STUDIES ON THE MECHANISM OF PORPHYRIA CUTANEA TARDA

by

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To my parents

ABSTRACT

The mechanism leading to porphyria cutanea tarda has been investigated using experimental porphyria caused by chlorinated aromatic hydrocarbons as a model. This lesion closely ressembles porphyria cutanea tarda in man and was useful in determining the biochemical lesion directly responsible for the defect in heme biosynthesis.

Methodology was developed for rapid and accurate measurement of urinary porphyrins by use of derivative spectroscopy. Based upon this technique, an extraction procedure and assay for urinary porphyrinogens was developed.

The above methodology made possible a detailed examination of excreted intermediates in rats during the development of porphyria caused by hexachlorobenzene. It was found that increased excretion of both porphyrinogens and porphyrins occurred, which disproved the theory that porphyria resulted from accelerated oxidation of porphyrinogens. Detailed analysis of urinary and fecal porphyrins supported the hypothesis that decreased activity of uroporphyrinogen decarboxylase was the direct cause of the porphyria.

Examination of the susceptibility of inbred strains of mice to porphyria caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin established a relationship between inheritance of responsiveness to induction of aryl hydrocarbon hydroxylase and susceptibility to porphyria. This was confirmed using a back-crossed strain which had been phenotyped for aryl hydrocarbon hydroxylase responsiveness. It was found that susceptibility to porphyria was inherited with the gene controlling aryl hydrocarbon hydroxylase responsiveness.

The requirement for non-heme tissue iron in the development of experimental porphyria was examined by depleting mice of stored iron prior to treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. It was found that iron deficiency protected against morphologic liver damage, depression of uroporphyrinogen decarboxylase activity, and skin lesions caused by the dioxin.

It was concluded that the development of porphyria caused by chlorinated aromatic hydrocarbons is attributable to decreased uroporphyrinogen decarboxylase activity which is brought about by a process in the liver which requires both iron and susceptibility to induction of aryl hydrocarbon hydroxylase.

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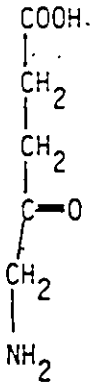
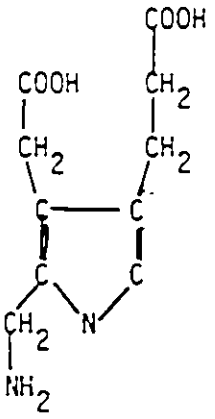
ABBREVIATIONS

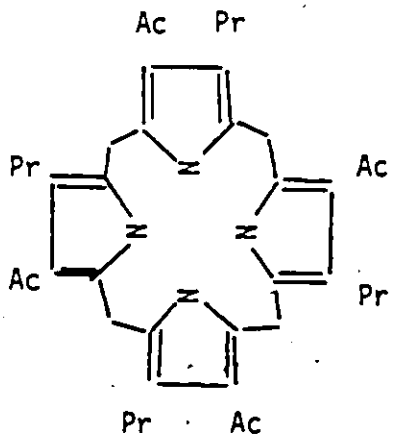
AIA	allyl-isopropyl acetamide
ALA	aminolevulinic acid
ALA-s	aminolevulinic acid synthetase
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
COPRO	coproporphyrin
COPROGEN	coproporphyrinogen
DDC	dihydro-dicarbethoxycollidine
GSH	reduced glutathione
HCB	hexachlorobenzene
ISO	isocoproporphyrin
3-MC	3-methylcholanthrene
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
PB	phenobarbital
PBG	porphobilinogen
PCT	porphyria cutanea tarda
TCA	trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Tris	tris(hydroxymethyl)-amino methane
URO	uroporphyrin
UROGEN	uroporphyrinogen
UV	ultra-violet



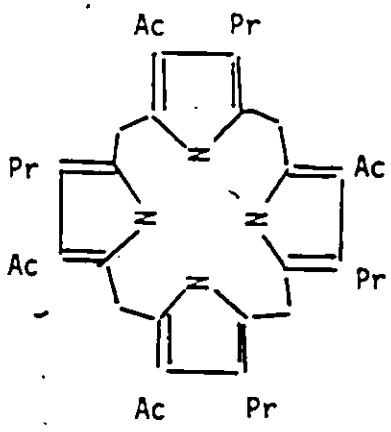
Aminolevulinic Acid



Porphobilinogen



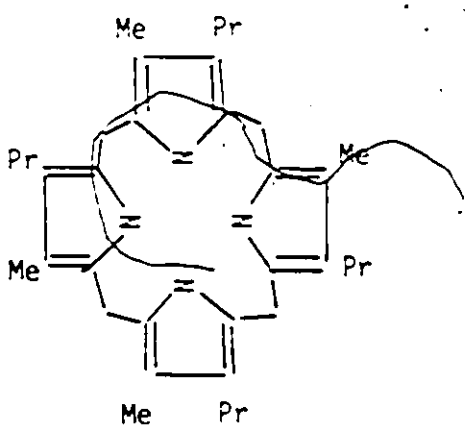
Uroporphyrinogen I



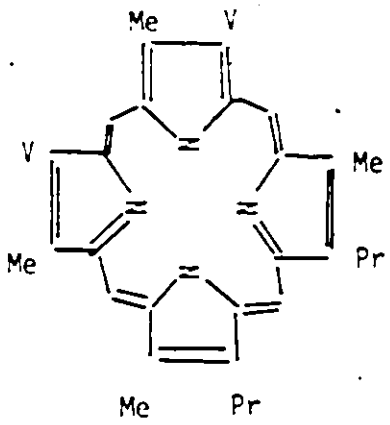
Uroporphyrinogen III

Ac = acetate (carboxymethyl)

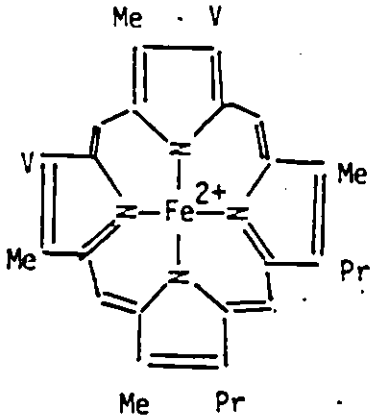
Pr = propionate (carboxyethyl)



Coproporphyrinogen III



Protoporphyrin IX



Heme

Me = methyl

V = vinyl

1. INTRODUCTION

The porphyrias are a group of diseases which develop as a result of defects which may occur in the biosynthesis of heme. They are characterized by the accumulation and excretion of excessive quantities of heme biosynthetic intermediates, especially porphyrins, from which the name arises, and are commonly classified according to the tissue in which the disease is manifest, i.e. the liver (hepatic porphyrias) or the bone marrow (erythropoietic porphyrias).

Porphyria cutanea tarda (PCT) is a particular type of hepatic porphyria which presents with cutaneous manifestations in exposed areas due to the accumulation of porphyrins under the skin and their subsequent absorption of sunlight which, in turn, leads to tissue damage. As explained in detail below, PCT, unlike all other types of porphyria, shows no clear pattern of inheritance but can definitely be acquired through exposure to certain toxins and it has been associated with such possible etiologic agents as ethanol, synthetic estrogens, and chlorinated aromatic hydrocarbons.

This makes PCT a unique and interesting problem since it indicates that at least one common event occurs in

the course of the hepatotoxicity of a wide range of chemically dissimilar molecules. Thus, studies on the mechanism of PCT could lead to a better understanding of the mechanism of toxicity of several different substances.

To gain an understanding of the nature of the metabolic defect which occurs in PCT it is useful to examine the heme biosynthetic pathway and compare PCT with the other abnormalities which are known to occur in heme synthesis.

1.1. THE HEME BIOSYNTHETIC PATHWAY

The heme synthetic pathway is illustrated schematically in Figure 1. The first and rate-limiting reaction occurs in the mitochondrion and is the condensation of succinyl-CoA and glycine to form aminolevulinic acid (ALA). This reaction is catalysed by aminolevulinic acid synthetase (ALA-s), an enzyme which is synthesized in the cytoplasm but resides there only briefly (half-life = 20 minutes; Hayashi et al, 1969) before it is modified and transported to the mitochondrion. The processing of the cytoplasmic enzyme results in a decrease in molecular weight from 178,000 to 113,000 (Hayashi et al, 1970; Whiting and Elliot, 1972).

An obligatory requirement for enzyme activity is the presence of the cofactor, pyridoxal phosphate, which binds

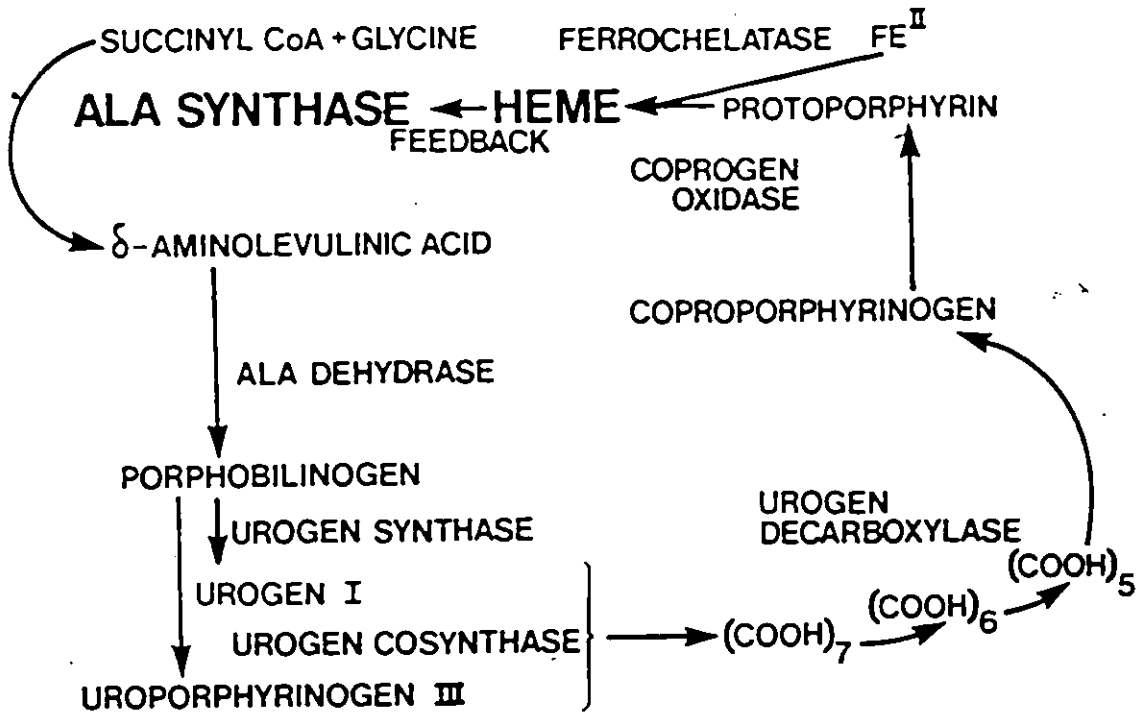


Figure 1: The heme biosynthetic pathway

tightly to ALA-s with Km of 3.0×10^{-6} M. Succinyl-CoA also has a high affinity for ALA-s ($Km = 7.0 \times 10^{-5}$ M) but glycine is required in much higher concentration for optimal enzyme activity ($Km = 1.0 \times 10^{-2}$ M) (Scholnick et al, 1972a). Such a high Km for glycine suggests that under some circumstances, the availability of glycine could determine the rate at which ALA and, therefore, heme, is synthesized (Sinclair and Granick, 1975).

Scholnick et al (1972b) have investigated the mechanism of the ALA-s reaction and confirmed that, following the reaction of pyridoxal phosphate with a thiol group on the enzyme, a Schiff base is formed with glycine which then condenses with succinyl-CoA. Loss of the glycyl carboxyl group accompanied by release of ALA completes the reaction.

Since ALA-S is the rate-limiting enzyme of heme biosynthesis, its regulation is very important in controlling the amount of heme produced in the cell. It is, therefore, not surprising to find that the end product, heme, plays a major role in determining the amount of ALA-s activity in the liver. The first and last steps of heme biosynthesis occur in the mitochondrion. This allows for a direct effect of heme on ALA-s and it has been shown that, <u>in vitro</u>, heme is inhibitory to ALA-s ($Ki = 2.0 \times 10^{-5}$ M) in partially purified enzyme preparations from rat liver

(Scholnick et al, 1969; 1972b).

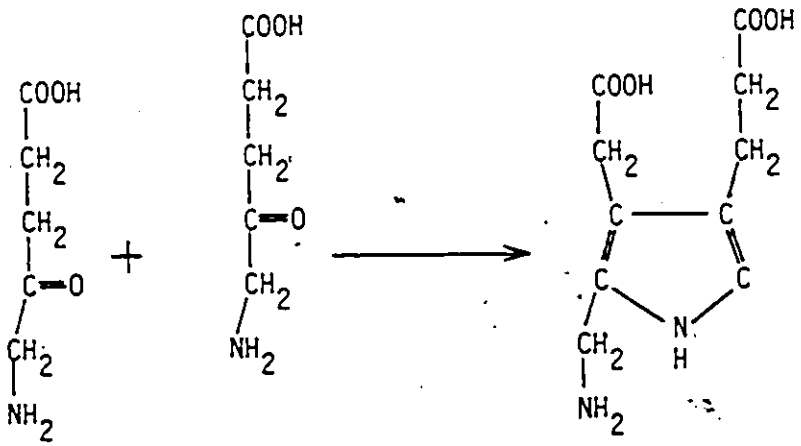
As well as having a direct effect on ALA-s activity, heme is believed to have an effect on the rate of synthesis of the hepatic enzyme. Since 1963, it has been known that certain drugs can cause an increase in ALA-s activity (Granick and Urata, 1963). Marks and co-workers have found that in the chick embryo liver system, ALA-s activity can be increased by a wide variety of aromatic and aliphatic amides and esters which have, as their distinguishing characteristics, lipophilicity and steric hindrance to hydrolysis (Murphy et al, 1975). Using an inhibitor of hydrolysis, it was demonstrated that the potency of these compounds as inducers correlated well with the lipophilicity of the molecule (Murphy et al, 1976). Whiting and Granick (1976) have shown, using immunoprecipitation techniques, that the rise in ALA-s activity results from increased synthesis of the enzyme and, therefore, represents true induction, rather than activation of existing enzyme. Drug-mediated induction of ALA-s is inhibited by administration of hemin (Sassa and Granick, 1970; Strand et al, 1972) and parenteral hemin has been shown to cause oscillatory changes in hepatic ALA-s levels (Waxman et al, 1966; Schacter et al, 1976).

Induction is presumed to be caused by depletion of a regulatory heme pool by drugs which block the heme pathway

(eg. dicarbethoxydihydrocollidine) and drugs which cause increased utilization of heme (eg. allyl isopropylacetamide and the barbiturates) (Marver, 1969). Padmanaban et al (1973) proposed a mechanism of positive translational control of ALA-s by apoprotein P-450 which is repressed by heme. Increased transcription may require a hormone metabolite, but the mechanism remains obscure.

Following formation of ALA, ALA-dehydrase catalyses the condensation of two molecules of ALA to form the monopyrrole precursor of all tetrapyrroles, porphobilinogen (PBG) (Figure 2). ALA-dehydrase is a cytoplasmic enzyme of molecular weight 280,000 which has been highly purified (Shemin, 1975) and found to consist of eight subunits (Doyle, 1971). It behaves like a typical thiol enzyme (i.e. inactivated by heavy metals, p-chloromercuribenzoate and iodoacetate, and activated by glutathione, β-mercaptoethanol and dithiothreitol) (Coleman, 1966); and also requires zinc for activity and can be reversibly inactivated by EDTA (Chen and Neilands, 1973).

ALA-dehydrase is inhibited by coproporphyrinogen (Satyanarayana Rao et al, 1970), protoporphyrin, and heme (Calissano et al, 1966) but it is unlikely that this inhibition plays any major role in regulation of heme synthesis since the tissue concentration of this enzyme is in approximately 80-fold excess relative to ALA-s (Meyer and



2 ALA PBG

Figure 2: Formation of porphobilinogen

Schmid, 1978).

Following formation of PBG, the next step in heme biosynthesis is the condensation of four molecules of PBG to form uroporphyrinogen. Although 13 isomers of uroporphyrinogen are possible, only 2 are found in nature, uroporphyrinogen I and uroporphyrinogen III. Only the III isomer can be converted to heme. For formation of uroporphyrinogen III, two enzymes are necessary, uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase. Uroporphyrinogen I synthetase will catalyse the conversion of four molecules of PBG to uroporphyrinogen I (Bogorad, 1958), but uroporphyrinogen III cosynthetase is inactive by itself. Recent studies indicate that the cosynthetase interacts physiochemically with uroporphyrinogen I synthetase. The cosynthetase modifies the sedimentation behavior of uroporphyrinogen I synthetase and uroporphyrinogen I synthetase associates with the cosynthetase after immobilization on Sepharose (Frydman et al, 1975); Higuchi and Bogorad, 1975). Both uroporphyrinogen III and its oxidized counterpart uroporphyrin III have been shown to be potent inhibitors of uroporphyrinogen synthetase in vitro (Levin, 1971).

The next enzyme in the heme synthetic pathway, uroporphyrinogen decarboxylase (UD), catalyses the conversion of uroporphyrinogen to coproporphyrinogen by

successively removing the four acetate carboxyl groups on uroporphyrinogen. This produces intermediates with seven, six, and five carboxyl groups but, under normal conditions, accumulation of these intermediates is minimal. Recently, Jackson et al (1976), in a very thorough study using chemically synthesized intermediates, suggested that uroporphyrinogen decarboxylase removes the acetate on uroporphyrinogen in a preferred order, i.e. clockwise from the asymmetric D ring. It has been suggested that more than one enzyme may be involved (San Martin de Viale et al, 1970; Benedetto et al, 1978) and that the reaction proceeds in two steps (Garcia et al, 1973), but purification has failed to separate these activities (Garcia et al, 1973; Elder and Tovey, 1977). The decarboxylase has been shown to be inhibited by ferrous iron in high concentration (Kushner et al, 1975) as well as by divalent mercury, copper, and manganese (Mauzerall and Granick, 1958). Oxygen inhibits the enzyme, probably by oxidation of porphyrinogens to poprphyrins, since the inhibition can be prevented by the addition of glutathione or cysteine to the reaction mixture.

Following the formation of coproporphyrinogen, the site of the next reaction in the heme synthetic pathway is again in the mitochondrion where coproporphyrinogen oxidase (specific for isomer III (Sano and Granick, 1961)) catalyses the conversion of coproporphyrinogen III to protoporphyrin

IX by converting the propionate (carboxyethyl) groups at positions 2 and 4 to vinyl groups. Again, a preferred order seems to exist, beginning with position 2 (Cavaleiro et al, 1973). It has recently been found that conversion of protoporphyrinogen to protoporphyrin involves another mitochondrial enzyme, protoporphyrinogen oxidase, requiring molecular oxygen for activity (Poulson and Polglase, 1975; Jackson et al, 1974).

The final step in heme synthesis is the insertion of ferrous iron into protoporphyrin IX to form heme. This is catalysed by the mitochondrial enzyme, ferrochelatase, which has been identified in liver and bone marrow (Lockhead et al, 1963; Porra and Jones, 1963). This enzyme is highly specific for protoporphyrin (Labbe et al, 1963) but is not as specific for ferrous iron since divalent cobalt and zinc can also be incorporated into protoporphyrin and inhibit the insertion of divalent iron (Wagner et al, 1976). As mentioned before, it is significant that the final step in heme formation is in the mitochondrion in close juxtaposition to the first and rate-limiting enzyme, ALA-s, which is, in fact, inhibited by heme.

1.2. ABNORMALITIES IN HEME BIOSYNTHESIS

As may be seen from the heme synthetic pathway,

several steps and intermediates are involved in the production of the final product, heme. Since heme is an essential molecule, complete blockage of the pathway at any stage is incompatible with life. Disturbances may arise, however, at several of the steps of synthesis which may be sufficiently severe to cause an accumulation of the proximal intermediate(s) without completely inhibiting formation of heme. When such an event occurs, porphyria is the result.

For a detailed review of the porphyrias the reader is directed to that of Meyer and Schmid (1978). Here, I shall merely indicate the points in the pathway at which abnormalities occur and the biochemical results which arise. Most disturbances of heme biosynthesis are the result of inherited deficiencies (usually autosomal dominant) of some enzyme in the pathway.

In acute intermittent porphyria uroporphyrinogen I synthetase activity has been demonstrated to be decreased to approximately 50% of normal levels in the liver, in fibroblasts, and in erythrocytes (Strand et al, 1972; Meyer, 1973). Manifestation of acute attacks in this disease shows periodicity, which implies that under normal circumstances the deficient enzyme is not rate-limiting. However, these patients are extremely sensitive to many lipophilic drugs which induce ALA-s (Murphy et al, 1975; 1976) and drugs such as barbiturates which cause induction of cytochrome P-450,

increasing the requirement for heme synthesis (De Matteis, 1973). An acute attack is accompanied by excretion of large quantities of PBG and ALA in the urine which is the predicted response for a situation in which uroporphyrinogen I synthetase becomes rate-limiting.

Acute intermittent porphyria may be contrasted with congenital erythropoietic porphyria which demonstrates autosomal recessive inheritance and manifests in the bone marrow rather than in the liver. The precise nature of the primary metabolic defect is in dispute but it is generally acknowledged that decreased activity of uroporphyrinogen III cosynthetase accounts for the dramatic increase in excretion of porphyrins of the isomer I series. The specificity of coproporphyrinogen oxidase for the III isomer prevents coproporphyrinogen I from being further metabolized and accounts for the increase in coproporphyrin excretion seen in this disease. Very large increases in excretion of uroporphyrin I compared with coproporphyrin I cannot be explained without involving uroporphyrinogen decarboxylase. Kinetic studies on this enzyme done in the course of this thesis may help to explain the pattern of porphyrin excretion seen in congenital erythropoietic porphyria. This will be dealt with in the Discussion section.

Although decreased activity of uroporphyrinogen III cosynthetase could be the primary defect in congenital

erythropoietic porphyria, another possibility which cannot, as yet, be ruled out is the hyperactivity of either ALA-s or uroporphyrinogen I synthetase. This theory was postulated by Miyagi et al (1976) based on the observation by Levin (1971; 1974) that uroporphyrinogen III cosynthetase is progressively inactivated in vitro in reaction mixtures containing uroporphyrinogen I synthetase and PBG. If this were also the case in vivo , decreased cosynthetase activity could be secondary to increased flux through the pathway.

Indirect evidence which mitigates against this theory that an increased flux through the pathway may be the primary disorder in congenital erythropoietic porphyria comes from the porphyrin excretion pattern seen in hereditary coproporphyria. In this hepatic disease, ALA-s activity is increased, presumably to compensate for a block in heme synthesis at the level of coproporphyrinogen oxidase. In patients with hereditary coproporphyria, coproporphyrinogen oxidase levels have been found to be decreased to approximately one-half the normal value in cultured fibroblasts (Elder et al, 1976) and leukocytes (Brodie et al, 1976; Grandchamp and Nordmann, 1977). However, in contrast to congenital erthropoietic porphyria, the accumulatd metabolite in this disorder is coproporphyrinogen III with very little increase in uroporphyrin excretion.

Another of the hepatic porphyrias, variegate porphyria, is inherited as an autosomal dominant characteristic which manifests with the excretion of large amounts of protoporphyrin and coproporphyrin in the feces. As in acute intermittent porphyria, acute attacks occur and patients with this disorder are similarly sensitive to barbiturates and other drugs which induce ALA-s (Eales, 1971). However, unlike acute intermittent porphyria, increased porphyrin excretion occurs even when patients are clinically asymptomatic. On the basis of the porphyrin excretion pattern, the enzymatic defect in variegate porphyria has been assigned to the steps between protoporphyrinogen and heme which are catalysed by protoporphyrinogen oxidase and ferrochelatase. Thus far, further differentiation has not been possible.

One type of porphyria which may involve both the liver and bone marrow is protoporphyria. Biochemically, it is characterized by increased quantities of protoporphyrin found in red cells and feces and by decreased ferrochelatase levels in erythrocytes (Bottomley et al, 1975) and other tissues (Bonkowsky et al, 1975). This disease follows, like most of the other porphyrias, an autosomal dominant mode of inheritance.

The disorders of heme synthesis described above are all clearly inherited as deficiencies in particular enzymes

in the heme biosynthetic pathway. For porphyria cutanea tarda (PCT), however, it has been difficult to demonstrate a pattern of inheritance. Most often, PCT is associated with long-standing liver disease, usually attributable to alcohol (Elder, 1977), and siderosis is a frequent, though not invarient finding (Elder et al, 1972; Joubert et al, 1973). Because of this, PCT has been considered secondary to liver disease.

While on the one hand, familial occurrence of PCT has not been apparent, on the other hand, it is clear that the disease can be acquired. The most dramatic instance of the acquired disease occurred in 1956 when several thousand individuals simultaneously presented with PCT (Schmid, 1960; Cam and Nigogoysan, 1963) months after eating grain which had been treated with a fungicide, hexachlorobenzene (HCB). Subsequently, Ochner and Schmid (1961) proved that HCB was a causative agent by producing a similar type of porphyria in rats by treating them with HCB in the diet. More recently, other halogenated aromatic hydrocarbons including polychlorinated biphenyls (PCB's) (Goldstein et al, 1974) and TCDD (Goldstein et al, 1973) have been shown to cause the same type of porphyria in animals.

The major biochemical abnormality found in PCT is the characteristic excretion in both urine and feces of large quantities of porphyrins with from eight to four

carboxyl groups, especially uroporphyrin, and excretion of isocoproporphyrin in the feces as a result of metabolism of pentacarboxyl porphyrinogen by coproporphyrinogen oxidase (Elder, 1972), which restricts the problem to the pathway between uroporphyrinogen and coproporphyrinogen. As mentioned above, uroporphyrinogen decarboxylase (UD) is responsible for catalysing this conversion and while it would be natural, given the enzymatic basis for the other types of porphyria, to suspect an inherited defect in this enzyme, the apparent lack of familial incidence of the disease, and its constant association with chronic liver injury, and the fact that it can be acquired from halogenated aromatic hydrocarbon poisoning have made the pathogenesis of PCT difficult to understand.

Recently, however, much activity has been focused on UD. Elder (1976) found reduced hepatic UD in rats made porphyric with HCB and Kushner and co-workers (Kushner and Barbuto, 1975; Kushner et al, 1976; Benedetto et al, 1978; Felsher et al, 1978) have found decreased activity of UD in both liver and erythrocytes of patients with PCT and in relatives of these patients. The finding of decreased UD in erythrocytes, and especially in erythrocytes of relatives of patients with PCT, is strongly suggestive of an inherited defect. However, this evidence has not been unequivocal. Other workers have confirmed the findings of decreased

hepatic UD in PCT but have found normal levels of the enzyme in erythrocytes of these same patients and their families (Blekkenhorst et al, 1976; Elder and Tovey, 1977; Elder et al, 1978). From the evidence available at this time, it appears that two types of PCT may exist: that which is purely acquired, and that which is brought about in people made unusually susceptible by an underlying genetic defect (Blekkenhorst et al, 1979).

The abnormalities of heme biosynthesis described above are all attributable to inherited or acquired deficiencies in particular enzymes of the heme biosynthetic pathway (Figure 3). Each enzyme deficiency is characterized by a specific pattern of heme precursor buildup and excretion. None, however, actually result in a demonstrable deficiency in heme. This is probably due to the great excess in capacity of the heme synthetic pathway and its regulation by heme which ensures that, if a partial block exists, flux through the pathway can be increased by induction of the initial enzyme, ALA-s.

1.3. EFFECTS OF CHLORINATED AROMATIC HYDROCARBONS ON THE LIVER

Although chlorinated aromatic hydrocarbons have been used extensively for many years, recognition of their

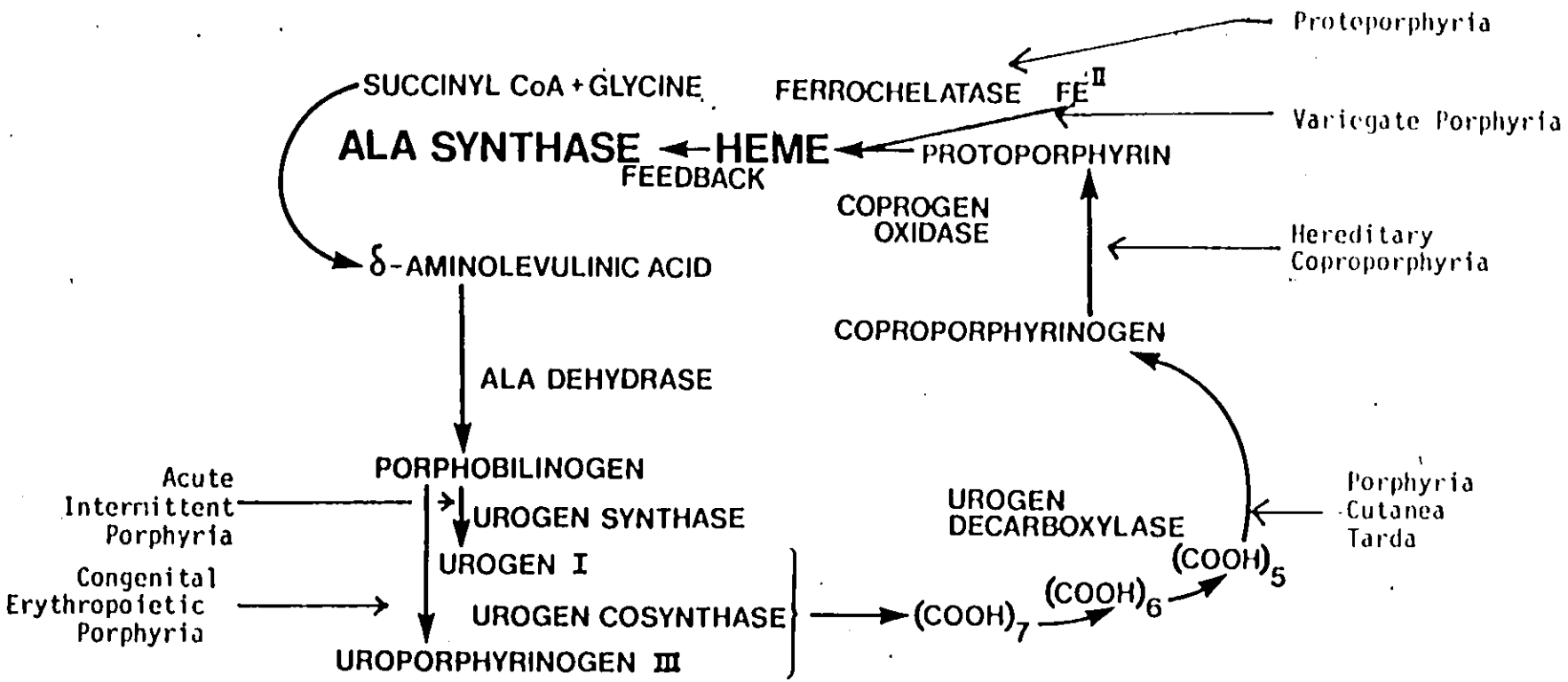


Figure 3: Abnormalities in heme biosynthesis

potential toxicity has been relatively recent. The discovery of a massive outbreak of porphyria in Turkey in 1956, later shown to be caused by HCB, has already been mentioned. In 1957, millions of chickens died of edema which was eventually traced to chlorinated dibenzodioxin present in a commercial diet (Firestone, 1973). In 1963, severe chloracne and porphyria erupted in workers in a 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) factory (Bleiberg et al, 1964) which abated when contamination with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was eliminated (Poland et al, 1971). In 1968, over 1,000 people exhibited a wide variety of symptoms (though, notably, not porphyria) following ingestion of rice bran oil contaminated with PCB's (Fishbein, 1974). In 1971, a number of horses and other animals became sick and died after riding arenas were sprayed with oil found to contain TCDD and PCB's (Kimbrough et al, 1977); and in 1976, the entire population of Seveso, Italy, was contaminated with TCDD following an explosion in a local 2,4,5-T plant (Hay, 1977).

Although the toxic manifestations of the chlorinated aromatic hydrocarbons involve many organs, I shall deal, here, only with those involving the liver.

1.3.1. HEPATOTOXICITY

The very fact that HCB, PCB's and TCDD are all capable of causing hepatic porphyria indicates that they have a deleterious effect on the liver. However, the precise nature of the toxicity remains obscure. HCB has been found to cause liver enlargement in all species tested and slight to marked morphologic damage as judged by the fatty infiltration, vacuolization and focal cellular necrosis seen in the livers of Japanese quail on a diet of 20 - 80 ppm for three months (Vos et al, 1971). Rats treated for a similar length of time with a diet consisting of 0.2% HCB showed disorganization of the liver lobule, focal cell necrosis and cellular enlargement, particularly toward the centre of the lobule. Electron microscopy showed proliferation of both rough and smooth endoplasmic reticulum (Campbell, 1963; Sweeney et al, 1971).

Although the effects of PCB's and TCDD on the liver have been the focus of much attention, the histologic findings have not been remarkably different from, nor more enlightening than, those documented for HCB. Enlargement of centrolobular cells, vacuolization of the cytoplasm, increased endoplasmic reticulum and focal necrosis are typical of the changes observed (Burse et al, 1974; Schwetz et al, 1973; Lucier et al, 1973; Hinton et al, 1978).

While HCB and PCB's are of considerable potency in causing the pathologic changes described above, TCDD is approximately four orders of magnitude more powerful (Poland and Kende, 1976). One of the most toxic small molecules known, TCDD has an LD50 in guinea pigs of approximately 1.0 ug/kg (Schwetz et al, 1973). Although reasons for the extreme toxicity of TCDD are not known, the potency of this molecule as a toxin and an etiologic agent for porphyria correlates well with its potency as an inducer of the microsomal mixed function oxygenase (MFO) system (Poland and Glover, 1973). HCB and PCB's also induce the hepatic MFO system, though mg/kg quantities of these are required as opposed to the ug/kg quantities of TCDD.

1.3.2. INDUCTION OF THE MIXED FUNCTION OXYGENASE SYSTEM

The hepatic microsomal mixed function oxygenase system is a multicomponent electron transport system which is responsible for the metabolism of endogenous substrates such as steroids as well as exogenous xenobiotics including many drugs and aromatic hydrocarbons. Fractionation of liver microsomes has shown that cytochrome P-450, an NADPH-dependent flavoprotein known as NADPH-cytochrome c (or P-450) reductase, and phosphatidylcholine are the necesary components for in vitro metabolism (Lu, 1976). The cytochrome P-450 moiety is the substrate binding component

and is not unique but, rather, consists of a class of at least six different proteins which may be distinguished spectrophotometrically (Hildebrandt and Estabrook, 1969), immunochemically (Thomas et al, 1976a; 1976b), by their different electrophoretic mobilities (Haugen et al, 1975), and by their different amino acid sequences (Botelho et al, 1979).

Treatment of experimental animals with various drugs causes induction of cytochrome P-450 which requires de novo protein synthesis and which can be blocked with either actinomycin D (Alvares et al, 1968) or cycloheximide (Poland and Glover, 1974). Two patterns of induction have been described: that characteristic of phenobarbital (PB) induction; and that characteristic of 3-methylcholanthrene (3-MC) induction. PB induction causes an increase in the amount of P-450 present in the liver and the cytochrome thus formed has spectral properties similar to the P-450 existing prior to induction, namely a carbon monoxide (CO) binding spectrum with a maximum at 450 - 452 nm and an ethyl isocyanide binding spectrum with peak ratios at 455/430 approximately equal to 0.6. On the other hand, 3-MC-induced cytochrome P-450 (also known as cytochrome P-448) has a CO binding spectrum with a maximum at 447 - 449 nm and ethyl isocyanide 455/430 peak ratios approximately equal to 1.2 (Mannering et al, 1969).

In addition to the spectral differences existing between the cytochrome P-450 induced by PB and that induced by 3-MC, differences in substrate specificity also exist. Cytochrome P-450 induced by PB is more active in metabolising benzphetamine and aminopyrine, and preferentially hydroxylates the 16-alpha position of testosterone, whereas P-448 induced by 3-MC has greater aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity and preferentially hydroxylates testosterone at the 7-alpha position (Conney et al, 1973).

Hexachlorobenzene, polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin have two properties in common. They all cause an experimental porphyria in animals which closely ressembles PCT in man, and they are all inducers of the hepatic MFO system. HCB (Lui et al, 1976) and mixtures of PCB's (Litterst et al, 1972; Alvares et al, 1973) have been shown to be inducers of both P-450 and P-448, but TCDD is a specific inducer of cytochrome P-448 (Poland and Glover, 1974).

The features which determine whether a molecule will induce P-450 or P-448 have been the subject of much work in recent years. Poland et al (1974) found that strains of mice which were genetically resistant to induction of AHH by polycyclic aromatic hydrocarbons such as 3-MC could be induced by TCDD, but a dose 10 to 15 times higher than that

required for responsive strains was needed. These authors suggested that the non-responsive strains of mice had an induction receptor with reduced affinity for the various inducers but, because of the very high affinity of TCDD for the receptor, TCDD (in sufficient concentration) could still bind sufficiently well to elicit a maximum induction response. Further studies revealed that the necessary conditions for a dioxin molecule to induce AHH were that chlorine or bromine atoms occupy at least three of the four lateral ring positions (2,3,7 and 8) and there must be at least one unhalogenated carbon atom.

The induction of both P-450 and P-448 by PCB's has been shown to be due to the presence of multiple isomers in arochlor mixtures. Several recent studies using pure PCB isomers have demonstrated that PCB's with symmetrical halogens at the ortho and para positions induce cytochrome P-450, while those with halogens at the meta and para positions induce cytochrome P-448 (Goldstein et al, 1976; 1977b; Bunyan and Page, 1978; Moore et al, 1978). Interestingly, an asymmetrical polybrominated biphenyl (2,4,5,3',4',5'-hexabromobiphenyl) has been reported to possess both P-450 and P-448 inducing properties (Dannan et al, 1978). Stereochemical studies by Poland and Glover (1977) showed that planar halogenated biphenyls with two adjacent halogens on lateral positions of each ring would

compete with TCDD for specific binding to an hepatic cytosol protein and the extent of competition was proportional to the potency of the molecule as an inducer of cytochrome P-448. The approximate dimensions of such molecules which were capable of displacing TCDD from the cytosol binding species were calculated to be 3 X 10 A.

Evidence that the binding species is the induction receptor for cytochrome P-448 and AHH has been further advanced by these same authors with the finding that specific binding is greatest in hepatic cytosol from mice homozygous for AHH responsiveness, intermediate in heterozygous responsive mice and least in homozygous nonresponsive mice (Poland and Glover, 1976). Also, it has been shown that nuclear uptake of TCDD is probably mediated by the putative receptor since uptake of TCDD into the nucleus is accompanied by a fall in the amount of specific binding in the cytosol (Greenlee and Poland, 1979).

1.4. SUMMARY AND EVALUATION

Porphyria cutanea tarda is an hepatic disease of man caused by a partial block in the pathway of heme biosynthesis between uroporphyrinogen and coproporphyrinogen. Unlike all other porphyrias, an underlying inheritance has been difficult to establish.

However, (again unlike all other types of porphyria) PCT may be acquired by ingestion of HCB, and various other etiologic agents are implicated including ethanol, synthetic estrogens, TCDD, and PCB's. The fact that PCT seems always to occur secondary to liver disease and that all the agents which are associated with the development of PCT in man and the corresponding experimental porphyria in animals are hepatotoxins of general clinical and environmental concern makes the study of the mechanism of PCT important from the point of view that it promises to add to the current understanding of the mechanism of hepatotoxicity of a diverse group of chemical substances.

Much new knowledge concerning PCT has been gained in recent years both from the work done in the course of this thesis and elsewhere. When this work was begun, two untested hypotheses existed to explain the development of PCT. One involved a failure of the liver to maintain porphyrinogens in the reduced state. This would result in oxidation to, and accumulation of, porphyrins with from eight to four carboxyl groups. The other hypothesis proposed a defect in uroporphyrinogen decarboxylase which would result in accumulation of porphyrinogens with from eight to five carboxyl groups.

It was decided that the first part of ths thesis would be devoted to the testing of the above two hypotheses.

Since one hypothesis predicted accumulation of porphyrins while the other predicted accumulation of porphyrinogens, one or other of the theories could be disproved by measuring urinary porphyrin and porphyrinogen excretion during the development of the disease. The experimental model of porphyria in rats caused by HCB was chosen because of the similarity of this model to PCT in humans (Stonard, 1974).

A precondition for such an experiment was the ability to measure urinary porphyrins rapidly and also to separate and quantitate porphyrinogens in urine. Development of such methodology was undertaken as the first task.

Following elimination of the oxidation hypothesis and the development of methodology for measurement of UD, focus was directed to the determination of which biochemical events were necessary for the development of experimental porphyria. The role of induction of cytochrome P-448 was examined using strains of mice which were genetically responsive or nonresponsive to induction. Also, the requirement for tissue iron in the pathogenesis of experimental porphyria was examined since it is known that, in man, depletion of iron stores results in clinical improvement in PCT (Ippen, 1962), and iron overload potentiates the porphyrogenic effect of HCB in rats (Taljaard et al, 1972a; 1972b).

2. EXPERIMENTAL PROCEDURES

2.1. MATERIALS AND REAGENTS

2.1.1. CHEMICALS

All reagents and solvents were of commercial reagent grade and were used without further purification except as specified for individual proceedures. Hexachlorobenzene (practical grade, 97 - 98%) was obtained from either Eastman Chemicals or Aldrich Chemical Company. When determining the effect of impurities on the efficacy of HCB as an etiologic agent for porphyria the Eastman product was used as supplied while the Aldrich HCB was recrystallized three times from hot benzene, which yielded long white crystals with a melting point of 230°- 231°C.

2.1.2. SPECIAL DIETS

Diets containing various concentrations of HCB which were used to produce experimental porphyria in rats were prepared from powdered laboratory chow (Purina Laboratory Chow) and HCB crystals which had been ground into a fine powder with a mortar and pestle. A weighed amount of HCB was thoroughly mixed with approximately 500 g of the powdered

chow which was, in turn, mixed with more powdered chow to a total weight of four kilograms. This diet was fed (in bowls) to the animals as a powdered mixture.

Iron deficient diet was purchased in powdered form from Nutritional Biochemicals (# 902199). To facilitate feeding to mice, this diet was mixed with water to a doughy consistency, pressed into aluminum pans approximately one-half inch deep and dehydrated overnight at 90°C. The resultant slab was broken into small pieces and fed to the mice in the same way as regular pelletized diet. When supplementation of the diet with iron was necessary, the appropriate amount of ferrous sulphate was dissolved in the water used to make the dough.

2.1.3. ANIMALS

Studies in experimental porphyria were conducted using both male and female rats as specified in individual experiments. In all cases young adult Wistars (Woodlyn Farms, Guelph, Ontario) weighing approximately 200 g at the beginning of the experiment were used.

All mice employed were purchased from Jackson Laboratories (Bar Harbor, Maine). Strains consisted of C57BL/6J, DBA/2J and a back-crossed variety, D2(B6D2)F1/J which was obtained by breeding the F1 progeny of C57BL/6J X

DBA/2J with DBA/2J. All breeding of mice was done by Jackson Laboratories.

2.2. PREPARATION OF PORPHYRIN STANDARDS

Standard solutions of uroporphyrin and coproporphyrin methyl esters were prepared by dissolving esterified porphyrin in chloroform and determining the concentration from the absorbance at the Soret maximum using the extinction coefficients of Falk (1964). Porphyrin free acids were obtained by hydrolysis of the methyl esters overnight in a small volume of HCl (7.5 M). Appropriate concentrations were obtained by dilution following measurement of the absorbance in HCl (1.0 M).

Partially decarboxylated porphyrins to be used as standards with five, six, and seven carboxymethyl groups were made from uroporphyrin I by heating at 180°C for 60 minutes, as described by With (1976). To obtain enriched mixtures of a given porphyrin, the heating time was increased to 180 minutes for 5-carboxyl porphyrin or decreased to 15 minutes for 7-carboxyl porphyrin. Individual porphyrins were purified by preparative thin layer chromatography described below. Standard solutions were prepared as above assuming a linear increase in the extinction coefficients of porphyrins with four carboxyl

groups (470 $mM^{-1} cm^{-1}$) to eight carboxyl groups (505 $mM^{-1} cm^{-1}$) (Tomio et al, 1970).

2.3. PREPARATION OF PORPHYRINOGENS

Porphyrinogens were prepared from porphyrins using either of two methods: reduction by sodium amalgam; or reduction by sodium borohydride. Using the sodium borohydride method, approximately 0.5 ml of a dilute NaOH solution of free porphyrin (ca. 0.25 mg/ml) was added to a vial (Reactivial) in an atmosphere of nitrogen. Five or six drops of a solution of sodium borohydride (1.0 M) were added and the solution shaken gently. HCl (0.5 M) was then added dropwise until the solution continued bubbling as hydrogen was produced in the reaction. At this point, a series of colour changes occurred from red to brown, green, yellow and, finally, to a clear colourless solution. Complete reduction was indicated when the solution showed no fluorescence in longwave ultraviolet light.

Reduction of porphyrins to porphyrinogens with sodium amalgam was based on the procedure of Mauzerall and Granick (1958). A small amount of sodium amalgam was added to 0.5 ml of a solution of porphyrin in dilute NaOH. The vial was flushed with nitrogen and sealed. After vigorous shaking, fluorescence disappeared indicating complete

reduction.

Porphyrinogens prepared by either of the above two methods were stable for some hours in the absence of light and oxygen. However, oxidation was rapid in aerobic solutions in the absence of reducing agents, especially under acidic conditions. Quantitative recovery of the porphyrin initially present could be obtained by oxidation with either iodine or hydrogen peroxide, provided that EDTA (1.0 mM) was incorporated into the solution.

2.4. QUANTITATION OF PORPHYRINS BY DERIVATIVE SPECTROSCOPY

The experiments planned to determine the pattern of porphyrin and porphyrinogen excretion during the development of HCB porphyria required quantitative porphyrin measurement in a large number of urine specimens. Methods available for such an analysis included solvent extraction procedures designed to separate a more polar porphyrin fraction, designated "uroporphyrins" and less polar constituents, "coproporphyrins" (Rimington, 1961); and quantitative thin layer chromatographic methods performed on derivatized porphyrins similarly extracted from the urines (Doss, 1971). These methods were unsatisfactory for two reasons: firstly, complete extraction from urine, separation and quantitation of material with the wide range of polarity shown by

uroporphyrin and coproporphyrin is difficult, especially for rat urine which contains protein which tends to cause emulsions; secondly, the time involved for such procedures makes the analysis of large numbers of samples impractical, if not impossible. It was decided, therefore, to attempt to develop a quantitative method in which porphyrin measurement could be made directly on urine without prior extraction and derivatization.

The absorption spectrum of porphyrins in HCl is characterized by two visible bands and a sharp Soret maximum in the near ultraviolet with a very high extinction coefficient. However, direct measurement of urine porphyrin using conventional absorptiometric techinques is not possible because of the presence of impurities conferring nonspecific absorbance which increases towards shorter wavelengths. Extraction of urine with, for example, ion exchange resin as recommended by Doss (1974) reduces but does not eliminate such nonspecific absorption in the region of the Soret band and quantitation requires correction of absorbance measurements using techniques suggested by With (1955). Such methods are suitable, clinically, as screens for abnormal porphyrin excretion but the inherent errors were judged to be too large and the time required too great for such methods to be useful in the experiments planned. Also, a distinction between coproporphyrin and uroporphyrin

is not readily apparent and this was an obligatory requirement.

The problem of increasing background absorbance incurred when scanning toward the ultraviolet may be overcome with the use of derivative spectroscopy since the slope, rather than the absolute absorbance is recorded. Absorbance peaks become more apparent since they appear as biphasic deflections in a flatter baseline rather than shoulders in the record of the absolute absorption spectrum.

Two similar methods have been developed for direct measurement of porphyrin in unextracted urine specimens. One procedure is based on the optically generated first derivative of the ultraviolet-visible absorption spectrum of acidified urine. This method is both rapid and accurate, avoids troublesome extraction procedures, and provides an estimate of the ratio of uroporphyrin to coproporphyrin in the solution. The recent introduction of units which generate first- and second-derivative spectra electronically from the output of scanning spectrophotometers made possible the development of a second method which eliminated the requirement for a dual wavelength spectrophotometer.

2.4.1. FIRST DERIVATIVE SPECTROSCOPY

The two requirements necessary in the methodology used to measure urinary porphyrins were: (a) an accurate and sensitive quantitation of total urine porphyrins and (b) a distinction between the two major porphyrin constituents, uroporphyrin and coproporphyrin. Both of these criteria were met by first derivative spectroscopy.

The procedure used for porphyrin measurement in urine specimens was the following: 1.0 ml of urine was added to 4.0 ml of HCl (1.25 M, containing EDTA, 1.0 mM); one drop of hydrogen peroxide (30%, w/v) was added to oxidize any porphyrinogens present (EDTA was found to prevent degradation of porphyrins in the presence of peroxide). The resultant solution was placed in a cuvette and scanned in the derivative mode from $(\lambda_1 + \lambda_2)/2$ = 450 nm to $(\lambda_1 + \lambda_2)/2$ = 375 nm using a Perkin-Elmer model 356 dual wavelength spectrophotometer operating in the derivative mode with a slit width = 1.0 nm half band-pass and $\Delta\lambda$ = 5.0 nm. The peak plus trough height was measured as well as the wavelength at which this record intersected a reconstructed baseline. For very dilute urine samples, 2.0 ml of urine was added to 1.0 ml of HCl (3.0 M). The limit of detection using this method was found to be approximately 12.0 ng of porphyrin per ml of urine (0.018 μM) corresponding to an

absorbance difference of 0.006 absorbance units.

The size of the optically determined first derivative deflection is directly proportional to the size of the absolute absorption peak and is, therefore, directly related to concentration. However, whereas the extinction coefficient for absolute spectra is a physical constant, the apparent "extinction coefficient" for derivative spectra is dependent upon instrument settings. By choosing appropriate instrument conditions, namely a slit width of 1.0 nm and a difference between the two wavelengths (reference and sample beams) of 5.0 nm, the size of the first derivative deflection for porphyrins was found not to be critically influenced by the precision of these settings.

An example of a solution of coproporphyrin scanned in both the absolute and derivative modes is shown in Figure 4. From this it may be seen that using the instrument settings described above the first derivative spectrum consists of a peak and a trough. The apparent "extinction coefficient" of coproporphyrin as measured from peak to trough was found to be 1.06 times the absolute extinction; for uroporphyrin the factor was 1.13 times. The point at which the spectrum intersects a reconstructed baseline corresponds to the wavelength at which the slope of the absolute spectrum is zero, i.e. at the absorption maximum. This feature of the derivative spectrum was used to

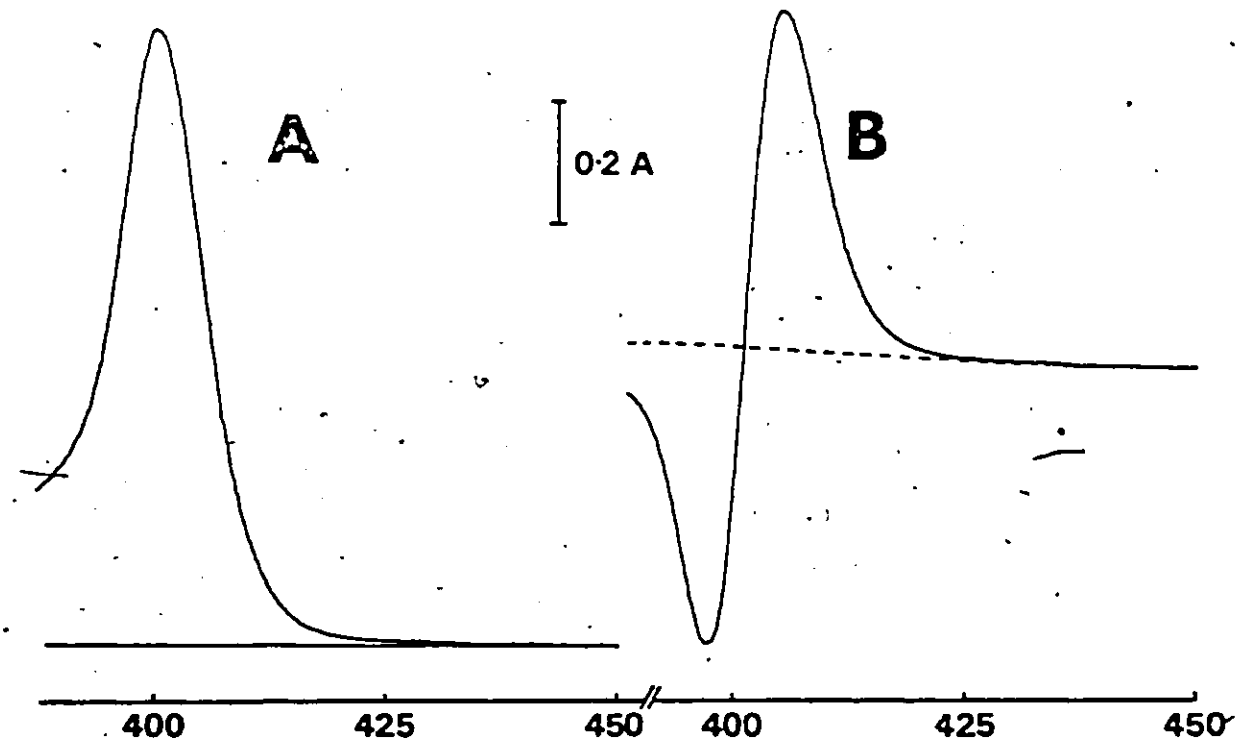


Figure 4: First derivative of porphyrin absorption spectrum. A solution of coproporphyrin (2.15 μM) in HCl (1.0 M) was scanned in conventional split (A) beam and derivative (B) modes using a Perkin-Elmer model 356 spectrophotometer.

distinguish between coproporphyrin and uroporphyrin.

Since the absorption maximum for coproporphyrin is 5.0 nm removed from that for uroporphyrin it should be possible to determine whether a sample contained either of the above porphyrins or a mixture of the two simply by observing the point at which the spectrum intersected a reconstructed baseline. As shown in Figure 5, this was found to be true.

Two other properties of derivative spectra are illustrated in Figure 5. One is that the apparent "extinction coefficients" of mixtures of coproporphyrin and uroporphyrin are lower than for either of the pure porphyrins. The other is that the wavelength at which the spectrum intersects a reconstructed baseline is a function of the composition of the porphyrin mixture but is not linear. Both of these properties are a result of the assymmetric broadening of the zero order absorption which occurs when mixtures of porphyrins are present.

A major advantage of direct measurement of porphyrins in a urine sample is that losses during the course of extraction procedures do not occur. Since it was found that whether purified porphyrin was added to dilute HCl or to acidified urine made no difference to the absorption spectrum or to its derivative, provided that

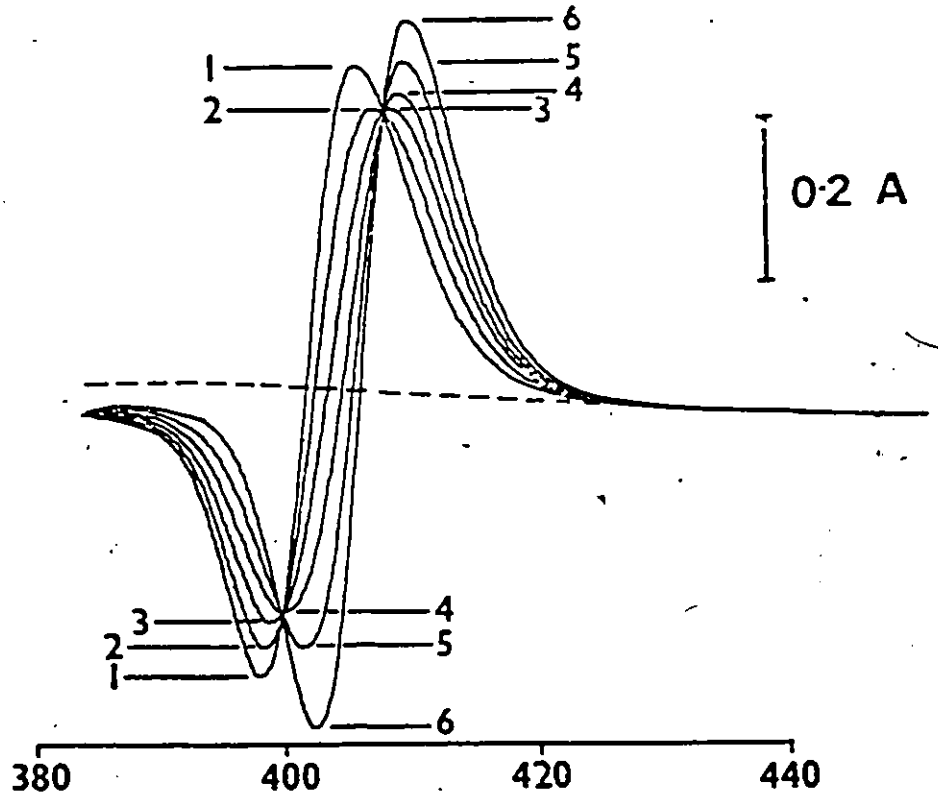


Figure 5: Approximate first derivative of the Soret band region of equimolar (2.15 μM) varying ratios of solutions of coproporphyrin III and uroporphyrin I in HCl (1.0 M). The composition of the mixtures were: coproporphyrin alone (1); 4:1 (2); 3:2 (3); 2:3 (4); 1:4 (5); and uroporphyrin alone (6).

acidified urine served as a reference solution for zero order measurements and the intrinsic urine porphyrin was subtracted from the derivative result, the measurement of porphyrins in urine made 1.0 M with respect to HCl, using the derivative technique, accurately reflected the porphyrin concentration in the sample.

2.4.2. SECOND DERIVATIVE SPECTROSCOPY

First derivative spectroscopy, as described above, provided a fast, accurate and economic method for measuring total urinary porphyrins. Since this method was developed, equipment has become available which is capable of generating first and second derivative spectra electronically from the output of conventional scanning spectrophotometers. This has made the derivative technique for porphyrin measurement much more widely applicable since it eliminated the requirement for a dual wavelength spectrophotometer. Evaluation of the use of such equipment in an assay for urine porphyrins led to the development of a procedure using second derivative, rather than first derivative, spectroscopy (Jones and Sweeney, 1979).

2.5. SEPARATION AND QUANTITATION OF PORPHYRINOGENS

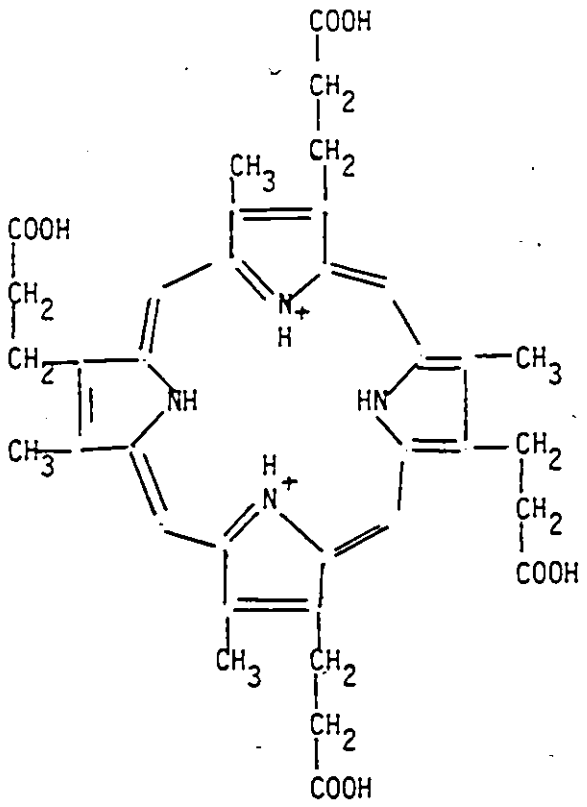
No methods were previously available for quantitation of porphyrinogens in urine. Two main problems had to be overcome to achieve this end: Firstly, porphyrinogens are unstable in urine and rapidly oxidize to porphyrins. Secondly, separation of porphyrinogens from porphyrins was required.

Premature oxidation of porphyrinogens was found to be prevented by the introduction of ascorbic acid into the urine to act as an antioxidant. Ascorbate was chosen because of its solubility in water and lack of absorbance in the region of the porphyrin Soret band and also because ascorbate is an endogenous constituent of rat urine and is present in large quantities in the urine of rats treated with inducers of the microsomal cytochrome P-450 (P-448) system. It was found that solutions of porphyrinogens containing ascorbic acid (ca. 10 mg/ml) and EDTA (1.0 mM) were stable for at least 30 minutes, even at pH 0.5.

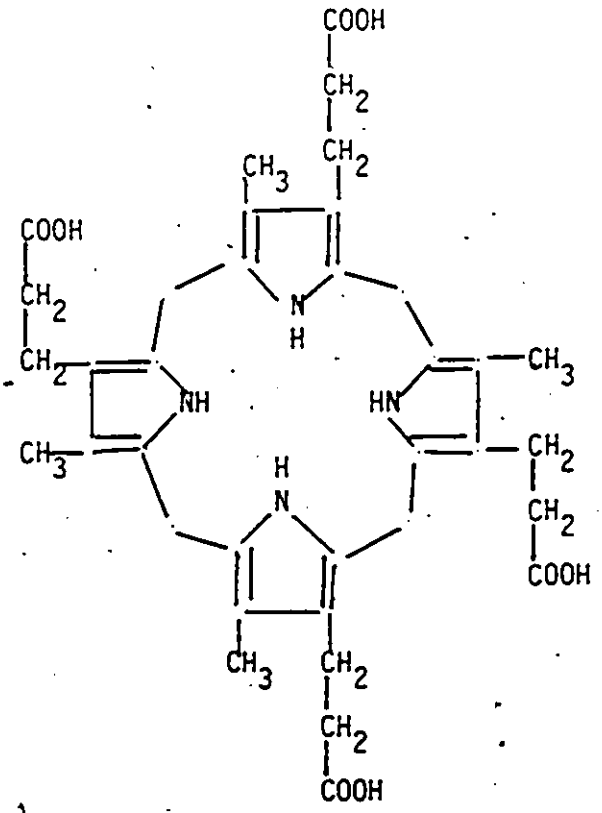
Separation of porphyrinogens from porphyrins was based on differences in the organic/aqueous partition coefficients at low pH. Because of the completely aromatic macrocycle in the porphyrins, two of the pyrrole nitrogens may be protonated at low pH and confer upon the molecule a positive charge (Figure 6a). The porphyrinogen macrocycle,

however, is not aromatic and all pyrrole nitrogens are equivalent (Figure 6b). Each nitrogen atom has its electrons in sp orbitals with two sigma bonds to carbon and one to hydrogen. The remaining pair of electrons is unavailable for sharing with a proton since it is conjugated with the system in the two double bonds of the pyrrole. Thus, while porphyrins develop a positive charge at low pH and favour an aqueous solvent, porphyrinogens remain uncharged and may be extracted into an organic phase.

The organic/aqueous partition coefficients for porphyrins and porphyrinogens were determined by the following procedure: an aqueous solution of uroporphyrinogen I (3.1 nmol) was added to about 2.0 ml of a solution of EDTA (1.0 mM) containing ascorbic acid (ca. 10 mg/ml). The pH was then adjusted with HCl (1.0 M) and/or saturated sodium acetate, the volume noted, and the extraction effected once with an equal volume of ethyl acetate. Complete phase separation was assured by centrifugation at 2,000 g in a bench-top centrifuge for 1.0 minute. To the ethyl acetate layer, two volumes of petroleum ether were added and the resultant solution was extracted three times with 1.0 ml aliquots of HCl (1.0 M), each containing EDTA (1.0 mM) and ~~one drop~~ of 30% hydrogen peroxide. The percentage of total porphyrinogen recovered as porphyrin was determined by comparing the spectrum with



(a) COPROPORPHYRIN III



(b) COPROPORPHYRINOGEN III

Figure 6

that of an equal amount of porphyrinogen added to 3.0 ml of HCl containing EDTA and peroxide. An identical procedure was used for coproporphyrinogen III. The oxidized forms of these porphyrins were treated in a similar fashion, except that re-oxidation with hydrogen peroxide was omitted.

Figure 7 shows the organic/aqueous partition coefficients between ethyl acetate and water of coproporphyrin III and coproporphyrinogen III at various pH. Whereas coproporphyrin was maximally extractable into organic solvent at about pH 3.2 and was completely unextractable at pH 0.5, coproporphyrinogen was best extracted from water at pH 0.5. Uroporphyrinogen I also proved to be most easily extracted into ethyl acetate at very low pH (Figure 8). Uroporphyrin I was not well extracted at any pH but formed a precipitate between pH 2.0 and 4.0.

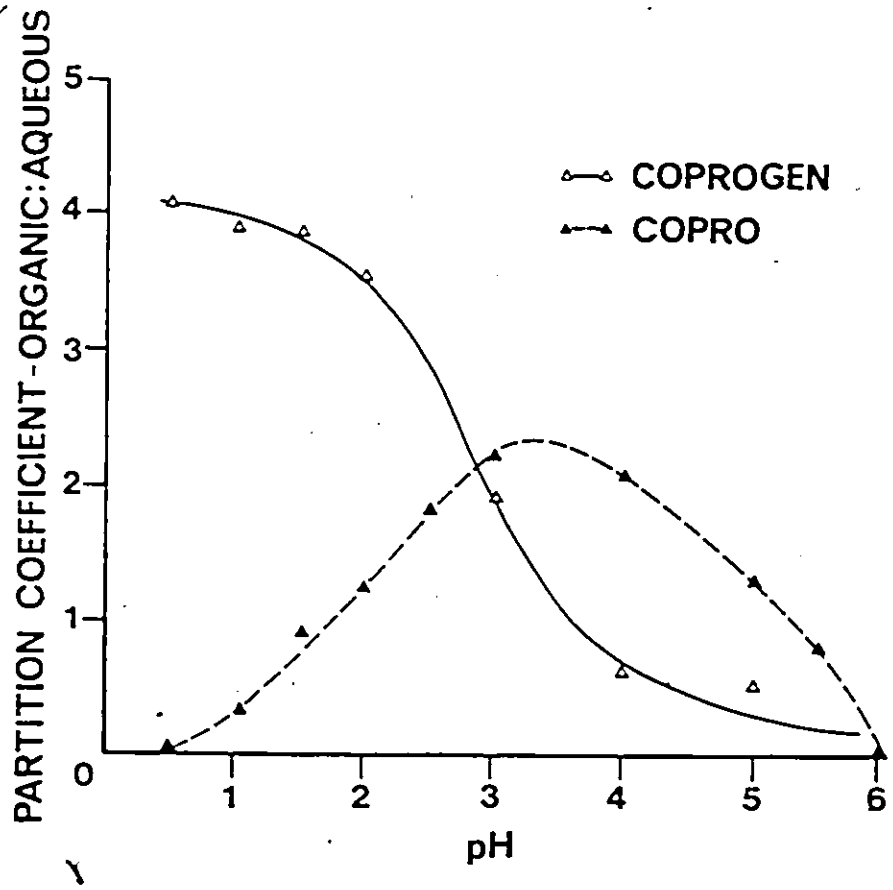


Figure 7: The pH dependence of the partition coefficients of coproporphyrinogen III (COPROGEN) and coproporphyrin III (COPRO) between ethyl acetate and aqueous phase

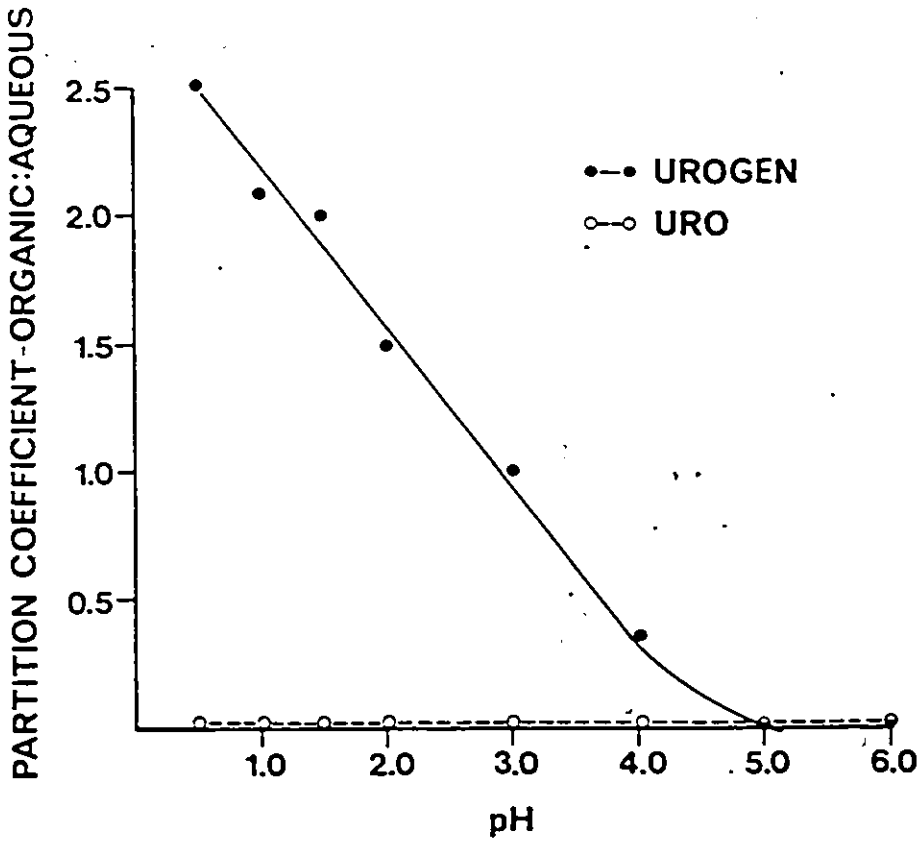


Figure 8: The pH dependence of the partition of uroporphyrinogen I (UROGEN) between ethyl acetate and aqueous phase. Uroporphyrin (URO) was practically insoluble in ethyl acetate over the range of pH examined.

On the basis of the above extraction profile the following procedure was used to extract porphyrinogens from urine: 1.0 ml of urine was added to 1.0 ml of a solution of EDTA (1.0 mM) and ascorbic acid (ca. 10 mg/ml). The mixture was then acidified to pH 0.5 with HCl (1.0 M) also containing EDTA (1.0 mM). With minimal delay, the solution was extracted three times with equal volumes of ethyl acetate. Two volumes of petroleum ether (boiling range 30°-60° C) were added to the combined extracts followed by two drops of ethanolic iodine (1.0%, w/v) to oxidize the porphyrinogens. The organic solvent was then reextracted three times with 1.0 ml aliquots of HCl (1.0 M) and the porphyrin present in the pooled extracts was measured using first derivative spectroscopy. Addition of one drop of 30% hydrogen peroxide to each of the HCl aliquots instead of iodine to the organic phase gave equally good oxidation and extraction.

Using the above procedure, the recovery of porphyrinogens from rat urine was investigated. Pooled urine samples from normal rats had from 0.078 to 0.78 nmol of uroporphyrinogen or 0.103 to 1.03 nmol of coproporphyrinogen in volumes of not more than 30 µl added to 1.0 ml aliquots of urine. Recoveries exceeded 87% in all instances and are given in Table 1. When porphyrinogen was added to normal urine in the absence of ascorbic acid and

Table 1: Recovery of porphyrinogens from urine

	COPROPORPHYRINOGEN					UROPORPHYRINOGEN				
Tube number	0	1	2	3	4	0	1	2	3	4
Added (nanomoles)	0	.103	.207	.517	1.034	0	.078	.156	.390	.780
Recovered (nanomoles)	.021	.123	.217	.506	.889	.014	.092	.171	.369	.760
% Recovery of added material	-	98.2	95.2	94.3	87.0	-	100	100	91.2	95.7

oxidized with hydrogen peroxide prior to extraction, no porphyrinogen was found in the extract.

2.6. ANALYSIS OF PORPHYRIN MIXTURES BY THIN LAYER CHROMATOGRAPHY

Separation of porphyrin methyl esters was carried out using thin layer chromatography (TLC). Quantitation of the separated porphyrin methyl esters was accomplished by scanning the TLC plates for absorbance at 405 nm. For urine, aliquots (3.0 ml) of 24 hour collections were quick-frozen in liquid nitrogen and freeze-dried in vacuo . Esterification was then effected with 15.0 ml of methanol-sulfuric acid (19:1, v/v) for 24 hours in the dark. The porphyrin methyl esters were then transferred to chloroform which was concentrated by distillation at 40°- 50° C under reduced pressure. The remaining chloroform was removed by heating on a sand bath at 40° - 50°C under a stream of nitrogen. If not used immediately, the porphyrin methyl esters were stored at -20°C in the dark.

For feces, 24 hour collections from rats were dried in vacuo over KOH in the dark and stored at -20°C until further processed. After weighing the dried samples, 0.5 g aliquots were powdered in a porcelain mortar, esterified (methanol-sulfuric acid, as above) and

transferred to chloroform. To reduce contamination with lipid, the mixtures were hydrolysed with HCl (7.5 M) (1.0 ml) for 24 hours at room temperature, in the dark. After-shaking with chloroform (3.0 ml) and centrifugation to obtain optimal phase separation, aliquots (0.10 ml) of the aqueous phase were taken to dryness in vacuo over KOH and the porphyrins dissolved in a minimum volume of tetrahydrofuran/pyridine (10:1, v/v). Reesterification followed the addition of an excess of ethereal diazomethane, prepared according to Vogel (1967).

Porphyrin methyl esters, dissolved in a known volume of chloroform, were spotted onto silica gel plates (E.M. Laboratories) with a 0.01 ml Hamilton syringe. Development was carried out in the dark in tanks previously equilibrated with solvent vapour. The solvent system used was modified from Doss (1970) and consisted of: benzene:ethyl acetate:methanol:kerosene (170:40:3:10, v/v). The kerosene played no part in the separation but was incorporated to keep the plates from drying out prior to quantitation of the porphyrins. Porphyrin-containing spots were quantitated from records of absorbance at 405 nm and standards (which were run simultaneously to determine recovery) using the TLC scanning attachment of an Aminco-Bowman spectrophotofluorometer operating in the transmission mode and recording the logarithm of the photometric output. The

extinction coefficients of 5-carboxyl, 6-carboxyl, and 7-carboxyl porphyrins were interpolated between those of uroporphyrin and coproporphyrin assuming a linear relationship between the number of carboxyl groups and the extinction coefficients (Tomio et al, 1970). Isocoproporphyrin was quantitated using the same physical constants as coproporphyrin.

An example of a scan of fecal porphyrins separated by TLC as described above is shown in Figure 9. Areas under the peaks were measured and the amount of each porphyrin was determined using the calibration curves shown in Figure 10.

2.7. SEPARATION OF PORPHYRINS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

A faster and more convenient method for separating and quantitating porphyrin methyl esters is by use of high pressure liquid chromatography (HPLC). This method is especially suitable for porphyrin samples which are relatively free of contaminating substances and was, therefore, used for quantitation of porphyrins in the assay for uroporphyrinogen decarboxylase.

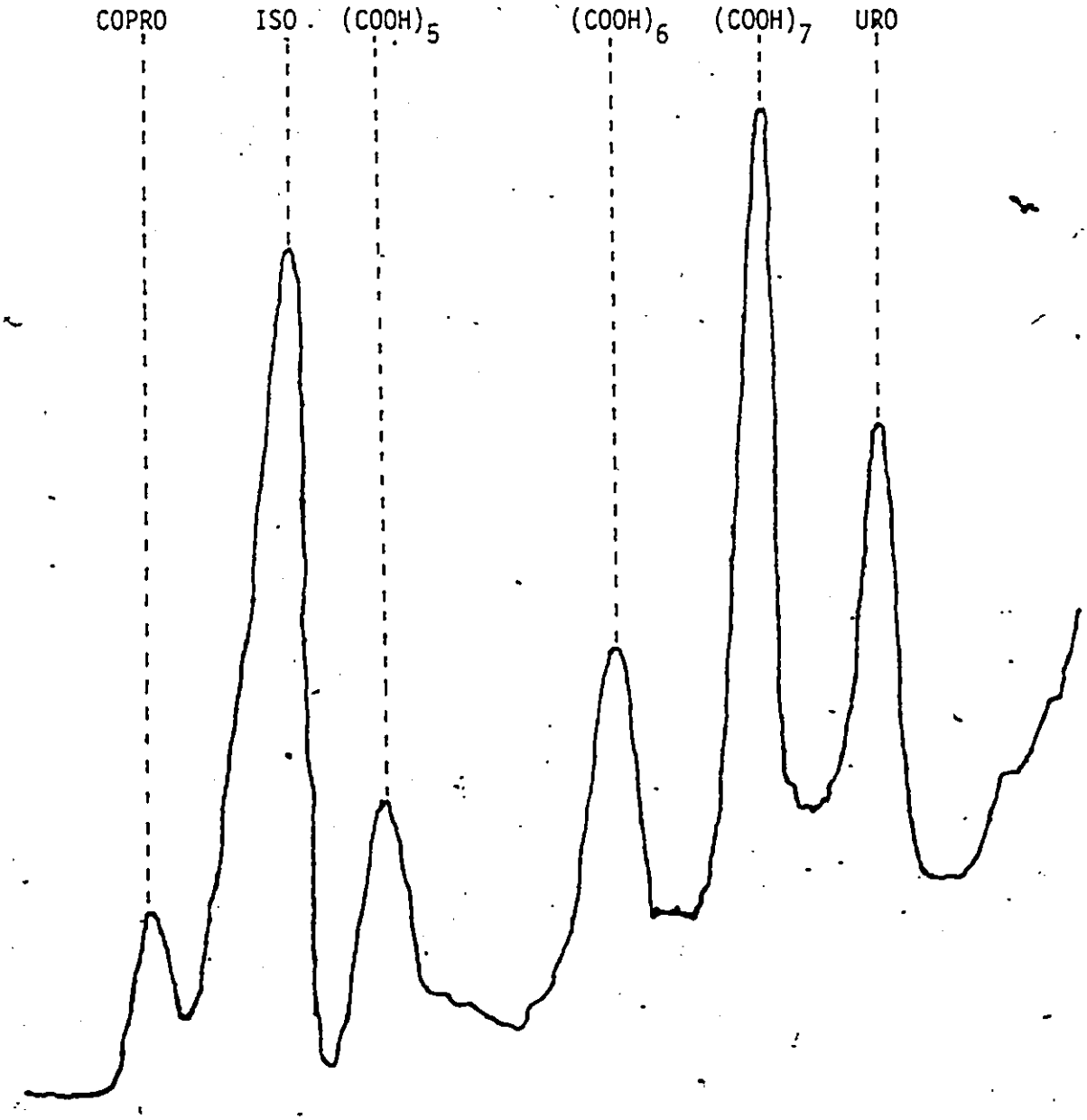


Figure 9: Example of fecal porphyrins from a patient with PCT. Esterification and separation by thin layer chromatography was effected as described in the text. Note the large peak for isocoproporphyrin. This peak is barely detectable in normal feces.

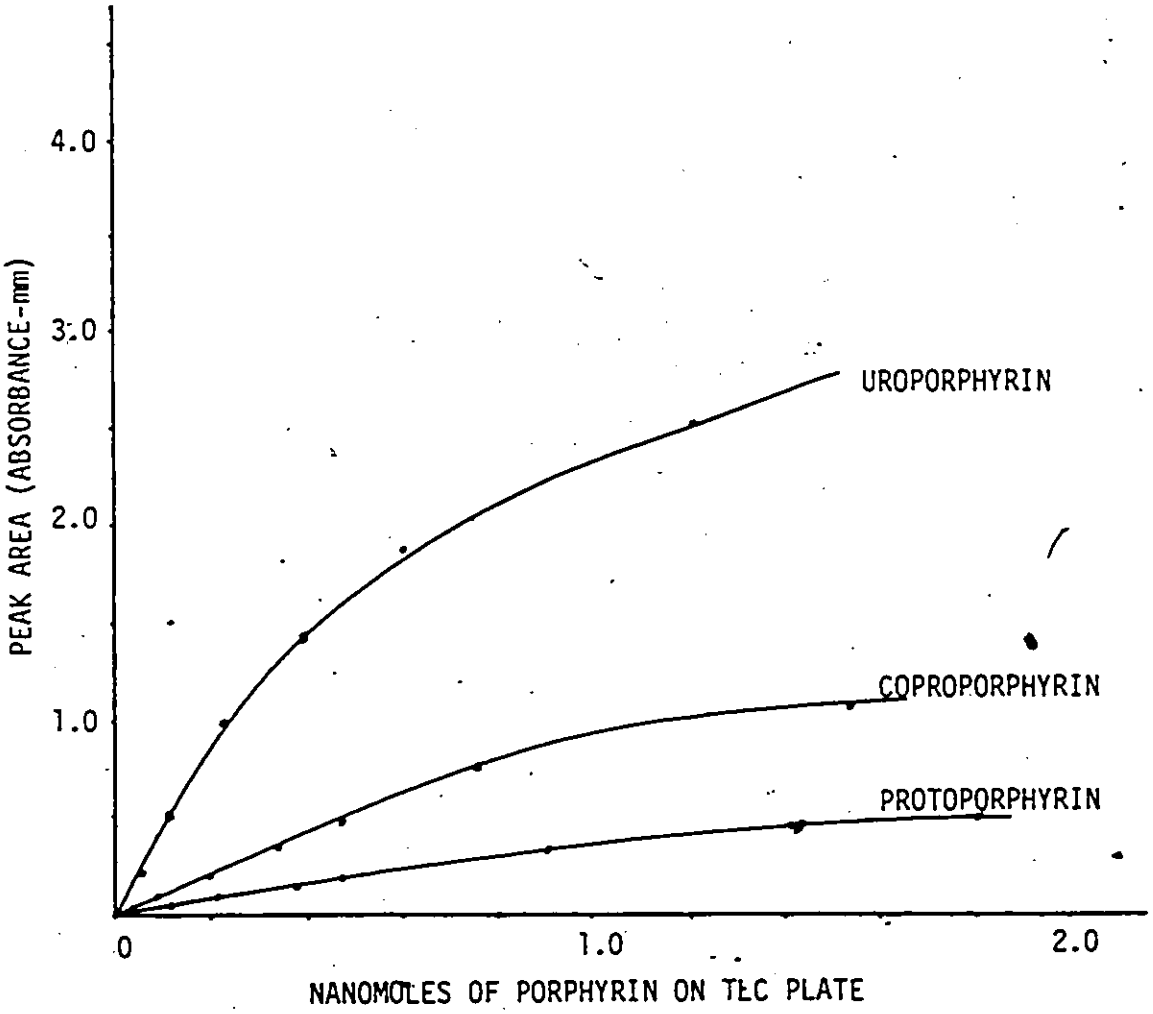


Figure 10: Calibration curves for porphyrins quantitated by thin layer chromatography. Known quantities of porphyrin methyl esters were applied to tlc plates which were subsequently developed in a modified Doss solvent and scanned for absorbance at 405 nm.

In all cases, a 10 µm silica column and a Waters model 440 Absorbance Detector (405 nm) were used. Two solvent systems were employed. For separating 5-carboxyl and 4-carboxyl porphyrin methyl esters the system recommended by Jackson et al (1976) consisting of cyclohexane:ethyl acetate (7:3, v/v) worked quite well, eluting both porphyrins, well separated, in approximately 15 minutes at a flow rate of 1.0 ml/minute. However, elution of more complex mixtures of porphyrin methyl esters took an inordinately long time (up to 60 minutes for uroporphyrin) and the more highly carboxylated compounds appeared as very broad peaks. Therefore, a new solvent system was developed consisting of: cyclohexane:methanol:ethyl acetate (166:20:14, v/v). This separated mixtures of porphyrin methyl esters with from eight to four carboxyl groups into sharp, well-defined peaks within 15 minutes at a flow rate of 1.0 ml/minute. An example of the separation is shown in Figure 11.

2.8. ASSAY FOR UROPORPHYRINOGEN DECARBOXYLASE

Uroporphyrinogen decarboxylase (UD) was measured in whole liver homogenate, 9000 g supernatant, 100,000 g supernatant, or whole blood hemolysates either by monitoring the conversion of 5-carboxyl porphyrinogen to coproporphyrinogen using a method modified from Elder (1976)

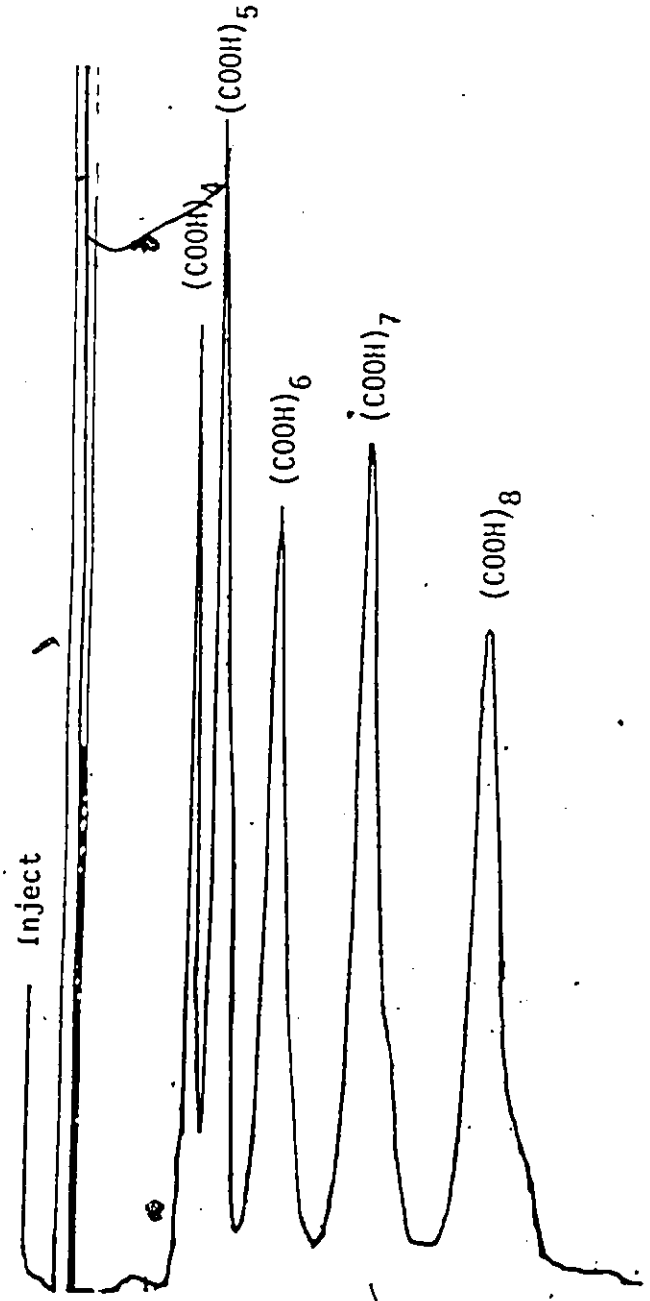


Figure 11: Separation of porphyrin methyl esters with from eight to four carboxyl groups by high pressure liquid chromatography

or by measuring the 7-carboxyl to 4-carboxyl products formed from uroporphyrinogen as a substrate.

A solution of substrate was prepared by reduction of the appropriate porphyrin with 3% sodium amalgam and titrated to pH 6.8 using HCl (0.5 M). 2.0 nmol. of the porphyrinogen (in a volume less than 50 ul) was added to a reaction mixture at 0°C consisting of 0.1 ml whole liver homogenate (10%, w/v) (or other enzyme source) and phosphate buffer (0.1 M, pH 6.8) containing EDTA (1.0 mM) and reduced GSH (1.0 mg/ml) to a total volume of 0.3 ml. The mixture was gassed with nitrogen and incubated anaerobically in the dark at 37° C (15.0 minutes for 5-carboxyl substrate; 30.0 minutes for 8-carboxyl substrate). The reaction was then stopped and the porphyrinogen oxidized by the addition of 1.0 ml of HCl (1.0 M) containing EDTA (1.0 mM) and one drop of 30% hydrogen peroxide. After sedimenting the protein by centrifugation at 5000 g for two minutes, the supernatant was adjusted to pH 3.0 to 3.2 and the porphyrin extracted with ethyl acetate (3.0 ml X 2). Esterification was effected with the addition of excess diazomethane and the solution was evaporated to a small volume (ca. 0.2 ml). Conversion of 5-carboxyl porphyrinogen to coproporphyrinogen was determined by measuring the relative peak heights produced after separaion by HPLC as described above. Standardized mixtures of porphyrin methyl esters were interspersed with

the samples as they were run.

When more complex mixtures of porphyrins were produced from an initial 8-carboxyl substrate, 1.0 nmol. of 6-carboxyl porphyrin was added as an internal standard immediately after the reaction was stopped with HCl. To determine the amount of endogenous 6-carboxyl porphyrin present, an identical sample was examined without addition of the internal standard. However, accumulation of 6-carboxyl porphyrinogen under the reaction conditions used was always found to be negligible.

2.9. DETERMINATION OF ARYL HYDROCARBON HYDROXYLASE ACTIVITY

Aryl hydrocarbon (benzo(a)pyrene) hydroxylase was measured using the method of Poland and Glover (1974) with slight modifications. The assay mixture contained a total volume of 1.0 ml which included 62.5 mol Tris-HCl buffer, pH 7.2; 3.75 mol $MgCl_2$; bovine serum albumin, 1.25 mg/ml; 0.1 ml 9000 g supernatant (equivalent to 5.0 mg of liver); and 50 µl of a solution of benzo(a)pyrene (2.0 mM, in methanol) which was added to initiate the reaction. The mixture was incubated for 10 minutes in a covered, shaking water bath at 37°C and was terminated by adding 1.0 ml of acetone and 3.25 ml of hexane. The mixture was shaken vigorously in the dark to extract benzo(a)pyrene and

metabolites. A 1.0 ml aliquot of the organic phase was extracted with 3.0 ml of NaOH (1.0 M), and the alkali extractable metabolites were examined in an Aminco-Bowman model 4-8202 SPF Spectrophotofluorometer. Fluorescence corresponding to 3-hydroxybenzo(a)pyrene was measured using excitation set at 388 nm and emission at 520 nm. The fluorescence of a sample blank, to which the benzo(a)pyrene was added immediately prior to the acetone and hexane, was subtracted from the fluorescence of each experimental sample. The fluorometer was calibrated with a standard solution of quinine sulphate and 3-hydroxybenzo(a)pyrene. One unit of AHH was defined as the amount of enzyme catalysing the formation of hydroxylated product causing fluorescence equivalent to that of 1.0 pmol of 3-hydroxybenzo(a)pyrene per minute, at 37°C.

2.10. ESTIMATION OF REDUCED GLUTATHIONE

Reduced glutathione was measured in liver homogenates following precipitation of protein with metaphosphoric acid. Solutions were adjusted to pH 8.0, and o-phthalaldehyde was added and allowed to react at room temperature, in the dark. Fluorescence was measured after 20 minutes using excitation and emission wavelengths of 350 nm and 420 nm, respectively (Cohn and Lyle, 1966).

2.11. ASSAY FOR AMINOLEVULINATE SYNTHETASE

Aminolevulinic acid synthetase (ALA-s) was measured in rat liver as described by de Matteis (1971). After incubation of a whole liver homogenate at 37° C in the presence of glycine and pyridoxal phosphate, proteins were precipitated with trichloroacetic acid (TCA) and a pyrrole was formed by reaction of the ALA with excess 2,4-pentanedione. Interfering thiols were eliminated by addition of N-ethylmaleimide; aminoacetone and excess acetyl acetone were extracted with ether. The pyrrole was measured by reading absorbance at 553 nm following reaction with modified Ehrlich reagent.

2.12. MEASUREMENT OF SERUM GLUTAMATE-OXALOACETATE TRANSAMINASE

Serum glutamate-oxaloacetate transaminase (SGOT) was measured spectrophotometrically according to the method described by Sigma (1975) following the disappearance of absorbance due to NADH at 340 nm in the reaction with oxaloacetate in the presence of excess malate dehydrogenase. Under conditions of excess malate dehydrogenase, the reaction of aspartate with α-ketoglutarate, catalysed by SGOT, becomes rate limiting, and the disappearance of NADH reflects the activity of SGOT.

2.13. HEMOGLOBIN MEASUREMENT

Hemoglobin was determined spectrophotometrically by measuring absorbance at 540 nm following conversion to cyanmethemoglobin with a solution of potassium ferricyanide and potassium cyanide (Drabkin and Austin, 1935).

2.14. IRON DETERMINATION

Iron was measured in tissue samples after digestion with nitric acid and hydrochloric acid. Evaporated samples were dissolved in water and then treated with trichloroacetic acid and hydrazine according to Henry et al. (1958) and colour was measured spectrophotometrically at 535 nm following addition of bathophenanthroline.

2.15. MEASUREMENT OF CYTOCHROME P-450

Cytochrome P-450 was assayed by measuring the carbon monoxide difference spectra of dithionite-reduced suspensions of microsomes according to the method of Omura and Sato (1964).

2.16. ETHYL ISOCYANIDE BINDING SPECTRA

Ethyl isocyanide binding spectra were obtained as difference spectra of dithionite-reduced suspensions of microsomes at pH 7.5 (Sladek and Mannering, 1966).

2.17. PROTEIN DETERMINATION

Protein was measured spectrophotometrically by the method of Sutherland et al (1949) using bovine serum albumin as the standard.

2.18. STATISTICS

Statistical significance was determined using the Student's t test.

3. RESULTS:

3.1. EFFECT OF IMPURITIES ON THE ABILITY OF HEXACHLOROBENZENE TO CAUSE EXPERIMENTAL PORPHYRIA IN RATS

After developing the methodology for rapidly measuring porphyrins in urine and separation and quantitation of porphyrinogens, an experiment was designed to follow the development of porphyria caused by hexachlorobenzene in rats.

Male Wistar rats, approximately 200 g in weight were divided into two groups of twelve and treated with a diet of either powdered laboratory chow (controls) or an identical diet containing recrystalized HCB (0.25%, w/w) (see Experimental Proceedures, Special Diets).

Urine was collected weekly from individual rats directly into containers which were cooled in liquid nitrogen and kept dark to minimize oxidation of porphyrinogens during the 24 hour collection period. Following collection of the urine, porphyrins and porphyrinogens were assayed with minimum delay using the proceedures described above.

It was found, however, that after twelve weeks of treatment with HCB porphyrin and porphyrinogen excretion was elevated only two to four times over the control value and that the predominant constituent was coproporphyrin(ogen). In other words, the rats had not developed the typical HCB porphyria which was expected.

Since experimental porphyria had previously been produced routinely by treating rats with HCB the lack of response in this case was puzzling. The only difference in treatment of the animals was that the HCB had been recrystallized three times from hot benzene, whereas previously, crude practical grade material had been used.

The finding that recrystallized HCB failed to cause porphyria in male rats whereas practical grade chemical had produced porphyria suggested that impurities present in the crude HCB were either directly responsible for the experimental porphyria, or they may augment the porphyrogenic effect of HCB. Experiments were designed to test this possibility.

To determine whether impurities in practical grade HCB contributed to the porphyrogenic effect of commercial preparations of this substance, crude HCB (Eastman) and HCB (Aldrich) which had been recrystallized three times from hot benzene were administered to rats in the diet, as described

above. However, females, rather than males, were used since they had been reported to be more susceptible to HCB-caused experimental porphyria (San Martin de Viale et al., 1970). Also, a range of doses (0.0%, 0.02%, 0.1%, 0.2%, 0.25%, and 1.0%, w/w) was employed. Rats were divided into groups of six and fed powdered chow containing the amounts of HCB (either recrystallized or crude) stated. The urine from individual animals was collected weekly and analysed for porphyrins.

After seven weeks of treatment, two results were apparent: Crude HCB (Eastman) was more potent in causing porphyria than recrystallized (Aldrich) HCB, and the practical grade HCB was more toxic (as judged by animal mortality). Four of six rats treated with the highest dose of crude HCB had died by the fifth week of treatment whereas no mortality occurred in any of the groups of animals to which recrystallized HCB had been administered.

Figure 12 shows the time-dependent porphyrin excretion of rats treated with doses of 0.1% and 0.25% either crude or recrystallized HCB. Weekly porphyrin excretion for each group of animals in the study is documented in Table 2. Since the porphyrin excretion curve for recrystallized HCB administered at a dose of 0.25% follows closely the curve for crude HCB at 0.1%, an approximation of the relative potencies of the preparations

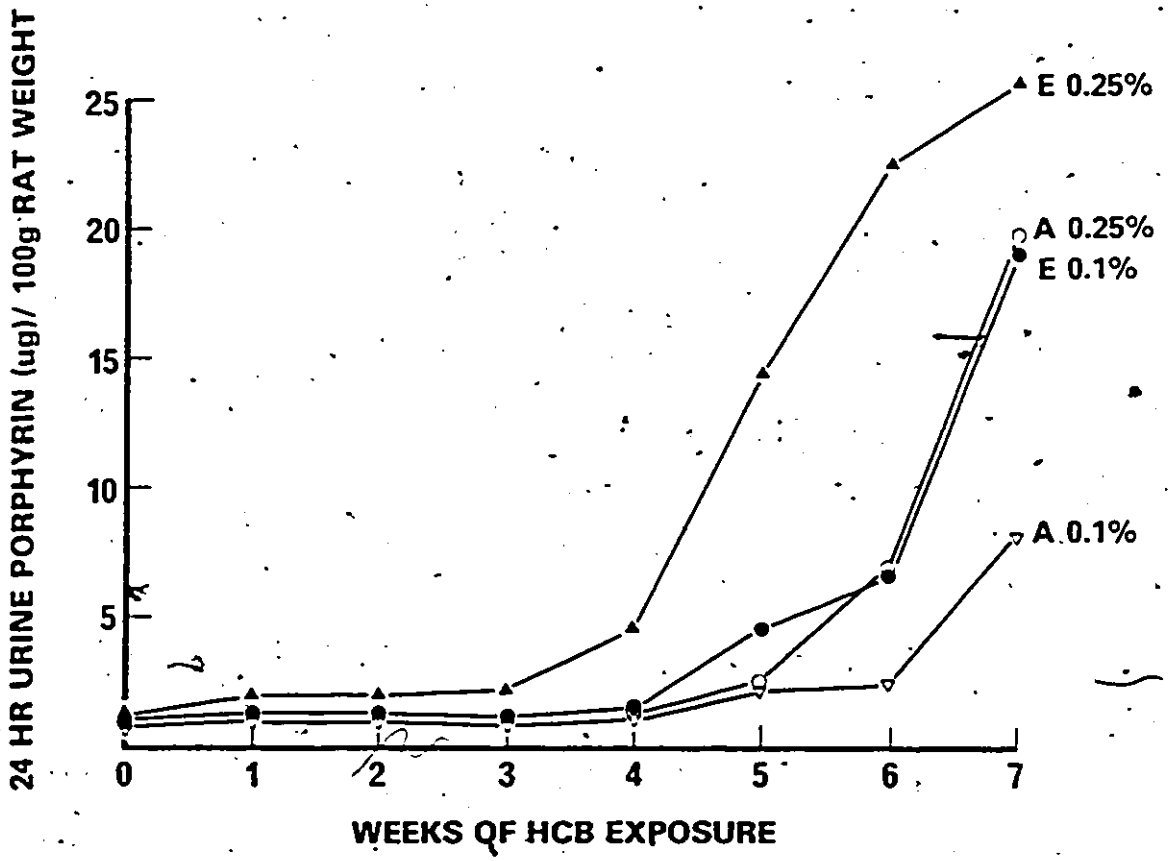


Figure 12: Urinary porphyrin excretion in response to treatment of rats with either recrystallized or crude HCB.
E 0.25% = Eastman crude HCB 0.25% (w/w) in the diet
E 0.1% = Eastman crude HCB 0.1% (w/w) in the diet
A 0.25% = Aldrich recrystallized HCB 0.25% (w/w) in the diet
A 0.1% = Aldrich recrystallized HCB 0.1% (w/w) in the diet

TABLE 2

24 Hour Porphyrin Excretion in Rats Treated with Crude and Purified Hexachlorobenzene (µg/100 g Rat Wt)

Group (dose)		Weeks Treatment with HCB 1	2	3	4	5	6	7
0.00% CON		1.13 ± 0.39	1.46 ± 0.47	1.27 ± 0.78	1.51 ± 0.68	1.08 ± 0.63	1.20 ± 0.54	1.35 ± 0.71
0.02%	A	1.29 ± 1.10	1.50 ± 1.22	2.26 ± 1.61	1.95 ± 1.14	1.87 ± 1.00	2.14 ± 1.56	4.83 ± 4.67
	E	0.94 ± 0.78	1.29 ± 1.14	1.46 ± 0.68	1.29 ± 0.81	2.07 ± 0.92	4.18 ± 3.76	9.93 ± 8.93
0.10%	A	1.26 ± 0.40	1.12 ± 0.96	1.05 ± 0.40	1.02 ± 0.36	2.34 ± 1.17	2.48 ± 0.88	8.21 ± 4.93
	E	0.99 ± 0.30	1.08 ± 0.22	1.06 ± 0.43	1.16 ± 0.60	5.97 ± 5.41	6.82 ± 3.47	19.79 ± 4.31
0.20%	A	0.65 ± 0.30	0.85 ± 0.25	1.09 ± 0.35	1.07 ± 0.47	3.15 ± 2.04	9.77 ±12.30	15.37 ± 8.82
	E	0.62 ± 0.18	0.84 ± 0.52	0.92 ± 0.42	1.37 ± 0.69	6.14 ± 4.51	14.84 ± 9.84	20.87 ±11.64
0.25%	A	0.91 ± 0.46	0.87 ± 0.32	0.73 ± 0.24	0.94 ± 0.41	2.71 ± 2.24	6.81 ± 3.82	19.03 ± 6.82
	E	1.74 ± 1.62	1.51 ± 1.13	2.00 ± 1.37	4.60 ± 4.70	14.76 ± 3.01	22.43 ±14.91	25.45 ±21.32
1.00%	A	0.71 ± 0.35	2.45 ± 1.63	1.73 ± 1.27	2.85 ± 1.58	8.08 ± 3.22	12.44 ± 9.21	18.30 ±10.41
	E	0.63 ± 0.42	1.90 ± 0.95	2.00 ± 1.50	6.96 ± 3.47	18.24* ±12.63		

Female Wistar rats were treated with HCB in the diet as described in the text. Values represent means ± SD for 6 animals.

*4 of 6 rats died between weeks 4 and 5.

would be 2.5/1 (crude/recrystallized).

3.2. SERIAL PORPHYRIN AND PORPHYRINOGEN EXCRETION DURING THE DEVELOPMENT OF HEXACHLOROBENZENE PORPHYRIA IN RATS

To determine whether experimental porphyria in rats caused by HCB results from a failure of the liver to maintain porphyrinogens in their reduced state, which results in oxidation to porphyrins; or whether decreased activity of uroporphyrinogen decarboxylase, resulting in a buildup of porphyrinogen intermediates was a more reasonable hypothesis, excretion of urinary porphyrins and porphyrinogens was followed during the course of the development of porphyria in rats treated with HCB.

Since a great deal of variation was found to exist in the time taken for individual animals in any group to develop porphyria, each rat was monitored individually. Females, rather than males, were used because of their greater susceptibility to HCB-caused porphyria. Collections of 24 hour urine specimens were made at weekly intervals from rats receiving 0.25% HCB (Eastman, practical grade) in the diet. As described above, care was taken to minimize oxidation of porphyrinogens during the collection process and extraction and measurement of porphyrinogens was proceeded with as expeditiously as possible. Concurrent

with collection of 24 hour urine samples, 24 hour fecal specimens were also collected and analysed for porphyrins, as described in Experimental Procedures (2.6.).

Figure 13 shows the weekly urine porphyrin excretion of two rats during the development of experimental porphyria. As previously mentioned, the time taken for individual rats to become porphyric is extremely variable and, as would be expected, the porphyrin excretion curves for different animals did not coincide closely. However, although the time frame for each rat was different, the overall development of the disease (as judged by the porphyrin and porphyrinogen excretion pattern) proceeded similarly in all animals. The two rats illustrated were chosen because they both became porphyric in a convenient period of time.

The increased urine porphyrin seen in Figure 13 was expected and merely confirmed that the rats developed porphyria. It was important to determine whether porphyrinogen excretion increased as the animals became porphyric. As can be seen in Figure 14, urinary porphyrinogen excretion increased concurrently with the elevation in porphyrin output although the magnitude of the porphyrinogen excretion was not as large as that for porphyrin, indicating that considerable oxidation of porphyrinogens occurred prior to excretion.

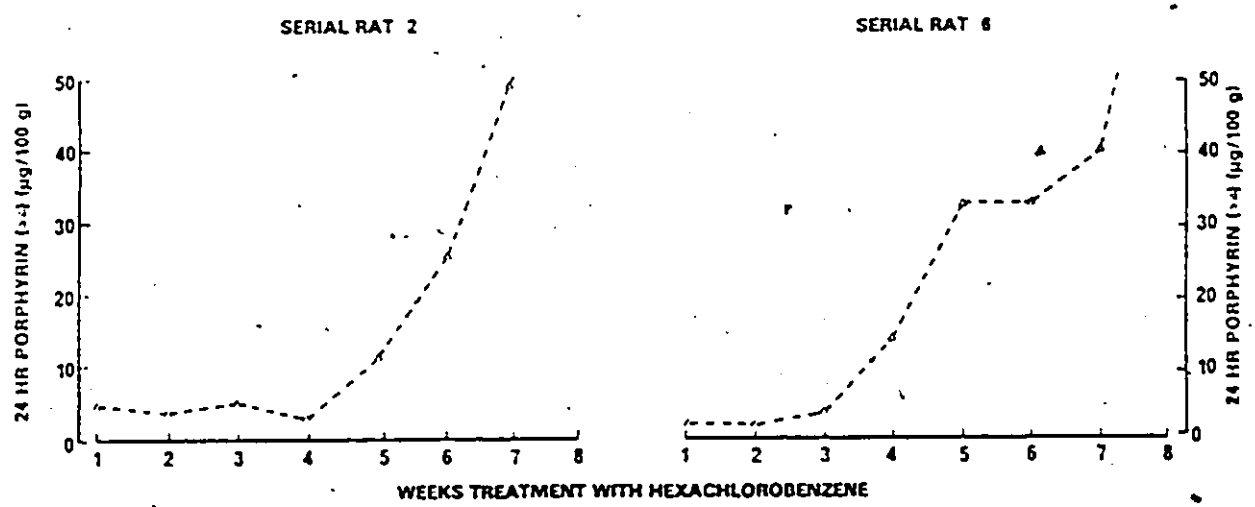


Figure 13: The time course for urine porphyrin excretion during treatment with hexachlorobenzene. Female Wistar rats were treated with HCB in the diet (0.25%, w/w). Urine was collected weekly and analyzed for for porphyrins by derivative spectroscopy.

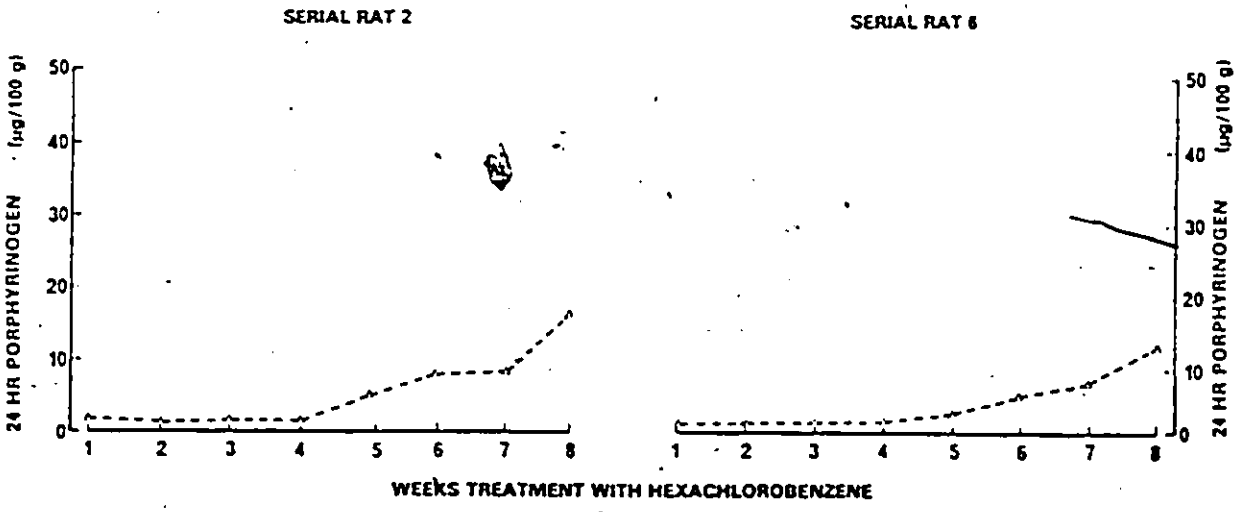


Figure 14: The time course for urine porphyrinogen excretion during treatment with hexachlorobenzene. Female Wistar rats were treated with HCB in the diet (0.25%, w/w). Urine was collected weekly and analyzed for porphyrinogens as described in Experimental Proceedures, section 2.5.

The composition of the porphyrinogen and porphyrin fractions present in the urine was also helpful in determining the nature of the metabolic lesion. Because loss of carboxyl substituents during conversion of uroporphyrinogen to coproporphyrinogen is associated with a shift in the porphyrin absorption peak toward the blue, the point at which the first derivative of the absorption spectrum of a mixture of porphyrins intersects a reconstructed baseline reflects the composition of the mixture. This technique was used to determine the relative contributions of the more highly carboxylated (uroporphyrin(ogen)) and less highly carboxylated (coproporphyrin(ogen)) fractions to the total porphyrin(ogen) in the urine. Figure 15 shows the coproporphyrin fraction as a percentage of the total porphyrin excretion during the development of porphyria. As can be seen, by the third or fourth week, when the total excretion of porphyrin began to increase, there was an increase in the relative contribution of the more highly carboxylated (uroporphyrin) fraction which occurred until the fifth or sixth week when a slight decrease occurred, but it remained elevated throughout the remainder of the experiment.

In contrast to the porphyrin excretion, the lower carboxylated porphyrinogens (coproporphyrinogen fraction)

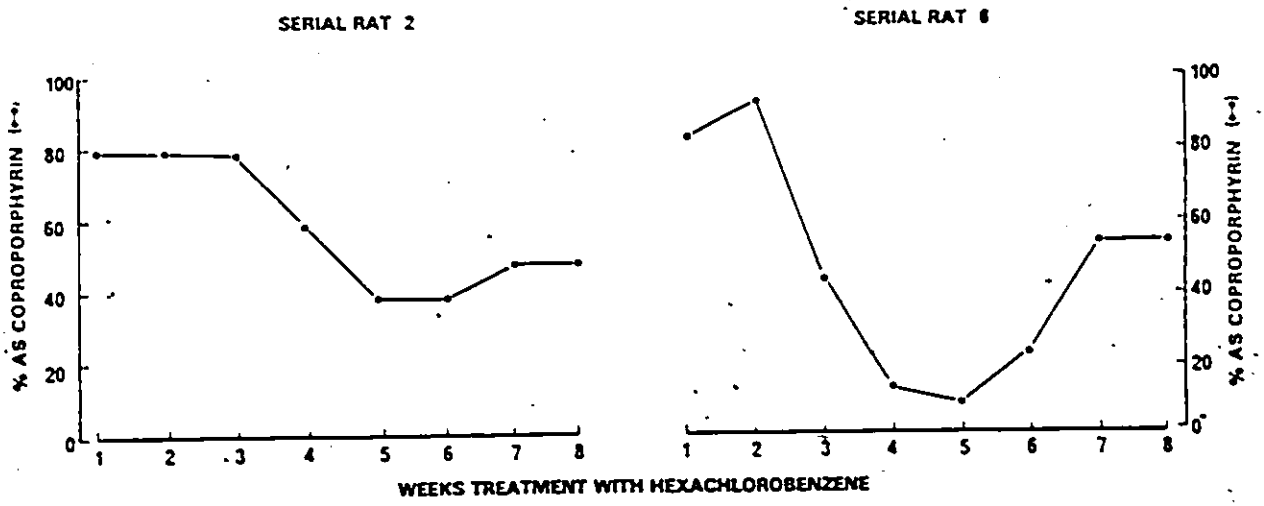


Figure 15: Variation in the composition of urinary porphyrins during treatment with hexachlorobenzene. Rats were treated with HCB as in Figure 13. The relative contributions of uroporphyrin and coproporphyrin were estimated using derivative spectroscopy as described in Experimental Proceedures, section 2.4.1.

remained the major constituents of the excreted porphyrinogens, although a slight decline in their contribution occurred as the porphyria continued to develop (Figure 16).

The parallel increase in porphyrin and porphyrinogen excretion described above suggested that a constant percentage of porphyrinogen was being oxidized to porphyrin prior to excretion, whether the animal was porphyric or not. This was investigated by comparing the percentage of the total urinary porphyrin + porphyrinogen which remained reduced in both porphyric and control animals (Figure 17). It was apparent that, indeed, no significant increase in the percentage of porphyrinogen oxidized to porphyrin occurred in porphyric animals.

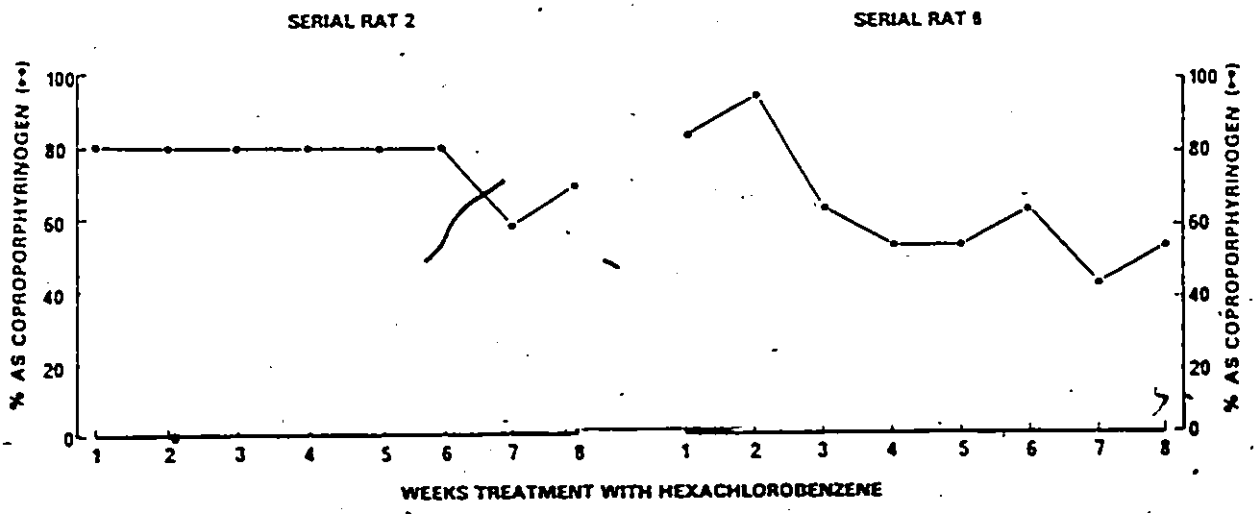


Figure 16: Variation in the composition of urinary porphyrinogens during treatment with hexachlorobenzene. Rats were treated with HCB as in Figure 14. The relative contributions of uroporphyrinogen and coproporphyrinogen were estimated by derivative spectroscopy after extraction of porphyrinogens as described in Experimental Proceedures, section 2.5.

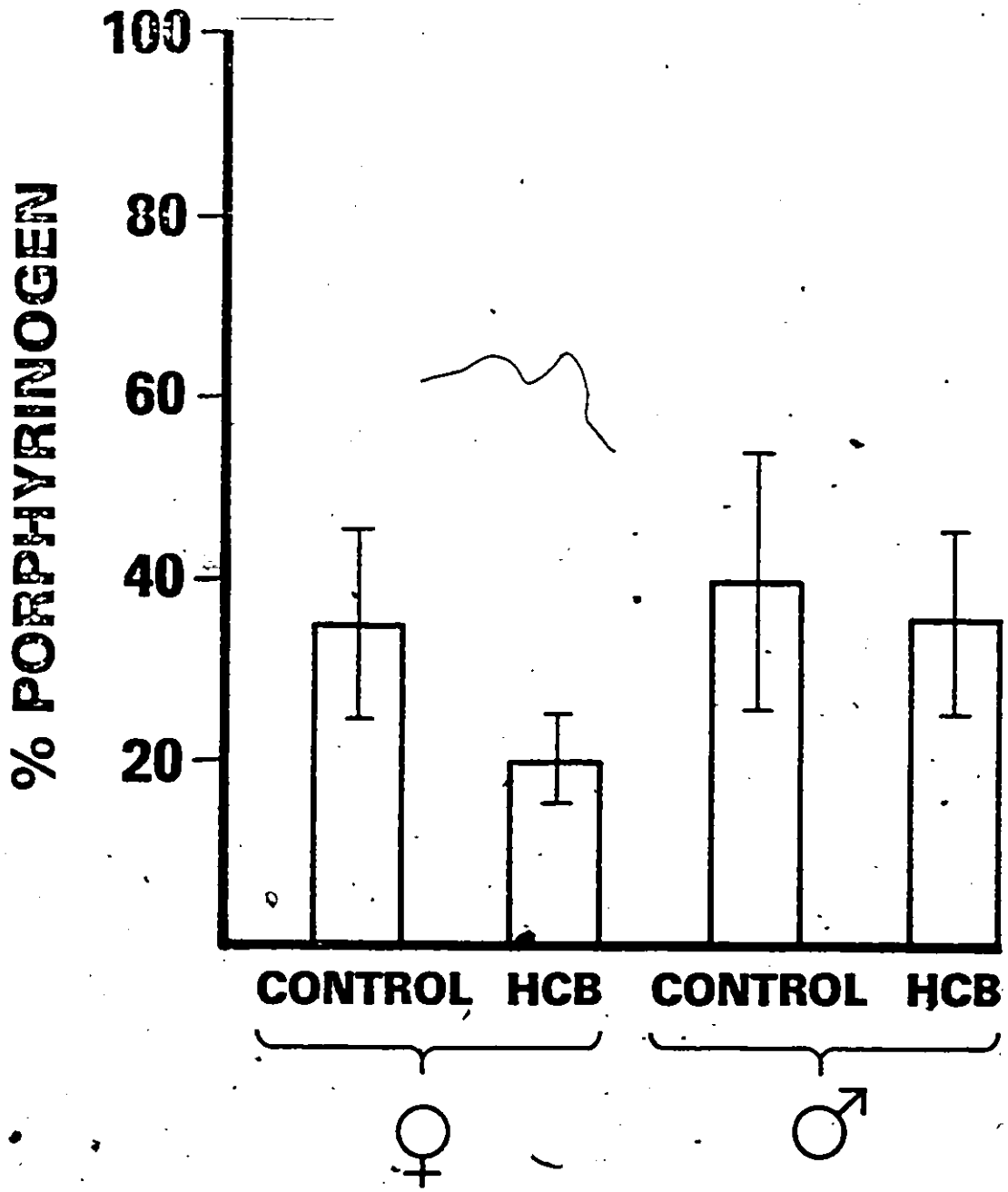


Figure 17: The percentage porphyrinogen of the total urinary porphyrin + porphyrinogen in normal and porphyric rats. Rats were made prophyric by treatment with HCB in the diet (0.25%, w/w). Porphyrinogens were extracted from the urine and quantitated as described in Experimental Proceedures, section 2.4.1. Values represent means and standard deviations for seven animals.

Thin layer chromatographic analysis of the porphyrins excreted in urine and feces revealed that the distribution of individual porphyrin species was quite asymmetric between these two routes, and this bears significantly on the interpretation of the uroporphyrin(ogen)/coproporphyrin(ogen) ratios in Figures 15 and 16. Figure 18 illustrates that urine was the predominant route of excretion for 8-carboxyl porphyrin (uroporphyrin) and 4-carboxyl porphyrin (coproporphyrin), while all other porphyrins occurred mainly in the feces and isocoproporphyrin was excreted exclusively via the fecal route.

In parallel with the studies on porphyrin and porphyrinogen excretion in the urine outlined above, a detailed analysis of total urinary and fecal porphyrin excretion was carried out following esterification, separation and quantitation of the individual porphyrins using thin layer chromatography, as described in the Experimental Procedures (section 2.6.). The detailed progression of 24 hour porphyrin excretion during the development of porphyria in the rats may be seen in Figure 19. It will be noted that the first observable increase in porphyrin excretion between weeks 3 and 4 affected coproporphyrin but within one to two weeks thereafter, uroporphyrin increased quite strikingly. After that time,

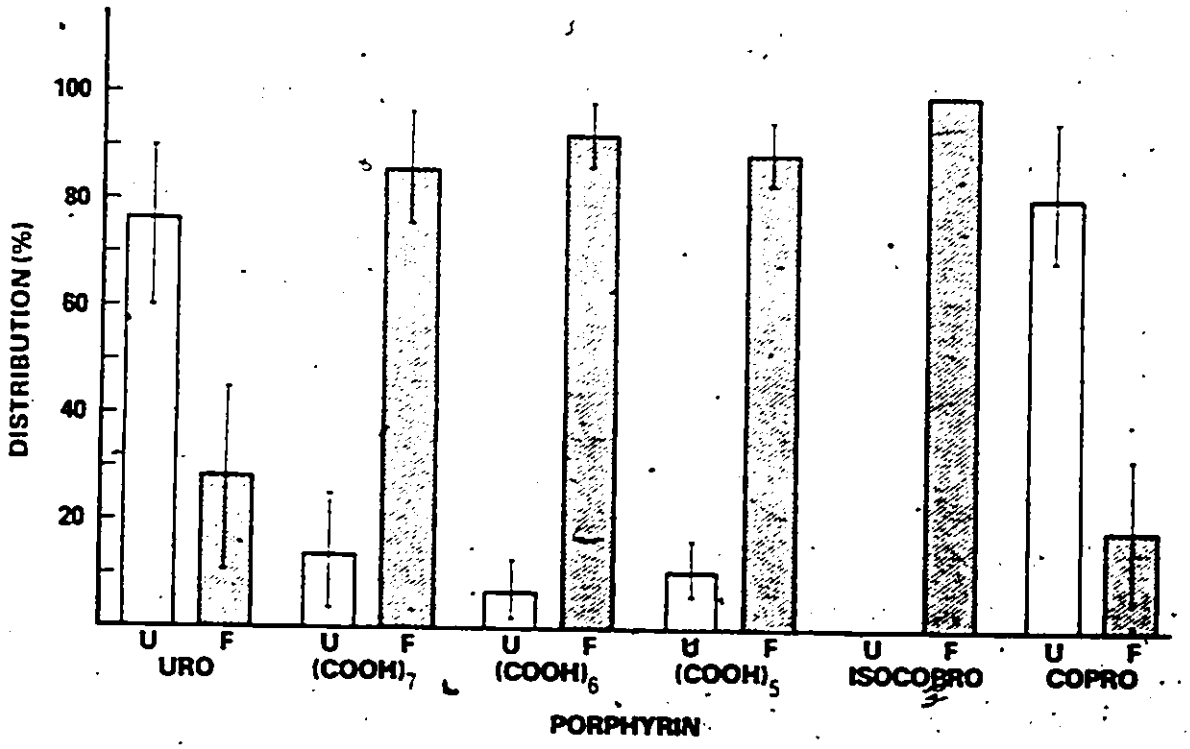


Figure 18: The distribution of the various porphyrins in urine and feces from rats made porphyric with hexachlorobenzene. 24 hour collections of urine and feces were analyzed for porphyrins by use of thin layer chromatography as described in Experimental Proceedures, section 2.6. Values represent means and standard deviations for six animals.

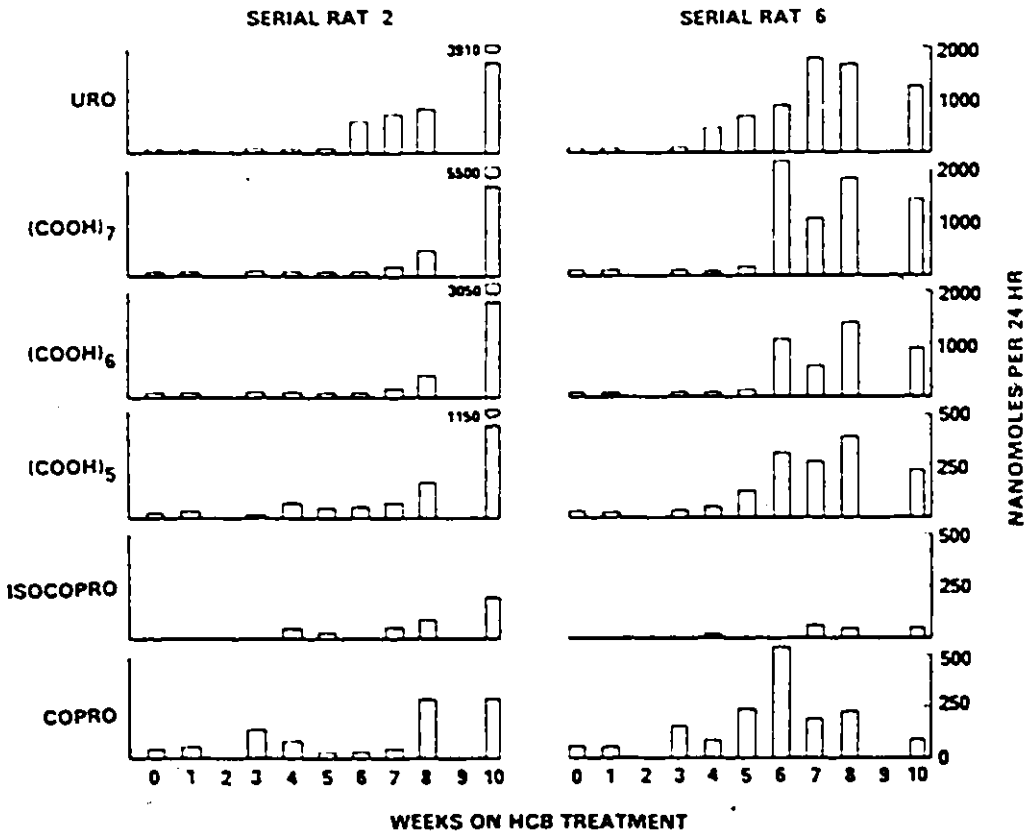


Figure 19: Progression of total porphyrin excretion during treatment with hexachlorobenzene. Thin layer chromatographic analysis of 24 hour collections of urine + feces was effected as described in Experimental Proceedures, section 2.6. Treatment was with HCB in the diet (0.25%, w/w).

by far the greater part of the increase in porphyrin excretion in combined urine and fecal analysis was attributable to porphyrins with 8, 7, and 6 carboxyl groups (note scale). In the serial analysis on rat 2, very high levels of excretion of all components except coproporphyrin were obtained at the tenth week; whereas porphyrins with 8, 7, 6, and 5 carboxyl groups could not be represented on the scales used, isocoproporphyrin had reached its highest level but coproporphyrin had decreased slightly. In rat 6, all fractions in the tenth week analysis were somewhat depressed, perhaps because of a small fecal sample collected, but the depression of coproporphyrin was most marked, while isocoproporphyrin remained at its highest level.

3.3. DIFFERENCES BETWEEN PORPHYRIC AND NON-PORPHYRIC RATS FOLLOWING TREATMENT WITH HEXACHLOROBENZENE

After ten weeks of treatment with HCB at the dose described above, some animals had only slightly elevated urine porphyrin levels. To determine whether any biochemical differences existed between the livers of porphyric and non-porphyric animals after ten weeks of identical treatment with HCB, rats were separated into "porphyric" and "non-porphyric" groups on the basis of their urinary porphyrin excretion and were sacrificed and the

measurements documented in Table 3 were made.

As would be expected, "porphyric" rats had higher levels of urinary porphyrin, ALA-S, ALA, and PBG than "non-porphyric animals. However, cytochrome P-450 was found to be lower in the "porphyric" group than in the "non-porphyric" group. This was unexpected since HCB is known to be an inducer of cytochrome P-450 and both groups of rats had been given identical treatment. Although there was too much scatter within groups to be able to achieve statistical significance, GSH was found to decrease progressively from control to "non-porphyric" to "porphyric" animals.

3.3.1. SUMMARY AND EVALUATION

Up to this point, development of methodology and experiments had been addressed to the question of whether the porphyria caused by HCB resulted from decreased activity of UD or from accelerated oxidation of porphyrinogens to porphyrins. Careful examination of the excreted intermediates of heme synthesis during the development of HCB porphyria supported the former hypothesis and effectively excluded the latter theory. This evidence, together with the recent finding of Elder (1976) that rats made porphyric with HCB had decreased levels of hepatic UD

Table 3

Progressive Effects of HCB Treatment

	CONTROL	NON-PORPHYRIC	PORPHYRIC
24 Hour Urine Porphyrin (µg)	1.84 ± 0.49	4.18 ± 2.33	109.9 ± 19.0
ALA-S (nmoles ALA/g liver/hour)	16.2 ± 4.6	30.6 ± 8.1	56.3 ± 16.7
ALA (µg/24 hours)	27.7 ± 11.3	54.0 ± 14.8	206.6 ± 165.5
PBG (µg/24 hours)	32.2 ± 10.8	48.8 ± 6.5	850.8 ± 965.2
Cytochrome P-450 (nmoles/g liver)	26.9 ± 5.7	46.9 ± 12.2	32.5 ± 6.4
GSH (µg/g liver)	337.9 ± 117.8	226.6 ± 86.3	159.3 ± 26.5

Table 3: Progressive effects of hexachlorobenzene treatment. Comparison of porphyric and non-porphyric rats after ten weeks of treatment with HCB. Values represent means and standard deviations for six animals.

activity compared with controls strongly suggested that decreased activity of this enzyme is directly responsible for the porphyria caused by HCB.

Focus was then switched to the broader issue of the mechanism of chlorinated aromatic hydrocarbon toxicity in the liver, especially as related to porphyria. For this work TCDD, rather than HCB, was used. This decision was based on two properties of TCDD: its potency as an hepatotoxin as well as an etiologic agent for porphyria, and its specificity for induction of cytochrome P-448. It was hoped that this potency and specificity of action would be useful in the determination of which actions attributed to the chlorinated aromatic hydrocarbons were important in leading to the particular toxicity which eventually resulted in porphyria.

3.4. EFFECT OF ARYL HYDROCARBON HYDROXYLASE RESPONSIVENESS ON THE SUSCEPTIBILITY TO THE PORPHYROGENIC EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

3.4.1. C57BL/6J STRAIN VERSUS DBA/2J STRAIN

The chlorinated aromatic hydrocarbons which cause an experimental porphyria in animals which ressembles PCT in man all share the feature of induction of the hepatic

microsomal mixed function oxygenase (MFO) system. Hexachlorobenzene and polychlorinated biphenyls are mixed inducers, i.e. they induce both cytochrome P-450 and cytochrome P-448, but 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is both the most powerful porphyrogenic agent and the most potent inducer of the MFO system, is specific for induction of cytochrome P-448, which is the cytochrome associated with aryl hydrocarbon (benzo(a)pyrene) hydroxylase (AHH) activity.

Certain inbred strains of mice have been shown to be resistant to induction of AHH by 3-MC, but they do respond to TCDD at a dose approximately 10 to 15 times higher than that required for induction of more responsive strains (Poland and Glover, 1974). This difference in susceptibility to induction of AHH by TCDD was exploited to determine whether susceptibility to induction of AHH correlated with susceptibility to the porphyrogenic effects of TCDD.

C57BL/6J (AHH responsive) mice were compared with DBA/2J (AHH nonresponsive) mice for susceptibility to porphyria caused by TCDD. Treatment consisted of weekly injections of either TCDD (25 µg/kg, i.p.) or vehicle (1,4-dioxane) alone (controls) for five weeks. This regimen had been shown by Goldstein (1973) to cause porphyria in the C57BL/6J strain whereas one-tenth of this dose was

ineffective. If susceptibility to porphyria reflected AHH responsiveness, DBA/2J mice would not have been expected to develop porphyria at the dose used.

Twenty-four hour pooled urine specimens were collected each week from the various groups and were analysed for porphyrins. As may be seen in Figure 20, only the C57BL/6J (AHH responsive) group developed porphyria as a result of treatment with TCDD. As well as an increase in the quantity of porphyrin excreted, the type of porphyrin changed in the C57BL/6J group treated with TCDD from predominantly coproporphyrin to mostly uroporphyrin, indicating that these mice had acquired the typical biochemical abnormality.

By this time, a convenient assay for uroporphyrinogen decarboxylase (UD) had been developed by Elder (1976), which was used with modifications (see "Experimental Procedures", section 2.8.) to determine the enzyme activity in the livers of the mice in this experiment. The results of this assay are shown in Figure 22. UD activity was found to be significantly decreased ($p < 0.05$) only in those mice treated with TCDD which were also AHH responsive.

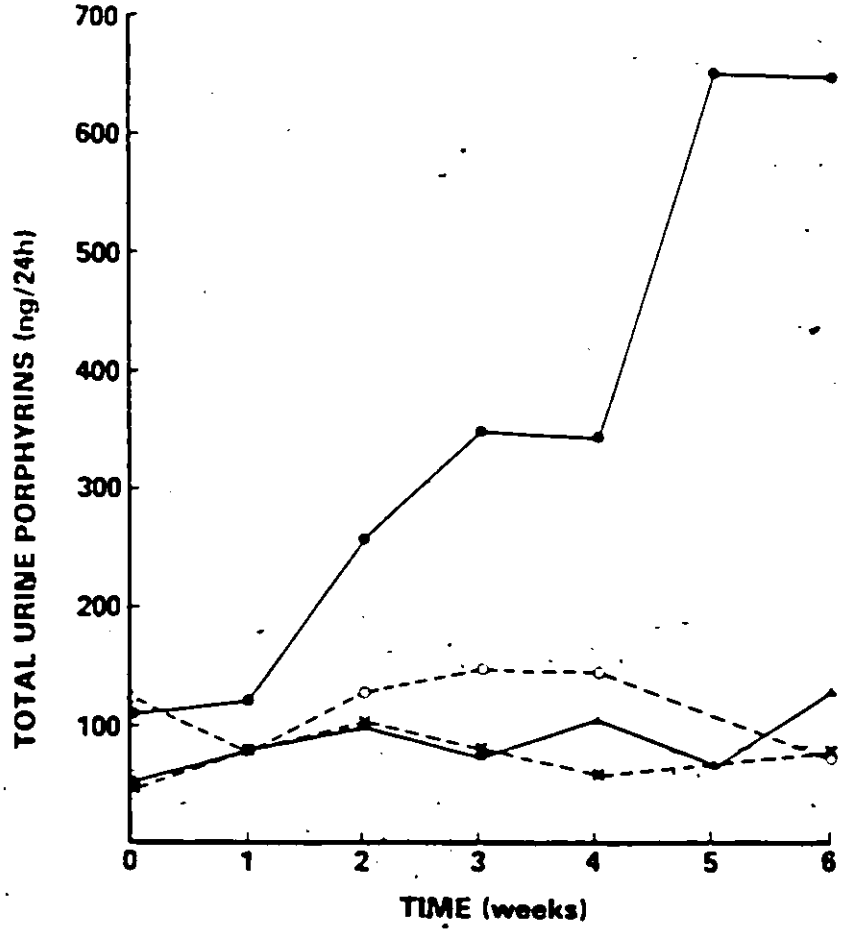


Figure 20: Effect of TCDD on urine porphyrin excretion in AHH responsive and nonresponsive mice. Treatment was with TCDD (25 µg/kg/wk) or 1,4-dioxane (controls). Urine porphyrin was quantitated by derivative spectroscopy as described in Experimental Proceedures, section 2.4.1.
C57BL/6J controls (o—o)
C57BL/6J TCDD (●—●)
DBA/2J controls (×—×)
DBA/2J TCDD (▲—▲)

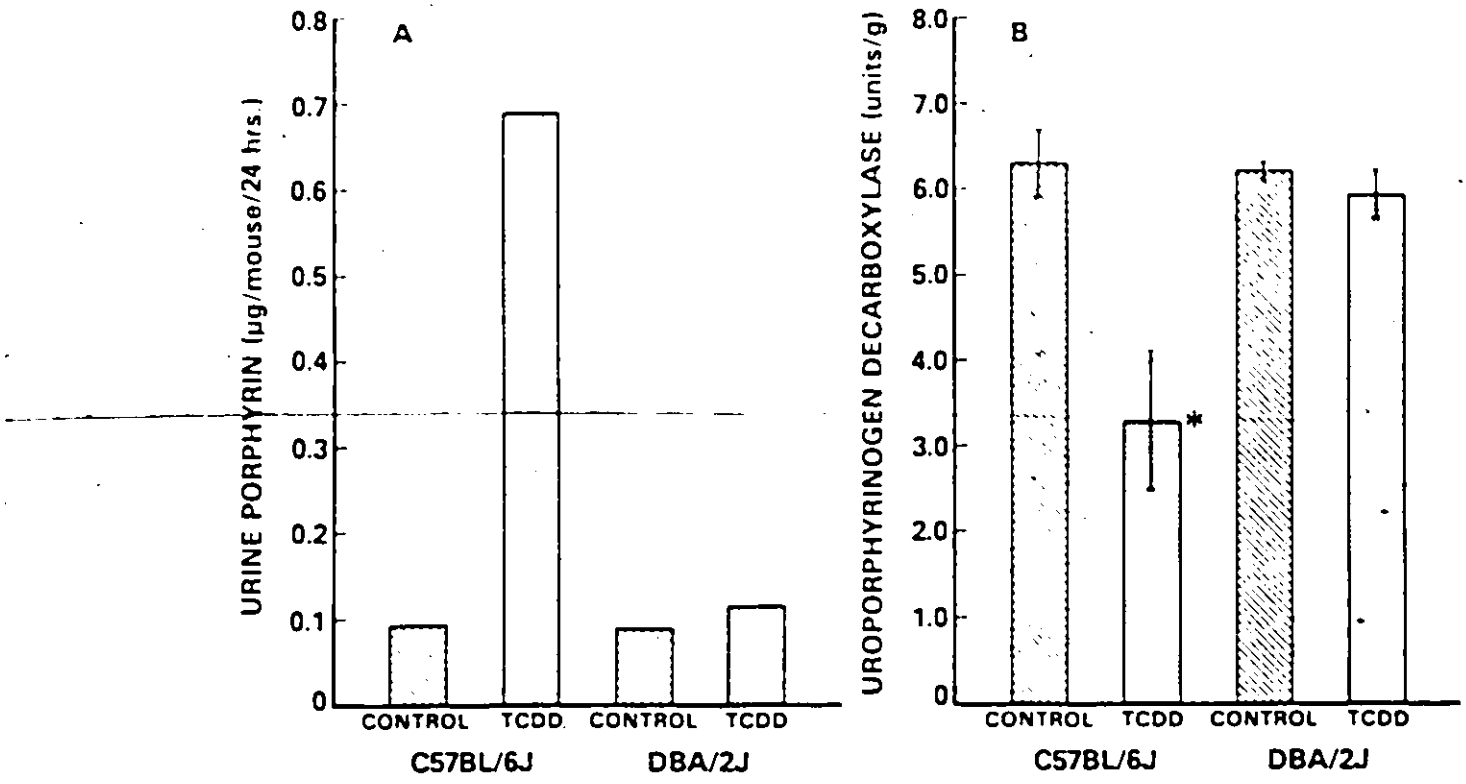


Figure 21: Urine porphyrin excretion (A) and uroporphyrinogen decarboxylase activity (B) in C57BL/6J and DBA/2J mice after six weeks of treatment with TCDD

* significantly different ($p < 0.05$)

3.4.2. D2(B6D2)F1/J STRAIN

The above experiments established that C57BL/6J mice were more susceptible to the porphyrogenic effects of TCDD than DBA/2J mice. To eliminate the possibility that a nonspecific strain difference, rather than a difference in AHH responsiveness was responsible for this result, a back-crossed strain, i.e. D2(B6D2)F1/J was used to repeat the experiment.

Inheritance of responsiveness to AHH induction has been shown to correspond most closely to the model of a simple autosomal dominant trait in the crosses and back-crosses between C57BL/6J and DBA/2J mice (Nebert and Gielen, 1972). C57BL/6J mice are attributed with a double dominant genotype (Ah/Ah) while DBA/2J have a double recessive (ah/ah). Thus, the first cross between these two strains would result in a single genotype (Ah/ah) which, phenotypically, is AHH responsive. When these mice, i.e. (B6D2)/J, are crossed with DBA/2J, the resultant strain is D2(B6D2)F1/J which has a genotype of (statistically) 50% Ah/ah (AHH responsive) and 50% ah/ah (AHH nonresponsive). Thus, by using the back-crossed strain D2(B6D2)F1/J, the genes of the two strains of mice used above were mixed for two generations and, by segregating the animals on the basis of AHH responsiveness, much of the potential nonspecific

strain difference could be assumed to be removed. Thus, D2(B6D2)F1/J mice, obtained from Jackson Laboratories were used to repeat the above experiment.

3.4.2.1. DETERMINATION OF PHENOTYPE OF BACK-CROSSED MICE

The procedure for determining AHH responsiveness in D2(B6D2)F1/J mice was based on the metabolism of zoxazolamine by cytochrome P-448 (Robinson and Nebert, 1974). Briefly, all mice were treated with 5,6-benzoflavone (80 mg/kg, i.p.) for 48 hours prior to a challenge with zoxazolamine (150 mg/kg, i.p.). Since zoxazolamine is metabolized by the P-448 (AHH) system, mice in which this system had been induced by the 5,6-benzoflavone would metabolize the zoxazolamine more rapidly than the AHH nonresponsive animals and would have a shorter paralysis time.

Results of the challenge with zoxazolamine are shown in Figure 22. As expected, mice segregated largely into two groups, responsive and nonresponsive, with average paralysis times of 11 minutes and 73 minutes, respectively. Five animals had intermediate paralysis times of 30 minutes to 45 minutes and were not used in further studies.

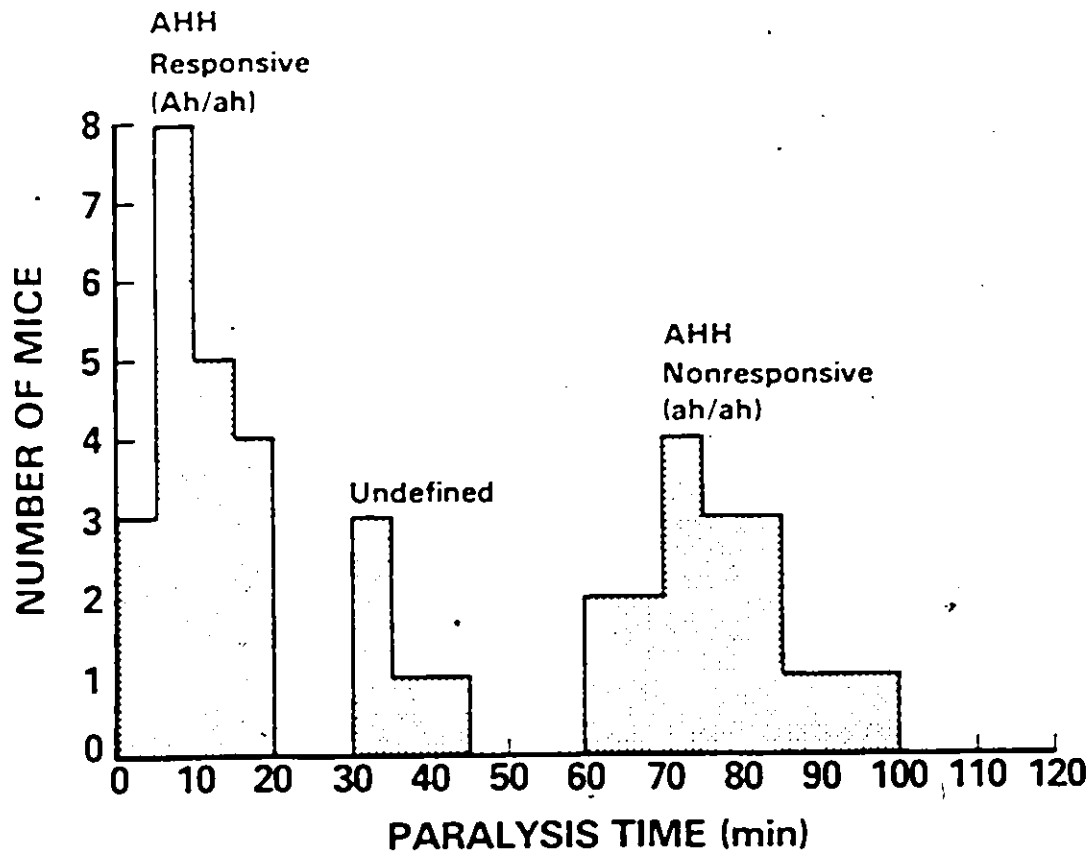


Figure 22: Zoxazolamine paralysis times of D2(B6D2)F1/J mice following pretreatment with 5,6-benzoflavone. Mice were treated with 5,6-benzoflavone (80 mg/kg, i.p.) 48 hours prior to injection of zoxazolamine (150 mg/kg, i.p.). The time required for each mouse to repeatedly right itself after being placed on its back was recorded.

3.5. TREATMENT WITH TETRACHLORODIBENZO-p-DIOXIN

C57BL/6J (Ah/Ah) mice as well as the back-crossed Ah/ah and ah/ah genotypes were further divided into control and TCDD treatment groups and, after a further interval of three weeks to permit recovery from the 5,6-benzoflavone, weekly intraperitoneal injections of either 1,4-dioxane (controls) or TCDD (25 µg/kg) were started. Urines were collected as a single pooled sample from each group of animals weekly over 24 hours and the total porphyrin excretion was measured using derivative spectroscopy.

Figure 23 shows that, of the six groups, only the Ah/Ah and the Ah/ah mice treated with TCDD showed any significant increase in urinary porphyrin excretion. As before, the increase was found to be predominantly due to uroporphyrin.

As shown in Table 4, the extent of depression of UD following 11 weeks of treatment with TCDD appeared to be dependent on the inheritance of the gene controlling AHH responsiveness. Ah/Ah mice were most affected, with an 80% decrease in enzyme activity ($p < 0.01$) followed by the Ah/ah group which demonstrated a 33% decrease ($p < 0.05$). The UD activity in the homozygous ah/ah group was least affected by TCDD treatment, showing only a 15% drop which was not statistically significant.

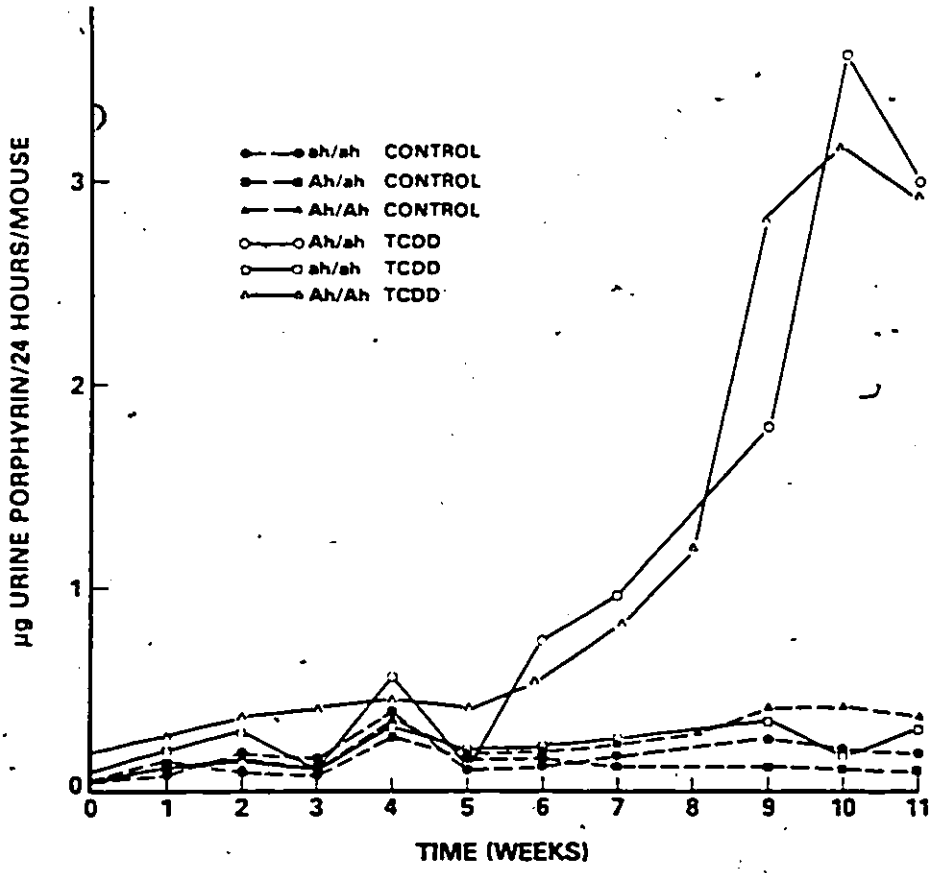


Figure 23: Effect of mouse genotype on urine porphyrin excretion in response to treatment with TCDD. Mice were treated with TCDD (25 µg/kg, i.p.). Pooled urine samples were collected weekly and quantitated for porphyrins by derivative spectroscopy.

Table 4

Effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin Treatment on Microsomal Cytochrome P-450, Aryl Hydrocarbon Hydroxylase, and Uroporphyrinogen Decarboxylase in Different Mouse Genotypes[a]

Group	n	Microsomal Cytochrome P-450[b]	AHH[c]	UD[d]
Ah/Ah Control	7	0.73 ± 0.13	4.09 ± 1.24	6.49 ± 1.16
Ah/Ah TCDD	10	1.73 ± 0.13[f]	100.12 ± 20.42[g]	1.28 ± 0.79[g]
Ah/ah Control	10	0.68 ± 0.16	4.10 ± 0.70	8.20 ± 0.34
Ah/ah TCDD	10	1.80 ± 0.36[f]	69.32 ± 20.18[g]	5.50 ± 1.29[f]
ah/ah Control	6	0.35 ± 0.09	6.56 ± 2.06	7.81 ± 0.41
ah/ah TCDD	8	1.46 ± 0.10[f]	93.46 ± 14.18[g]	6.64 ± 0.67

[a]Treatments and assays were performed as described under Methods

[b]Nanomoles per milligram of protein

[c]Units per milligram of liver

[d]Units per gram of liver

[f]Statistically different ($p < .05$) compared to control mice

[g]Statistically different ($p < .01$) compared to control mice

It will also be noted from Table 4 that all groups treated with TCDD had increased microsomal cytochrome P-448 and AHH activity, including the AHH nonresponsive ah/ah group. This was not unexpected since the cumulative dose, following several weeks of TCDD treatment at 25 µg/kg would be sufficient to cause AHH induction in homozygous nonresponsive mice.

It was possible to induce AHH in the homozygous nonresponsive mice with TCDD because the "nonresponsiveness" is merely a decrease in sensitivity which can be overcome with a sufficiently high dose of TCDD. It might then be expected that, given an adequate total dose of TCDD, it would be possible to cause porphyria in nonresponsive mice. To this end, eight DBA/2J mice were treated weekly with 250 µg/kg of TCDD for eight weeks. By the eighth week, six of the eight mice had died without evidence of porphyria. However, 2 animals lived for twelve weeks and, at necropsy, the livers showed brilliant red fluorescence under ultraviolet light, indicating the presence of porphyrin.

3.6. EFFECT OF IRON DEFICIENCY ON THE PORPHYROGENICITY OF TCDD

Since 1962, PCT in man has been treated by venesection (Ippen, 1962) with satisfactory results, i.e.

when the patient is depleted of stored iron, porphyrin excretion falls until he is asymptomatic (skin fragility and sensitivity to sunlight disappears). Figure 24 illustrates the progressive decrease in porphyrin excretion in a patient with PCT who was treated with phlebotomy.

To determine whether depleted iron stores would prevent the porphyrogenic effect of TCDD in experimental animals, C57BL/6J mice were placed on an iron deficient diet and were made iron deficient by twice weekly removal of 0.2 to 0.25 ml of blood from the orbital sinus for four weeks. At this time, hemoglobin, which was measured in pooled blood samples, had dropped to 5.5 g/dl. The iron deficient diet was maintained for these mice and the groups of animals on iron deficient and regular diets were each subdivided into two treatment and control groups. Treatment was with either TCDD (25 µg/kg/wk, i.p.) or vehicle (1,4-dioxane) alone (controls) and 24 hour pooled urine samples were collected weekly from each group and assayed for porphyrins.

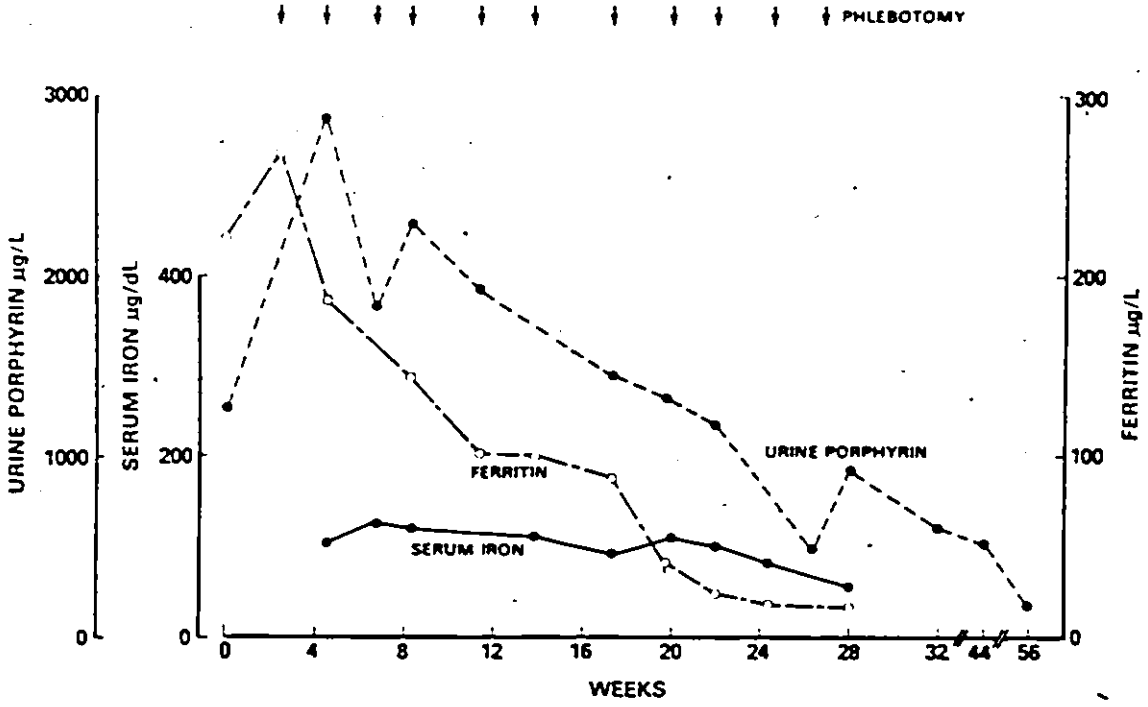


Figure 24: Biochemical course of a patient with PCT treated by phlebotomy (from Sweeney and Jones, 1979).

Figure 25 shows that only the group which was maintained on a normal iron diet and treated with TCDD developed porphyria, i.e. iron deficiency protected the mice from the porphyrogenic effects of TCDD. Measurement of hepatic UD activity (Figure 26) revealed that this enzyme retained a normal level of activity in TCDD-treated animals with depleted iron stores, but activity was decreased in the normal iron TCDD-treated group.

Upon sacrifice it was found that after eleven weeks hemoglobin levels in the iron deficient animals had risen to values comparable with the mice on the normal diet (Table 5). However, total liver iron was significantly decreased in both groups maintained on an iron deficient diet. Although liver weights in the iron deficient animals were lower than those in the normal iron groups, this was a reflection of the smaller size of the animals as a whole, due to a slight retardation of their growth without sufficient iron. When liver weight was expressed as a percentage of body weight, the values compared well with the mice maintained on a normal diet. Also, the increase in liver weight in response to TCDD treatment was similar for both normal iron and iron deficient mice. Table 5 shows that indices of MFO activity were similarly affected by TCDD in both normal iron and iron deficient mice.

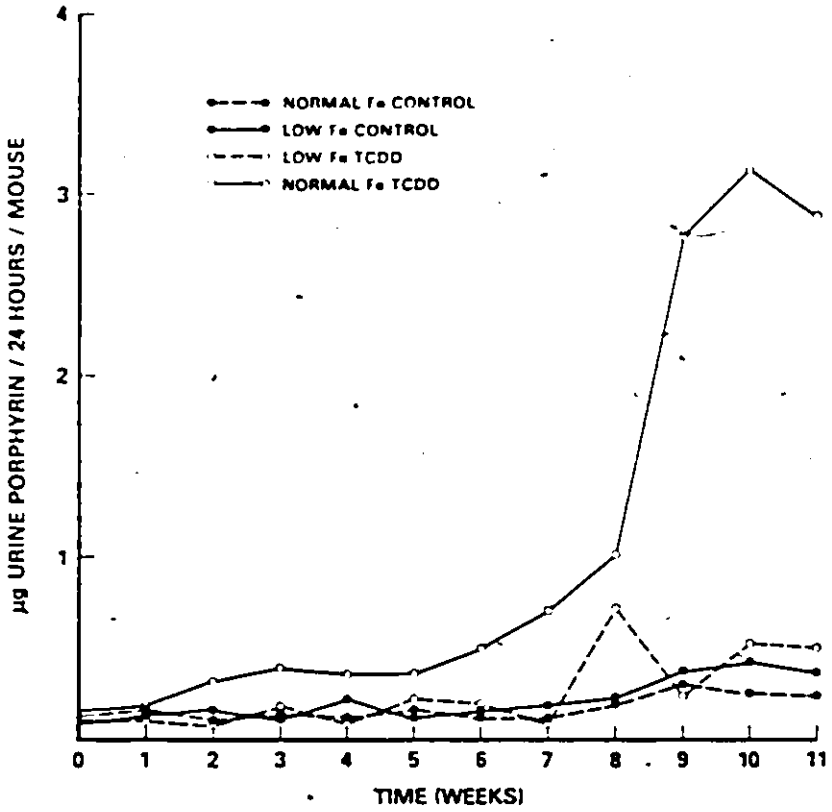


Figure 25: Effect of iron deficiency on urine porphyrin excretion in response to treatment with TCDD. Mice were treated with TCDD (25 µg/kg/wk, i.p.). Pooled urine samples were collected weekly and quantitated for porphyrins by use of derivative spectroscopy (Experimental Proceedures, section 2.8.).

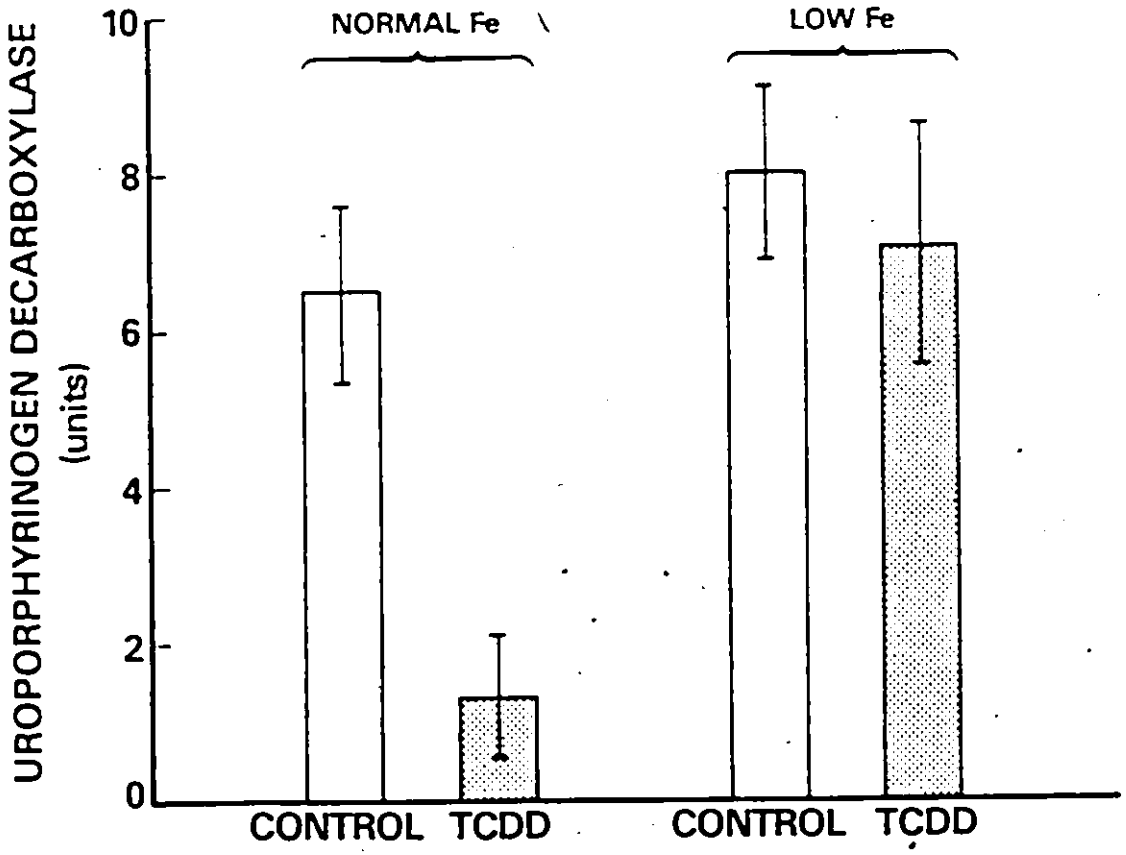
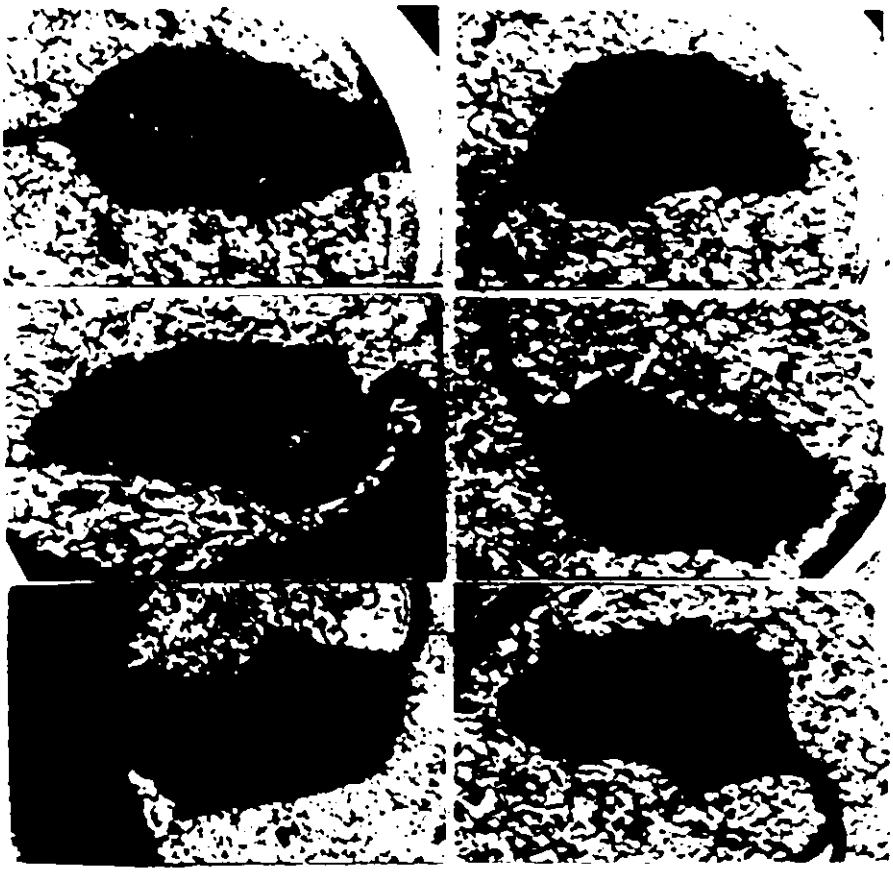


Figure 26: Effect of iron deficiency on uroporphyrinogen decarboxylase activity in response to treatment with TCDD. Twelve weeks after beginning treatment with TCDD (25 µg/kg/wk, i.p.) mice were sacrificed and UD was measured in the 9000 g supernatant fraction of liver as described in Experimental Proceedures, section 2.8.

Table 5 Effect of TCDD on Normal and Iron-deficient Mice

	Iron Deficient Mice				Control Mice (normal Iron)			
	(A) Controls (N=5)		(B) TCDD (N=9)		(C) Controls (N=7)		(D) TCDD (N=10)	
	Mean ± S.D.		Mean ± S.D.		Mean ± S.D.		Mean ± S.D.	
Weight: Body (grams)	26.85	2.02	23.64	1.59	33.6	2.58	30.9	3.67
Weight: Liver (grams)	1.38	.19	1.90	.27	1.72	.23	2.34	.35
% Liver/Body	5.17	.80	8.10	.94	5.14	.68	7.53	.50
Hemoglobin (g/dl) (pooled specimens)	13.4		10.22		12.50		11.68	
Total liver iron (µg/g)	70.18	11.28	86.19	5.79	121.12	9.25	144.76	10.98
Cytochrome P-450 (nanomoles/mg protein)	.785	.086	1.44 (increase x 1.83)	.137	.727	.131	1.727 (increase x 2.38)	.132
Cytochrome b_5 (nanomoles/mg protein)	.350	.047	.653 (increase x 1.87)	.029	.312	.039	.479 (increase x 1.54)	.058
NADPH Cytochrome c reductase (µmoles/mg protein/min)	.333	.042	.456 (increase x 1.37)	.079	.355	.044	.435 (increase x 1.23)	.052
Ethoxyresorufin-O-deethylase (nanomoles/g protein/min)	.220	.034	28.26 (increase x 128.5)	2.46	.291	.038	20.98 (increase x 72.1)	2.54
Ethoxycoumarin-O-deethylase (nanomoles/g protein/min)	4.19	.590	24.09 (increase x 5.75)	3.98	3.103	.649	24.19 (increase x 7.80)	2.52
Aryl hydrocarbon hydroxylase (Units/g liver)	5.76	1.71	60.42 (increase x 10.5)	11.01	4.09	1.24	100.12 (increase x 24.5)	20.42
Uroporphyrinogen decarboxylase (nanomoles/g liver/min)	7.99	1.16	7.02 (decrease x 1.14)	1.65	6.49	1.16	1.28 (decrease x 5.07)	0.79

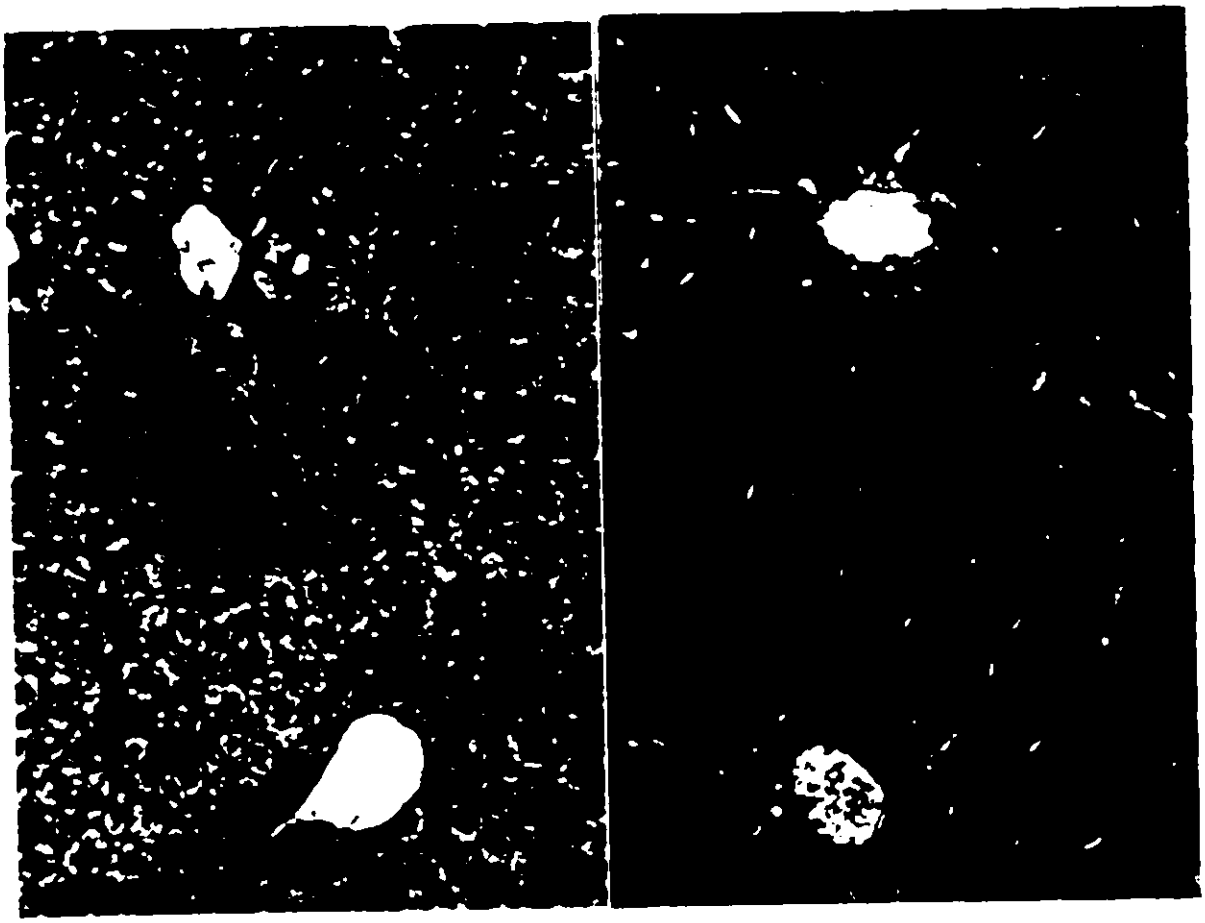
Before sacrifice, it was noted that the iron deficient mice which had been treated with TCDD appeared to be physically much more active than the similarly treated normal iron mice, and their coats were much sleeker (Figure 27). This suggested that iron deficiency may have protected the mice from other toxic effects of TCDD besides the development of porphyria and decreased UD activity. Histology of the livers after sacrifice revealed that, indeed, the pathological changes in the liver associated with TCDD were absent in mice which were iron deficient. The TCDD-treated animals on a normal iron diet showed a severe degree of disarray of the hepatic lobular architecture due to swelling of the parenchymal cells. Cytoplasm was markedly vacuolated and this change was more marked in the mid and peripheral zones of the lobules (Figure 28 A). The central area showed a striking eosinophilia of the cytoplasm, and throughout the lobule there were focal areas of individual cell necrosis with an associated mononuclear inflamatory cell infiltrate. Reticulo-endothelial elements (Kupffer cells) were prominent and increased in numbers. There was no cirrhosis but a diffuse increase of delicate reticulin fibres was noted, particularly in and around the portal tracts. No fat was demonstrated and stainable iron was absent. In contrast, the livers of TCDD-treated animals fed iron deficient diet were normal histologically (Figure 28 B) except for an



Normal Iron - TCDD
A

Low Iron - TCDD
B

Figure 27: Effect of iron deficiency on skin lesions caused by TCDD. Normal (A) and iron deficient (B) mice were treated with TCDD (25 µg/kg/wk, i.p.) for 12 weeks.



Normal Iron - TCDD

A

Low Iron - TCDD

B

Figure 28: Effect of iron deficiency on changes in liver morphology in response to treatment with TCDD. Normal (A) and iron deficient (B) mice were treated with TCDD (25 µg/kg/wk, i.p.) for 12 weeks. After sacrifice, portions of liver were fixed in Carnoy's fluid, embedded in parafin, sectioned at 5 µm, and stained with hematoxylin and eosin. Magnification X 285.

increase in cytoplasmic eosinophilic material, consistent with hypertrophy of the smooth endoplasmic reticulum.

3.7. STUDIES ON UROPORPHYRINOGEN DECARBOXYLASE

The assay used for UD in the above experiments was modified from that of Elder (1976). This particular assay was chosen because it utilized a 5-carboxyl substrate for the reaction which yielded a single product and had the advantage of greatly facilitating the interpretation of the results. However, Kushner (1975) has used uroporphyrinogen generated from PBG as a substrate to measure the same enzyme. The problem which arose during the course of this thesis was that these two workers reported diametrically opposite results based upon their respective assays. Briefly, Kushner found evidence for an inherited defect in UD and documented decreased activity of the enzyme in liver and red cells of patients with PCT (Kushner et al., 1976) while Elder and Tovey (1977) have found decreased UD in liver of patients with PCT but normal activity of the enzyme in red cells. Benedetto et al. (1978) suggested that UD may be more than one enzyme and this might explain the discrepancy in the results of these two groups.

Because of the ambiguity in the literature and the possible doubt cast upon the interpretation of the results of some of the preceeding experiments, it was decided to compare uroporphyrinogen and 5-carboxyl porphyrinogen as substrates for hepatic UD and also to examine red cell UD in

four patients with PCT using both substrates.

3.7.1. UROPORPHYRINOGEN VERSUS PENTACARBOXYL PORPHYRINOGEN AS SUBSTRATE

Since the same qualitative results have been obtained by the above authors in liver biopsies from patients with PCT and the discrepancy lies in the results obtained in blood hemolysates, the first experiment consisted of a comparison of the UD activity found in hemolysed blood from five control subjects and four patients with PCT testing uroporphyrinogen versus pentacarboxyl porphyrinogen as substrate. The enzyme activity was measured as described in the Experimental Procedures. The results of this comparison are shown in Table 6. From this it may be seen that decarboxylation of 5-carboxyl porphyrinogen proceeded three to four times as rapidly as uroporphyrinogen and, also, that a good correlation existed between the results obtained with the 5-carboxyl substrate and those obtained with the 8-carboxyl substrate both in the blood from control subjects and that from patients with PCT. The other result which is apparent is that three patients had red cell UD activity which was within the range found in control subjects, while one patient had UD activity which was 65% to 72% of the average found in controls, depending on which substrate was used.

TABLE 6

DECARBOXYLASE ACTIVITY
(nmol/min/g hemoglobin)

	$(COOH)_5$		$(COOH)_8$	
CONTROLS				
1	21.1		6.56	
2	17.5		3.93	
3	21.4	19.8 ± 1.5	4.94	5.3 ± 1.1
4	19.2		6.65	
5	20.1		4.85	
PATIENTS				
GH	18.0		4.84	
RH	14.4		3.51	
GC	21.3		5.40	
EC	17.8		4.05	

3.7.2. HEAT INACTIVATION STUDIES

Besides differences in substrate specificity, another possible method of determining whether UD activity is catalysed by more than one enzyme is by heat inactivation. Evidence that one enzyme activity was heat inactivated at a different rate than the other activity would be evidence that more than a single enzyme was involved. Therefore, 100,000 g supernatant from rat liver was incubated at 43°C in the buffer used for the UD assay according to Romeo and Levin (1971) who used this method in an attempt to distinguish between decarboxylation of isomers I and III of uroporphyrinogen. Eight aliquots were removed at 3 minute intervals and quick frozen in liquid nitrogen and stored frozen in liquid nitrogen until the enzyme was assayed using both 5-carboxyl and 8-carboxyl substrates. However, only 40% inactivation was achieved using this procedure, so the experiment was repeated at 60°C.

The results of the assays are depicted in Figure 29. Although only 40% inactivation occurred at 43°C, in contrast to the 90% inactivation described by Romeo and Levin (1971), 60°C caused complete inactivation of both activities within 10 minutes. The rate of inactivation at either temperature did not distinguish between these two enzyme activities.

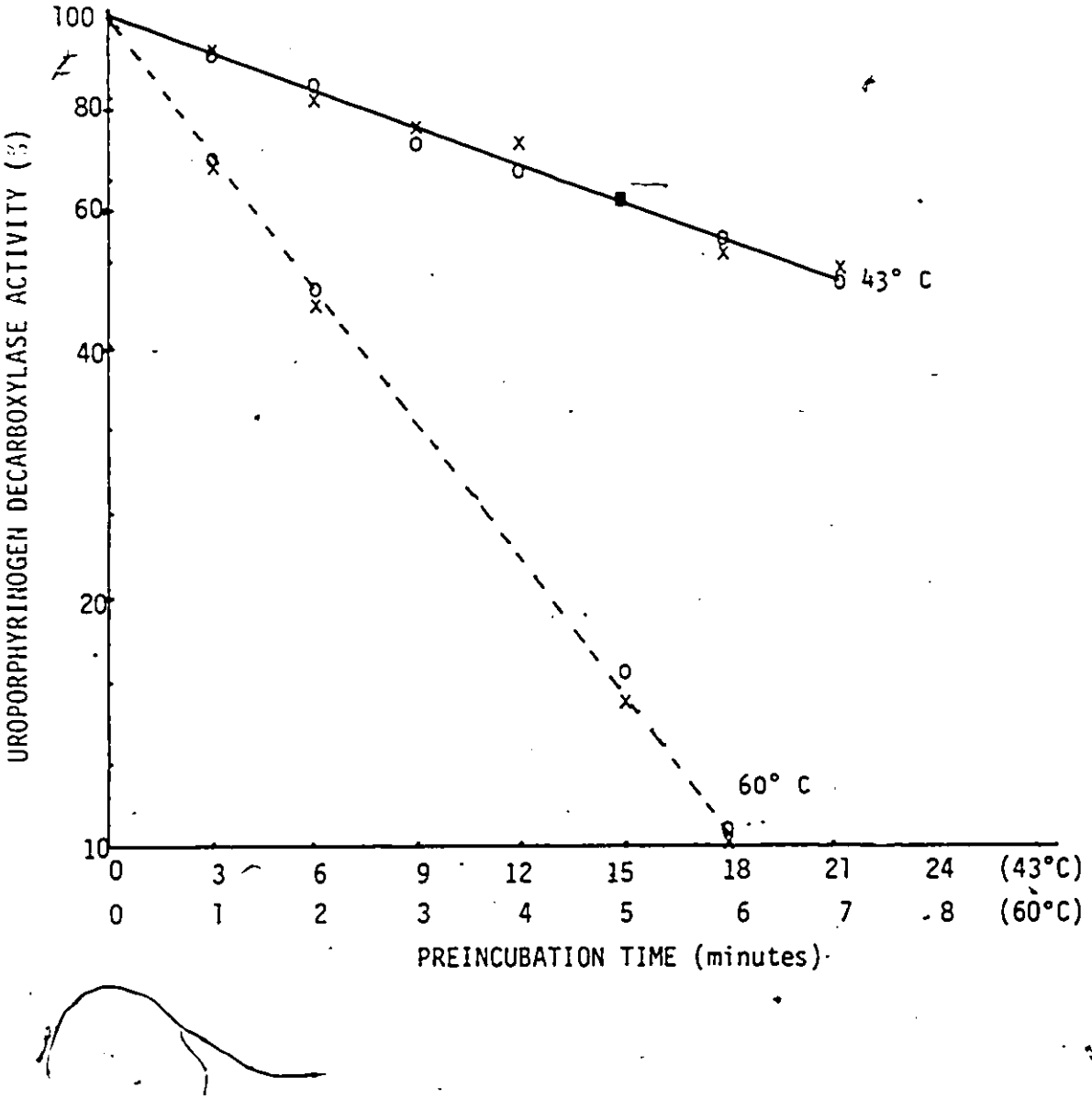


Figure 29: Heat inactivation of uroporphyrinogen decarboxylase using either 8-carboxyl (x) or 5-carboxyl (o) porphyrinogen as substrate. Points represent means of duplicate determinations.

3.7.3. SUBSTRATE-SUBSTRATE INTERACTIONS

The above experiments on UD attempted to distinguish between activities of the enzyme toward 5-carboxyl and 8-carboxyl substrates. The results were consistent with a single enzyme being responsible for metabolism of both substrates. If a single enzyme were responsible for catalysing the decarboxylation of four different substrates, one might expect the different substrates to interfere with each other's reaction. To determine whether this occurred, varying concentrations of one substrate were used in the presence of a constant concentration of the other substrate and the enzyme activities were measured. The results are shown as Lineweaver-Burke plots in Figures 30 and 31. From these figures it is apparent that the two substrates competed with each other for reaction with the enzyme. Also, the Vmax for the 5-carboxyl substrate (10.0 nmol/min/g liver) was approximately four times as high as for the 8-carboxyl substrate (2.5 nmol/min/g liver), although the Km for both substrates was very close to 1.0 μM.

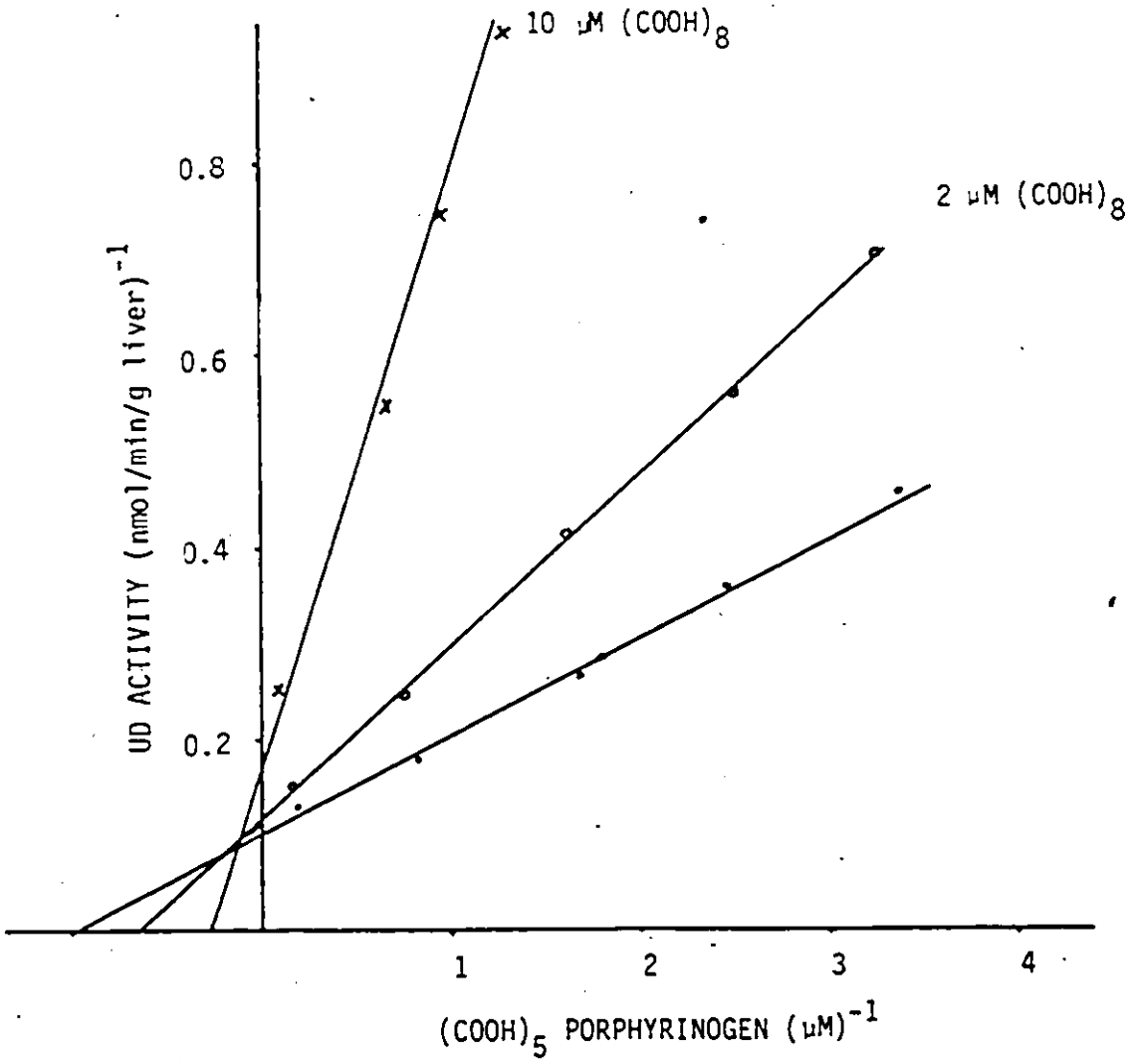


Figure 30: Effect of 8-carboxyl porphyrinogen on the rate of decarboxylation of 5-carboxyl porphyrinogen. Points represent means of duplicate determinations.

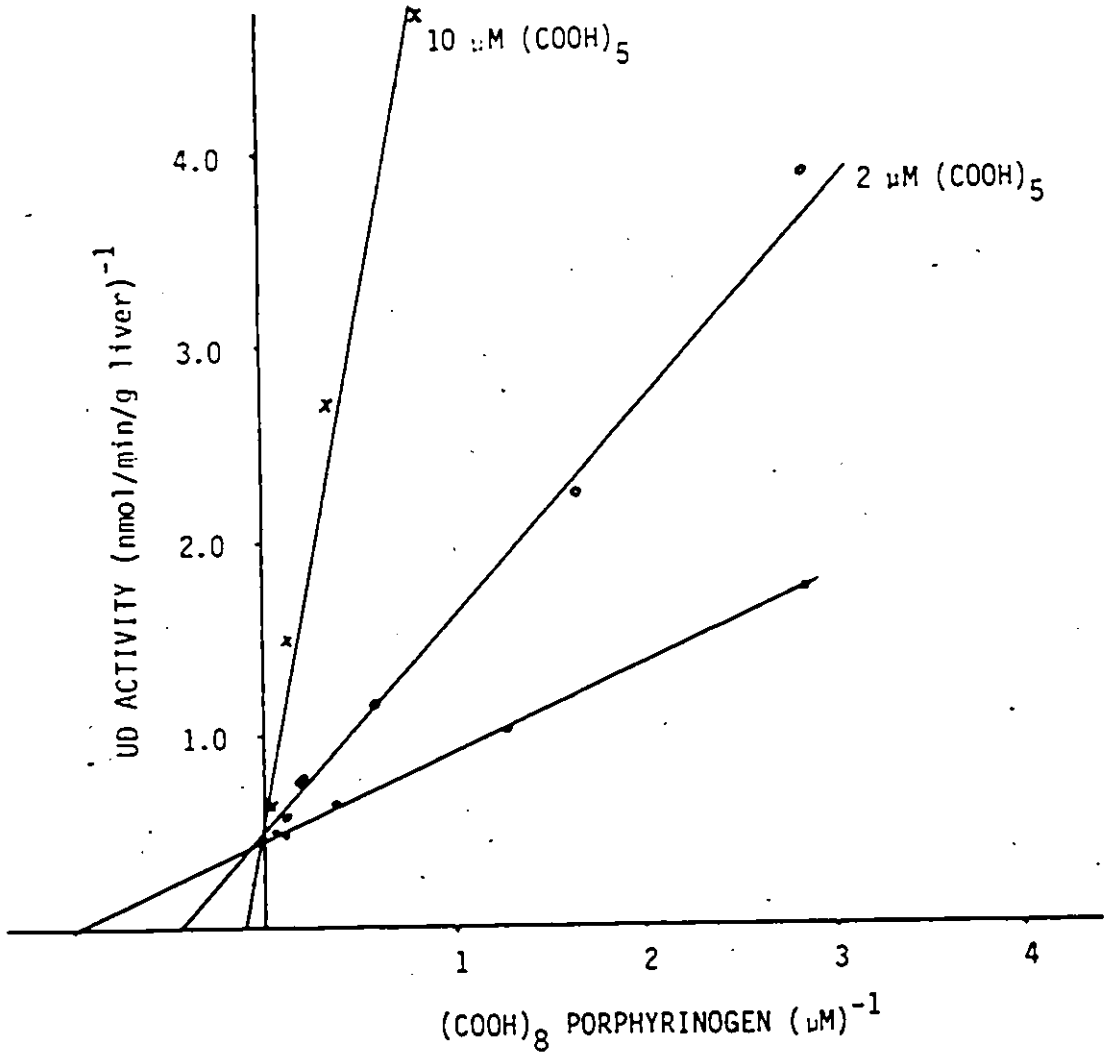


Figure 31: Effect of 5-carboxyl porphyrinogen on the rate of decarboxylation of 8-carboxyl porphyrinogen. Points represent means of duplicate determinations.

3.7.4. INHIBITION BY UROPORPHYRIN

Since the various substrates for UD can affect the rate of reaction of other substrates, it was of interest to determine whether uroporphyrin could affect UD activity since significant hepatic accumulation of uroporphyrin occurs in PCT. Figure 32 illustrates the effects of different concentrations of uroporphyrin on the activity of UD toward 5-carboxyl porphyrin. Although the inhibition was not as marked as for uroporphyrinogen, there was, nonetheless, inhibition of UD which could be significant in PCT, since large quantities of uroporphyrin accumulate in the liver during the course of this disease.

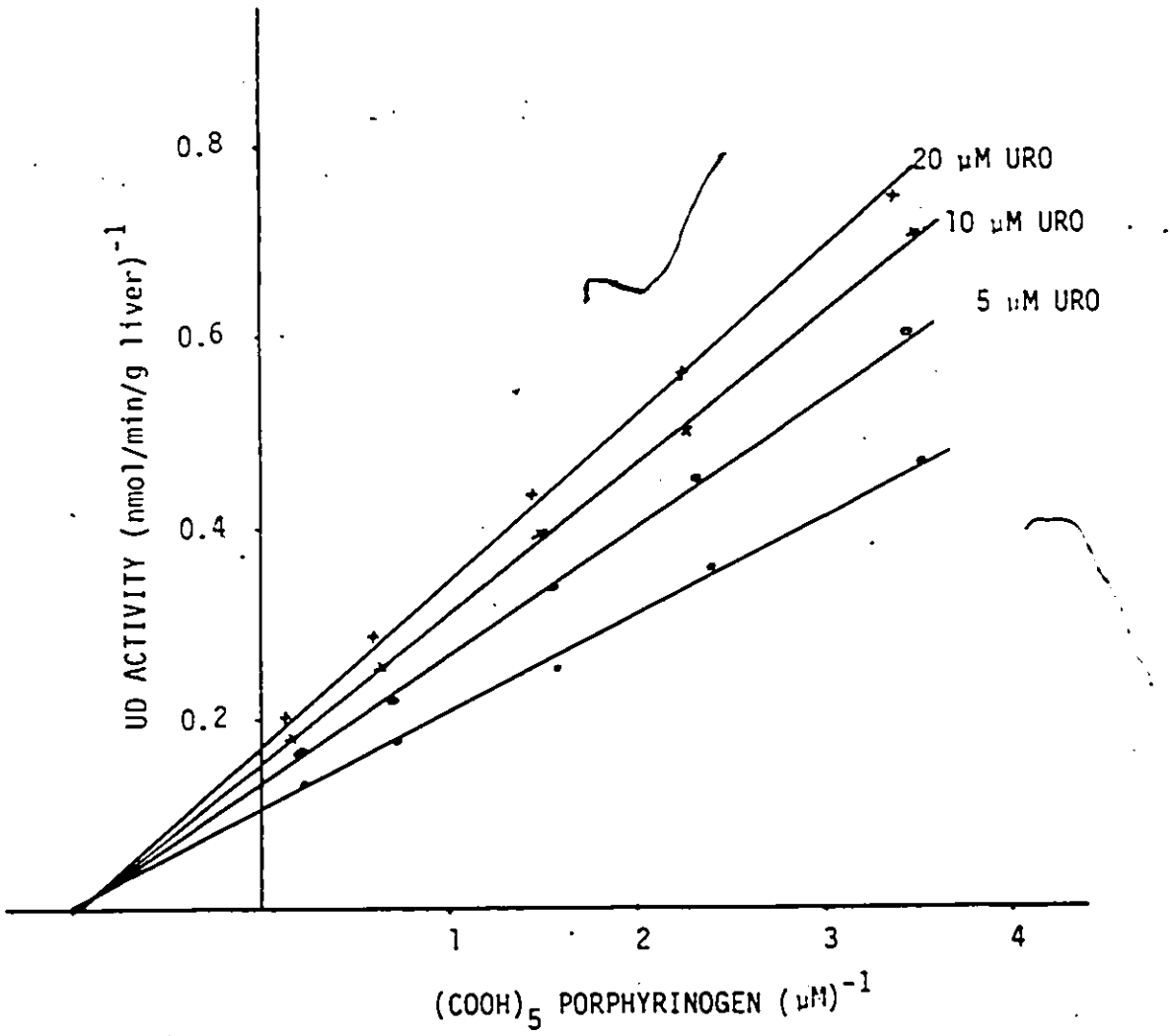


Figure 32: Effect of uroporphyrin on the rate of decarboxylation of 5-carboxyl porphyrinogen. Points represent means of duplicate determinations.

4. DISCUSSION

Porphyria cutanea tarda, as mentioned in the introduction, is a skin disease of man which can be acquired, and this makes it different from all other types of porphyria which depend upon genetically inherited enzyme deficiencies. Since a variety of different etiologic agents apparently cause PCT in man, and some of these have been shown to produce an equivalent metabolic defect (on the basis of the porphyrin excretion pattern and decrease in UD activity) in experimental animals, it seems reasonable to assume that at some point a common event must occur which results in accumulation and excretion of the particular porphyrins which typify this disease.

The chemical diversity of the etiologic agents associated with PCT in man (ethanol, synthetic estrogens, chlorinated aromatic hydrocarbons) suggests that a rather nonspecific event occurs which, in certain individuals, leads to the specific manifestation of this particular type of porphyria. This view is supported by two characteristics of the disease. One is that only a minority of people exposed to most of the presumed causative substances eventually acquire porphyria. The second is that a relatively long lag time exists which (in the experimental

model) cannot be overcome simply by increasing the dose of the etiologic agent; with increased doses of TCDD or HCB an increased incidence of death of the animals occurs but porphyria still is not apparent for several weeks.

The fact that HCB (in man) and certain other chlorinated aromatic hydrocarbons (in animals) have been found consistently to produce the characteristic biochemical abnormality typical of PCT suggests that whatever is the pathologic process which results in PCT, it is a common and reproducible feature in animals treated with HCB, TCDD, and certain PCB's. These compounds, therefore, are suitable for use as experimental probes to elucidate the mechanism by which PCT occurs.

4.1. THE EFFECT OF IMPURITIES ON THE PORPHYROGENICITY OF HEXACHLOROBENZENE

Since 1961, when Ockner and Schmid demonstrated that HCB caused porphyria in rats, commercial preparations of HCB have been used to produce experimental porphyria in animals as a model for PCT in man. It was, therefore, surprising to find that rats treated for twelve weeks with HCB which had been recrystallized three times did not develop porphyria. Purification of the HCB was suspected as the reason for the decrease in porphyrogenicity since recrystallization of the

practical grade material represented the only change from past procedures which had resulted in porphyric animals. The subsequent demonstration (Figure 12) that, indeed, crude HCB was approximately 2.5 times as potent at causing porphyria as the recrystallized chemical implied that impurities in the practical grade HCB were either completely responsible for the porphyrogenic properties of the commercial HCB, or that they at least contributed toward this effect.

The nature of the contaminating substances which affected the porphyria-producing properties of the HCB is unknown but Villanueva et al. (1974) have found evidence of chlorodibenzodioxins and chlorodibenzofurans in commercial preparations of HCB. This situation may be analogous to that described by Goldstein et al. (1977a) in which porphyria caused by pentachlorophenol was attributed to contamination with chlorinated dibenzofurans and dibenzodioxins.

Although Goldstein et al. (1978) have recently demonstrated that samples of technical grade and highly purified HCB did not differ in their abilities to cause porphyria, the technical grade HCB used in their experiments was from a different manufacturer than that used here and did not contain measurable quantities of chlorodibenzodioxins; also only 4.0 ppm

octachlorodibenzofuran were detected whereas the crude HCB used in the experiments described in this thesis was found to contain numerous impurities (Lui et al, 1976). The experiment of Goldstein et al (1978) demonstrated, however, that HCB, itself, is capable of causing porphyria in experimental animals and suggests that the contaminants in the crude HCB used above were not entirely responsible for the porphyrogenic effects of the chemical.

It may be concluded that purified HCB is, in itself, capable of causing experimental porphyria. Also, impurities present in some commercial preparations of HCB add to the potency of the chemical as an etiologic agent for porphyria. Since the impurities were in much lower concentrations than the HCB, they must be much more potent than HCB, itself. It is also possible that they could bioaccumulate much more efficiently than HCB.

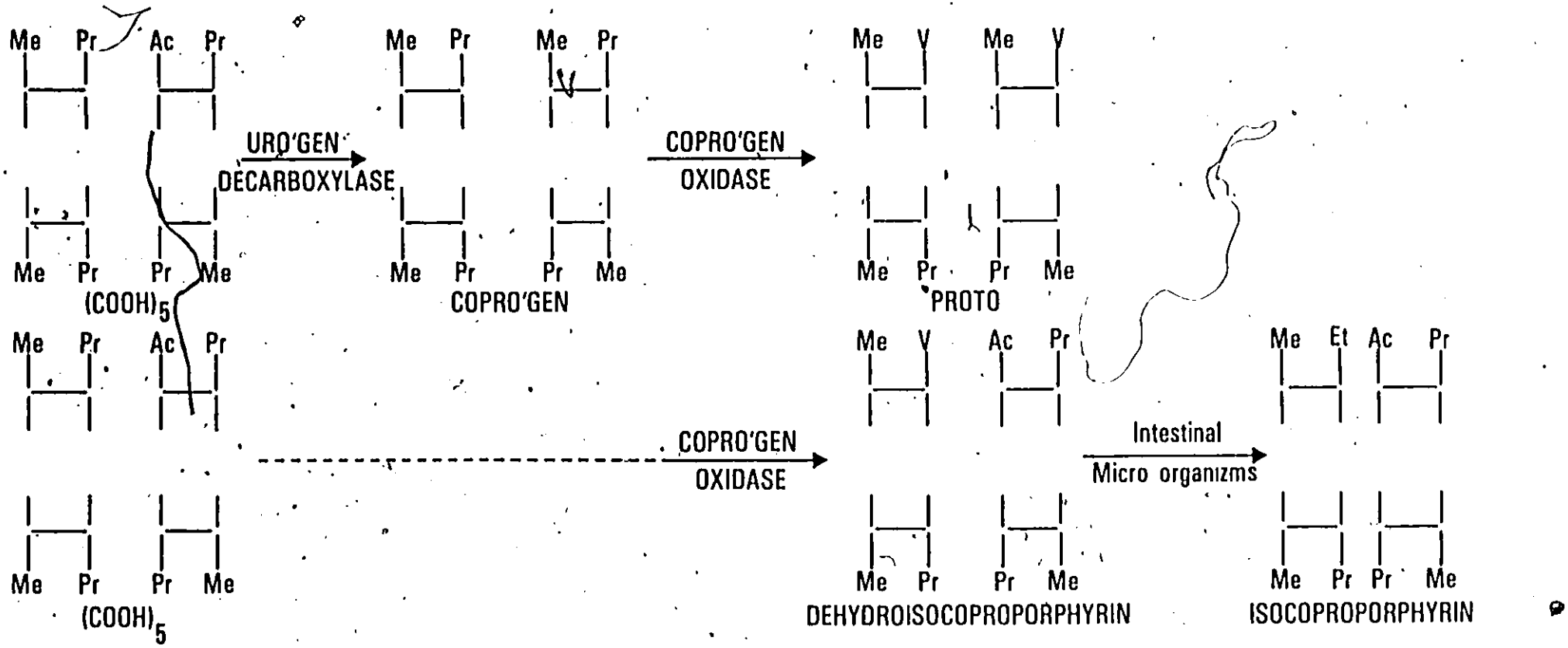
4.2. PORPHYRIN AND PORPHYRINOGEN EXCRETION DURING THE DEVELOPMENT OF HEXACHLOROBENZENE PORPHYRIA

From the pattern of porphyrin excretion seen in PCT (increased excretion of porphyrins with from eight to four carboxyl groups), it is clear that the problem in this disease lies in the steps of heme biosynthesis between uroporphyrinogen and coproporphyrinogen. The two hypotheses

which existed to account for the presence of increased quantities of 8-carboxyl to 4-carboxyl porphyrins proposed that either premature oxidation of porphyrinogens, due to a failure of the liver to maintain these intermediates in their reduced state, or decreased activity of uroporphyrinogen decarboxylase was responsible.

The hypothesis that PCT resulted from increased oxidation of porphyrinogens to porphyrins would account for the increase in all porphyrins with from eight to four carboxyl groups. Decreased uroporphyrinogen decarboxylase activity, on the other hand, could account for increased quantities of porphyrinogens with eight to five carboxyl groups but could not, by itself, be responsible for increased coproporphyrin(ogen). However, this could be explained if 5-carboxyl porphyrinogen were to compete with coproporphyrinogen for metabolism by coproporphyrinogen oxidase (Figure 33).

In any case, the two hypotheses may be distinguished by their predictions of increased excretion of either porphyrins (oxidation theory) or porphyrinogens (UD theory). The results of serial porphyrin and porphyrinogen measurements carried out on the urine of rats as they developed porphyria caused by hexachlorobenzene (Figures 13 and 14) may, therefore, be interpreted in terms of the above hypotheses. Although porphyrin, rather than porphyrinogen,



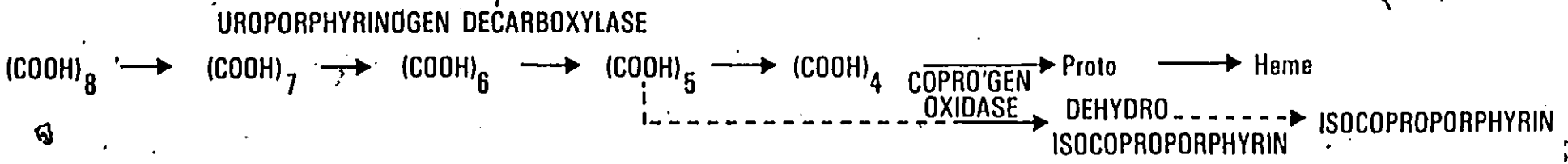


Figure 33: Formation of isocoproporphyrin

represented the major fraction excreted in the urine, the quantity of porphyrinogens excreted increased in parallel with urinary porphyrins. Such an increase in accumulation and excretion of porphyrinogens effectively excluded the possibility of oxidation as the primary cause of the porphyria.

The composition of the porphyrin and porphyrinogen fractions excreted in the urine diverged as the porphyria progressed (Figures 15 and 16). The oxidized intermediates were initially predominantly coproporphyrin (80 - 90%) until approximately six weeks into the treatment period. At that time, the percentage of coproporphyrin increased again until, by eight weeks, fifty percent of the porphyrin was coproporphyrin. In contrast, the porphyrinogen fraction remained predominantly coproporphyrinogen, though a decrease to approximately sixty percent was apparent after eight weeks of HCB treatment.

These results are consistent with those expected as a result of a decrease in UD activity and may be explained as follows: A primary decrease in UD activity would result initially in a gradual accumulation of porphyrinogens with from eight to five carboxyl groups. Oxidation of porphyrinogens to porphyrins can be assumed to be a continuous process. However, once oxidized, an intermediate cannot be further decarboxylated (Mauzerall and Granick,

1958). Oxidation, then, would predominantly favour an increase in the more highly carboxylated intermediates. Since heme biosynthesis is regulated by the end product, heme, only sufficient quantities of porphyrinogens would be made in order to maintain the rate of heme synthesis required by the cell. As UD becomes rate-limiting, the concentrations of both porphyrinogens and porphyrins would increase. When pentacarboxyl porphyrinogen reached a sufficiently high concentration to compete effectively with coproporphyrinogen for the enzyme, coproporphyrinogen oxidase, dehydroisocoproporphyrin would be made from the pentacarboxyl porphyrinogen (Figure 33) as described by Elder (1972), and increased quantities of coproporphyrinogen would accumulate, with some of this oxidizing to coproporphyrin. The compromise in heme biosynthesis brought about by decreased UD activity and competition between pentacarboxyl porphyrinogen and coproporphyrinogen for coproporphyrinogen oxidase would compound the problem by increasing the rate of flux through the pathway due to decreased feedback repression by heme on ALA-s, as the regulatory pool of heme is decreased. Thus, in advanced porphyria, one would expect to see increased excretion of ALA and PBG as well as porphyrins and porphyrinogens. If heme biosynthesis were sufficiently impaired, hemoproteins, such as cytochrome P-450, might also be affected.

These events are summarized in Figure 34 and account for the porphyrin and porphyrinogen excretion patterns shown in Figures 13 to 19. This scheme also explains the finding (shown in Table 3) of increased ALA and PBG excretion in "porphyric" rats in conjunction with decreased levels of cytochrome P-450 as compared with "nonporphyric" animals following identical treatment with hexachlorobenzene.

Although, as mentioned previously, oxidation of porphyrinogens to porphyrins most certainly occurs to a significant extent, and porphyrins, rather than porphyrinogens, are the major metabolites found in the urine of porphyric rats, there is no evidence that oxidation proceeds at an abnormally high rate. In fact, as shown in Figure 17, porphyrinogens, expressed as a percentage of the total porphyrin + porphyrinogen excreted, were found not to be significantly reduced in porphyric animals as compared with controls. Although a statistically non-significant decrease was noted in porphyric female rats, this was not considered to be important since no decrease was evident in porphyric males and there was no reason to believe that the etiology of the porphyria was different in the two sexes.

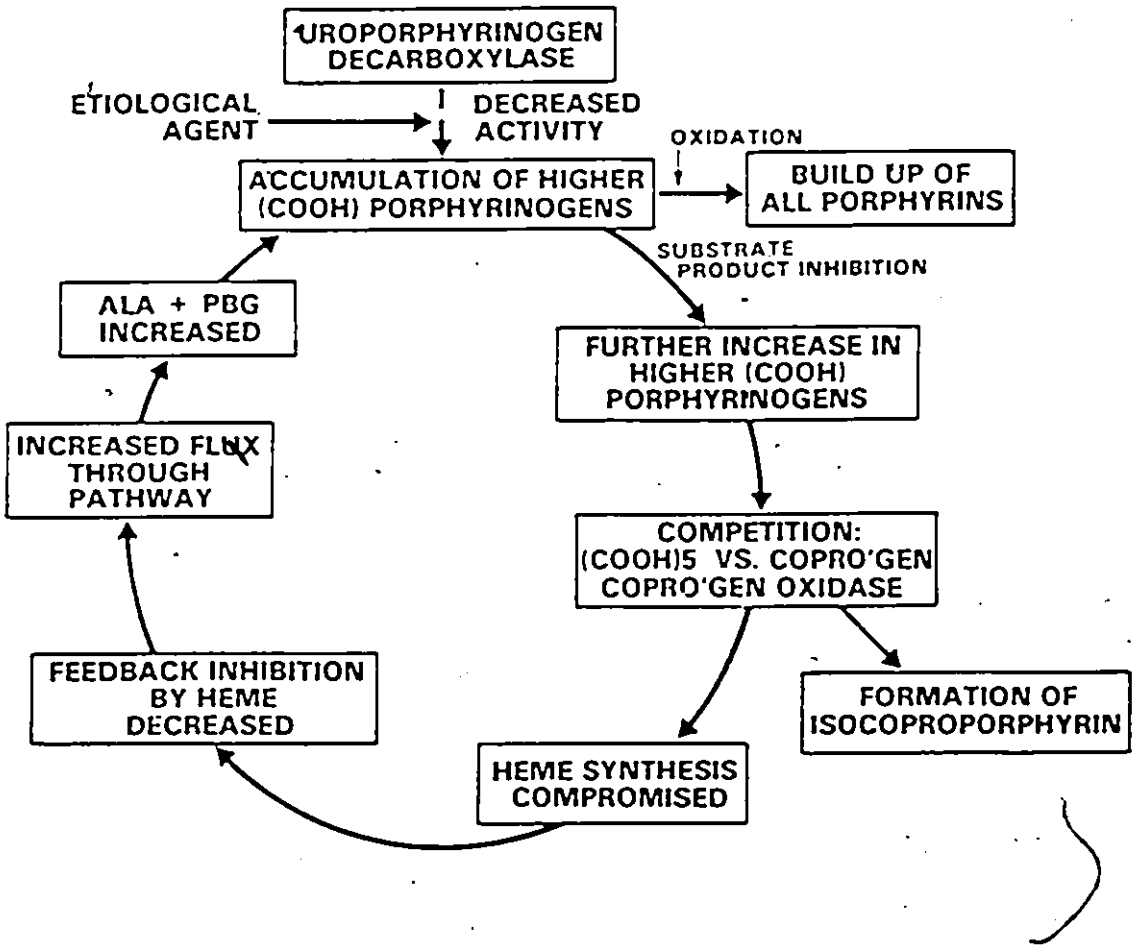


Figure 34: Progression of events in PCT

4.3. EFFECT OF ARYL HYDROCARBON HYDROXYLASE RESPONSIVENESS ON THE SUSCEPTIBILITY OF MICE TO THE PORPHYROGENIC EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

It will be recalled that the chlorinated aromatic hydrocarbons which cause PCT (or the equivalent animal lesion) are all inducers of the hepatic MFO system. HCB and PCB's cause an increase in the carbon monoxide binding spectrum of cytochromes P-450 and the ratio of peaks at 455 nm and 430 nm in the presence of ethyl isocyanide indicates induction of a mixture of cytochromes P-450 and P-448 (Alvares et al, 1973). MFO-related enzyme activities also indicate that HCB and PCB's induce a mixture of cytochromes.

TCDD, however, has been shown to be specific for induction of cytochrome P-448 and its potency as an inducing agent has been associated with two properties of the molecule: its affinity for a cytosol "receptor", which has been demonstrated to be a consequence of its stereochemical structure, and its resistance to metabolism which gives it a very long half-life, particularly at the site of action in the liver (Poland and Glover, 1976).

As well as being by far the most powerful and specific inducer of cytochrome P-448 and its related enzyme activities (including AHH), TCDD is also the most potent porphyrogenic molecule known. Clearly, if any relationship

exists between induction of MFO activity and PCT, induction of cytochrome P-448, rather than both P-450 and P-448, is sufficient to fulfill the MFO induction requirement. Also, it is unlikely that induction of P-450 could replace P-448 in view of the fact that no pure P-450 inducer has ever been associated with either experimental porphyria in animals, or PCT in man, despite the fact that phenobarbital (a classic P-450 inducer) is widely used on a chronic basis in the treatment of epilepsy.

Responsiveness to induction of AHH by polycyclic aromatic hydrocarbons has been shown to be inherited as an autosomal dominant trait among the offspring of the appropriate crosses between C57BL/6J and DBA/2J mice (Gielen et al, 1972; Nebert and Gielen, 1972; Nebert et al, 1975), although, in some strains, lack of responsiveness has been found to be dominant (Nebert, 1978). The structural genes for AHH are apparently intact and current evidence suggests that the gene product of the Ah locus (which has been defined as the gene controlling AHH responsiveness) is the induction receptor for AHH (Poland and Glover, 1976). "Nonresponsiveness" results from a decreased affinity of the induction receptor for AHH inducers such as 3-MC. The very potent inducer, TCDD, has sufficiently high affinity for the receptor that AHH can be induced to the same maximal extent in nonresponsive strains as in responsive strains when TCDD

is used, though a dose approximately fifteen times higher than that required for responsive mice is necessary (Poland and Glover, 1974).

It was shown (Figure 21) that, given identical treatment with TCDD, C57BL/6J mice (AHH responsive) excreted elevated levels of urinary porphyrins and demonstrated decreased hepatic UD activity (Figure 22), while the DBA/2J strain had normal levels of both urinary porphyrins and UD activity in the liver. These results confirm that a relationship does exist between the responsiveness to induction of AHH and susceptibility to the porphyrogenic effect of TCDD in these mice. However, the possibility that nonspecific strain differences between these two groups of mice was responsible for the difference in susceptibility to porphyria caused by TCDD could not be ruled out on the basis of this evidence.

Since responsiveness to AHH induction is inherited as an autosomal dominant trait in C57BL/6J mice (homozygous responsive (Ah/Ah)) and DBA/2J mice (homozygous nonresponsive (ah/ah)) the F1 hybrid (B6D2)F1/J is heterozygous (Ah/ah) and phenotypically responsive to AHH induction, and the back-cross of this strain with DBA/2J results in a strain (D2(B6D2)F1/J) which is, statistically, 50% heterozygous responsive (Ah/ah) and 50% homozygous nonresponsive (ah/ah) to AHH induction. Homozygous and

heterozygous mice in the D2(B6D2)F1/J strain were, therefore, used to reduce nonspecific strain differences between AHH responsive and nonresponsive mice when testing for an association between susceptibility to porphyria caused by TCDD and AHH responsiveness.

D2(B6D2)F1/J mice were phenotyped for AHH responsiveness by measuring zoxazolamine-mediated paralysis times (Robinson and Nebert, 1974) following treatment with 5,6-benzoflavone (an AHH inducer of potency comparable with 3-MC (Boobis et al, 1977)). Subsequent treatment of these animals with TCDD confirmed that the susceptibility to the porphyrogenic effect of TCDD segregates with the gene controlling AHH responsiveness. Thus, either expression of the same gene is necessary for both events, i.e. the gene coding for the putative induction receptor, or genes controlling the two events are closely linked.

The term "nonresponsive" when applied to these animals is relative and indicates only a decreased sensitivity to induction of AHH since TCDD, when used in adequate amounts, causes the same maximal AHH induction in both nonresponsive and responsive mice (Poland and Glover, 1975). The theory put forward to explain their findings is that nonresponsive mice have a defect in an induction receptor such that affinity for inducing compounds is diminished. This theory is supported by the finding of a

stereospecific, high affinity binding site in hepatic cytosol of C57BL/6J mice, a lesser capacity for high affinity binding in the hybrid (B6D2)F1/J strain, and least in the DBA/2J strain.

If the induction receptor for AHH also determines the susceptibility to the porphyrogenic effect of TCDD, one would expect the susceptibility of the Ah/ah mice used in this study to porphyria caused by TCDD to follow the same dominant pattern of inheritance found for other characteristics associated with the Ah locus. Using 24 hour urine porphyrin excretion at an indicator, no distinction could be made between Ah/Ah and Ah/ah groups (Figure 24). When studying the underlying enzyme defect, a progression in the extent of decreased UD activity from Ah/Ah through Ah/ah to ah/ah genotypes was noted but care should be taken to avoid overinterpretation of this data since it is well recognized that individual animals treated with chlorinated aromatic hydrocarbons develop porphyria at different rates. The fact that both Ah/Ah and Ah/ah groups became porphyric while the ah/ah group did not is significant but the progression of decreased UD activity may have been fortuitous.

This interpretation is in line with the evidence available for induction of AHH in homozygous and heterozygous strains of mice. Although Poland and Glover

(1975) have reported an intermediate dose-response effect in the heterozygous (B6D2)F1/J mouse strain as compared with C57BL/6J and DBA/2J strains, in fact, the dose-response curves for the homozygous and heterozygous AHH responsive groups were very close together and a distinction was made only after normalizing the curves. This distinction resulted from the fact that a greater maximal AHH response occurred in the heterozygous (B6D2)F1/J strain. Nebert and coworkers have noted no distinction in the phenotypic response between homozygous and heterozygous AHH responsive animals (Gielen et al, 1972; Nebert et al, 1975; Nebert, 1978).

Since AHH nonresponsive mice can be induced by a large dose of TCDD (approximately 10 to 15 times that required for responsive strains), one might reasonably expect DBA/2J mice to acquire porphyria if they were treated with a dose of TCDD ten times larger than that required for the C57BL/6J strain. In the experiment in which DBA/2J mice were treated with TCDD at 250 μg/kg/wk for 8 weeks, six of the eight treated animals died without any evidence of porphyria before the seventh week of treatment. However, two mice lived for 12 weeks and at necropsy the livers showed brilliant red fluorescence under ultraviolet light, indicating an accumulation of porphyrins. Porphyria, then, as with AHH induction, can be acquired by AHH nonresponsive

mice provided that a sufficiently high dose of TCDD is administered.

The Ah locus has been found to be responsible for the responsiveness to induction of at least 20 monooxygenase activities as well as several other non-oxygenase enzymes (Nebert, 1978). Which (if any) of these phenotypic characteristics is responsible for the decreased UD activity which results in porphyria remains obscure. It is worth remembering that induction of the activities associated with the Ah locus preceeds the development of porphyria by several weeks. Maximal AHH induction (given an appropriate dose of TCDD), for example, occurs within 48 hours (Boobis et al, 1977) while porphyria is not apparent for six to twelve weeks. Whatever is the direct cause of decreased UD activity, the process is time dependent and cannot be greatly accelerated by administration of a larger dose of TCDD. Cytotoxicity, as evidenced by hepatocellular necrosis and animal mortality may be achieved more quickly with increased TCDD, but not porphyria.

4.4. THE REQUIREMENT FOR TISSUE IRON IN EXPERIMENTAL PORPHYRIA AND HEPATOTOXICITY CAUSED BY 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

Treatment of patients suffering from PCT with

phlebotomy was originally begun by Ippen (1962) on the premise that removal of hemoglobin in these patients would divert the excess production of heme precursors into the synthesis of hemoglobin, rather than the accumulation of porphyrins. Although, as later stated by Ippen himself (1977), this hypothesis ignored the distinction between the hepatic and erythropoietic porphyrias, the treatment was, nevertheless, successful.

The success of venesection as a treatment for PCT has been attributed to the removal of storage iron. Several lines of evidence suggest that this is the case: Although hepatic siderosis is not usually accompanied by PCT, approximately 80% of patients with PCT demonstrate abnormally high levels of iron in the liver (Felsher et al, 1973). Isolated instances of PCT have been found to develop following prolonged administration of ferrous sulfate (Welland and Carlsen, 1969) or numerous blood transfusions (Felsher and Redeker, 1966). Also, removal of packed red blood cells has been demonstrated to result in clinical remission of PCT while administration of parenteral iron to these same patients resulted in relapse of the porphyria (Felsher et al, 1973).

It has been demonstrated (Taljaard et al, 1972a; 1972b; Joubert et al, 1973) that rats which have been pretreated with parenteral iron are more sensitive to the

porphyrogenic effects of HCB. An extrapolation of the effects of iron overload suggests that non-heme tissue iron may be a necessary condition for the development of porphyria caused by chlorinated aromatic hydrocarbons. If this were the case, TCDD-mediated experimental porphyria should be preventable by depletion of tissue iron. It was found (Figure 26) that, indeed, C57BL/6J mice which were maintained on an iron deficient diet and had been depleted of body iron stores by repeated removal of blood until the mean hemoglobin level fell to 5.5 g/dl did not demonstrate increased urinary porphyrin excretion in response to treatment with TCDD whereas mice with normal iron stores treated identically with TCDD showed the expected increase in urinary porphyrin excretion.

Whatever is the mechanism by which TCDD causes decreased activity of UD in the liver, it is likely that iron is involved in the pathologic process since the activity of this enzyme was found to be normal in the livers of iron deficient animals treated with TCDD but decreased in normal iron mice treated identically with TCDD (Figure 26). Since hepatic cytochrome levels were similar in normal iron and iron deficient animals it is probable that a pool of non-heme iron participates in the pathogenesis of porphyria. Further, it is likely that this pool of iron participates in other aspects of the toxicity of TCDD since the general

vigor of the iron deficient animals treated with TCDD in this experiment was greater than that of the normal iron TCDD-treated group. They were physically more active and paid more attention to their fur than the normal iron mice. More direct evidence of the protection conferred by iron deficiency was found when the histopathologic changes caused by TCDD were compared. The livers from iron deficient animals treated with TCDD were normal histologically (Figure 28 B), apart from a proliferation in eosinophilic material which was consistent with an increase in smooth endoplasmic reticulum which would be expected from the increase in microsomal cytochrome P-450 (P-448) found in these mice. However, extensive histopathologic changes were evident in normal iron TCDD-treated mice (Figure 28 A). (Figure 29 A). It is probable, therefore, that non-heme iron is essential for the hepatotoxicity of TCDD.

4.5. STUDIES ON UROPORPHYRINOGEN DECARBOXYLASE

Measurement of UD activity in erythrocytes from control subjects and patients with PCT using both 8-carboxyl and 5-carboxyl porphyrinogen as substrate confirmed the findings of Elder (1977) that normal activity exists in erythrocytes of patients with PCT. These results are, however, in conflict with those of Kushner and coworkers (Benedetto et al, 1978). It is, of course, possible that

two types of PCT exist: an inherited form, and an acquired type (Blekkenhorst et al, 1979). However, these results cannot be accounted for by the suggestion (Benedetto et al, 1978) that two or more enzymes may be responsible for the conversion of uroporphyrinogen to coproporphyrinogen since both 5-carboxyl and 8-carboxyl substrates were found to yield similar results.

Further evidence that UD is a single enzyme comes from heat inactivation studies on the enzyme using both 5-carboxyl and 8-carboxyl substrates. Both activities disappeared concurrently when preincubated at either 43°C or 60°C indicating that either the same enzyme catalyzed both activities or, fortuitously, both enzymes were equally susceptible to heat inactivation.

Substrate-substrate inhibition experiments demonstrated that 5-carboxyl and 8-carboxyl porphyrinogens compete with each other for metabolism by UD which was the result predicted, assuming one enzyme to be responsible for both reactions.

The finding that different substrates of UD are mutually inhibitory has relevance for congenital erythropoietic porphyria as well as for PCT. It will be recalled that in congenital erythropoietic porphyria an inherited deficiency in uroporphyrinogen III cosynthetase

results in decreased synthesis of uroporpohyrinogen III which, in turn, leads to decreased heme synthesis and subsequent induction of ALA-s activity. The increase in ALA-s results in greater production of ALA and PBG and, consequently, both uroporphyrinogens I and III. Since UD can metabolize both isomers (Jackson et al, 1976) it would be expected that these substrates would be converted to coproporphyrinogens I and III, respectively. Thus, the expected accumulation would be coproporphyrin(ogen) I since no further metabolism of this isomer is possible. However, uroporphyrin I accumulates even more than coproporphyrin I.

The effectiveness of 5-carboxyl porphyrinogen as an inhibitor of the decarboxylation of the 8-carboxyl substrate (Figure 31) suggests that, in a situation in which flux through this pathway is greatly increased, significant inhibition of uroporphyrinogen metabolism would occur in favour of the less highly carboxylated substrates. This would lead to greater accumulation of uroporphyrin I in congenital erythropoietic porphyria.

4.6. SUMMARY AND EVALUATION

Porphyria cutanea tarda is an acquired (or, apparently, in some instances, inherited) disorder of heme biosynthesis characterized by the excretion of large

quantities of porphyrins with from eight to four carboxyl groups, and isocoproporphyrin. Etiologic agents implicated in this disease include alcohol, chlorinated aromatic hydrocarbons, synthetic estrogens, and iron. When this work was begun, it was uncertain whether the increase in excretion of intermediates of heme synthesis resulted from accelerated oxidation of porphyrinogens to porphyrins, or from decreased activity of uroporphyrinogen decarboxylase; no association was apparent between the known biochemical effects of any of the etiologic agents and PCT; and the reasons for the success of venesection as a treatment for this disease were completely obscure.

Careful examination of the excreted intermediates of heme biosynthesis during the development of experimental porphyria in rats caused by hexachlorobenzene (which was made possible by the development of improved methodology for measurement of urinary porphyrins and for extraction and measurement of porphyrinogens) eliminated the possibility that oxidation was the primary cause of the porphyria. Elder (1976), simultaneously, found decreased hepatic UD activity in rats made porphyric with HCB, confirming that decreased activity of this enzyme was probably the direct cause of the porphyria.

The association between the ability of chlorinated aromatic hydrocarbons to induce aryl hydrocarbon hydroxylase

and their capacity to cause experimental porphyria was examined in strains of mice which were either "responsive" or "nonresponsive" to induction of AHH. TCDD, rather than HCB, was used as the porphyrogenic agent because of its specificity for induction of P-448. It was found that C57BL/6J mice, which were "responsive" to AHH induction were more susceptible to the porphyrogenic effects of TCDD than were DBA/2J mice which were "nonresponsive" to induction of AHH. A further examination of this association was carried out in a back-crossed strain (D2(B6D2)F1/J) after phenotyping the animals for AHH responsiveness. The results confirmed that susceptibility to porphyria caused by TCDD segregated with the gene controlling AHH responsiveness.

The requirement for tissue iron in the pathogenesis of experimental porphyria caused by TCDD was examined by depletion of iron stores in mice prior to treatment with TCDD. The results of the experiment revealed that not only was tissue iron required for porphyria, but also that iron was an obligatory requirement for the more generalized morphologic liver damage and skin lesions caused by TCDD.

The last point is, perhaps, the most revealing and the one which helps to bring into perspective many of the facts known about PCT. It is worth remembering that, while chlorinated aromatic hydrocarbons do cause PCT, most cases of this disease have a much less well defined origin and

tend to be rather loosely associated with prolonged consumption of alcohol, iron overload, and/or estrogen therapy (Elder, 1977; Sweeney and Jones, 1979).

It seems likely that PCT is a specific disorder which may arise from a more generalized and chronic pathologic process occurring in the liver. The process must involve iron and can be brought about reproducibly by certain chlorinated aromatic hydrocarbons which have in common the ability to induce cytochrome P-448. The chronic nature of the process leading to decreased UD activity is probably of significance since no etiologic agent is capable of causing porphyria in the short term, though acute toxicity and death may result.

When considering toxic processes in the liver which involve both MFO activity and iron, the most obvious possibility is the production of toxic metabolites produced by the cytochrome P-450 (P-448) system. For most chlorinated aromatic hydrocarbons, the metabolites which come to mind are aryl epoxides of the chemicals themselves, but this is not possible with TCDD since there is no evidence that significant metabolism of this molecule occurs (Piper et al, 1973). Another possibility which cannot so easily be be ruled out is the formation of reactive metabolites of oxygen. It is well recognized that stimulation of MFO activity results in increased lipid

peroxidation (Orrenius et al, 1969) and such reactive forms of oxygen as superoxide and peroxide complexes are postulated intermediates of MFO reactions (Estabrook and Werringloer, 1977).

Non-heme iron has been shown to be an obligatory requirement for microsome-mediated lipid peroxidation (Willis, 1969; Pederson et al, 1973). The role of iron in this process is probably to catalyze the production of free radicals which could then participate in the peroxidation process. Evidence for such radical formation comes from the work of Pfeifer and McCay (1971) who showed that reactive species generated during NADPH-dependent lipid peroxidation in microsomal preparations caused hemolysis of erythrocytes. Formation of toxic radicals is common in phagocytes (review by Babior, 1978a; 1978b) which utilize them to kill bacteria. Weis et al (1973) have found evidence suggesting that one of the radicals involved is the extremely reactive hydroxyl radical which may be generated from superoxide and peroxide.

It is conceivable that prolonged induction of the hepatic MFO system could lead to an overproduction of toxic radicals which could, in turn, cause generalized liver toxicity, including decreased activity of uroporphyrinogen decarboxylase. However, the specific role for cytochrome P-448, as opposed to P-450, remains unexplained by this

hypothesis. It is, of course, possible that some other product controlled by the expression of the Ah locus is involved in the process leading to PCT. These possibilities remain to be tested.

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