets and Strik 1979). It has been well established that polychlorinated biphenyls (PCB's) and polybrominated biphenyls are metabolized to hydroxylated derivates (phenols) via highly reactive arene oxide intermediates, which are formed by cytochrome P450, oxygen and $NADPH^+$(Debets and Strik 1979).

Van Ommen (1986) elegantly demonstrated that in the absence of $NADPH^+$ and at low oxygen concentrations rat liver microsomes do not form HCB phenolic metabolites. Formation of these metabolites was also associated with a relatively high percentage of covalent binding to protein, which he interprets as a possible mechanism by which URO-D can be damaged.

Other arguments for this radical hypothesis is the finding of sulfur containing metabolites, (the result of glutathione conjugation), the aggravation of porphyric action of HCB by glutathione depleting agents as diethymaleate, and the prevention of HCB porphyria by the inhibitors of cytochrome dependent hepatic drug metabolism, SKF 525A and piperonylbutoxide (Debets 1979, Debets 1980, Stewart 1986). In contrast, acetylcystein does not prevent HCB induced porphyria (Lissner 1985), although gluthathion inhibits microsomal oxidation of HCB in vitro (Van Ommen 1986).
One of the phenolic metabolites, pentachlorophenol, was shown to inhibit the electron transport between a flavin and cytochrome P450 in the microsomal MFO system in vitro. This inhibition can result in the type of reaction by which electrophilic intermediates are formed. However, direct porphyric action of the main phenolic and sulfur containing compounds of HCB has not been demonstrated (Goerz 1978, Koss 1979).
Pentachlorophenol and tetrahydroquinone both aggravate the porphyric effect of HCB (Debets 1980, Carpenter 1985a).

A very remarkable development is the discovery of the Ah gen which codes for the Ah receptor .The susceptibility of mouse strains for TCDD and HCB induced porphyria is determined by this receptor. Mice with the allele $Ah^b$ had significantly higher hepatic porphyrin levels than those with $Ah^d$ (Dencker 1985, Hahn 1988). So inherited factors also play a part in the development of HCB induced porphyria.

### 1.4.9.7. Radical intermediates and lipid peroxidation in relation to HCB induced porphyria and iron.

Metabolism of HCB wil lead to formation of radical intermediates as is pointed out above. An increase of porphyrinogenicity of HCB has been observed when it was given concurrently with iron (Taljaard 1972a,1972b). Desferrioxamine on the other hand decreases HCB toxicity (Wainstock 1986ab), while TCDD is not toxic in iron deficient mice (Sweeney 1979,Jones 1981). This was correlated to a synergism of iron and hexachlorobenzene concerning the inhibition of URO-D (Smith 1983), which has been demonstrated to be the affected enzyme in TCDD-induced porphyria as well (De Verneuil 1983b). Maines (1980) has found evidence for the involvement of endogenous iron in lipid peroxidative destruction of heme by allylisopropamide which causes acute porphyria. Lipid peroxidation is a process in which the unsaturated fatty acids, mainly localized in the phospholipids of the membranes are peroxidized (Mead 1976). In this process the fatty acids are broken down to aldehydes and carbonyl compounds, resulting in membrane damage. During this process cross-

linking of membrane proteins also occurs. This leads to the formation of high molecular weight proteins (HMW proteins). Iron catalyzes the proces of lipid peroxidation according to the Haber-Weiss reaction and is therefore of great importance (Freeman 1982). It is also claimed that iron performs its catalysation of lipid peroxidation through perferryl iron (Svingen 1979). The role of iron in the process of lipid peroxidation has been the subject of an excellent review by Aust and Svingen. This subject is further discussed in chapter 6 and 7.

**1.4.10. The Role of Iron**

**1.4.10.1.General remarks**

The human body contains about 4 gram of iron. About 70 % of the iron is located in hemoglobin, 20% is bound to ferritin in the liver, spleen, bone marrow and gut; 4% is used as myoglobin. Only 0.3% is part of cell hemin, cytochromes and catalase; 0.1% is transport iron coupled to transferrine and 1 % is formed by hemosiderin. The binding to proteins for transport and storage is essential because of the very poor solubilty of iron ,that occurs under aerobic circumstances as $Fe(OH)_3$. The solubility product of $Fe(OH)_3$ is only $4x10^{-38}$, which means that in an aerobic environment the concentration of free ferri-ions is $10^{-17}$ mol /l (Van de Heul 1981). In the hepatocyte iron is stored mainly as ferritin. Normal daily loss is about 1mg, normal iron absorption about the same. Iron absorption can increase to 3-5 mg in iron deficiency. In primary hereditary hemochromatosis iron absorption is 1,5 -5 mg per day despite already increased iron stores (Kreeftenberg 1986)

**1.4.10.2. Iron in relation to PCT.**

In 1961 Ippen published his results of repeated phlebotomy (4 - 6 liters in total) in patients with PCT, which induced complete remission of this disease, both clinically and biochemically. He had based his therapy on the observation that values of serum iron, erythrocytes and hemoglobin were slightly increased in patients with PCT. Between 1956 and 1958 Brugsch reported 8 patients with PCT, hyperpigmentation of the skin, diabetes mellitus and considerable siderosis of the liver. In 1960 Tuffanelli reported a similar case of PCT and signs of iron overload in the U.S. Lamont (1960) reported in 12 of 15 patients of Bantu origin an increased plasma iron load and hepatic siderosis but the histological findings were different from the classical Bantu siderosis.
At that time most patients that presented with PCT used significant amounts of alcohol, so the next question was if the iron load was primarily due to the use of alcohol, to PCT or an iron overload disorder associated with PCT.

With isotope labelling Charlton (1964) demonstrated that 22,6% of 5 mg ferrichloride was absorbed in combination with alcohol , compared to 4% in the control group who took the iron with water. For ferrous ascorbate he found no difference. Since especially wines contain significant amounts of iron (5-9 mg/l), a high alcohol in-

take can cause an significant increase of iron stores and siderosis. Dramatic siderosis has been decribed in South African Bantus, who drink beer containing 150 mg iron / liter brewed in iron kettles (Bothwell 1960, Bothwell 1988).
Turnbull (1973) measured iron absorbtion with $Fe^{59}$ in 6 patients with PCT and liver cirrhosis and found values between 10 en 36 % (Normal 10%); but in other laboratories values are regarded normal between 10 and 30 %). In the 12 patients with PCT, but without cirrhosis he found values between 0 and 14 %. He found normal to moderate increased values of plasma iron in both groups. In all these patients he found significant amounts of stainable iron in the liver parenchym and in the Kuppfer cells.
Mild hepatic siderosis is seen up to 91 % in large series (Cortes 1980, see 1.4.8.) In a series of forty patients with PCT increased serum iron values were found in 62 % (Grossmann 1979.) Disler found increased ferritin in 6 out of 9 patients (1984b). In a series of 60 patients serum ferritin levels in PCT patients were significantly higher compared to normal controls (Rocchi 1986). Quantitative measurement of liver non-heme iron in 4 PCT patients revealed levels up to 1000 µgr liver wet weight were found clearly above normal values (300mu gr /gr liver wet weight ) but definitely lower than the amounts found in hemochromatosis (Zuyderhoudt 1983).
Total body iron stores are only occasionally raised more then two to three fold in PCT patients and are normal in 25% (Turnbull 1973, Elder 1985b)
Kushner (1982b, 1985) has postulated that in the family of a patient with sporadic PCT, mild iron loading existed as familial trait associated with the presence of the HLA haplotype A3 B7, which is associated with heriditary hemochromatosis, an autosomal recessive disease. He suggested that in this patient, in whom only URO-D deficiency in the liver could be demonstrated, a single allele for HLA linked hereditary hemochromatosis was responsible for hepatic siderosis which resulted in the manifestation of the PCT.
Later studies confirmed the association of sporadic PCT with heriditary hemochromatosis associated HLA types, A3 and DR7 (Kushner 1985, Elder 1985, Beaumont 1987, Edwards 1988a, 1988b, 1989).
A hypothetical explanation for this association according to Kushner is as follows: In sporadic PCT the URO-D defect is confined to the liver and inherited as autosomal recessive trait. He further suggests that this defect becomes only manifest in the presence of iron overload, which occurs in about 20-30% of the heterozygote carriers of the hemochromatosis gene.
The gene for URO-D is located at chromosome 1 (see 1.4.5.4), while chromosome 6 is thought to carry the hemochromatosis gene close to the HLA-A locus.
The joint hereditary transmission of both sporadic PCT and the heterozygote trait of hemochromatosis can only be explained by coincidence. Although hereditary hemochromatosis is an uncommon disease, some studies indicate that the frequency of the heterozygote state is about 8-10 % (Cartwright 1979, Bothwell 1989).
Several authors have assumed that URO-D is damaged by a direct effect of iron. This theory is based on in vitro experiments, in which a decreased activity of URO-D was demonstrated after incuba-

tion with $Fe^{2+}$ ions. However unphysiological concentrations of iron up to 0,1 M were used in the reported experiments (Kushner 1975, Mukerji 1984, 1986).

### 1.4.11. Associated Factors

The most important factors that are associated with PCT are alcohol,iron and estrogens. The role of iron is discussed in 1.4.11.
Most patients with PCT described in the fifties and sixties were ingesting large quantities of alcohol (Waldenstrom 1957 Ippen 1960) Alcohol induces the synthesis of ALA-S (Shanley 1969, Doss 1978).
In this way the production of porphyrin precursors and uropophyrinogen increases, and an underlying deficiency of urodecarboxylase can become manifest. Only recently it has been shown that a specific cytochrome P450IIE1 is induced by alcohol in rat liver microsomes. There is also evidence that a specific cytochrome, cytochrome P450j, is induced by alcohol in man (Lieber 1988).
In later series the use of oral contraceptives appeared to be an important precipitating factor of PCT in women of fertile age (Fiedler 1981, Zaumseil 1982,1983, Sixel 1985), while postmenopausal hormonal replacement therapy has been described as causal factor in older women (De Salamanca 1982a). Patients with PCT after estrogen therapy for prostatic cancer have also been described. The estrogen Stilbestrol has been shown to induce hepatic ALA-S in males with prostatic cancer (Levere 1966). Intriguing is that PCT can also become manifest in pregnancy and post-partum, indicating that ALA-S is induced as well by endogenous estrogens (Goerz 1983 ,Rajka 1984, and Malina 1988).
Other drugs like rifampicin that induce the mixed function oxygenase system have been associated with the activation of PCT, but drug induced acute hepatic syndrome as in the acute porphyrias does not occur (Millar 1980, Manzione 1988).
The drugs tetracycline, nalidixic acid, furosemide and naproxen can induce a photosensitive skin disease known as pseudoporphyria, without abnormalities in porphyrin metabolism (Kappas 1989, Judd 1986).

### 1.4.12. Associated Disorders

#### 1.4.12.1. Porphyria Cutanea Tarda associated with chronic renal failure and mantainance hemodialysis.

Eleven patients on maintainance hemodialysis have been described with PCT, with elevated levels of uroporphyrin in urine and plasma and increased levels of isocoproporphyrin in feces. These case reports have been reviewed by Goldman (1983). Ten of them had significant increases of plasma, urine and/or fecal porphyrins. Eight other cases have been described more recently (King 1983, Harlan 1983, Seubert 1985, Anderson 1987). Goldman (1983) has also collected 31 patients from the literature with a pseudoporphyria or PCT-like syndrome, who developed typical bullous skin disease with increased skin fragility and milia, but who had normal porphyrins in urine and/or feces. However plasma porphyrins were measured in only 13 patients.

In addition to this group of pseudoporphyria, several patients have been reported, in whom the symptoms could also have been due to nalidixic acid or furosemide (Goldstein 1983).
Although slightly increased plasma levels of uroporphyrin from 10 to 39 nmol/l have been found in about 60% of the patients on maintainance hemodialysis, increased levels of heptacarboxylporphyrin have not been found (Anderson 1987). Although there is some overlap of total plasma porphyrin levels in hemodialysis patients with those found in PCT, in PCT the levels tend to be higher (Poh-Fitzpatrick 1982). The PCT cases on hemodialysis (Goldsman 1983) are also related to the use of iron and/ or alcohol, but the mechanism of the slighly elevated uroporphyrin levels in these patients is not clear. Changes in porphyrin metabolism in chronic renal failure are not well defined.
Decreased renal excretion seems to be an important factor.
In normals uroporphyrin is cleared by the kidneys at a rate of 5 ml/min, while coproporphyrin is cleared at a rate of 200 - 5000 ! ml/min. This difference is not due to protein binding, which is even stronger for coproporphyrin, but to the tubular secretion of coproporphyrin (possibly in part from local synthesis). The amount that reaches the urine by glomerular filtration seems to negligible. On the other hand coproporphyrin is also excreted in the feces, which is an alternative pathway for excretion in renal failure (Day 1981). Although uroporphyrin can be demonstrated in dialysate, during hemodialysis differences in plasma concentration could not be shown pre- and post- dialysis (Poh-Fitzpatrick 1978). In one patient a high permeability membrane appeared to be effective in clearing uroporphyrin (Garcia-Parilla 1980). Charcoal hemoperfusion was not effective in a patient with hemodialysis related porphyria (McColl 1986). Resin hemoperfusion was effective in one well-documented patient (Horak 1985).
Although more complicated to administer than low dose chloroquine therapy desferrioxamine seems a serious option in patients on chronic dialysis (Praga 1987). The use of erythropoeitin, together with repeated small volume plebotomy as described by Riccioni (1987), may also be a suitable approach in these patients. Interestingly, small volume plasmapheresis led to remission of PCT in a patient on chronic intermittent hemodialysis (Disler 1982, Miyauchi 1983).
Other observations of disturbances in porphyrin metabolism in patients on hemodialysis have also been reported. Increased plasma levels of PBG and ALA, and raised erythrocyte zinc protoporphyrin have been reported. Erythrocyte ALA-D activity is decreased in these patients, while ALA-S is increased (Buchet 1987). The serum level of porphobilinogen was increased. PBG-D activity was found to be normal in this study but reported to be decreased in an earlier paper (Andriolo 1980).
The decrease of ALA-D and the increase of erythrocyte zinc protoporphyrin is positively correlated with raised serum aluminium levels, which are frequently seen in patients on chronic hemodialysis due to aluminium contamination of the dialysis fluid and especially to the use of aluminium hydroxyde containing phosphate binders (Anderson 1987, Buchet 1987). The effects of aluminium show a remarkable resemblance to those of lead (see 1.3.1).

Very intriguing is the observation of increased HCB levels in uremic patients versus controls, but in this studies uroporphyrin was not measured so that no conclusions concerning a possible relationship can be made (Rutten 1988).
In conclusion, both increased synthesis and decreased excretion seem to play a role in the accumulation of porphyrins and their precursors in chronic renal failure.

**1.4.12.2. PCT and hepatoma.**

Tio (1957) described a patient with a liver adenoma and a PCT, who was cured after resection of the tumor (Tio 1957). After this report several studies have been published in which an association between PCT, chronic liver disease and hepatocellular carcinoma have been described.
Two separate entities of disease can be discerned. There is a paraneoplastic form, in which the tumor produces large quantities of porphyrins, while the liver tissue produces normal quantities of porphyrins. Only a few patients are well documented (Tio 1957, Thompson 1970, Solis 1982). Difference in porphyrin production of tumor and liver tissue can be evaluated by looking at the fluorescence under Wood's light.
PCT caused by tumor pophyrin overproduction is also probable if PCT disappears after tumor resection (Tio 1957, Combe 1982). It is assumed that the tumor tissue is deficient in URO-D, without evidence of a genetically predisposed deficiency in other tissues but this has not been confirmed by enzyme studies.
The second type is hepatocellular carcinoma associated with chronic liver disease, especially cirrhosis, in PCT. In this type increased porphyrins are seen in the entire liver (Solis 1982, O'Reilly 1988). A well designed prospective study has not been done and would indeed be difficult to perform. It is especially difficult to assess the extra risk of PCT in a population which already has risk factors for HCC, including cirrhosis, hemochromatosis, alcohol and chronic liver disease caused by hepatitis B and C (Schonland 1985).
Early studies of Braun and Bergman (1959) demonstrated hepatocellular carcinoma in 8 of 15 PCT patients, of whom 8 of 10 had cirrhosis. Kordac (1972) followed a series of 342 PCT patients, 65 died in the course of 17 years and 36 of them were autopsied. In 23 (64%) cirrhosis was found and in 17 (47 %), who all had cirrhosis, HCC was present.
Topi (1980) failed to detect HCC in 96 patients with PCT by scintigraphy and alpha-fetoprotein determinations.
Solis (1982) reported HCC in 10 of 138 PCT patients (7.2%), versus 20 out of 358 (5.6%) non-porphyric cirrhotics, which was not significantly different. When he corrected his figures for sex and underlying disease, he found an increased risk in male cirrhotics with PCT, the incidence of HCC in the porphyric group being 24 % versus 7 % in the non-porphyric group. All patients with PCT and HCC were negative for hepatitis B surface antigen. Serum iron levels were normal in this group of PCT patients, but ferritin was not measured. Other studies indicate that iron overload contributes to the risk of HCC (Powell 1985).

Salata (1985) reported HCC in 13 out of 83 patients (16%), all males with cirrhosis. Patients with a HCC had a significantly longer history of PCT than patients without HCC. No differences in HBV markers and alcohol intake were found, although a high prevalence (54%) of past HBV infection was found in both groups. The risk of HCC increased with age.
The cause of the increased of HCC in PCT, if one assumes it is increased from the data presented above, is not certain. Increased genome and chromosome mutations have been reported in PCT (Lukanov 1981), and the damage to the chromosomes can hypothetically be caused by lipid peroxidation under influence of iron and uroporphyrins.
HCC associated with PCT can be complicated by rupture as other large liver tumors (Combe 1982).
Besides HCC and a single adenoma (Tio 1957) a hemangioma has also been described (Iwamura 1983).

**1.4.12.3. PCT and hematological disease**

Six patients with a lymphoma and PCT have been described (Rayhanzadeh 1975, Maughan 1979, Lai 1984). These lymphomas were of different histological types. There were 5 non-Hodgkin lymphomas, three of high grade malignancy, and one Hodgkin's disease. Perhaps an increase of PBG-D that has been described in hematological malignancy provokes the outbreak of the disease in patients with a hereditary predisposition, a deficiency of URO-D. Unfortunately URO-D activity has not been measured in any of these patients.
A patient with multiple myeloma and PCT was described, who had a normal URO-D activity in erythrocytes, but that does not exclude a hepatic URO-D deficiency (Maier 1984).
A remarkable case was reported by Dopfer (1986), who described PCT in a girl with chronic myelogenous leukemia (Philadelphia chromosome positive), who developed PCT after a bone marrow transplantation donated by her sister. Both the patient, her father (who had overt PCT) and her sister had URO-D deficiency of the erythrocytes. Although it is not sure if the erythrocytes of the recipient were her own or her sisters, it has to be assumed that this girl had a genetic predisposition of PCT, since she developed symptoms and must also have a hepatic deficiency of URO-D.
PCT has also been reported in agnogenic myeloid metaplasia, idiopathic myelofibrosis, and thalassemia due to iron overload by multiple transfusions (Fivenson 1984, De Rosa 1988, Adjarov 1984).

**1.4.12.4. PCT and iron overload.**
PCT is associated with hereditary hemochromatosis and secondary iron overload. The association of PCT and HLA-antigens A3 and B7 can be ascribed to the association with hemochromatosis. This subject is discussed in chapter 1.4.11.

**1.4.12.5. PCT and other diseases**
Although the association of PCT and SLE has been reported, the incidence appears to be quite low. Clemmensen (1982) found no case of SLE in 55 patients with PCT. He also reviewed 13 patients with reported association of SLE and PCT. Only five seemed to fulfill the

American Rheumatism Criteria. Four other patients had a discoid lupus erythematodes. Another patient with discoid LE has been reported by Weatherhead (1985). It has been stressed that symptoms and signs like joint pain, leukopenia, thrombocytopenia, and hypergammaglobulinemia might be due to chronic liver disease.
PCT has also been associated with other diseases of autoimmune origin
Cases of rheumatoid artritis and Sjögren's disease, and recently a patient with hypothyroidism, vitiligo, alopecia universalis and high titers of microsomal thyroid and parietal antibodies have been reported (Nyman 1972, Ramasamy 1982, Saban 1991).

**PCT and Sarcoidosis.**
PCT has been reported in a patient with sarcoidosis. She came into remission after treatment with prednisolon and phlebotomy. She developed an exacerbation after the dose of prednisolon was tapered down.
This patient was also using phenytoin and phenobarbital, which can also explain the outbreak of disease (Mann 1982)

**PCT and the acquired immune deficiency syndrome (AIDS)**
Several patients are now reported with both AIDS and PCT. The mechanism by wich this occurs is not clear (Wissel 1987, Conrad 1988).

**PCT and viral hepatitis**
Although the association of porphyria with viral hepatitis was described previously, this was well documented by the first time by Coburn (1985) who reported a patient with PCT and hepatitis A.

**Closing remarks**
The etiological factors which cause the manifestation of PCT in viral infections and autoimmune diseases are not known. It is possible certain lymphokines like TNF that decrease the cytochrome P450 content of the liver play a role, but evidence is still lacking.
Finally, there are reports of diseases associated with PCT that are probably coincidental like Wilson's disease, peptic ulcer and carpal tunnel syndrome ( Chesney 1981, De Salamanca 1982b, Massey 1981).

### 1.4.13. Treatment

The standard treatment of PCT for several years has been phlebotomy (one of the few remaining modern indications) or chloroquine. Ippen described the effect of phlebotomy in 1960. Increased serum iron levels in absence of anemia in some PCT patients inspired him to do so, but his main argument was that excess porphyrins would be used for hemoglobin synthesis after repeated venesections, so in retrospect his discovery was at least partly serendipitous.
Large series of patients has been treated since then (Ippen 1965, 1977; Epstein 1965,1968; Lundvall 1968,1971,1982). A randomized controlled trial has never been performed. Hickmann in a small series recorded remissions in three patients after repeated phleboto-

my while in three control patients the disease persisted (Hickman 67).
Although in the different studies treatment schedules are not exactly the same, good results can be obtained by weekly or biweekly removal of 500 ml of blood, until the hemoglobin leve begins to drop below 7.0 - 7.5 mmol/l. One of the best documented series is the Grossman study (1979),who treated 32 patients by venasection. A mean of 5.4 phlebotomies (s.d. 0.7) of 400 to 500 ml was performed. In all patients clinical remission was achieved, in three biochemical remission was never achieved.
Blistering diminishes usually first within 3 to 6 months, when porphyrin excretion drops below 1200 nmol/24h ( Lundvall 1982). Skin fragility improves usually only later. Hypertrichosis and hyperpigmentation can persist for two years, when biochemical remission already has occurred. Milia may take years to disappear (Muhlbauer 1979), while sclerodermoid plaques may persist (Grossmann 1980). Mean clinical remission was achieved in 6.2 months and biochemical remission in 13.5. (range 7 to 72 months) (Grossman 1979). In most patients a drop in urinary porphyrin excretion can be seen 1 to 2 months after initiating therapy, in some a rapid normalisation in four months is observed, while in others a more gradual improvement occurs. In 10 patients in Grossman's series clinical and biochemical relapse occurred, in 7 patients this could be ascribed to alcohol (5) or estrogens (2). In three the cause of relapse was not clear.
Although total porphyrin excretion in the urine may decrease to normal, slightly elevated amounts of uroporhyrin and heptacarboxyl porphyrin may persist (De Salamanca 1980b, Di Padova 1983). Liver histology in a series of 23 patient with PCT before and one year after phlebotomy, showed a significant decrease of siderosis and fatty degeneration, compared to a control group of 12 which was not treated. In a minority of the treated patients there was progression of the liver disease, but this was not significantly different from the control group (Di Padova 1983). An earlier study of Chlumsky (1973) also mentions the regression of siderosis and fatty changes.
It has to be emphasized that as first step in the treatment, factors such as alcohol, estrogens and iron have to be eliminated. In fact, remissions have been described in several patients after withdrawal of alcohol and some drugs (e.g. isoniazide) without venasection being performed.
Topi (1984) described 14 patients who improved after stopping drinking , but he had also given these patient "liver protecting " drugs as silymarin and folinic acid. Topi collected these patients during 14 years, during wich he saw 320 patients with PCT. This does not necessarily mean that spontaneous remissions are rare , but it can also be explained by the fact that PCT patients do not always stop drinking.
After the initial reports of Colomb (1957 and Teodorescu (1959) , the effect of chloroquine in PCT in five patients was reported by Sweeney in 1962. It was tried because of its effect in lupus erythematosus, where photosensitivity is also a symptom. It soon became clear that after the administration of 625 mg to 4 g of chloroquine, pyrexia occurred together with massive porphyrinuria

resulting in red urine and elevated transaminases. Some patients had headache, nausea, and abdominal pain. Liver cell necrosis, severe inflammation and mitochondrial disruption can be seen in biopsies taken during chloroquine treatment (Grossman 1980, Tamayo 1985). After taking 1.0 to 2.5 g chloroquine orally for 2 to 6 days uroporphyrin excretion reached a peak after six days, dropping to normal within 3 months (Cripps 1962 , Felsher 1966).
Low dose chloroquine phosphate, 125 mg twice weekly proved to be effective and safe. As in high dose therapy, an initial peak excretion of uroporphyrin was seen after 2 to 6 days, accompanied by a rise in serum transaminases, but the rise was much lower than that seen with high dose therapy . Moreover, other high dose chloroquine side effects as pyrexia do not occur (Ashton 1984). However, patients have to be warned to stay out of the sun during the first weeks of therapy. Low dose chloroquine therapy is also safely given to children (Del Batlle 1987), while using higher doses could lead to severe liver cell necrosis, resulting in fibrosis after therapy (Tamayo 1985).
Wennersten (1982) treated 21 patients with a combination of phlebotomy and chloroquine. After one or two blood lettings a one week course of 250 mg chloroquine daily resulted in slight nausea and subfebrile temperature in a few patients.
A randomized controlled trial comparing phlebotomy (31 patients) and low dose chloroquine therapy (30 patients), demonstrated that choroquine normalized porphyrin excretion both quantitatively (below 200 microgram/ 24h) and qualitatively (normal excretion pattern) in 22 patients , versus 8 patients in the phlebotomy group ($p<0.01$) (Cainelli 1983). However ,activity of liver disease judged by the extent of necrosis, inflammation and fibrosis increased in 12 chloroquine treated patients and 7 of the phlebotomy group. Both the chloroquine group and phlebotomy group showed a significant reduction in siderosis and fatty changes.
The mechanism by which chloroquine works has not been completely elucidated. Scholnick (1968) demonstrated the formation of a chloroquine-uroporphyrin complex, its release from lysosomes and mitochondria, and its exretion in the urine. Moreover iron excretion in urine is increased. Besides these effects, sunprotecting properties, strong anti-inflammatory qualities including inhibition of chemotaxis of polymorphonuclear cells, antagonism of histamine and serotonin, inhibition of prostaglandin synthesis and their interference of complement mediated antigen-antibody reaction have to be considered (Peyron 1986).In HCB induced porphyria in the rat chloroquine appears to reverse the HCB-induced increase of ALA-S, and the induction of cytochrome P450 and aminopyrine-N-demethylase (Goerz 1985).
Many other therapies have been described. Desferrioxamine is the most important one. It was tried in PCT already in the early sixties (Holzmann 1963). This iron chelator has proven to be effective in decreasing iron stores in severe hemochromatosis (Anonymous 1984b). Slow subcutaneous infusion of 1.0 to 1.5 g of desferrioxamine during 8 to 10 hours, five nights a week effectively resolved skin symptoms within three months and uroporphyrin excretion within 3 to 12 months (Gibertini 1984). A lower dose of desferrioxamine proved to be less effective then chloroquine in a randomi-

zed controlled trial and less effective than the treatment schedule mentioned above (Marchesi 1984). Although more complicated than the low dose chloroquine therapy desferrioxamine is a serious option in the treatment of patients on chronic dialysis (Praga 1987). The use of erythropoeitin, together with repeated small volume plebotomy as described by Riccioni (1987), may also be a suitable approach in these patients.
Other chelators as sodium calcium ethylene-diamine-tetra-acetic acid (EDTA) and British Anti-Lewisite (BAL) are now obsolete in the treatment of PCT (Peters 1957 and 1960, Woods 1961, Donald 1970, Hunter 1967 and 1970 ).
The use of adenosine mono phosphate was advocated by Gajdos (1961,-1967,1968) on weak theoretical grounds. A depletion of glycine was suggested because of an increased production of delta ALA, leading to a depletion of the purine adenine. However a controlled trial proved its ineffectiveness (Wetterberg 1970).
For a long time alkalization of the urine by oral administration of bicarbonate has been advocated, but only the excretion of coproprphyrin is increased in this way (Bourke 1966, Copemen 1970).
Dhar (1975) described one patient treated as an acute porphyria with hematin, which had no effect. Vitamine E has been tried with success in some patients, but no controlled data are available (Ayres 1978).

### 1.5. Hepatoerythropoietic Porphyria

Hepatoerythropoietic porphyria (HEP)is characterized clinically by severe photosensitivity ,skin fragility ,hypertrichosis and scleroderma-like changes of the sun exposed areas of the skin
Mutilation of face and acra, erythrodontia ,hemolytic anemia, hepatosplenomegaly and cirrhosis of the liver occur less frequently
HEP can not be discriminated from congenital erythropoietic porphyria (CEP) on clinical grounds. CEP is biochemically characterized by massive URO I excretion in the urine and deficiency of the enzyme Uroporhyrinogen-III-cosynthetase (See 1.3.1).
HEP however is characterized by an increased urinary excretion of Hepta III and URO III, and isocoproporhyrin in the feces. Moreover increased levels of protoporphyrin in the feces are found (Gunther 1967, Pinol 1969 , Eriksen 1974) Later a severe decrease of URO-D activity to only 7 to 8 % of normal in erythrocytes of patients with HEP was demonstrated (Elder 1981, Lim 1984). In the heterozygous state, in familial PCT, an activity of about 50% is seen in patients and carriers.
Lazaro (1984) published the first study concerning two generations and demonstrated an URO-D activity of about 50% in the erythrocytes of the parents of the propositus. Others have shown erythrocyte URO-D deficiency in three generations of a propositus with HEP. So HEP is considered to be the homozygous form of PCT (Toback 1987).
De Verneuil showed at molecular level that HEP is a heterogenous group of diseases (See 1.4.5.)
A remarkable patient with HEP has been described by Kushner (1982b). This 51 year old male had a classical clinical picture of CEP , with massive overproduction of Hepta III and URO III, and a

deficiency of URO-D activity at a level of about 50%. The massive porphyrin overproduction was due to the dyserythropoietic anemia type I this patient was suffering from. This anemia is characterized by hemolysis and ineffective erythropoiesis.The red-orange nuclear fluorescence of normoblasts was a particular feature that was not observed in patients with this rare dyserythropoietic anemia without porphyria.
In a patient with HEP charcoal appeared to be the most effective treatment compared with blood transfusions and cholestyramine, to lower plasma and skin porphyrin concentrations (See 1.3.1).

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## 2. FAMILIAL PORPHYRIA CUTANEA TARDA: THE PATTERNS OF PORPHYRINS FORMED FROM PORPHINOBILINOGEN BY HEMOLYSATES

### Familial Porphyria Cutanea Tarda: The Pattern of Porphyrins Formed from Porphobilinogen by Hemolysates

### 2.1. Summary

Porphyria cutanea tarda is thought to result from an inherited deficiency of uroporphyrinogen decarboxylase (EC 4.1.1.37) in some patients. Present methods for determining uroporphyrinogen decarboxylase activity are time consuming, so we examined the pattern of porphyrins formed from porphobilinogen by hemolysates as a possible marker for hereditary porphyria cutanea tarda. After the hemolysates are incubated with porphobilinogen, the porphyrins are converted to their methyl esters and examined by liquid chromatography, with fluorometric detection. The porphyric patients examined, and some of their relatives, showed a characteristic pattern of porphyrin production, with high uroporphyrin/coproporphyrin and (uroporphyrin + heptacarboxylic porphyrins)/coproporphyrin ratios, at least partly ascribable to increased uroporphyrinogen I synthetase (EC 4.3.1.8) activity in patients' hemolysates, and also to a relative deficiency of uroporphyrinogen decarboxylase. Examination of the pattern of porphyrins produced from porphobilinogen by hemolysates is a suitable technique for detecting asymptomatic individuals with porphyria cutanea tarda.

**Additional Keyphrases:** heritable disorders . genetic screening . fluorometry . chromatography, liquid . urine . feces

### 2.2. Introduction

Porphyria cutanea tarda (PCT) is a disease usually manifest in adult life as blistering and increased fragility of areas of the skin exposed to sunlight. Biochemically, PCT is characterized by marked increases in uro- and heptacarboxylic porphyrins in the urine, and some increase in urinary excretion of hexa-, pentacarboxylic- and coproporphyrin (Nacht 1970, Dowdle 1970). Large amounts of isocoproporphyrin are present in the feces (Elder 1975). The cause of the disease is not completely understood. PCT is associated with exposure to alcohol (Waldenström 1957, Watson 1960), estrogens (Becker 1965, Taylor 1975), iron excess (Felsher 1960), and polyhalogenated aromatic hydrocarbons such as hexachlorobenzene (Schmid 1960, Cam 1963), vinylchloride (Doss 1979), and 2,3,7,8-tetrachlorodibenzodioxin (Centen 1979). Liver disease, ranging from chronic hepatitis to cirrhosis or hepatoma, is often present (Tio 1957, Keczkes 1976, Topi 1978). All this suggests that PCT is an acquired disease.

On the other hand, several authors have reported the familial occurrence of PCT (Waldenström 1957, Dehlin 1973, Kushner 1976, Topi 1977, Benedetto 1978, Day 1979). Kushner et al (1976) were able to demonstrate a deficiency of uroporphyrinogen decarboxylase (URO-D; uroporphyrinogen III carboxylase, EC 4.1.1.37) activity in

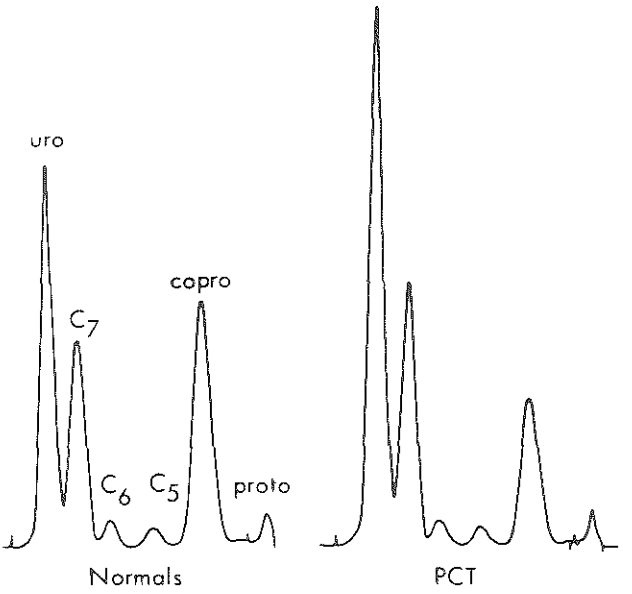


**Fig 2-1 Chromatograms of porphyrin methylesters obtained after the incubation of PBG with erythrocyte lysates.**
Proto,protoporphyrin; other abbreviations as in table 2-1

liver tissue and erythrocytes of patients with PCT, and showed that some of their relatives had a similar deficiency of erythrocyte URO-D, despite a normal porphyrin excretion.This has been confirmed by others (De Verneuil 1978). Patients with a negative family history may have normal URO-D activity in their erythrocytes but some such patients demonstrated low hepatic URO-D activity (Elder 1978).
Apparently there are two forms of PCT, one inherited, the other acquired. This inherited form is not always manifest, clinical or biochemical abnormalities being dependent on exposure to exogenous factors such as estrogens or alcohol.
Methods currently used for measuring URO-D activity involve pentacarboxylic porphyrinogen (De Verneuil 1978, Elder 1978) as substrate or preincubation with uroporphyrinogen synthetase (EC 4.3.1.8) (Kushner 1976, Benedetto 1978). Neither of these reagents is readily available. We therefore decided to examine the possibility that the pattern of porphyrins produced from porphobilinogen (PBG) by erythrocyte hemolysate might be characteristic of PCT and of use in family studies.

## 2.3. Materials and Methods

**Materials.** Kieselgel 60 (70-230 mesh) and Merckosorb SI 100 (20 $\mu$m particle diameter) were obtained form Merck, Darmstadt, F.R.G.

**Table 1. Urinary Porphyrins [a] in PCT Patients and Their Relatives**

| Patient no. | Urinary excretion rate, nmol/24 h | | | | |
|---|---|---|---|---|---|
| | Uro | 7-COOH | 6-COOH | 5-COOH | Copro |
| 1 | 104 | 35 | 8 | 5 | 47 |
| 2 | 20 | 14 | 11 | 0 | 131 |
| 3 | 30 | 18 | 10 | trace | 91 |
| 4 | 18 | 11 | 5 | 3 | 56 |
| 5[b] | 572 | 105 | 10 | 4 | 123 |
| 6[b] | 221 | 36 | 6 | 4 | 120 |
| 7 | 29 | 20 | 26 | 6 | 223 |
| 8 | 14 | 13 | 10 | 3 | 128 |
| 9 | 30 | 17 | 11 | 6 | 125 |
| 10 | 39 | 20 | 5 | 8 | 258 |
| Reference values | <26 | <15 | <15 | <5 | 55–256 |

Table 2-1 a: From left to right :Uroporphyrin,heptacarboxylic-,hexacarboxylic, pentacarboxylic and coproporphyrin. b: Patient with atypical pattern of PCT as judged by urinary porphyrin excretion. Patient no. is same as in fig 2-5.

The methyl esters of protoporphyrin, coproporphyrin I, and uroporphyrin I were obtained from Sigma Chemical Co., St.Louis, MO 63178; their purity was checked by measuring the molar absorptivity in chloroform by spectrophotometry and also by "high-pressure" liquid chromatography. The various porphyrins formed in the assays were quantitated by the latter method as their methyl esters. Because methyl ester standards of hepta-, hexa-, and pentacarboxylic porphyrins were not available, we standardized pentacarboxyl porphyrin against the coproporphyrin methyl ester, and the hepta- and hexaporphyrins against the uroporphyrin methyl ester (Wilson 1978).
It is essential that fluorometer readings be appropriately standardized and calibrated with porphyrin solutions of known concentrations and molar absorptivities (Chisholm 1975). All other reagents used were of analytical grade.
**Methods.** Heparinized venous blood was washed three times with isotonic saline. (NaCl, 9 g/L) at 4 °C; the buffy coat was discarded. The erythrocytes were hemolyzed by freeze-thawing four times. The hemolysate, 0.25 mL, was incubated with 1 mL of Tris-PBG solution immediately afterwards for 60 min, at 37 °C and in the dark. The final reaction mixture contained, per liter, 40 mmol of Tris.HCl (pH 8.0), 240 μmol of PBG, and about 150 mg of protein. The reaction was stopped by freezing in a mixture of acetone and solid $CO_2$. After the hemolysate was lyophilized overnight, we dissolved the

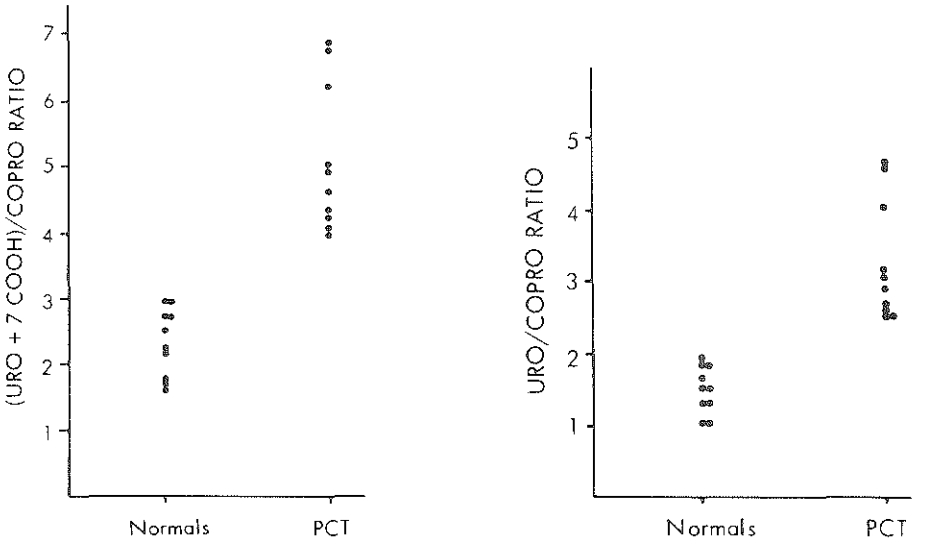


Fig 2.2 Uro- and heptacarboxyl porphyrin/ coproporphyrin ratios (left) and uroporphyrin /coproporphyrin ratios (right) in erythrocyte lysates after incubation with PBG.
The difference between mean values of normals and PCT subjects is statistically significant ($p < 0,01$, Wilcoxon test).

residue in 1 mL of chloroform, added 5 mL of a solution of concd. $H_2SO_4$ in methanol (100 mL/L), and incubated the mixture for 60 min in the dark at 37 °C. We then added 10 mL of water, transferred the solution to a separatory funnel, and added chloroform to give a total volume of 35 ml.The two phases were vigorously mixed and centrifuged, and the water (upper) phase was removed. To neutralize the chloroform phase, we added 8 mL of a 50 g/L solution of $NaHCO_3$ in water, and gently mixed. The procedure was repeated if the pH of the water phase was < 7. The chloroform phase was then washed twice with 10 mL of water, filtered over paper, and evaporated under reduced pressure at room temperature.
The porphyrin methyl esters so produced were then dissolved in chloroform and apllied to a silica gel column (Merckosorb SI 100, 20-$\mu$m particle size; Merck) for high-pressure liquid chromatography. A linear gradient of tetrahydrofuran/heptane, increasing from 1/4 by vol to 1/1 by vol in 15 min, was used as eluent. The porphyrins were measured with a fluorometric detector (L 1000; Perkin-Elmer, Beaconsfield, England); excitation 400 nm, emission 625 nm, band width 8 nm at half peak height.
We methylated 20 mL of lyophilized (for 48 h) urine, as described above. The feces were methylated as described above after overnight lyophilization. To remove other pigments of dietary and biliary origin, it is necessary to pass the extract through a column of

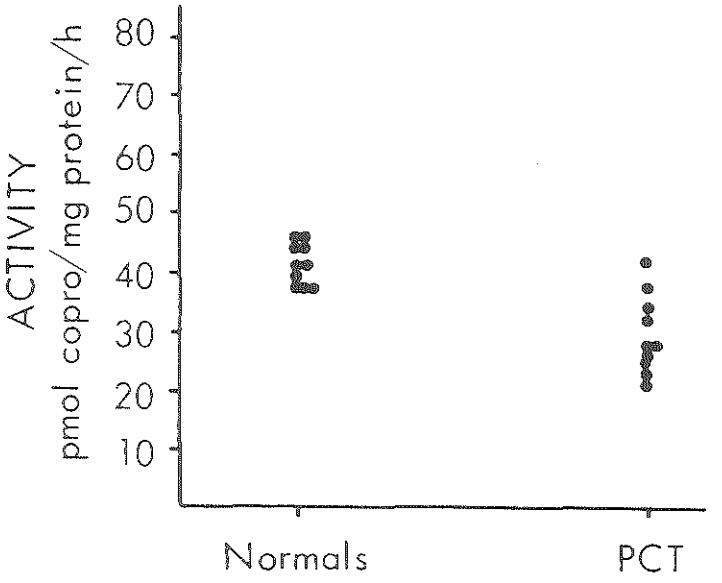


Fig 2-3 Urodecarboxylase activity, as measured by coproporphyrin production, in erythrocytes of normals and PCT subjects.The difference of the meean values is statistically significant (p <0,01, Wilcoxon test).

kieselgel and wash with dichloromethane/ethyl acetate/methanol (99/5/05 by vol) as described previously (Wilson 1978).
**Patients.** We examined members of four generations of the families of two young women with PCT. The symptoms of PCT had become manifest while the patients were taking oral contraceptives. At thetime of our investigation they had no symptoms, having stopped oral contraceptives and having been treated by repeated phelobotomy, but they showed the typical biochemical abnormalities of PCT in their urine. Normal values were obtained for 10 ostensibly healthy subjects who clearly free of PCT.

**2.4. Results**

The urine analysis of the two propositae (patients 5 and 6), who as we have said were clinically in remission, showed the typical pattern of PCT, with a predominant increase in uro- and heptacarboxylporphyrin (Table 2-1). Five other family members had a slight increase of uroporphyrins and heptacarboxyl porphyrins in urine.
After incubation of hemolysates with PBG, a mixture of uro-, heptacarboxyl, hexacarboxyl-, pentacarboxyl-, and coproporphyrin was observed. There was a marked difference between the patterns for the normal controls and the patterns for the PCT patients and some of their relatives (Figure 2-1). For the normal persons the ratio of uroporphyrin plus heptacarboxyl- to coproporphyrin is 2.42 (SD = 0.45) and of uroporphyrin to coproporphyrin 1.52 (SD = 0.32).

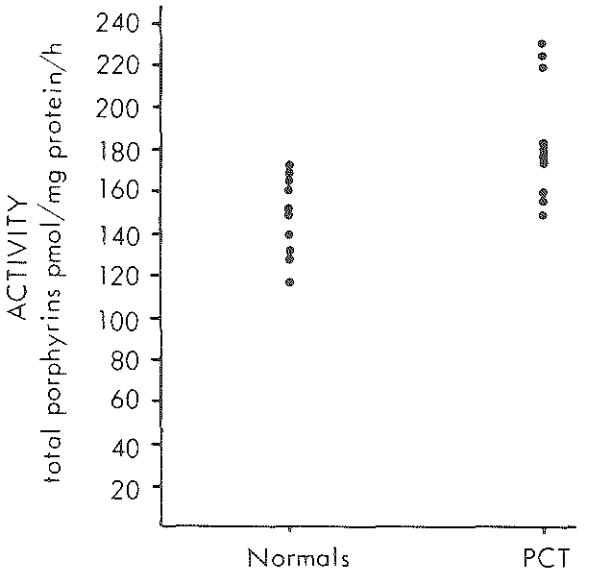


Fig 2-4 Uroporphyrinogen I synthetase activity in erythrocytes (as measured by total porphyrin production) in normals and PCT subjects. The difference of the mean values is statistically significant ($p < 0,01$ Wilcoxon test).

In the PCT group both these ratios were significantly greater (Figure 2-2). In this test system we demonstrated also a significant decrease in mean URO-D activity, but there was some overlap between normals and PCT subjects (Figure 2-3). Mean uroporphyrin I synthetase activity was significantly increased in the PCT group (Figure 2-4), but also in this case there was some overlap.
Each of these parameters alone will not safely discriminate between PCT and normals because of this overlap, but the ratio of porphyrins as described above can be used to discriminate between normals en persons with latent PCT who have minimal or no abnormalities in urine porphyrin excretion, for there was no overlap between these groups.
In this family we could establish the diagnosis of PCT in one of these forms in members of each of three generations (Figure 5).

## 2.5. Discussion

The increased ratio of uroporphyrin to coproporphyrin and of uro- plus heptacarboxyl porphyrins to coproporphyrin formed on incubation of hemolysates from PCT patients with PBG can partly be explained by a decrease in URO-D activity. Moreover, in the PCT group, uroporphyrin I synthetase activity was high as compared with normals, in agreement with Brodie (1977). These facts together can

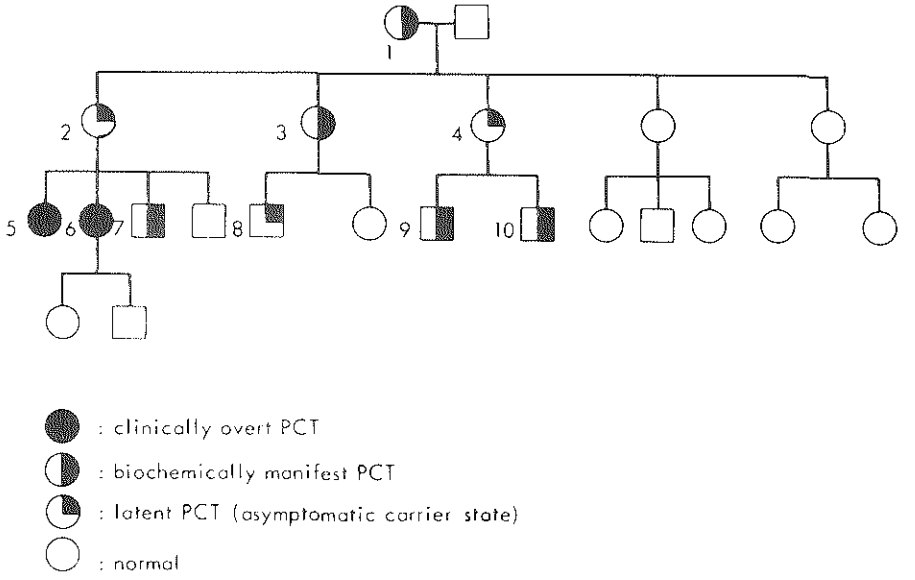


Fig 2-5 Family tree of two PCT patients. Clinically overt patients had skin lesions in addition to abnormal concentrations of urinary porphyrins without complaints. Latent PCT subjects had an abnormal erythrocyte test,with normal urinary porphyrins.

explain the relative increase in uro- and heptacarboxylic porphyrins seen in PCT. An increase in uroporphyrinogen I synthetase activity is also observed in liver tissue in hexachlorobenzene-induced PCT in rats (Von Tieperman 1980).
Our results suggest that the pattern of porphyrins produced from PBG is characteristic for PCT. If so, this is a valuable addition to clinical examination and biochemical analysis of urine. In our family study, eight persons in the PCT groups had abnormal urinary porphyrin excretion rates, while two had only a slight increase in heptacarboxyl porphyrin exretion. In subjects with only slight or no changes in urinary porphyrin exretion rates, this test will provide a diagnosis. This is important because it has consequences for these carriers.
Use of alcohol and of oral contraceptives is contraindicated. Because of the relative simplicity of using PBG as substrate, this test may by substituted for more complex procedures in the investigation of PCT patients and their families.

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## 3. EXOGENOUS FACTORS INFLUENCING PORPHOBILINOGEN DEAMINASE ACTIVITY IN RAT LIVER

### 3.1. Summary

Porphobilinogen deaminase has the next lowest endogenous activity of the enzymes of heme synthesis after delta-aminolevulinic acid synthase, and is a possible second control point in the heme pathway. As little is known about factors influencing hepatic porphobilinogen deaminase activity, we studied the effects of lead and phenobarbital on the activity of this enzyme in livers of male Wistar rats, as well as the effects of fasting. Lead + phenobarbital caused an increase in porphobilinogen deaminase activity, which could not be prevented by hematin . We thus could not demonstrate a negative feed back regulation by heme for hepatic porphobilinogen deaminase.

### 3.2. Introduction

Porphobilinogen deaminase (PBG-D, EC 4.3.1.8, hydroxymethylbilane synthase) catalyzes the conversion of porphobilinogen (PBG) to hydroxymethylbilane, which is an essential step in the synthesis of heme. After the initial enzyme of the heme biosynthetic pathway, delta-aminolevulinic acid synthase (ALA-S, EC 2.3.1.37), PBG-D has the next lowest endogenous activity of all of the eight enzymes in the heme pathway (Kappas et al, 1989), and PBG-D has been suggested to be a possible second control point in the heme pathway (Brodie et al, 1977). PBG-D activity is reduced in patients with the disease, acute intermittent porphyria (Strand 1972, Granick, 1972, Kappas et al, 1989). In healthy individuals and in most biological systems, the formation of delta-aminolevulinic acid (ALA) is the rate limiting step in heme synthesis. ALS-S is subject to feed-back inhibition by heme, and its activity in the liver can be induced by a wide variety of drugs or endogenous products which bind to, or increase the turnover of hepatic cytochromes (Maxwell 1978, Tschudy 1978). In patients with acute intermittent porphyria, during periods of ALA-S induction, PBG-D becomes rate-limiting. The low PBG-D level acts as a metabolic block, leading to accumulation of the porphyrin precursors, PBG and ALA. Increased levels of ALA are thought to related to the neurological symptoms of the acute porphyric attack and thought to inhibit brain Na-K-ATP-ase.
Little is known about factors influencing hepatic PBG-D activity. We therefore decided to study PBG-D activity in rat liver under some of the conditions which affect heme biosynthesis. The following conditions were chosen.
Fasting is known to cause an exacerbation in patients with acute intermittent porphyria, and the inducibility of ALA-S in vivo is increased by fasting and reduced by the administration of glucose (Tschudy 1978).
Lead inhibits ALA dehydratase and the incorporation of iron into heme, and the effects of such partial blocks in heme synthesis on ALA-S activity are greatly enhanced when the lead is given in combination with phenobarbital (Maxwell 1976).

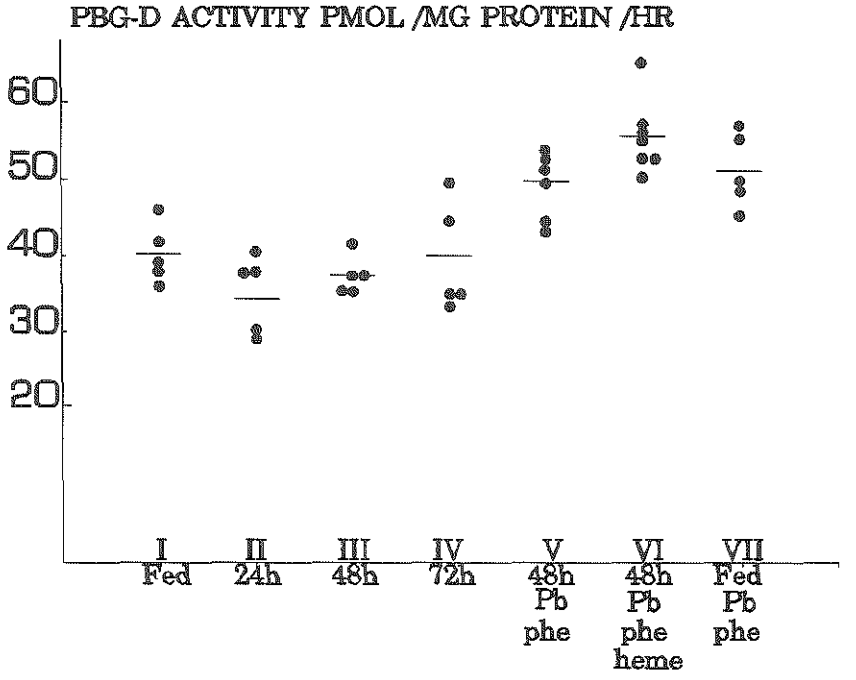


**Fig 3-1 PBG-Deaminase activity in rat liver tissue in pmol /mg protein /hr. For statistics: See table 3-1.**

### 3.3. Materials and Methods

Male Wistar rats, weighing between 200 and 250 g, were obtained from TNO, Zeist, The Netherlands. They were fed standard laboratory chow (Hope Farms, Woerden, The Netherlands) unless otherwise stated. During the fasting experiments, the rats were allowed free access to water. Lead intoxication was induced by the administration of 25 mg lead acetate per kg intravenously together with phenobarbital 50 mg/kg intraperitoneally Hematin was given intraperitoneally simultaneously with the lead acetate in a dosage of 6 mg/kg. The rats were killed by stunning after ether narcosis .The livers were removed immediately and kept at -20°C until assay. At the time of the experiment livers were homogenised in a Potter-Elvehjem homogeniser
PBG-D activity in liver homogenates was measured according to a modification of the method of Strand (1972) by Wilson et al (1986) using 50 mM Tris HCL pH 8.0 buffer in all experiments.
For the determination of PBG-D activity one gram of rat liver was homogenized in 9 ml Tris-Cl buffer (pH 8.0, 50 mM) with a Potter-Elvehjem homogenizer at 4°C. One ml of this homogenate was incuba-

ted with 1.0 ml of Tris-PBG solution for 60 min at 37°C in the dark. The final reaction mixture contained Tris-Cl (50 mmol/l, pH

| | I Fed | II 24h | III 48h | IV 72h | V 48h Pb pheno | VI 48h Pb pheno Heme | VII Fed Pb pheno |
|---|---|---|---|---|---|---|---|
| X | 40,23 | 35,30 | 37,50 | 39,73 | 49,31 | 55,50 | 51,0 |
| SD | 3,82 | 5,30 | 3,47 | 6,90 | 4,44 | 4,64 | 4,61 |

STATISTICAL ANALYSIS : WILCOXON RANK SUM.

I ~ II -IV : n.s.
I ~ V : p = 0,02
I ~ VI: p = 0,005
I ~ VII: p = 0,02
II ~ III-IV : n.s
II ~ V : p = 0,006
II ~ VI: p = 0,005
II ~ VII: p = 0,009

III ~ IV : n.s.
III ~ V : p = 0,02
III ~ VI : p = 0,005
III ~ VII: p = 0,02
IV ~ V : p = 0,04
IV ~ VI : p = 0,005
IV ~ VII: p = 0,03

V ~ VI : p = 0,042
V ~ VII: n.s.
VI ~ VII: n.s.

KRUSKAL WALLIS TEST
H ( 6, N = 38) = 22,61
p = 0,0003

**Table 3.1 mean values, standard deviations and main statistical results.**

8.0) and PBG 0.15 mmol/l, and about 17 mg protein. The enzyme reactions was stopped by freezing the solution in a mixture of acetone and solid $CO_2$, followed by freeze drying overnight. Total porphyrins were measured with a fluorometer (Perkin Elmer Beaconsfield, England) Instrument parameters : excitation wavelength 400nm, emission wavelength 625, band width 20nm. Coproporphyrin I was used as the standard. Activities of PBG-D are expressed as pmol total porphyrin\mg protein\hr. Protein was determined by the method of Lowry (1951).
The statistical significance of differences between groups of experimental animals were evaluated by the Wilcoxon Rank Sum test and the Kruskal Wallis test.

**3.4. Results**
The results are shown in figure 3.1, the statistical data are shown in table 3.1 We could not find a significant change in hepatic PBG-D activity after fasting. The administration of both lead acetate + phenobarbital was associated with a significant increase of PBG-D activity in both in fed and 48 h fasting rats. Fasting had no influence on the increase of PBG -D activity induced by lead and phenobarbital. Simultaneous administration of heme had no effect on the rise of PBG-D , caused by lead and phenobarbital. In contrast PBG-D activty appeared significantly increased in the group treated with hematin.

### 3.5. Discussion

These results indicate that fasting does not influence PBG activity. However lead acetate given in combination with phenobarbital causes an increase in PBG- D activity wich could not be prevented by simultaneous adminstration of hematin - in contrast we observed a significant rise of PBG-D with hematin. These findings do not support the hypothesis that there is a second site for negative feed back regulation by heme biosynthesis of heme in addition to ALA-S. Apart from these experimental results, increased levels of PBG-D activity have been found in porphyria cutanea tarda in liver tissue (Kushner et al 1976, Felsher et al 1982), in neoplastic and regenerating tissue (Epstein et al 1983),and in experimental HCB induced porphyria in the rat (Von Tieperman 1980, De Wainstok 1984 ,1986). In chapter 4 the lack of effect of hematin on on liver PBG-D activity in HCB induced porphyria is shown.
The mechanism underlying the increase in PBG-D activity is not clear. It is possible that the PBG-D activity is regulated by ALA and PBG, as has been suggested from experiments with mutant yeasts, deficient in ALA-S (Labbe-Bois 1980). Another possibility is that the presence of substrate (PBG) stabilises the enzyme, reducing catabolism (Beaumont et al 1986, Siersema et al 1990).
Of interest is the finding that fasting does not change PBG-D activity, in contrast to the well known increase of ALA-S in that condition.
Fasting is associated with exacerbations in patients with acute intermittent porphyria (Tschudy (1978). The deficiency of active PBG-D becomes manifest, when ALA-S increases in the fasting patient up to 10 -20 fold. A lack of increase of PBG-D in response to fasting may contribute to the severity of the accumulation of PBG in this disorder.
Finally, we want to discuss our findings of an increase in PBG-D activity following in-vivo administration of lead. These are at variance with the inhibitory effects of lead on PBG-D found in **vitro**. Piper and Van Lier have shown that the inhibitory effect of lead in-vitro is antagonised by a dialyzable factor which is probably pteroylhexaglutamate. This factor however is only effective to lead concentrations of 1-10 mmol/l (Piper 1977). It is possible that this or other factors, protect PBG-D against inhibition by lead in-vivo.

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## 4. HEXACHLOROBENZENE-INDUCED PORPHYRIA IN THE RAT: THE EFFECTS OF IRON AND OF HEMATIN ON PORPHOBILINOGEN DEAMINASE ACTIVITY AND PORPHYRIN PRODUCTION.

### 4.1. Summary

Experimental porphyria was created in female Wistar rats by the intraperitoneal injection of hexachlorobenzene. The effects of iron and hematin administration on hepatic and urinary porphyrin levels, and hepatic porphobilinogen deaminase activity were studied. 5-aminolevulinic acid synthetase activity is known to be the rate limiting enzyme in heme synthesis, and is regulated through negative feedback repression by heme. Using iron to aggravate hexachlorobenzene-induced porphyria, we found an increase in porphobilinogen deaminase activity in rat liver tissue. Iron administration alone did not lead to a measurable increase in porphobilinogen deaminase, but did cause an increase in urinary uroporphyrin excretion.
Instead of the expected decrease of uroporphyrin excretion in porphyric rats, after the administration of hematin we observed a slight, but not statistically significant increase of uroporphyrin excretion. We therefore could not demonstrate a negative feedback repression by heme of porphobilinogen deaminase activity or of porphyrin production in hexachlorobenzene induced porphyria.

### 4.2. Introduction

Heme synthesis is normally controlled through changes in the activity of the enzyme 5-aminolevulinic acid synthetase (ALA-S). This enzyme is the rate limiting step in heme synthesis under most circumstances and is subject to negative feedback repression by heme at translational levels (Sinclair 1975, Sassa 1970), Tyrrell 1972). Increased activity of this enzyme in the liver has been described in the acute hepatic porphyrias, erythrohepatic protoporphyria and by some, but not all studies of porphyria cutanea tarda (Zail 1968 ,Moore 1972 ,Brodie 1977, Kappas et al 1989), and is responsible for the increased production and excretion of heme precursors seen in these conditions. The hepatic porphyrias are a group of hereditary diseases in which there is a deficiency of one of the enzymes of heme biosynthesis, which results in a relative block in the pathway. During periods of increased heme turnover - such as fasting or following the administration of inducers of microsomal cytochromes - the deficient enzyme becomes the rate limiting step, and levels of its substrate accumulate.
In many patients, the levels of the porphyrin precursors 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) also accumulate, because the enzyme porphobilinogen deaminase (PBG-D) has the lowest capacity of the remaining enzymes of heme synthesis (Kappas 1989). Raised levels of ALA and PBG are, however, usually not found in patients with porphyria cutanea tarda, a disorder resulting from decreased activity of uroporphyrinogen decarboxylase (URO-D); (Kushner 1976, De Verneuil 1978/b, Elder 1978). In these patients, but not in the other forms of porphyria, the activity of erythrocyte PBG-D has been found to be increased (Brodie 1977), Alleman 1982, Siersema 1990). Hexachlorobenzene (HCB) administration causes a

reduction in URO-D activity and a porphyria cutanea tarda syndrome (Von Tiepermann 1980 , De Wainstok 1986).
HCB-induced porphyria is exacerbated by iron administration (Taljaard 1972), a factor which has been implicated in the provocation of porphyria cutanea tarda (PCT) in humans. HCB-porphyria thus seems to be a good animal model of PCT (Von Tiepermann 1980). We decided to study the effects of iron and of HCB administration on the porphyrin levels in the liver and urine of rats, and on the activity of hepatic PBG-D.
Hematin administration causes a marked reduction in porphyrin and porphyrin precursor excretion in patients with acute porphyric attacks. Analogously we studied the effects of hematin administration on porphyrin levels in HCB-induced porphyric rats, to see whether hematin could influence porphyrin production in this type of porphyria.

**Table 4.1**

TOTAL DELTA-ALA PRODUCTION IN URINE IN NMOL / MMOL CREATININE IN RATS TREATED WITH HEMATIN ( N = 5 , GROUP I ) AND TREATED WITH DEXTROSE ( N = 5 ) AFTER ADMINISTRATION OF ALLYLISOPROPAMIDE AT DAY 0

| | heme | $\bar{x}$ | s.d. | | dextrose | $\bar{x}$ | s.d. |
|---|---|---|---|---|---|---|---|
| I | day 1 | 8549 | 2088 | II | day 1 | 23279 | 8549 |
| Ia | day 5 | 13239 | 2922 | IIa | day 5 | 44593 | 13239 |
| Ib | day 13 | 15094 | 2907 | IIb | day 13 | 44763 | 20565 |

statistics: wilcoxon rank sum
I ~ II : p = 0,009
Ia ~ IIa: p = 0,009
Ib ~ IIb: p = 0,009

wilcoxon signed rank
I ~ Ia : p = 0,04 II ~ IIa: 0,03
I ~ Ib : p = 0,04 II ~ IIb: 0,04
Ia ~ Ib : n.s. IIa ~ IIb: n.s.

## 4.3. Materials and Methods

Animal procedures: Female Wis-tar rats with an initial weight of about 75 g were obtained from T.N.O., Zeist. Hexachlorobenzene was suspended in olive oil (75 mg HCB/ml) and the rats received 37.5 mg

of HCB intraperitoneally on day 1, and 75 mg on day 14. One group received 25 mg iron dextran (Imferon) s.c. at the same time. Another group received 25 mg iron dextran only. All animals were fed standard laboratory chow and were given water ad libitum. The animals were places in metabolic cages and urine was collected over a 24 hour period immediately before sacrifice, which was performed on day 45. The liver was removed immediately and placed on melting ice. In a second experiment all the rats were given both HCB and iron as described above. In one group, we implanted hematin pellets (50 mg) under the abdominal skin on day 45. The rats were sacrificed on day 55. Again urine samples were collected before implantation of the hematin and just before sacrifice. HCB was a synthetical grade and obtained from Merck,Darmstadt, Germany. Hematin from Sigma was pelleted by Dr. P.J.H. Eggels of the University Hospital Pharmacy. In preliminary experiments these pellets, implanted subcutaneously, were shown to suppress the stimulatory effects of allyl isopropylacetamide on ALA synthesis by the rat, for a period of at least 13 days (Table 4.1).

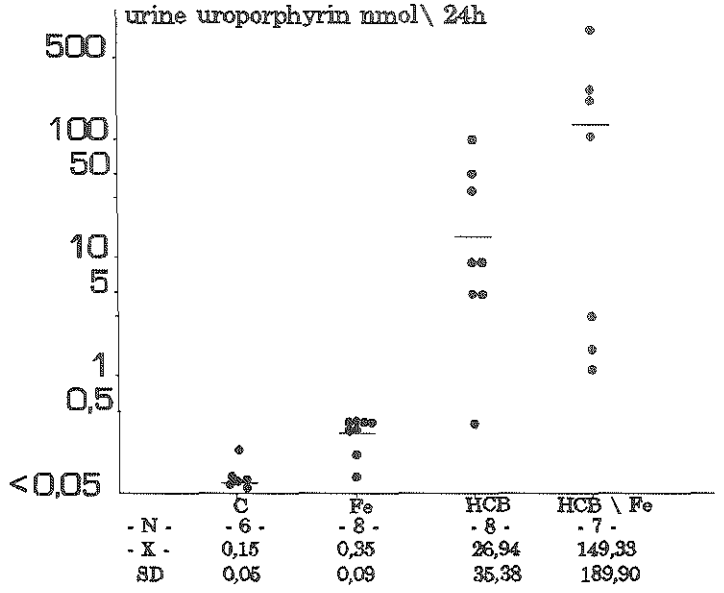


**Fig 4.2 Uroporphyrin in urine (nmol / 24hr).**

**Statistics, Wilcoxon rank sum test:**
**C ~ FE :p=0,005 C ~HCB :p=0,002 C ~ HCB+ FE :p=0,003**
**FE ~ HCB:p=0,004 FE ~ HCB+ FE :p=0,002 HCB ~HCB+FE: n.s**
**Kruskal Wallis test: H (3, N=29) = 22,00 p=0,0002.**

Enzyme activities: For the determination of PBG-D activity one gram of rat liver was homogenized in 9 ml Tris-Cl buffer (pH 8.0, 50 mM) with a Potter-Elvehjem homogenizer at 4°C. One ml of this homogena-

te was incubated with 1.0 ml of Tris-PBG solution for 60 min at 37°C in the dark. The final reaction mixture contained Tris-Cl (50 mmol/l, pH 8.0) and PBG 0.15 mmol/l, and about 17 mg protein. The enzyme reactions was stopped by freezing the solution in a mixture of acetone and solid $CO_2$, followed by freeze drying overnight. Quantitation of the porphyrins was by HPLC as described below.
URO-D activity was calculated from the amount of copro formed during the incubation of liver homogenates with PBG.
PBG-D was not measured separately but was calculated from this same incubation experiment by summation of the total amount of porphyrins formed. The endogenous porphyrin content of the liver homogenate was determined in samples incubated without the substrate PBG.

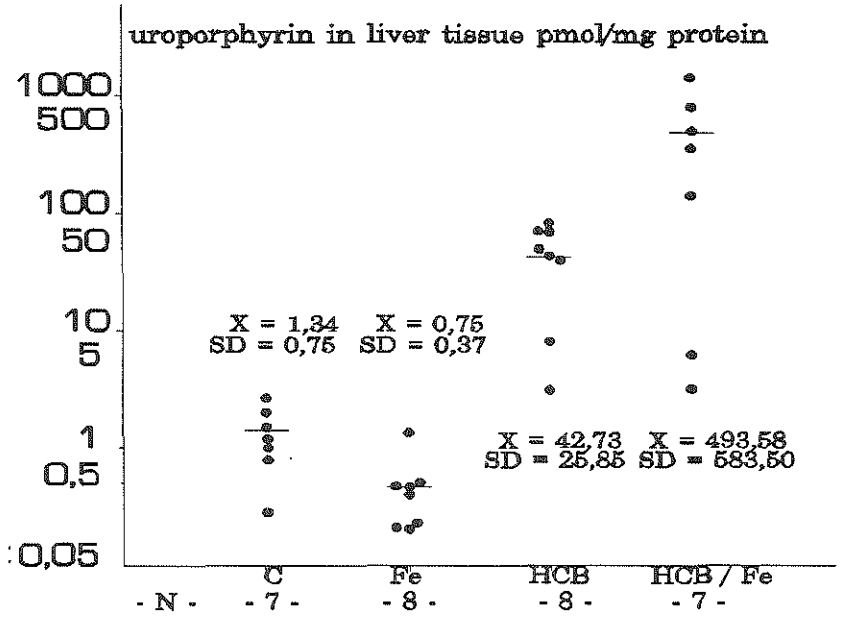


**Fig 4.3 Uroporphyrin in liver tissue pmol /mg protein.**

**Statistics, Wilcoxon rank sum test:**
**C ~ FE :p=0,03 C ~ HCB :p=0,002 C ~ HCB+FE :p=0,002**
**FE ~ HCB :p=0,001 FE ~ HCB+FE :p=0,002 HCB ~ HCB+Fe :n.s**
**Kruskal Wallis test: H (3, n=30)= 23,77 p=0,0001**

Chromatographic (HPLC) separation and quantitation of porphyrins: The freeze dried material (either liver homogenates or urine) was methylated with sulfuric acid 10% in methanol and extracted with chloroform as described before (Wilson (1978)). HPLC was performed

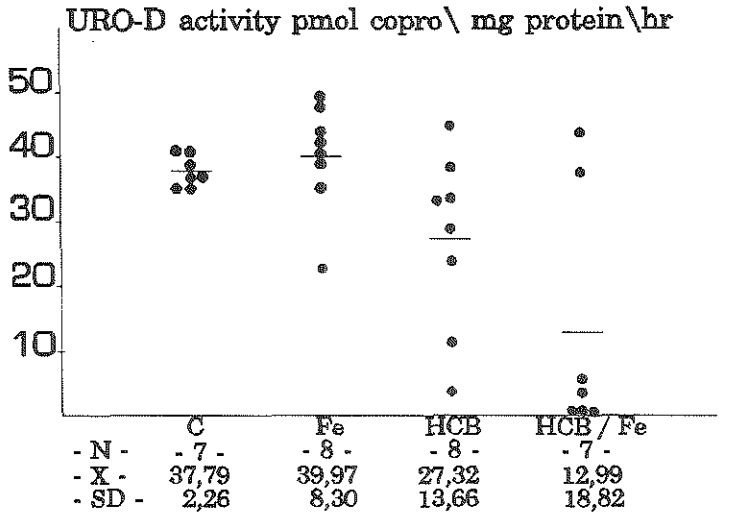


Fig 4.4 URO-D activity in liver tissue pmol copro/mg protein/hr.

**Statistics: Wilcoxon rank sum test.**
**C ~ FE :n.s C ~ HCB :0,046 C ~ HCB + FE :n.s.(p=0,08)**
**FE ~HCB:p=0,025 FE ~ HCB+FE :p=0,031 HCB ~ HCB+FE: n.s.**
**Kruskal Wallis test: H (3, N=30)=10,17 p=0,017**

on a silicic acid column. This material Merckosorb S.I.100 (20 um particle diameter) was obtained from Merck, Darmstadt, Germany. Detection of porphyrins was done with a fluorometer (Perkin Elmer, L2000; Perkin Elmer, Beaconsfield, England). Instrument parameters were as follows: excitation wavelength 400 nm, emission wavelength 625 nm, band width 8 nm at half peak height. As reference material the methyl esters of protoporphyrin, coproporphyrin I and uroporphyrin I were used (obtained from Sigma Chemical Co.,St. Louis, MO, USA). Their purity was checked by HPLC.Because methylester standards of hepta-, hexa- and pentacarboxyl porphyrin were not available, pentacarboxyl porphyrins were compared against the coproporphyrin ester and the hepta- and hexacarboxyl porphyrins against uroporphyrin methylester.
Statistical methods: Groups were compared by means of the Wilcoxon rank sum test. A probability of less than 5% was regarded as significant.

**4.4. Results**

Effects of HCB and iron: Porphyria developed about 40 days after the first intraperitoneal injection of HCB in both the HCB as well as the HCB plus iron group. The difference in urinary uroporphyrin excretion between the HCB and HCB plus iron group compared to the control group was significant (Fig.4.2). The increased uroporphyrin excretion as the result of HCB plus iron seemed more pronounced than that of HCB alone, but the difference of these two groups was not significant due to an interindividual variation within the groups. The uroporphyrin concentration in the liver was also significantly increased in the HCB and HCB plus iron groups compared to the other groups (Fig 4.3).

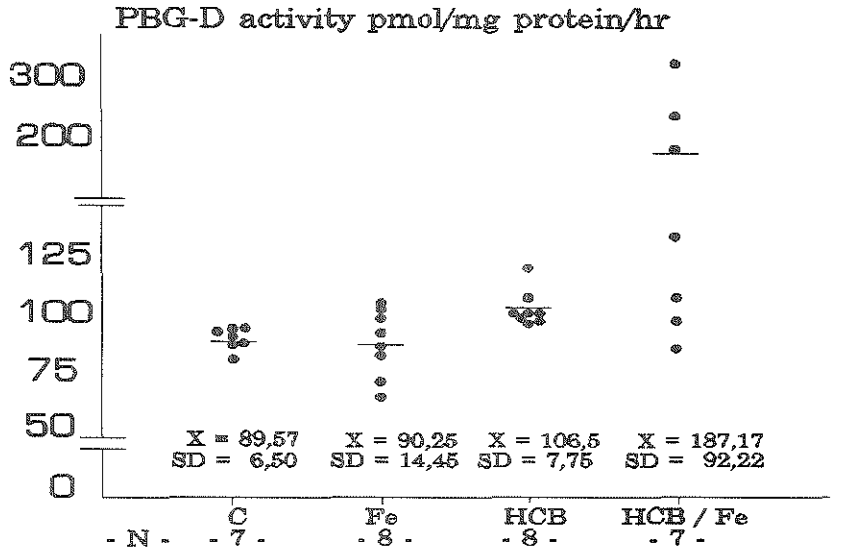


**Fig 4.5 PBG-D activity in liver tissue pmol\porphyrin\mg protein hr.**

**Statistics: Wilcoxon rank sum**
**C ˜FE :n.s C ˜HCB :0,002 C ˜ HCB+FE :p=0,007**
**FE ˜HCB:p=0,03 FE˜HCB+FE :p=0,02 HCB˜HCB+FE :n.s.**
**Kruskal Wallis test: H (3, n=30): 14,727 p=0,0025**

Heptacarboxyl porphyrin excretion in urine was significantly increased in the HCB and HCB plus iron groups compared to the control group (Figures not shown).

URO-D activity was significantly **decreased** in the HCB group (Fig 4.4). In the HCB plus iron group the level was significantly decreased compared to the iron group, but just not significant compared with controls.
PBG-D activity was significantly **increased** in the HCB and HCB + Fe group, compared with the controls and iron group (4.5)
Remarkably iron administration by itself resulted in an increase of uroporphyrin excretion, while significantly lower uroporphyrin concentrations in liver tissue have been observed, and no change in PBG-D activity could be measured.
Effects of hematin: PBG-D activity and URO-D activity in the liver were not significantly different between the treated group and the rats that did not receive hematin. In a group of HCB plus iron induced porphyric rats, extra hematin application seemed to increase uroporphyrin excretion in urine but the difference was just not statistically significant (p=0.0511 ,Fig 4.6)

**4.5.Discussion**

We found an increase in PBG-D activity in HCB-induced porphyria, in addition to the well known decrease in URO-D activity. These

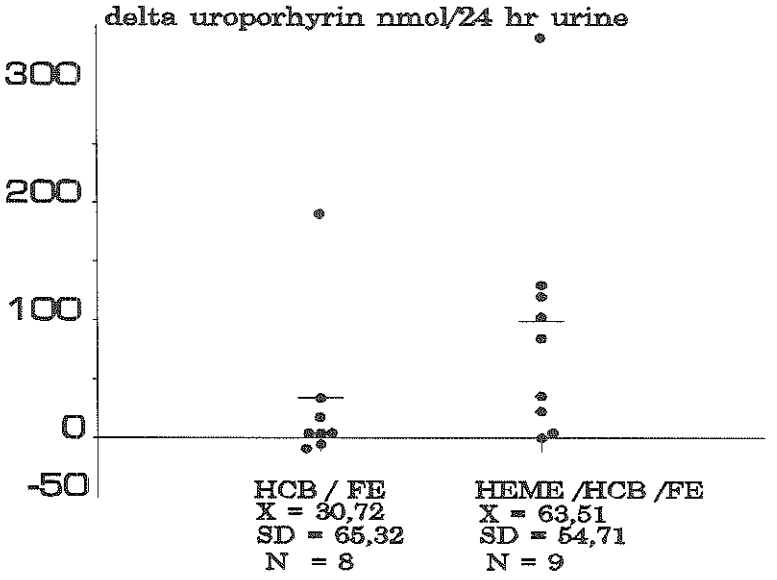


**Fig 4.6 Difference in excretion of uroporhyrin (Delta-URO) in urine samples taken one day before giving hematin pellets and and 11 days afterwards. Statistics: Wilcoxon rank sum : n.s (p=0,051).**

results confirm the finding of Von Tiepermann (1980) and de Wainstok (1984, 1986). Hematin, which was given as subcutaneously implanted pellets to produce a prolonged suppressor effect on ALA synthetase, did not reverse the PBG-D increase, but seemed to increase porphyria as reflected in the increased uroporphyrin excretion in the urine, but the difference was just not significant (p=0,051). Thus, in this porphyria model we did not show a negative feedback inhibition of heme on PBG-D activity.
Iron administration has been shown to aggravates the porphyric effects of hexachlorobenzene (Taljaard 1972a). In this study the urinary excretion rates of uro- and heptacarboxyl porphyrin were not statistically different in the HCB and HCB plus iron groups due to interindividual variation. The activity of URO-D and PBG-D activity in the liver did not differ significantly between these groups.
Iron deficiency reduces the porphyric effects of TCDD and of HCB (Sweeney 1979, Alleman 1985).Iron therefore seems to be a necessary factor enabling polyhalogenated compounds to exert their toxic effect on the enzyme URO-D. The increase of PBG-D could be secondary to an increased production of ALA and PBG as the enzyme PBG-D has been shown to be more stable in the presence of its substrate.
Apart from these relations between iron and HCB, we have also found that lipid peroxidation, mediated by iron, could play a role by increasing cytochrome P450 turnover and heme turnover (De Matteis 1978, Alleman 1985).
The increased porphyrin excretion in rats treated with iron only is intriguing. This suggests a stimulatory influence of iron on the PBG-D activity in vivo, in a direct or indirect way, but we could not demonstrate this by PBG-D measurements in these experiment, in contrast with Kushner, who has found that iron increases PBG-D activity in **vitro**. In his experiments however high concentrations of free iron and ferritin were used (Kushner (1972). Additionally, we found significantly lower uroporphyrin concentrations in liver tissue in the iron group.
These findings could be explained by increased oxidation of uroporphyrinogen to uroporphyrin under the influence of iron, which is known to catalyse oxidative processes (Austin 1982). Recently Mukerji (1990) showed that oxidation of uroporphyrinogen occurs in vitro under influence of ferrous iron, which could be blocked by the iron chelator 1,10 phenylanthroline, although this study can also be criticized, because non-physiological concentrations of iron were used. Despite this criticism, increased oxidation of uroporphyrinogen can be important factor in the pathogenesis of HCB induced porphyria.

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## 5. THE INVOLVEMENT OF IRON AND LIPID PEROXIDATION IN THE PATHOGENESIS OF HCB INDUCED PORPHYRIA.

### 5.1. Abstract

Hexachlorobenzene (HCB) induces a porphyria characterized by a diminished activity of the enzyme uroporphyrinogen decarboxylase (URO-D), presumably due to inactivation by reactive metabolites of HCB.
We studied the effect of iron on HCB porphyria in female rats, to determine whether the iron dependent process of lipid peroxidation was involved in the pathogenesis of porphyria. We showed that malondialdehyde formation is increased in rat liver tissue of porphyric rats and that high molecular weight proteins due to cross-linking are formed. We also showed that the induction of porphyria by HCB is dependent on the presence of iron. Our findings suggest that lipid peroxidation is involved in the toxicity of HCB and that the aggravating effects of iron in HCB are mediated by lipid peroxidation.

### 5.2.Introduction

It is well known that hexachlorobenzene (HCB) is a potent inducer of porphyria in man as well as in rats (Courtney 1979). This type of porphyria is similar to the disease porphyria cutanea tarda and is characterized by photosensitivity and an increased urinary excretion of uro- and heptacarboxyl porphyrins (Cam 1963, Cripps 1980).In porphyria cutanea tarda a decreased activity of the enzyme uroporphyrinogen decarboxylase (URO-D) has been observed; in the inherited form both in erythrocytes and in liver tissue, in the acquired form only in liver tissue (De Verneuil 1978).
In HCB-induced porphyria in the rat this decrease in activity of URO-D also has been found (Taljaard 1971, Von Tiepermann 1980). The exact mechanism by which HCB exerts its toxicity is not known. Recently this subject was extensively reviewed by Debets (1979, 1980). It is thought that electrophilic products appearing during the metabolism of HCB react with the catalytic site of URO-D, this leading to the inactivation of the enzyme (Debets (1979, Debets (1980). This is supported by the finding of Elder that immunoreactive URO-D is unchanged in HCB induced porphyria (Elder 1982).
Administration of iron together with HCB gives an enhancement of the toxicity of HCB (Taljaard 1972/ab). This is further substantiated by the finding that 2,3,7,8-tetrachlorodibenzodioxine (TCDD), another polyhaloginated aromatic compound (PHA) is not toxic in iron deficient mice (Sweeney 1979, Jones 1981). Controls with a normal iron status develop porphyria. Furthermore Maines found evidence for the involvement of endogenous iron in lipid peroxidative destruction of heme by allylisopropylacetamide, which induces acute porphyria (Maines 1980). Lipid peroxidation is a process in which the unsaturated fatty acids, mainly localized in the phospholipids of the membranes are peroxidized (Mead 1976). In this process the fatty acids are broken down to aldehydes and carbonyl compounds, resulting in membrane damage. During this process cross-linking of membrane proteins also occurs. This leads to the forma-

Table 1. Mean uroporphyrin content of the liver measured by HPLC

| Group | nmol/g net weight $\bar{X}$ | S.E.M. | * | † | ‡ |
|---|---|---|---|---|---|
| I Normal chow | 0.03 | 0.02 | | | |
| II Iron deficient | 0.00 | 0.00 | ns | — | |
| III Iron deficient + HCB | 0.00 | 0.00 | ns | — | |
| IV Iron excess | 0.07 | 0.01 | ns | 0.005 | |
| V Iron excess + HCB | 368.14 | 55.54 | 0.005 | ns | |
| VI Normal chow + HCB | 17.93 | 13.50 | ns | | 0.05 |

* P compared to I.
† Compared to VI.
‡ V Compared to VI.

**Table 5-1**

tion of high molecular weight proteins (HMW proteins). Iron catalyzes this process of lipid peroxidation according to the Haber Weiss reaction and is therefore of great importance (Freeman 1982). It is also claimed that iron performs its catalysation of lipid peroxidation through perferryl iron (Svingen 1979).
The role of iron in the process of lipid peroxidation has been the subject of an excellent review by Aust and Svingen (Aust 1982). Considering these findings in relation to iron we decided to investigate whether lipid peroxidation is involved in the development of HCB porphyria.

## 5.3. Materials and Methods.

### Animal Procedures

Female Wistars rats with an initial weight of about 75 g were obtained from TNO (Zeist). Hexachlorobenzene was suspended in olive oil (100 mg HCB/ml). Six groups of rats under various conditions were studied. Three groups of rats (groups III, V, VI) received 100 mg HCB intraperitoneally on day 8. One of these groups (III) was fed iron deficient food from the first day during the whole experiment (Hope Farms, Alphen a/d Rijn, Holland) and were bled three times by cardiac puncture. One ml was taken on day 1, 2 and 3. Another group (V) received 25 mg iron dextrans s.c. on day 1 and received normal food. The third group received no special treatment. Three controle groupes, normal chow (group I), iron deficient group (group II) and iron overload (groups IV) received no HCB. THe rats were sacrified on day 60.

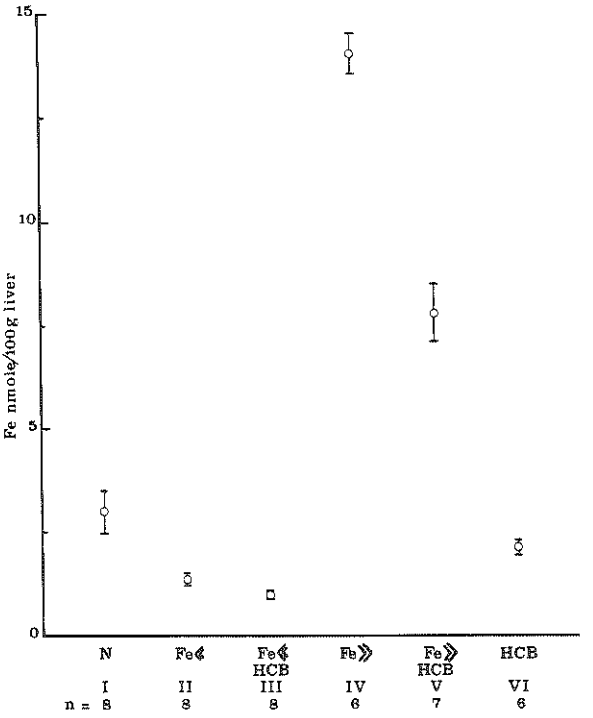


Fig.5.1 Liver iron content of the various groups; as tabulated in table I. Statistics (student-t-test)

I ~ II : p<0.009 I ~ V : p<0.0005 VI ~ V : p<0.0005
I ~III : p<0.003 I ~ VI : n.s. V ~VI : p<0.0005
I ~ IV : p<0.0005

**Chromatographic Quantification and Separation of Porphyrins in Rat Liver Tissue.**

For the determination of porphyrin concentration 1 g of rat liver was homogenized in 9 ml NaCl 154 mM/l with a Potter-Elvehjem homogenizer at 4°. The homogenate was freeze dried. The material was methylated with sulphuric acied methanol and extracted with chloroform as described before (Wilson 1978, Alleman 1982). HPLC was performed on a siliconic acid column. Merkosorb S.I. 100 (20 $\mu$m particle diameter) was obtained from Merck (Darmstadt, Germany). Detection of porphyrins was done with a fluorometer (Perkin Elmer, L200-0, Beaconsfield, U.K.). Instrument parameters were as follows: excitation wavelength 400 nm emission height. As reference material the methylesters of protoporphyrin, coproporphyrin I and uroporphyrin I were obtained from Sigma Chemical Co. (St.Louis, MO, U.S.A.). Their purity was checked by measuring the molar absorptivity in chloroform and also by high pressure liquid chromatography. Because methyl ester standards of hepta-, hexa- and pentacarboxyl porphyrins were not available to us, we standardized

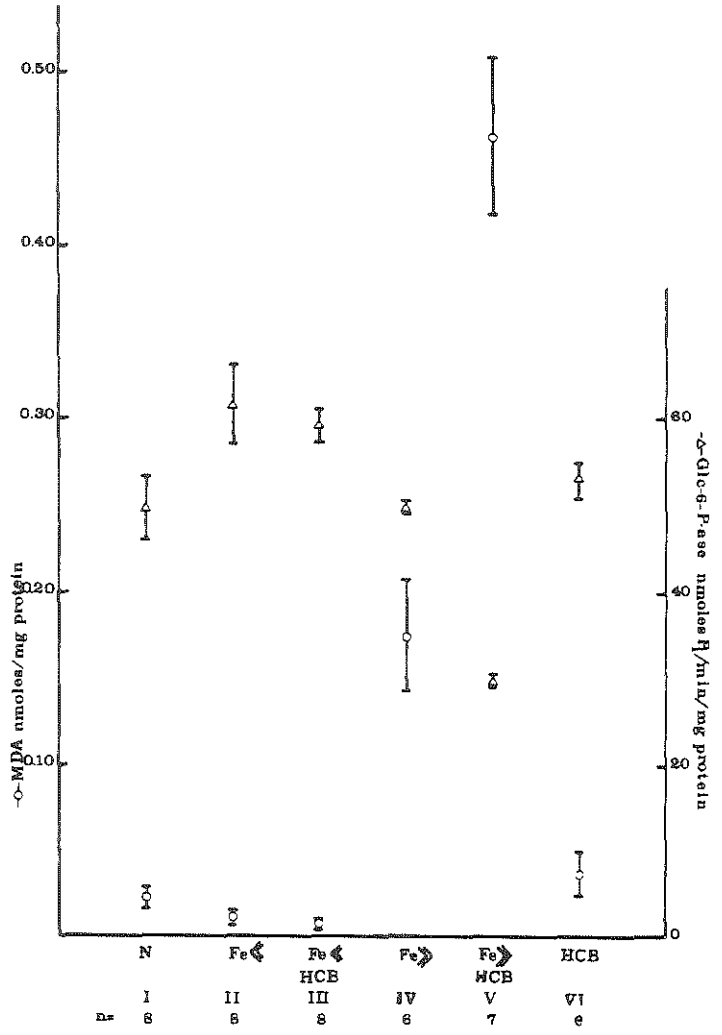


Fig.5.2 Malondialdehyde formation and glucose-6-phosphatase activity in liver homogenates of the various groups; as tabulated in table I.

statistics (student-t-test)

M.D.A. : I ~ II : $p<0.05$ IV ~ V : $p<0.002$
I ~ III: $p<0.0005$ V ~ VI : $p<0.0005$
I ~ IV : $p<0.005$
I ~ V : $p<0.0005$
I ~ VI : N.S.

G-6-P-ase : I ~ II : N.S. IV ~ V : $p<0.0005$
I ~III : $p<0.05$ V ~VI : $p<0.0005$
I ~ IV : p<N.S. I ~ VI : p N.S.

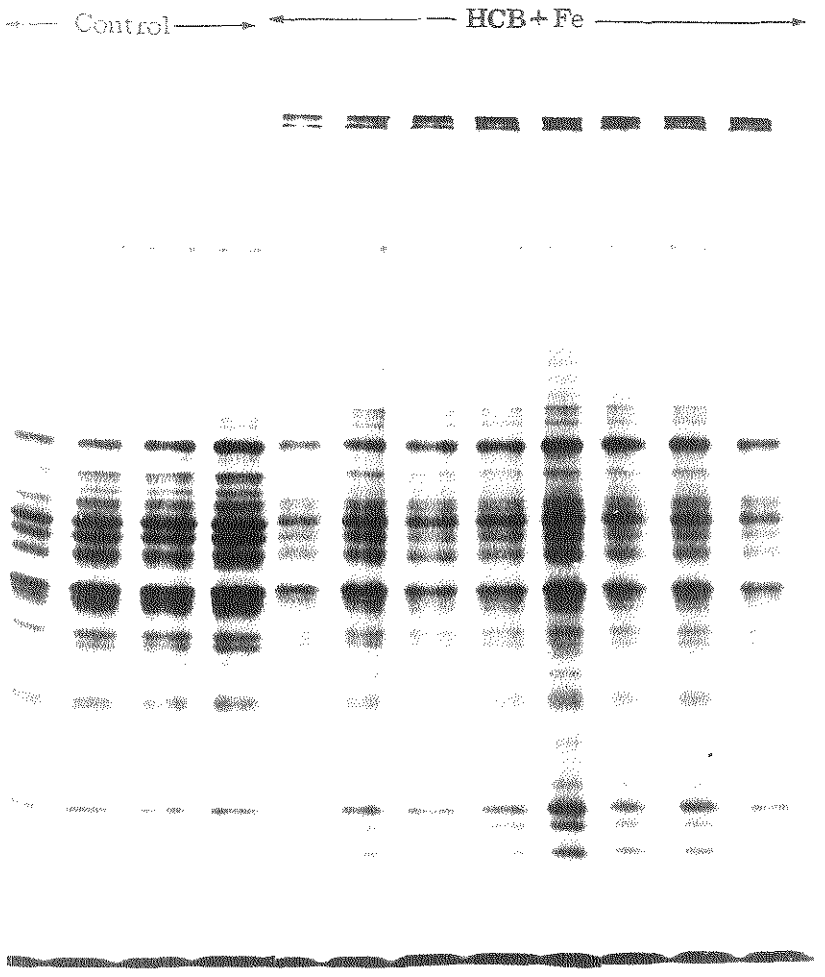


Fig.5.3 The SDS polyacrylamide gel electrophoresis of the liver homogenates from control group (I) and the HCB plus iron group (V). The upper band in group V represents the high molecular weight proteins.

pentacarboxylporphyrin against the coproporphyrin ester and the hepta- and hexacarboxyl porphyrins against uroporphyrin methyl ester.

**Determination of Liver Iron.**
This method has been described previously (Van Eijk 1974).

**Lipid Peroxidation**
Malondialdehyde (MDA) measurement. A 20% liver homogenate was prepared in 0.15 M KCl-5 mM Tris maleate, pH 7.4.
The amount of malondialdehyde was determined as described previously [Lowry (1951).
In short: to 0.5 ml liver homogenate 0.3 ml 20% TCA, 0.6 ml TBA (0.05 M), 0.1 ml butylated hydroxy-toluene (0.2%) and 0.5 ml homogenate buffer was added. After centrifugation the supernatant was heated at 100° for 8 min, cooled and the absorbance was read at 535 nM. The MDA content was expressed as nmol/mg protein using the extinction coefficient of 156.000. Protein was determined according to Lowry (1951).
High molecular weight proteins (HMWP). High molecular weight proteins were detected with S.D.S. polyacrylamide gel electro- phoresis as described previously (Koster 1980).
Glucose-6-phosphatase (G-6-P-ase). Glucose-6-phosphatase activity was measured according to Harper (1965).

**5.4. Results**

Figure 1 shows the various iron contents of the livers obtained from iron deficient, iron overloaded and normal rats. It can be seen that the iron overloaded rats have a significantly higher content in the liver. In agreement with Schäfer (1982) rats overloaded with iron and also given HCB had a significantly lower iron content in the liver compared to the iron overload control. Schäfer suggests that this is due to reduced absorption of iron from the small intestine following HCB administration. In contrast to iron deficient rats, the iron overloaded rats developed HCB porphyria. The liver porphyrin content was significantly increased in the HCB-iron overloaded group (V) compared to the HCB control (VI) ($P \leq 0.05$) (Table 5-1).
To investigate if lipid peroxidation had occurred in the liver, the MDA content and the glucose-6-phosphatase activity was measured. MDA is the end product of the breakdown of peroxidized polyunsaturated fatty acids. During the process of lipid peroxidation the enzyme G-6-P-ase is inactivated. We found that iron overloading resulted in an increase of MDA formation (group IV), but the microsomal enzyme G-6-P-ase remained in the control range (Fig. 2). For the HCB-iron overloaded rats (group V) a tremendous increase in the MDA level occurred with a concomitant decrease in G-6-P-ase activity. The rise in MDA and the fall of G-6-P-ase activity are considered to be the result of lipid peroxidation.
Lipid peroxidation of rat liver microsomes leads not only to the MDA formation, but also to cross-linking of membrane proteins. Figures 5-3 and 5-4 show the SDS polyacrylamide electrophoresis of

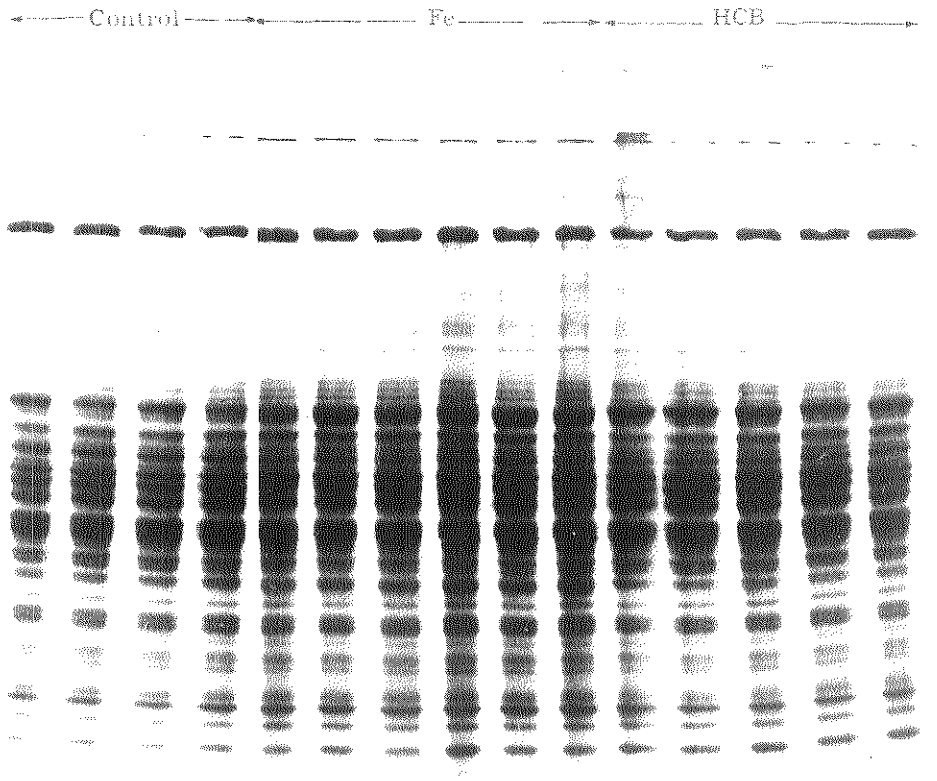


**Fig.5.4 The SDS polyacrylamide gel electrophoresis of the liver homogenates from control group (I), the iron overloaded group (IV) and the HCB group (VI).**

the liver homogenates of groups, I, IV, V and VI. All the animals of group V (HCB plus iron) showed high molecular weight protein formation, but the controle groups did not. It should be mentioned that in group VI (HCB control) one rat showed formation of cross-linking of proteins while two other rats showed some formation of HMWP. It was not found in the other rat livers of this group.

## 5.5. Discussion

It has been known for several years that iron aggravates experimental HCB-induced porphyria and it has also been described that the activity of URO-D is inhibited in the presence of iron in HCB por-

phyria. Our experiments show that iron is a neccessary factor for the porphyrinogenic properties of HCB. This substantiates the results of Sweeney and Jones, who have found that iron deficient mice, who recieved TCDD, another polyhalogenated compound, did not develop porphyria, in contrast to the mice on a normal diet (Sweeney 1979, Jones 1981).
Although it was known that iron plays an important role to both the induction of HCB porphyria and the process of lipid peroxidation, these two phenomena have not been associated. In the process of lipid peroxidation MDA is formed as the end product of the breakdown of peroxidized polyunsaturated fatty acids, located in the phospholipid fraction of biomembranes. During this process also cross-linking of proteins occurs leading to the formation of high molecular weight proteins. It has previously been shown that in the process of lipid peroxidation the activity of the enzyme G-6-P-ase is diminished (Koster 1980, Wills 1971). Our data show that all these phenomena are present in HCB porphyria in the rat. Thus the concurrence of porphyria and lipid peroxidation appears to be dependent on the presence of iron.
Electron microscopic studies (Debets 1979) of rat liver tissue have shown that damage of membranes takes place during HCB intoxication. In the cytoplasm "whorl"-like structures are found which appear to have been built up concentrically from membrane-like structures. In the centre of these structures fat drops are often found. It is possible that these electron microscopical changes are the result of membrane damage by lipid peroxidation.
It is known, that with the induction of HCB porphyria the activity of the enzyme URO-D is inhibited. This inhibition is enhanced by the presence of iron (Taljaard 1971). Is seems reasonable to assume, as suggested by Debets and Strik, that destruction of the enzyme URO-D by HCB radicals is involved in the pathogenesis of HCB induced porphyria (Debets 1979, Debets 1980). Our experiments suggest that these radicals can also be responsible for the initiation of lipid peroxidation. Although its mechanism is still in debate (Aust 1982), it is known that iron catalyses lipid peroxidation. This will lead to an increased destruction of cytochrome P450, which results in an increase of haem synthesis by induction of the enzyme ALA synthetase (De Matteis 1975). Thus the interplay between inactivated URO-D and the effects of an increased lipid peroxidation aggravates the porphyria induced by HCB. If radical formation does play an important role in the pathogenesis of HCB induced porphyria, antioxidants could theoretically modulate the disease process.
Debets et al.(1981) found in in vitro experiment with chick embryo liver cell cultures, a protective effect of vitamin E (DL-alpha-tocopherol) and other anti-oxidants against the porphyrinogenic action of HCB. However, vitamin E given orally did not prevent toxicity of HCB in Agus rats. In patients with porphyria cutanea tarda, however, remissions have been described after administration of vitamin E (Nair 1971).

Possibly these conflicting results can be explained by the erratic absorption of vitamin E after oral administration. This subject warrants further study because the iron dependency of toxicity of

PHA compounds and the possible protective effect of vitamin E can have important implications for prevention of toxicity of PHA compounds, which increasingly pollute our environment.

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# 6. THE EFFECT OF ALPHA-TOCOPHEROL ON THE PORPHYRINOGENICITY OF HEXACHLOROBENZENE

## 6.1. Introduction

The environmental pollutant HCB is a potent inducer of porphyria in man as well as in rat (Courtney 1979). It causes a type of porphyria that strongly resembles the disease porphyria cutanea tarda and is characterized by photosensitivity and an increased production of uroporphyrin and heptacarboxylporhyrins in the liver, which are excreted in the urine (Cam 1963, Cripps 1980, 1984). Decreased activity of the enzyme uroporphyrin decarboxylase (URO-D) has been observed in both the erythrocytes and liver tissue in the inherited variety of this disease while in the sporadic, or acquired form this could only be demonstrated in liver tissue ( De Verneuil 1978). In this disease an increase of pophyrinogen deaminase (PBG-D) activity also has been observed in erythrocytes. In HCB induced porphyria in the rat both decreased URO-D and increased PBG-D activity has been found (Taljaard 1971, Von Tieperman 1980, Wainstok 1984,1986). It is thought that electrophilic metabolites of HCB react with the catalytic site of URO-D and inactivate it (Strik 1979). However immunoreactive URO-D is unchanged in this condition (Elder 1980).
Iron has proven to be an enhancing factor for the toxicity of HCB and 2,3,7,8-tetrachlorodibenzo-p-dioxine, another porphyrinogenic compound.
We have shown that the development of HCB induced pophyria is associated with the presence of iron and with lipid peroxidation, which was demonstrated by the formation of Malondialdehyde (MDA) and High Molecular Weight proteins, and a decreased activity of the enzyme G-6-P-ase (Alleman 1985).

Lipid peroxidation is a process in which polyunsaturated fatty acids ,mainly localized in the membrane phospholipids are peroxidized.During this proces fatty acids are broken down to aldehydes and carbonyl compounds, resulting in membrane damage. Lipid peroxidation can be initiated by free radicals, which can induce the formation of superoxide $O^-_2$. According to the Haber Weiss reaction which is catalyzed by iron, superoxide and hydrogen peroxide may produce a hydroxyl radical,which is a very potent biological oxidant (Koster 1980 ,Freeman 1982, Aisen 1982)

$$Fe^{3+} + O^-_2 \longrightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^\cdot \quad (2)$$

The hydroxyl radical that is formed will attack virtually any organic molecule to obtain an electron to become a hydroxyl ion. When this occurs in a polyunsaturated fatty acid of a membrane a lipid peroxy radical is formed by which the reaction is propagated. The chain reaction proceeds until it stops by the reaction of two radicals with each other or by restoration of the electron pairing by antioxidants as DL-alpha-tocopherol (Kornbrust 1979, Aisen 1982, Niki 1987). One of the possible forms in which iron facilitates the

Haber Weiss reaction is perferryl ion $(FeO_2)^{2+}$ in complex with ADP. Reduction of ADP-$Fe^{3+}$ is therefore necessary. This occurs both non enzymatically e.g. by ascorbate, as enzymatically by NADPH-Cytochrome P450 reductase and cytochrome P450 (Wills 1971, Svingen 1979 Morehouse 1988). Cytochrome P450 has been shown to catalyze the propagation of lipid peroxidation, but is destroyed during this process (De Matteis 1978, Svingen 1979).
This process of lipid peroxidation in HCB induced porphyria is thought to be initiated by HCB radicals. There is evidence that these are formed during the metabolism of HCB including the finding of sulfur containing metabolites, (the result of glutathione conjugation), the aggravation of porphyric action of HCB by glutathione depleting agents as diethymaleate, and the prevention of HCB porphyria by the inhibitors of cytochrome dependent hepatic drug metabolism, SKF 525A and piperonylbutoxide (Debets 1979, Debets 1980, Stewart 1986). In contrast, acetylcysteine does not prevent HCB induced porphyria (Lissner 1985), although gluthathione inhibits microsomal oxidation of HCB in vitro (Van Ommen 1986).
In in vitro experiments with chick embryo liver cell cultures, Debets found protective effects of vitamine E, DL-alpha-tocopherol (Debets 1980 ). When given orally to Agus rats, vitamin E did not prevent the toxicity of HCB (Debets 1981). The porphyrinogenicity of allylisopropamide in rats, however could be prevented by vitamine E in a single oral dose of 100 mg (Murty 1969).
In some patients with porphyria cutanea tarda remissions have been described after administration of Vitamine E (Nair 1971, Ayres 1978).
We decided to investigate the effect of intraperitoneal administration of DL-alpha-tocopherol on the development and the course of HCB induced porphyria.

**6.2. Materials and Methods**

Female Agus rats with a weight of about 180 gr were obtained from TNO (Rijswijk, The Netherlands). At day 0 ,7 and 14 these rats were given 50mg HCB in 0,5 ml olive oil and 50 mg DL-alphatocopherol intraperitoneally. Moreover, they were given 25 mg iron dextran (Imferon) subcutaneously. Another intraperitoneal injection of 50 mg HCB in 0,5 ml olive oil was given at day 29.
The rats were placed in metabolic cages to collect urine for 24 hr at about 6 weekly intervals from day 82 (43 days after the last HCB injection).
Three rats were sacrificed at day 121 to measure liver uro- and heptacarboxylporphyrin concentration, malondialdehyde (MDA) concentration and glucose-6-phosphatase activity (G-6-P-ase). The other six rats were sacrificed at day 181, after the fourth urine collection.
Porphyrins. Separation and measurement of porphyrins in liver homogenates and urine was performed as described before (Alleman 1982 and 1985).
Malondialdehyde (MDA). A 20% liver homogenate was prepared in 0.15 M KCl-5 mM Tris maleate, pH 7.4. The amount of malondialdehyde was determined as described previously (Lowry 1951) In short: to 0.5 ml liver homogenate 0.3 ml 20% TCA, 0.6 ml TBA

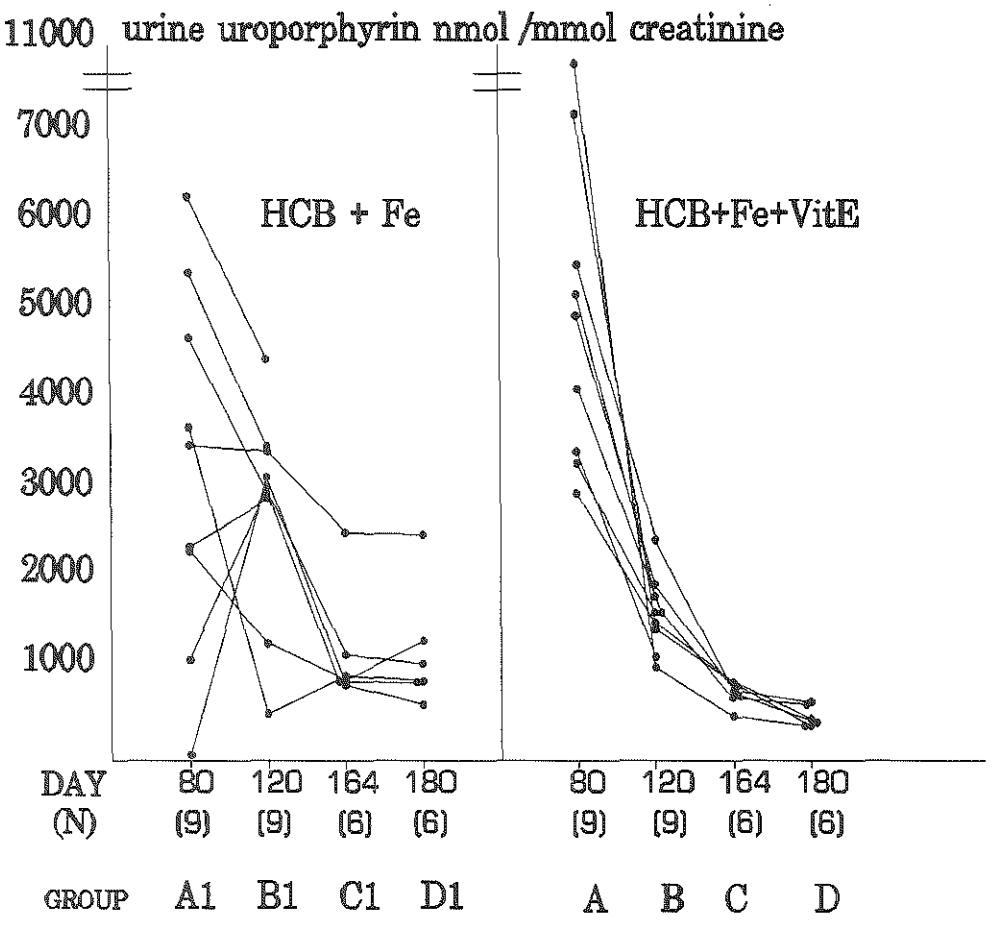


Fig 6.1 Uropophyrin excretion in urine in nmol/mmol creatinine in rats with porphyria induced by HCB and Iron. One group(right) was treated with vitamin E.
Statistics:

| Wilcoxon signed rank test | | Wilcoxon rank sum test |
|---|---|---|
| A ~ B: p=0,008 | A1 ~ B1:n.s. | A ~ A1:n.s |
| A ~ C: p=0,026 | A1 ~ C1:n.s | B ~ B1:p=0,029 |
| A ~ D: p=0,026 | A1 ~ D1:n.s | C ~ C1:p=0,01 |
| B ~ C: p=0,026 | B1 ~ D1:n.s | D ~ D1:p=0,01 |
| B ~ D: p=0,026 | C1 ~ D1:n.s | |
| C ~ D: n.s.(p=0,071) | C1 ~ D1:n.s. | |

(0.05 M), 0.1 ml butylated hydroxy-toluene (0.2%) and 0.5 ml homogenate buffer was added. After centrifugation the supernatant was heated at 100° for 8 min, cooled and the absorbance was read at 535 nM. The MDA content was expressed as nmol/mg protein using the extinction coefficient of 156.000. Protein was determined according to Lowry (1951).
Glucose-6-phosphatase (G-6-P-ase). Glucose-6-phosphatase activity was measured according to Harper (1965).
Statistical analysis: The Wilcoxon rank sum test was used to compare the vitamin E group and the control group.
The Wilcoxon signed rank test (paired) was used to compare the results of the uroporhryrin excretion within one group. As at day 121 three rats were sacrificed, only six rats remained at day 164 and 180. Statistical analysis was performed only with the six same rats at day 82 and 120.

**6.3. Results**

The results of the uroporphyrin excretion at day 82, 120, 164 and 180 of both the vitamin E group and the control group are presented in Fig 5.1. At day 82 the uroporphyrin excretion in the urine did not differ in both groups. At day 120, 164 and 180 significant lower quantities of urine uroporphyrin have been found in the vitamin E group.
In the vitamin E group a significant decrease of uroporphyrin was observed at day 120 and 164, at day 180 no further decrease was shown. In the control group a significant decrease was only seen between day 120 and day 164, and not to the same (low) level as in the vitamin E group.
At day 121 there seemed to be a larger amount of MDA in the vitamin E group livers, but the number of rats investigated at this time of experiment was too small to draw firm conclusions . At day 181 no statistical difference could be found for MDA (Figure 5.2). On day 121 MDA seemed to be higher in the Vitamin E group, but the numbers were to small for statistical evaluation.
G-6-P-ase activity did not show a significant difference (Vitamin E group - mean: 37,5 nmol $P_i$ /min/mg protein S.D: 5,95; HCB+Fe group - mean: 48,6 S.D: 7,83).
Liver uroporphyrin did not demonstrate a significant difference either: Vitamin E group - mean: 942 pmol/mg protein, S.D. 525;
HCB +Fe group- mean: 1170 pmol/mg protein, S.D. :244.

**6.4. Discussion**

Although DL-alpha-tocoferol does not prevent HCB-induced porphyria it appears that the treated rats recovered more rapidly than the controls. Since the same values of MDA were found in the vitamin E group as in the control group it indicates that the breakdown of polyunsaturated fatty acids due to lipid peroxidation is the same, despite the intraperitoneal administration of 150 mg vitamin E. In the three rats that had been sacrificed at day 81 the formation of MDA appeared to be more pronounced in the Vitamin E group than in the controls. It would be of interest to see if in that stage of

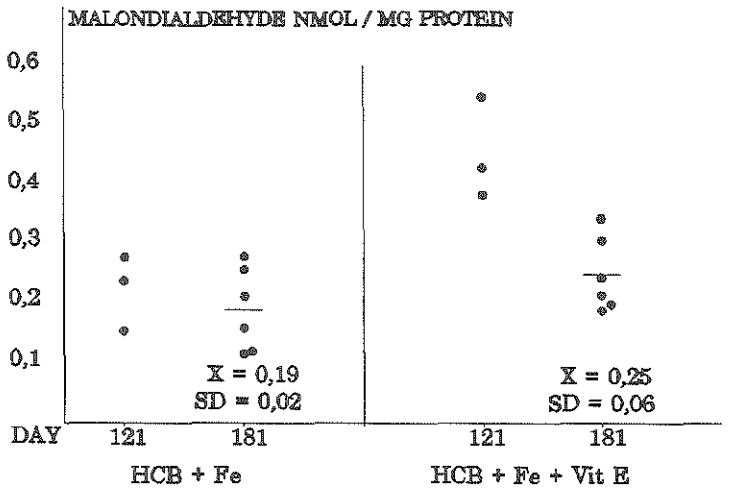


Fig 6.2 Malondialdehyde in liver tissue in nmol/mg protein.There was no significant difference at day 121 in the liver between the HCB + Fe and the HCB + Fe + Vit E group.

the disease DL-alpha-tocoferol has in fact an aggravating effect on the lipid peroxidation induced by HCB porphyria, which would be in contrast to the expected protective effect. We have not investigated if simultaneous intraperitoneal administration of HCB and Vitamin E influences the transport of HCB to the liver.
When both given orally no difference was seen by Debets (1981)
Former investigations could not demonstrate any effect of water soluble radical scavengers as acetylcysteine on the porphyrinogenic effect of HCB, but the porphyrinogenic effect of HCB is aggravated by glutathione depleting agents.
It would be of interest to investigate the combinated effect of a lipophilic radical scavenger like DL-alpha-tocopherol and a hydrophylic antioxidant like acetylcysteine on the toxicity of HCB and iron. Such a combination could have an inhibitory effect on the development of porphyria and is possibly more effective than either of these apart, because protection is offered both in the aqueous phase as in the lipid membranes (Niki 1987).

The effect of the antioxidant ascorbate on the toxicity of HCB is difficult to predict because this substance can reduce $Fe^{3+}$ , and known to enhance the effects of iron on lipid peroxidation (Aitken 1982). In the study of Debets (1981) that failed to show a protective effect of antioxidants in rats a combination of ascorbate and DL-alpha-tocopherol was used. This could have been of influence on the results.
Until now reducing iron stores by bleeding and an iron deficient diet or iron chelation by desferrioxamine is the mainstay of prevention of HCB toxicity in rats (Alleman 1985, Wainstok 1986).

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## 7. GENERAL DISCUSSION AND SUMMARY

In this thesis we have reviewed the literature on the disease PCT and HCB-induced porphyria. PCT is a disorder of the heme synthesis pathway, which leads to accumulation of uroporphyrin and heptacarboxyl porphyrin and the excretion of very high levels of these two porphyrins in the urine. Similar metabolic findings are observed in HCB-porphyria, and HCB-induced porphyria has been used by many researchers as an animal model of PCT, to obtain insights into the pathogenesis of PCT. Our first studies were on the enzymatic changes in human PCT. PCT, although generally a disease of adulthood, appears in some instances to have a genetic background, and the familial occurrence of PCT was described many years ago. The inherited enzymatic defect in these families is a reduction in URO-D activity. We developed a simple method to evaluate URO-D status in family studies (chapter 2).

In familial PCT occurring in three generations we have shown that the pattern of porphyrins formed from porhobilinogen by hemolysates is a suitable technique for detecting asymptomatic individuals with this disease. Using PBG as substrate it is a cheaper and quicker assay than using uroporphyrinogen. Recently Siersema and coworkers (1990) showed that it can be used to discriminate sporadic and hereditary PCT.
With this method we could also confirm that URO-D deficiency in rat liver homogenates can be induced by HCB and iron (Chapter 4).
Not all patients with PCT have a reduced level of URO-D in their erythrocytes. There is only one gene for URO-D, and at present there is no clear evidence for tissue-specific gene products or gene regulation. In sporadic PCT although URO-D activity is normal in erythrocytes, it is reduced in the liver. This means that local hepatic changes must play a role in the pathogenesis of sporadic PCT. It is indeed likely that such factors are also important in familial PCT, as not all individuals with the genetic trait develope manifest porphyria. One possible additional factor which we encountered was an increased activity of the enzyme PBG-D, which is responsible together with URO-III-cosynthetase for the production of uroporphyrinogen.
We found that PBG-D activity of erythrocytes in patients and carriers with hereditary PCT was increased in agreement with Brodie (1977). This PBG-D rise could also be found in rat liver homogenated in HCB induced porphyria according to Von Tieperman (1980).
The mechanism underlying this increase in PBG-D activity has not been elucidated.
We suggest that this rise of PBG-D activity is secondary to the URO-D deficiency, that results in a negative feedback stimulation of ALA-S, which finally results in an increased PBG production. The ability to increase PBG-D activity in fact determines that PCT is not an acute porphyria because it prevents accumulation of porphyrin precursors. Meissner has suggested that the occurrence of acute attacks in hereditary coproporphyria and variegate porphyria is due to an inhibitory effect of high levels of coproporphyrinogen and protoporphyrinogen IX respectively on PBG-D activity, and has shown that this is in fact the case in vitro. In vitro, uroporphyrin and

uroporphyrinogen have no effect on PBG-D activity (Meissner, personal communication).
In further studies using rats, we examined the possiblity that PBG-D activity, like ALA-S, was affected by heme and by fasting.
We investigated this in rats with acute lead intoxication, which was aggravated by phenobarbital.
This treatment caused a significant increase in hepatic PBG-D activity. However, intraperitoneal administration of hematin did not reduce the increase of PBG-D in these rats, but in contrast, it earned a significantly further increase (Chapter 3).
Moreover, subcutaneous inplanted hematin pellets had no inhibiting effects on PBG-D activity in HCB-induced porphyria in the rat (Chapter 4). We could not demonstrate a change of PBG-D in fasting rats, in contrast to the well known increase of ALA-S in fasting (Chapter 3).
These findings are compatible with an increase in PBG-D activity during prolonged ALA-S induction with overproduction of the precursors ALA and PBG, possibly by stabilization of the PBG-D enzyme. However other mechanisms, including an increased translation or transcription cannot be ruled out.
In the following chapters we reported our studies on the interaction between iron and porphyrin metabolism in HCB-porphyria. These studies have potential clinical implications in view of the fact that in human PCT mild to moderate increases in hepatic iron stores are universally observed, and most patients respond to a reduction in body iron stores by venasection, with clinical and biochemical improvement.
In Chapter 5 and 6 we investigated the effect of iron on the toxicity of HCB. Iron depletion protects against the toxicity of HCB After bleeding and administration of an iron restricted diet rats did not develop the slightest biochemical change pointing to HCB-induced porphyria. Development of porphyria was related to the amount of iron in the liver and was associated with the process of lipid peroxidation as measured by the formation of malondialdehyde, glucose-6-phosphatase, and by the formation of HMW proteins.
We share the opinion with Debets that HCB radicals are involved in the damage of URO-D. The fact that HCB -like TCDD- is not toxic in iron-deficient animals indicate that the development of porphyria is iron dependent. The process of lipid peroxidation initiated by HCB radicals and catalyzed by $Fe^{2+}$ may play an important role in the development of HCB induced porphyria (Chapter 5).
The possibility that HCB and iron promote the formation of porphyrin radicals, which in turn damage the catalytic site of URO-D, requires further study. It seems likely that the effect of the putative HCB-radical or porphyrin radical is relatively specific as uroporphyria is not a feature of other forms of liver damage associated with lipid peroxidation such as carbontetrachloride induced hepatic necrosis.
Lipid peroxidation could cause additional problems by oxidizing the uroporphyrinogen to uroporphyrin, which is not a substrate for URO-D, and would therefore accumulate.
In view of the constant finding of evidence for lipid peroxidation in both HCB-porphyria and in the very small number of patients with PCT in which we were able to look for HMW proteins in liver biop-

sies, we considered the possibility that a free-radical scavenger such as alpha-tocopherol might have a beneficial effect.
We expected that we could inhibit the development HCB-induced porphyria in the rat with DL-alpha-tocopherol, but porphyria was not prevented by this radical scavenger, although it appeared to accelerate recovery.
In conclusion, both PCT and HCB-induced porphyria are iron-dependant disorders. In HCB-induced porphyria there is clear evidence that this iron-dependence is associated with the process of lipid peroxidation in the liver. Further work has to be done in human PCT to determine whether lipid peroxidative processes also play an important role in converting a biochemically and clinically symptomfree individual with a genetically low URO-D activity into a PCT patient. In addition much work remains to be done on the underlying genetic defects of the URO-D deficiency, and on the pathogenesis of sporadic PCT.

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## 8. SUMMARY IN DUTCH: SAMENVATTING

In dit proefschrift werd een overzicht gegeven van de literatuur over de ziekte porphyria cutanea tarda (PCT) en de door hexachlorobenzeen (HCB) geïnduceerde porphyrie. PCT is een ziekte die berust op een stoornis in de heemsynthese, hetgeen leidt tot stapeling van uroporphyrine en heptacarboxylporphyrine en sterk verhoogde uitscheiding van deze twee porphyrinen in de urine. Een vergelijkbare metabole stoornis wordt waargenomen bij de door HCB geïnduceerde porphyrie. Dit heeft er toe geleid dat deze vorm van experimentele porphyrie door vele onderzoekers wordt gebruikt als een diermodel voor PCT om inzichten te verkrijgen in de pathogenese van PCT.
Onze eerste studies waren gericht op de enzymatische verandering in PCT bij de mens. Ofschoon PCT over het algemeen een ziekte is die zich op volwassen leeftijd manifesteert, blijkt deze aandoening in sommige gevallen een genetische oorsprong te hebben. Het familiair voorkomen van PCT werd dan ook al vele jaren geleden beschreven. Het aangeboren enzymatische defect in deze families is een afname van de activiteit van uroporphyrinogeendecarboxylase (URO-D). Een eenvoudige methode werd ontwikkeld die geschikt was om bij familieonderzoek de URO-D status vast te stellen (hoofdstuk 2).
Bij porphyria cutanea tarda die in één familie voorkwam in drie generaties, werd aangetoond dat het patroon van porphyrines dat gevormd werd uit porphobilinogeen door hemolysaten een geschikte techniek is om asymptomatische individuen met deze ziekte op het spoor te komen. Omdat deze methode uitgaat van porphobilinogeen als substraat in plaats van uroporphyrinogeen is het een goedkopere en snellere bepaling. Onlangs toonden Siersema en medewerkers aan dat deze methode eveneens gebruikt kan worden om sporadische en familiaire porphyria cutanea tarda van elkaar te onderscheiden (Siersema 1990).
Met deze methode kon eveneens bevestigd worden dat URO-D deficientie in rattenlever homogenaten geïnduceerd kan worden door hexachlorobenzeen en ijzer (hoofdstuk 4). Niet alle patienten met PCT hebben een afgenomen activiteit van URO-D in hun erythrocyten. Er is slechts één gen voor URO-D en op dit moment is er geen duidelijk bewijs voor weefselspecifieke genprodukten of genregulatie. In sporadische PCT is de URO-D activiteit afgenomen in de lever, terwijl de activiteit in erythrocyten normaal is. Dit betekent dat plaatselijke veranderingen in de lever een rol moeten spelen in de pathogenese van sporadische PCT. Het is eveneens waarschijnlijk dat zulke factoren een belangrijke rol spelen bij hereditaire PCT, omdat niet alle individuen met de erfelijke aandoening een manifeste porphyrie ontwikkelen. Een factor, die zou kunnen bijdragen aan het manifest worden van PCT, is de toegenomen activiteit van het enzym porphobilinogeendeaminase PBG-D, dat samen met URO-III cosynthetase verantwoordelijk is voor de produktie van uroporphyrinogeen. We vonden, evenals Brodie (1977), dat de PBG-D activiteit van erythrocyten in patienten en dragers van de erfelijke aandoening was toegenomen, evenals Brodie (1977). Deze toename van PBG-D kon eveneens worden gevonden in rattenlever homogenaten bij de door HCB geïnduceerde porphyrie. We bevestigden daarmee de bevindingen van von Tieperman (1980). Het mechanisme dat ten grondslag ligt aan de toename van PBG-D activiteit is nog niet opgehelderd.

Ons inziens is deze toename van PBG-D activiteit secundair aan de URO-D deficiëntie, die resulteert in een negatieve feedback stimulatie van delta-aminolevulinezuur synthethase (ALA-S), hetgeen uiteindelijk resulteert in een toegenomen PBG produktie. Het vermogen om de PBG-D activiteit te doen toenemen bepaalt in feite dat PCT niet een acute porphyrie is, omdat het voorkómt dat er stapeling optreedt van porphyrine precursors. Meissner heeft gesuggereerd dat het voorkomen van acute aanvallen in hereditaire copropophyrie en porphyria variegata te wijten is aan een remmend effect van hoge concentraties van respectievelijk coproporphyrinogeen en protoporphyrinogeen IX op de PBG-D activiteit. Hij heeft dit aangetoond in vitro. Bovendien is in vitro gebleken dat zowel uroporphyrine als uroporphyrinogeen geen effect hebben op de PBG-D activiteit (Meissner, persoonlijke mededeling). In verdere studies, waarbij we gebruik maakten van ratten hebben we de mogelijkheid onderzocht dat PBG-D activiteit, evenals het ALA-S, werd beinvloed door heem en door vasten. We onderzochten dit in ratten met acute loodintoxicatie die werd verergerd door fenobarbital. Dit leidde tot een significante toename van PBG-D activiteit. Intraperitoneale toediening van hematine deed deze toename van PBG-D in deze rattten niet afnemen. Integendeel, de PBG-D activiteit in de lever van deze ratten nam alleen maar verder toe (hoofdstuk 3).
Subcutaan geïmplanteerde hematine pellets hadden eveneens geen remmend effect op PBG-D activiteit in de door HCB geïnduceerde porphyrie in de rat (hoofdstuk 4). We konden geen verandering aantonen van PBG-D in ratten na vasten. Dit in tegenstelling tot de welbekende toename van ALA-S na vasten (hoofdstuk 3).
Deze bevindingen zijn compatibel met een toename in PBG activiteit gedurende langdurige ALA-S inductie met overproduktie van de precursors ALA en PBG, mogelijk door stabilisatie van het enzym PBG-D. Andere mechanismen waaronder een toegenomen translatie of transcriptie, kunnen echter niet worden uitgesloten.
In de volgende hoofdstukken worden onze studies besproken die betrekking hebben op de interactie tussen ijzer en porphyrine stofwisseling bij de door HCB geïnduceerde porphyrie. Deze studies hebben mogelijk klinische implicaties met het oog op het feit dat bij humane PCT een lichte tot matige toename bestaat van het leverijzer en omdat de meeste patiënten zowel klinisch als biochemisch een remissie krijgen, als de ijzerdepots door middel van aderlating of therapie met desferrioxamine worden verminderd.
In hoofdstuk 5 en 6 hebben we het effect van ijzer op de toxiciteit van HCB onderzocht. IJzergebrek beschermt tegen de toxiciteit van HCB. Na bloedafname en onder toediening van een ijzerbeperkt dieet ontwikkelden ratten niet de geringste biochemische verandering die zou kunnen wijzen op een door HCB geïnduceerde porphyrie. De ontwikkeling van porphyrie was gerelateerd aan de hoeveelheid ijzer in de lever en werd geassocieerd met het proces lipide-peroxydatie. Dit werd gemeten aan de hand van de vorming van malondialdehyde, de activiteit van glucose-6-fosfatase en door de vorming van eiwitten met een hoog moleculair gewicht (HMW eiwitten).
We zijn met Debets van mening dat HCB radicalen betrokken zijn bij de beschadiging van URO-D. Het feit dat HCB -evenals TCDD- niet toxisch is in proefdieren met een ijzergebrek indiceert dat de ontwikkeling van porphyrie ijzerafhankelijk is. Het proces lipide-

peroxydatie wordt geïnitieerd door HCB radicalen en wordt gekatalyseerd door $FE^{2+}$ .Dit proces kan een belangrijke rol spelen in de ontwikkeling van HCB geïnduceerde porphyrie (hoofdstuk 5). De mogelijkheid dat HCB en ijzer de vorming van porphyrine radicalen veroorzaken, die op hun beurt de katalytische plaats van het enzym URO-D beschadigen, vereist verdere studie. Het lijkt waarschijnlijk dat het effect van het vermeende HCB radicaal of porphyrine radicaal betrekkelijk specifiek is omdat uroporphyrie niet een kenmerk is van andere vormen van leverbeschadiging die geassocieerd zijn met lipide-peroxydatie zoals de door koolstoftetrachloride geïnduceerde levernecrose.
Lipide-peroxydatie zou additionele problemen kunnen veroorzaken door uroporphyrinogeen tot uroporphyrine te oxideren. Uroporphyrinogeen is geen substraat voor URO-D en kan daarom accumuleren.
Omdat we aanwijzingen vonden voor lipide-peroxydatie bij zowel de door HCB geïnduceerde porphyrie en in het hele kleine aantal patiënten met PCT dat we konden onderzoeken op HMW eiwitten in de lever, overwogen we de mogelijkheid dat een vrije radicalen scavenger zoals DL-alpha-tocoferol wellicht een gunstig effect zou kunnen hebben.
We verwachtten dat DL-alpha-tocoferol de ontwikkeling van de door HCB geïnduceerde porphyrie in de rat wellicht zou kunnen voorkomen. DL-alpha-tocoferol evenwel voorkwam de ontwikkeling van de porphyrie niet, alhoewel deze vitamine het herstel bleek te versnellen.
Concluderend zijn zowel PCT als de door HCB geïnduceerde porphyrie ijzerafhankelijke ziektes. Bij de door HCB geïnduceerde porphyrie is er duidelijk bewijs dat deze ijzerafhankelijkheid geassocieerd is met lipide-peroxydatie in de lever. Verder werk moet worden gedaan in humane PCT om te bepalen of het proces lipide-peroxydatie eveneens een belangrijke rol speelt bij de verandering die plaats vindt van een zowel biochemisch als klinisch symptoomvrij individu met een genetisch bepaalde lage activiteit van URO-D tot een patiënt met een volledig ontwikkeld beeld van een porphyria cutanea tarda. Bovendien moet er nog veel onderzoek gedaan worden naar het onderliggende genetische defect van de URO-D deficiëntie en naar de pathogenese van sporadische porphyria cutanea tarda.

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## 10.CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 10 maart 1950 te Amsterdam. In 1969 behaalde hij het einddiploma gymnasium $\beta$ aan het Aloysius College te 's Gravenhage. In datzelfde jaar maakte hij een aanvang met de studie Geneeskunde aan de Rijksuniversiteit te Leiden. In 1974 werd het Doctoraalexamen afgelegd en werd de studie voortgezet aan de faculteit der geneeskunde van de Erasmus universiteit te Rotterdam, alwaar in 1975 het artsdiploma werd behaald. Tijdens het vervullen van de militaire dienstplicht van 1-3-1976 t/m 31-5-77 was hij als eerste-luitenant arts gedetacheerd op de afdeling immunohematologie van het Academisch Ziekenhuis te Leiden (Hoofd Prof Dr J.J. van Rood).
Op 1-6-1977 begon hij zijn opleiding tot internist op de afdeling Interne Geneeskunde II (Hoofd Prof Dr M. Frenkel). Per 1-6-1982 volgde zijn registratie als internist. Vanaf 1-7-1982 maakt hij deel uit van de internistenmaatschap van het Ziekenhuis De Weezenlanden te Zwolle.